INTERACTIVE INFLUENCE OF APOLIPOPROTEIN E GENOTYPE AND DIETARY CHOLESTEROL ON CHOLESTEROL SYNTHESIS IN HUMANS

by

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ABSTRACT

Apolipoprotein (apo) E is a 299 amino acid polypeptide that mediates the uptake of apo E containing lipoproteins by receptor-mediated endocytosis. Allelic differences at the apo E gene locus are responsible for as much as 8% of the variance of low density lipoprotein (LDL) cholesterol. Due to the association between LDL cholesterol levels and atherosclerosis, it has been suggested that apo E polymorphism may play a role in determining the risk of coronary artery disease. A method for apo E genotyping was developed using the polymerase chain reaction (PCR) with allele-specific oligonucleotide primers. The fourteen phenotypes identified by isoelectric focusing methods were consistent with the fourteen genotypes identified by the PCR method. Seven E2/− and six E4/− volunteers were selected for a dietary study investigating the interactive effects of apo E polymorphism and dietary cholesterol levels on cholesterol synthesis rates. Volunteers underwent two dietary treatments consisting of a low cholesterol diet (250 mg/day) and a high cholesterol diet (800 mg/day). At the end of each diet period an oral dose of deuterium was given at 0.7 g/Kg body water to determine fractional synthetic rate (FSR) of cholesterol. The apo E4/− group had a significantly higher serum total cholesterol level than the E2/− group throughout both dietary treatments. Cholesterol FSR was not significantly different between apo E groups when comparing dietary cholesterol levels. The apo E4/− did respond to fasting state differently from the E2/− group by not
Abstract

depressing cholesterol synthesis while on a low cholesterol diet. This suggests E4/- individuals may not have a sensitive endogenous feedback mechanism to suppress cholesterol synthesis while on a low cholesterol diet.
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1.0 INTRODUCTION

Hypercholesterolemia is strongly associated with the risk of coronary heart disease. Studies instituting dietary changes to correct hypercholesterolemia have shown positive results in reducing the incidence of coronary heart disease. Therefore, it is of central importance both to identify individuals with elevated cholesterol levels and to institute diet therapies which will reduce circulating cholesterol levels.

Apolipoprotein (apo) E polymorphism influences serum total and low density lipoprotein cholesterol levels. Individuals possessing the $E_4$ allele characteristically have elevated cholesterol levels while individuals possessing the $E_2$ allele have lower than normal serum cholesterol levels. Genetic studies considering dietary changes have found that individuals with the $E_4$ allele respond to dietary therapy more positively than do individuals with the $E_2$ allele.

The project objectives were: i) to develop a rapid, simple technique to identify apo E genotypes and ii) to investigate cholesterol synthesis rate variations in apo E genotyped volunteers possessing $E_2/-$ or $E_4/-$ alleles after dietary cholesterol intake manipulations. The first objective may help to identify individuals at high risk of heart disease as well as increase our knowledge about the population distribution of apo E genotypes. The second phase of the experiment investigates why people of different genetic backgrounds
1.0 Introduction

respond to therapeutic diets differently. Increasing our knowledge of how diet and apo E effect plasma cholesterol levels may help to target preventative dietary measures aimed at reducing plasma cholesterol levels.
2.0 Literature Review

2.1 APOLIPOPROTEIN E

Apolipoprotein E (apo E) is a 299 amino acid, 34,000 molecular weight glycoprotein found in human plasma (Paik et al. 1985, Shore et al. 1973). In humans apo E is synthesized in liver, spleen, kidney, adrenals, gonads, brain, macrophages and astrocytic cilia (Blue et al. 1983). Apo E is a surface component on plasma lipoprotein particles including chylomicrons and chylomicron remnants, very low density lipoproteins (VLDL), and a sub-population of high density lipoproteins (HDL), HDL-1 or HDLc (Havel et al. 1987).

2.1.1 Lipoproteins

Utermann et al. (1985) were the first to observe the impact of apo E allelic variation on plasma cholesterol levels. Because apo E is associated with the cholesterol carrying lipoproteins, it is an important component of cholesterol metabolism (Davignon et al. 1988, Utermann et al. 1986).

In chylomicrons and chylomicron remnants, which transport dietary cholesterol from the intestine to the liver, apo E mediates the hepatic uptake of dietary cholesterol. Chylomicron remnants are not present in the circulation of healthy humans because they are rapidly taken up by
the liver. This hepatic endocytosis is a receptor mediated process involving a highly specific cell surface receptor. In this class of lipoproteins apo E is directly required for removal of cholesterol from dietary sources. The second class of lipoproteins in which apo E is a component is VLDL, which are transport vehicles for endogenous cholesterol and triglycerides assembled in the liver. Functional apo E molecules are required for the uptake of VLDL remnants (intermediate density lipoproteins (IDL)) and their conversion to LDL. The role apo E plays in this conversion of IDL to LDL is presently undefined.

The subgroup of HDL called HDL-1 or HDLc is the third class of lipoproteins associated with apo E (Utermann et al. 1986). This class of lipoproteins is responsible for the movement of cholesterol from peripheral tissues to the liver. Specific hepatic receptors remove apo E carrying HDL particles (Havel 1984).

2.1.2 Polymorphism

Family and population studies have shown the structural gene locus for apo E to be polymorphic (Zannis et al. 1981) and controlled by three common alleles (E2, E3, E4) which code for three common isoforms (E2, E3, E4) found in plasma (Sing et al. 1985). These 3 alleles produce 3 homozygous (E2/2, E3/3, E4/4) and 3 heterozygous (E2/3, E2/4, E3/4) phenotypes.
The 3 common isoforms of apo E differ by amino acid substitutions at one or both of two sites (residues 112 and 158) (Rall et al. 1982). The alleles $E_4$ and $E_2$ contain arginine and cysteine at both sites, respectively, while the $E_3$ allele contains cysteine at site 112 and arginine at site 158 (Rall et al. 1982). As the $E_4$ isoform has one more positive charge than $E_3$, while $E_2$ has one less positive charge than $E_3$, each isoform has a different isoelectric point due to these substitutions.

2.1.3 Population Distribution

Across the general population, the frequency of occurrence varies greatly for $E_4$, $E_3$, $E_2$ alleles (Sing et al. 1985, Wardell et al. 1982, Davignon et al. 1988, Enholm et al. 1986, Utermann 1986) (Figure 1). In all populations studied, the $E_3$ allele is most commonly observed. Generally, $E_4/4$ and $E_2/2$ phenotypes make up the smallest proportion of the population (Enholm et al. 1986). In comparing 9 different studies Enholm et al. found a range of 11-23% for $E_4$, 72-78.3% for $E_3$, and 4-17% for $E_2$ alleles. Comparison of populations indicates that relative allele frequencies vary significantly between different ethnic and cultural populations (Davignon et al. 1988).
Figure 1 Serum cholesterol distribution in apo E phenotypes

from Utermann, 1986
Table 1: Apolipoprotein E gene frequencies (± SD) in different populations

<table>
<thead>
<tr>
<th>Country</th>
<th>E4</th>
<th>E3</th>
<th>E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finland</td>
<td>0.227 ± 0.0125</td>
<td>0.733 ± 0.0170</td>
<td>0.041 ± 0.0150</td>
</tr>
<tr>
<td>Germany</td>
<td>0.150 ± 0.0081</td>
<td>0.773 ± 0.0137</td>
<td>0.077 ± 0.0125</td>
</tr>
<tr>
<td>Germany</td>
<td>0.139 ± 0.0080</td>
<td>0.783 ± 0.0139</td>
<td>0.078 ± 0.0128</td>
</tr>
<tr>
<td>USA</td>
<td>0.110 ± 0.0291</td>
<td>0.720 ± 0.0586</td>
<td>0.170 ± 0.0556</td>
</tr>
<tr>
<td>USA</td>
<td>0.175 ± 0.0307</td>
<td>0.740 ± 0.0484</td>
<td>0.085 ± 0.0441</td>
</tr>
<tr>
<td>USA</td>
<td>0.149 ± 0.0302</td>
<td>0.757 ± 0.0515</td>
<td>0.095 ± 0.0474</td>
</tr>
<tr>
<td>New Zealand</td>
<td>0.141 ± 0.0123</td>
<td>0.739 ± 0.0217</td>
<td>0.120 ± 0.0202</td>
</tr>
<tr>
<td>Scotland</td>
<td>0.150 ± 0.0130</td>
<td>0.770 ± 0.0220</td>
<td>0.080 ± 0.0201</td>
</tr>
<tr>
<td>France</td>
<td>0.152 ± 0.0262</td>
<td>0.770 ± 0.0472</td>
<td>0.078 ± 0.0424</td>
</tr>
</tbody>
</table>

Table adapted from Enholm et al. 1986.

Davignon et al. (1988) have found that the contribution of the apo E gene locus to the genetic distance between Caucasian and Chinese/Japanese populations was primarily attributable to the relative frequency of the ε3 allele. The frequencies of the ε2 and ε4 alleles were similar when comparing the Caucasian and Chinese/Japanese populations. In addition, the heterogeneity among the Caucasian populations is associated with differences in the relative frequencies of the ε2 and ε4 alleles. A good example of this statistically significant variation in allele frequencies among Caucasian populations is the E4 frequency in the Finnish population. The relative frequency of E4 is 50% higher than that of other Caucasian populations (Enholm et al. 1986).
2.1.4 Cholesterol

Apo E is an essential component of cholesterol metabolism due to its critical role in mediating hepatic cholesterol uptake. Therefore any variations in the structure of apo E may result in perturbations of plasma cholesterol levels. This trend was first observed by Utermann et al. when examining cholesterol levels in E2/2 subjects (Utermann 1985, Utermann et al. 1975). Subjects in the studies carrying the E2 allele had lower levels of plasma total and LDL cholesterol than people with the E3 allele. The serum cholesterol levels of these individuals were approximately 40 mg/dl lower than E3/3 subjects (Utermann et al. 1979).

Utermann proposed a mechanism explaining the cholesterol lowering effect of the E2 allele from the findings that E2/2 individuals have a very low LDL cholesterol concentration (Utermann 1986). Because apo E2/2 is structurally unable to efficiently bind to apo E receptors an up-regulation of LDL receptors on the liver occurs, resulting in more efficient removal of LDL cholesterol. This process is proposed to result in reduced LDL levels in the circulation.

Conversely, Bouthillier et al. (1983) found that individuals possessing the E4 allele had significantly higher levels of cholesterol and LDL cholesterol compared with subjects with the E3 allele. A difference of 60 mg/dl mean total cholesterol was observed between
individuals of apo E2/2 and apo E4/4 phenotypes. Several studies show that the cholesterol lowering effect of apo E2/2 is approximately twice as strong as the raising effect of apo E4/4 (Utermann et al. 1979, Bouthillier et al. 1983).

2.1.5 Genetic Variability and Circulating Cholesterol Levels

Three separate study groups (Rall et al. 1982, Boyles et al. 1985, Schaefer et al. 1986) have determined that approximately 50% of the variability in normal serum cholesterol levels is due to genetic differences among individuals. Furthermore, it was estimated that as much as 16% of the genetic variance (8% of total variance) of LDL cholesterol was due to allelic differences at the apo E gene locus (Sing et al. 1985). Sing et al. found that in an Ottawa sample group as much as 7% of total phenotypic variance for serum cholesterol could be due to the apo E gene allele. These data emphasize the important role of apo E in determining plasma cholesterol levels.

2.2 Apolipoprotein E Identification

A rapid procedure for determining apo E genotype in large groups could prove very useful in both clinical and research settings. It is of central importance to have a rapid technique for genotyping because
apo E polymorphism plays a major role in lipoprotein metabolism and plasma cholesterol levels.

2.2.1 Apo E Phenotyping

Polyacrylamide Gel Electrophoresis

Isoelectric focusing on a single polyacrylamide cylindrical gel has been the method of choice in apo E phenotyping in the past (Warnick et al. 1979, Roe et al. 1991). The methodology was initially devised by Bouthillier et al. (1983) and Warnick et al. (1979). Analytically, the method requires ultracentrifugation of plasma to separate VLDL followed by incubation of VLDL apo E with optional use of neuroaminidase to cleave off sialic acid residues. The delipidation of VLDL is followed by a polyacrylamide electrophoresis (PAGE) step which requires 17 hours before focused protein bands can be stained and visualized (Warnick et al. 1979). The shortcomings of this procedure include: i) the large initial sample of blood (12 ml), ii) the high labour intensity, iii) the limited sample throughput, and iv) the extended sample throughput interval.
Immunoblotting

A more recent alternative approach to diagnosing apo E phenotypes is by use of a combination of isoelectric focusing of delipidated serum and immunoblotting (Menzel et al. 1986, Havekes et al. 1987, Hill et al. 1990). This approach does not require the long ultracentrifugation step but is still limited to a moderate number of samples per week and is labor intensive. Anti-apo E antibodies are initially used followed by gold labelled anti-IgG antibodies (Hill et al. 1990). For the immunostaining technique to work properly the antibodies must be very specific to detect a difference of only a single amino acid differentiating individual alleles.

2.2.2 Apo E Genotyping using the Polymerase Chain Reaction

Genotyping techniques identify single nucleotide variations that exist between the various apo E alleles. The polymerase chain reaction (PCR) technique is used to address sensitivity and specificity problems encountered in the characterization of nucleic acids. The PCR method allows specific amplification of DNA fragments which aids in detection of discrete nucleotide variations.

PCR Methodology

Genomic DNA can be extracted from buccal epithelial cells or leukocytes (Lench et al. 1988, Miller et al. 1988) and then amplified by
PCR. The DNA is extracted from white blood cells using a lysis buffer solution and purified for amplification using a protein and lipid extraction procedure (Wilson, K - personal communication). This amplification is capable of producing a selective enrichment of a specific DNA sequence by a factor of 1 million (Oste 1988, Mullis and Faloona 1987). The selective amplification procedure aids in the recognition and differentiation of the various apo E alleles. The PCR technique mimics a natural DNA replication process in that the number of DNA molecules increases exponentially with each cycle (Oste 1988).

The PCR method is based on the repetition of three steps. The double stranded DNA is first denatured by incubation at a high temperature (96°C). As a result, the two strands dissociate and remain free in solution until the temperature is lowered (Saiki et al. 1988a).

The next step is carried out at a lower temperature (62°C) which allows the extension oligonucleotide primers to anneal to DNA sites by flanking the region to be amplified. These primers hybridize to opposite strands of the target sequence and are oriented so DNA synthesis by the *Thermus aquaticus* (Taq) DNA polymerase enzyme proceeds across the region between the primers (Saiki et al. 1988b). The last step, the extension process, is carried out by Taq I polymerase at a slightly elevated temperature compared to that of annealing.
The three steps, denaturation, annealing, and extension, are referred to as a cycle. In the PCR technique, temperature cycling is performed in a temperature controlled heating block, and the repetition of this cycle (20-40 fold) results in exponential growth of the specific target fragment.

Allele Specific Oligonucleotide Probes

The amplification of the apo E allele can be followed by a hybridization process to identify the different apo E alleles. This occurs when 32-P labelled allele specific oligonucleotide probes hybridize with the apo E allele and form stable duplexes at the correct temperature and solution concentration (Funke et al. 1986, Smeets et al. 1988). These duplexes can be visualized on photographic plates using an autoradiography technique (Maxam and Gilbert 1980). Weisgraber et al. (1988) found the potential throughput of this entire process to be approximately 40-50 subjects over a 3 to 4 day interval. Using the PCR technique 67 subjects were identified in the study of Weisgraber et al. 1988.

Restriction Enzyme Isotyping with Hha I

Restriction isotyping of apo E is another method that uses oligonucleotides to amplify the apo E gene (Hixson and Vernier 1990, Kontula et al. 1990). The PCR products are digested with the restriction enzyme HhaI and then separated by electrophoresis on
polyacrylamide gels. The HhaI apo E genotypes can be directly visualized after gel staining. This technique requires fewer oligonucleotides than the previous mentioned probe technique and does not require the use of radioisotopes.

Allele Specific Primers

Most recently a technique using the PCR with allele specific oligonucleotide primers was developed to identify the six common apo E genotypes (Wenham et al. 1991, Syvanen et al. 1990). This technique uses four allele specific primers with each primer being specific for the single base nucleotide change which results in either Cys or Arg at positions 112 and 158. Details of this technique are described in the experimental design section of this thesis.

2.3 DIET, SERUM CHOLESTEROL LEVELS, and APO E PHENOTYPE

2.3.1 Serum Cholesterol Variability in Response to Dietary Challenges

It is well established that in either steady state conditions on a metabolic ward or in outpatients there is a large inter-patient variability in response to a dietary cholesterol challenge (McNamara et al. 1987). The type of dietary fat ingested is also a controlling factor in cholesterol metabolism (Grundy et al. 1986, 1988, Vega et al. 1982, Brussard et al. 1980, Bowman et al. 1988, Kohlmeier et al. 1988,
Reynier et al. 1988). McNamara et al. found that the major metabolic changes to dietary cholesterol alteration are suppression of endogenous synthesis and/or excretion of already absorbed dietary cholesterol. The patient to patient variability seen in the type and degree of response in cholesterol homeostasis could be in part due to the influence of apo E (Miettinen et al. 1988, Tikkanen et al. 1990, Mantarri et al. 1991).

In a controlled study of cholesterol homeostasis in man, McNamara and co-workers (1987) found no evidence to support the notion that reducing dietary cholesterol intake lowered plasma cholesterol levels. In that study, the dietary cholesterol intake was reduced from 450 mg/d to less than 300 mg/d. This led to the conclusion that most individuals (69%) were able to compensate by maintaining consistent plasma cholesterol levels in the face of a dietary cholesterol change. In a similar study Quintao and coworkers (1985) combined 31 studies to investigate the effect of diet cholesterol fluctuations on blood cholesterol levels in subjects of varying plasma cholesterol level (Beynen et al. 1987). Results indicated 1) cholesterol production is similar in normal and hypercholesterolemic individuals in the absence of dietary cholesterol; 2) the magnitude of the compensatory mechanisms was similar in normals and in hypercholesteroleemics.
2.3.2 Dietary Fat

Bowman and co-workers (1988) conducted a study on the effect of cholesterol and dietary fat on plasma lipid levels and lipoprotein fraction concentrations in normolipidemic men. This experiment varied the amount of fat and cholesterol ingested by four groups of male volunteers. The outcome of this study agrees with that of McNamara et al. (1987) in that the plasma lipids were not affected by dietary cholesterol, nor by any interaction of dietary fat with cholesterol. However, it was found that a diet low in fat reduced total cholesterol levels by 17±2 mg/dl (SD) and HDL cholesterol by 10±1 mg/dl (SD). An analysis of these results still indicates a large patient-to-patient variability.

2.3.3 Apo E and Diet

In an attempt to explain the inter-individual variability in serum cholesterol responses, Miettinen et al. (1988) considered apo E phenotype in a dietary challenge investigation. The hypothesis tested was to see if dietary cholesterol feeding might increase serum cholesterol more in E2 than in E4 allele possessing normolipidemic males. Normal, low fat, low cholesterol (150-200 mg/day) and low fat, high cholesterol (900 mg/day) diets were tested. Results showed a reduction in total plasma cholesterol level in both groups when changed.
from normal to low fat and cholesterol. However total cholesterol was significantly increased when cholesterol was added to diets of subjects possessing the €4 allele only. Recently, in similar studies, investigators have found that volunteers with the €4 allele exhibit a greater reduction in serum cholesterol levels following dietary counselling (Mantarri et al. 1991, Tikkanen et al. 1990, Weisgraber 1991). Further investigation in this area is required in order to understand apo E’s role in cholesterol homeostasis.

2.4 CHOLESTEROL SYNTHESIS MEASUREMENTS

A variety of procedures have been used to measure in vivo cholesterol synthesis in laboratory animals and humans. In humans non-invasive, non-hazardous methodologies are needed to measure the rate of whole body and organ cholesterol synthesis, thus the number of procedures is limited.

2.4.1 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Activity

Because the liver and small intestine are the primary sites of cholesterol formation, in vitro studies of these tissues have been popular in animal models. The measurement of 3-hydroxy-3-methylglutaryl
coenzyme A (HMG-CoA) reductase activity was used to determine rates of hepatic cholesterol synthesis \textit{in vitro} (Brown et al. 1979). HMG-CoA reductase is the rate limiting step in cholesterol formation, thus, by determining HMG-CoA reductase activity in the liver one can assess the potential formation rate of cholesterol.

2.4.2 Precursor Measurement

Parker and co-workers (1984) employed a method measuring mevalonic acid (MVA) concentrations in plasma. They compared plasma MVA concentrations to cholesterol synthesis rates measured by sterol balance. The sterol balance method relies on the assumption that a steady state of cholesterol metabolism exists and that losses of endogenous cholesterol occur only in the feces (Bowman et al. 1988, Quarfordt 1977). Parker found plasma MVA concentrations were directly related to the rate of whole body cholesterol synthesis over a wide range of cholesterol synthesis rates. It was concluded that levels of this cholesterol precursor (MVA) in plasma reflect HMG-CoA reductase activity during cholesterol synthesis. This conclusion may not be valid because there is a delay between the uptake of precursor and product formation.
2.4.3 Radiolabelled Precursors

Another way to measure cholesterol synthesis is by the use of radiolabelled precursors such as $^{14}$C-mevalonate or $^{14}$C-acetate (Ferezou et al. 1982). Ferezou employed these labelled precursors to measure the rate of formation of labelled cholesterol (Ferezou et al. 1982). This procedure was used to determine the fractional synthetic rate of cholesterol by measuring the rate of formation of labelled cholesterol (Andersen et al. 1979). A major drawback of this technique is that it requires a long term data collection period of up to 28 weeks. Another obvious hazardous feature is the use of radioactive materials in humans.

2.4.4 Radiolabelled Cholesterol

Whole body and tissue cholesterol turnover was studied in four baboons (Dell et al 1985). This procedure required the injection of $^{14}$C- and $^3$H-cholesterol. The study was carried out to determine the turnover rates of cholesterol in various tissues throughout the body. Labelled cholesterol was injected into subjects and the specific radioactivity of serum cholesterol was measured over several weeks. The $^{14}$-C cholesterol injection method has been used successfully in humans for monitoring long term changes in cholesterol synthesis rates (Goodman
et al. 1980, 1983). Goodman and coworkers studied cholesterol turnover and metabolism in 54 normal and hyperlipidemic subjects. This long term study using the three pool model provided information on the quantitative aspects of whole body cholesterol production and of the movement of cholesterol between the three mathematical compartments in the body. A set of highly predictive equations that describe some of the body cholesterol kinetic parameters in intact humans was developed in this study (Goodman et al. 1980). However, this type of procedure requires 30-40 weeks of consecutive blood sampling and cannot detect changes in rate of cholesterol synthesis over short periods.

2.4.5 Deuterium Incorporation Methodology

An accurate, safe, short term method for measurement of human cholesterol synthesis would eliminate problems encountered by other previous methods. The deuterium incorporation method, originally developed by Rittenberg and Schonheimer in 1937 and then refined by Jones et al. in 1988, appears appropriate for these types of measurements.

History

In 1937 deuterium was first used as an indicator in the study of fat and cholesterol formation (Rittenberg and Schonheimer 1937). In
1966, Taylor and co-workers measured human cholesterol synthesis using a deuterium label. Deuterium oxide (D$_2$O), a stable isotope of water which occurs naturally in the body, was administered orally. A cholesterol-free diet and a cholesterol rich diet were fed to 5 subjects. The objective of the study was to determine whether a change in cholesterol synthesis rates could be detected between diets. Mass spectrometric techniques were not very sensitive during this time and therefore the protocol required a large initial dose of D$_2$O (Taylor et al. 1966). Most subjects experienced severe vertigo following this first large priming dose. Results indicated no change in cholesterol synthesis rate while consuming different diet cholesterol levels.

Sensitivity

Mass spectrometer analytical sensitivity has increased and Schoeller et al. (1983) have developed a double-comparison method for mass spectrometric determination of hydrogen isotopic abundances. This allows deuterium enrichment analysis to be carried out with better precision. Jones and co-workers (1988) found labelling of the body water pool at 0.05 atom % excess was sufficient to determine cholesterol deuterium enrichment over a 12 hour period. This has important applications in being able to detect short-term fluctuations in rates of cholesterol synthesis.
Three-Pool Model

Accurate use of the deuterium incorporation methodology requires the body's cholesterol to be compartmentalized into three pools. The 3 pool model is a mathematical model which separates various tissue pools of the body containing cholesterol (Goodman et al. 1973). The 3 pools are distinguished by the rate at which their exchangeable body cholesterol equilibrates with plasma cholesterol.

The first compartment is the pool which equilibrates rapidly with plasma cholesterol. This pool includes plasma, red blood cells, liver, intestine, pancreas, spleen, kidney, and lungs. The second pool is an intermediate equilibrator and includes cholesterol in peripheral tissues and some viscera. Pool 3 has the slowest turnover rate and is made up of cholesterol located in muscle and adipose tissue (Goodman et al 1973).

Assumptions

Several assumptions must be made when using the 3-pool model and deuterium incorporation methodology. Given these assumptions this method can accurately measure perturbations in cholesterol synthesis rates over a short term.
Firstly, new cholesterol enters only pool 1 by absorption through diet or from endogenous synthesis. The second assumption is that pool 1 conducts the exchange of cholesterol between pools 2 and 3 (Goodman et al. 1973). The next is that interpool exchange is slow. If all of these assumptions are correct then the production rate of cholesterol in pool 1 is approximately equivalent to the total body turnover rate (Lieberman et al. 1982).

Calculation of plasma cholesterol fractional synthetic rate (FSR) using the results from the mass spectrometer require additional assumptions. The first, described by Dietschy and Spady (1984), states nearly all cell membranes are permeable to deuterated water and therefore intracellular enrichment is equal to that of plasma. Next, the same number of H atoms from water will be taken up in cholesterol synthesizing tissues, independent of metabolic state.

Dell et al. (1985) and have confirmed that the two previous assumptions are valid using tritiated water in animals. They found turnover rates of tissues to fall into three theoretical categories designated fast, intermediate, and slow rates of cholesterol turnover. The fast, intermediate, and slow rates represent pools 1, 2 and 3 respectively.

The rapid, accurate identification of apo E genotypes will allow a large number of individuals to be screened and increase our knowledge about apo E population distribution. Measurement of cholesterol FSR
under different dietary cholesterol levels will demonstrate if there is
an interaction between dietary cholesterol and apo E genotype on
cholesterol synthesis.

The objectives of this study were: i) to develop a rapid, simple
technique to identify apo E genotypes and ii) to investigate
cholesterol synthesis rate variations in apo E genotyped volunteers
possessing C2/- or C4/- alleles after dietary cholesterol intake
manipulations.
3.0 EXPERIMENTAL DESIGN

3.1 GENOTYPE SCREENING

Phase 1 of the thesis included the development of the PCR genotyping technique and the subsequent screening of individuals apo E genotypes. Past studies have identified the phenotypes of 131 subjects using the IEF or immunoblot techniques.

3.1.1 Subjects

Individuals genotyped using allele-specific primers were previously phenotyped by IEF or immunoblot analysis. This group included 4 E3/2, 7 E3/3, 2 E3/4, and 1 E4/4. After verification of the methodology, 30 additional subjects were genotyped indicating 22 E3/3, 6 E3/4, and 2 E3/2 genotypes.

3.1.2 Oligonucleotides

Allele-specific oligonucleotide primers were synthesized on an Applied Biosystems 391A - PCR Mate synthesizer. Primers were designed with the nucleotide change at the 3' end and a deliberate mismatch 3 nucleotides in from the 3' end. The primer sequences and their location within the apo E gene are presented in Figure 2.
**Figure 2**: Allele-specific oligonucleotide primers with 3' base pair mismatch.
3.1.3 Genomic DNA preparation

DNA was isolated from leukocytes or buccal epithelial cells. Also, sufficient DNA for ten PCR reactions was obtained from a pin prick method for obtaining blood. The cells were first lysed with 0.5 ml of 0.23 M sucrose, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, and then digested for a minimum of two hours in 10 mM Tris, pH 8.0, 2 mM EDTA, pH 8.0, 10 mM NaCl, 1% SDS, 0.4 mg/ml proteinase K, and 8 mg/ml dithiothreitol (DTT). The digest was then extracted with phenol-chloroform followed by a 95% ethanol DNA precipitation. The DNA was air dried, then resuspended in 10 ul of water.

3.1.4 Amplification of genomic DNA

Alleles were determined using the primers described in Figure 2. PCR amplification was performed in an automated thermocycler (Perkin-Elmer Cetus) as follows. Reactions were carried out in a total volume of 50 ul containing 1 ug DNA, deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), each at 2mM. The dNTP stock solution was prepared by adjusting the magnesium chloride concentration to 20 mM in 5 mM dNTP. The 10 X buffer consisted of 670 mM Tris-HCl, 15 mM magnesium sulphate, 166 mM ammonium sulphate, and 100 mM B-mercaptoethanol. Also added were 10 % dimethyl sulphoxide (DMSO) and 20
pmol of each primer. In addition to apo E primers, all tubes contained prothrombin primers (see Table 2) used as internal controls. Samples were overlaid with mineral oil (Sigma) and the DNA was denatured for ten minutes at 96°C. Four units of *Thermus aquaticus* (Taq) polymerase was added to each sample prior to the PCR. Samples underwent 25 cycles with each cycle consisting of a 10 second denaturation step at 96°C, a 30 second annealing step at 58°C, and a 60 second extension step at 65°C.

Table 2 Oligonucleotide primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>TACTCGACCAGGCCGCCGCC</td>
</tr>
<tr>
<td>B</td>
<td>TACTCGACCAGGCCGCCGCA</td>
</tr>
<tr>
<td>C</td>
<td>ACTGCACCAGGCCGCCGCC</td>
</tr>
<tr>
<td>D</td>
<td>TACTGCACCAGGCCGCCGTC</td>
</tr>
<tr>
<td>E</td>
<td>TACTGCACCAGGCCGCCCTCA</td>
</tr>
<tr>
<td>F</td>
<td>GCCTGGTACACTGCCAGTCG</td>
</tr>
<tr>
<td>G</td>
<td>GCCTGGTACACTGCCAGTCA</td>
</tr>
<tr>
<td>H</td>
<td>AAGGAGTTGAAGGCCCTACAAAT</td>
</tr>
<tr>
<td>I</td>
<td>ACAGAATTCTGGGGATGAGCTATGTC</td>
</tr>
<tr>
<td>J</td>
<td>ACACTGGAGATAATTCTTTTCACGCGCTT</td>
</tr>
<tr>
<td>K</td>
<td>CGGGGCCCCGGCTGGTACACTGCCAGTCA</td>
</tr>
</tbody>
</table>

Primers A and B contain the differentiating nucleotides at the 3' end while primer C has the mismatch one nucleotide in from the 3' end. Primers D and G are designed similarly to A and B but have a second deliberate mismatch (G-T) three nucleotides in from the 3' end. Primer H is the common primer. Primers I and J are the human prothrombin internal controls. Oligonucleotide K is a 29-nucleotide primer similar to primer F.
3.2 Diet, Genotype and Cholesterol Synthesis

3.2.1 Subjects

Thirteen healthy male volunteers were selected after apo E genotype screening. Of the thirteen volunteers, seven possessed the E2 allele and six possessed the E4 allele with each group having one homozygote. Some of the requirements the individuals had to meet to be included in the study were: a) total plasma cholesterol levels between 110-210 mg/dl, b) no history of lipid disorders, c) non-obese, d) free of restrictive food allergies or other diet limitations. Physical characteristics and fasting cholesterol levels are in Table 3. Subjects gave informed consent prior to the investigation and the protocol was approved by the Ethical Review Committee of the University of British Columbia.

3.2.2 Dietary Protocol

Subjects were randomly distributed into groups of two to four and underwent two separate studies of three weeks duration. The two studies were separated by a five week interval during which time the volunteers were permitted to consume their normal diet.

One study consisted of the volunteers consuming a low fat (30%), low cholesterol (250 mg) diet and the other period required the subjects to consume a low fat (30%), high cholesterol diet (800 mg). During the three weeks the volunteers consumed their prescribed diet at home while
<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Weight (lbs)</th>
<th>Height (in)</th>
<th>%Fat</th>
<th>TChol (mmol)</th>
<th>LDL-C</th>
<th>HDL-C</th>
<th>TGL (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>44</td>
<td>135.2</td>
<td>68</td>
<td>10</td>
<td>4.87</td>
<td>2.79</td>
<td>1.65</td>
<td>0.94</td>
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<td>70</td>
<td>20</td>
<td>3.96</td>
<td>2.26</td>
<td>1.20</td>
<td>1.11</td>
</tr>
<tr>
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<td>68</td>
<td>7</td>
<td>3.63</td>
<td>1.85</td>
<td>1.42</td>
<td>0.79</td>
</tr>
<tr>
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<td>23</td>
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<td>71.5</td>
<td>17</td>
<td>3.42</td>
<td>2.32</td>
<td>0.84</td>
<td>0.57</td>
</tr>
<tr>
<td>WH</td>
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<td>74</td>
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<td>0.83</td>
</tr>
<tr>
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<td>69.7</td>
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<td>3.78</td>
<td>2.01</td>
<td>1.60</td>
<td>0.63</td>
</tr>
<tr>
<td>PG</td>
<td>29</td>
<td>166.5</td>
<td>69.5</td>
<td>22</td>
<td>3.48</td>
<td>2.17</td>
<td>0.93</td>
<td>0.84</td>
</tr>
<tr>
<td>Mean =</td>
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<td>161.8</td>
<td>70.1</td>
<td>16</td>
<td>4.01</td>
<td>2.42</td>
<td>1.22</td>
<td>0.86</td>
</tr>
<tr>
<td>± STD</td>
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<td>21.0</td>
<td>1.9</td>
<td>6</td>
<td>0.48</td>
<td>0.39</td>
<td>0.35</td>
<td>0.20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Weight (lbs)</th>
<th>Height (in)</th>
<th>%Fat</th>
<th>TChol (mmol)</th>
<th>LDL-C</th>
<th>HDL-C</th>
<th>TGL (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP</td>
<td>44</td>
<td>187.0</td>
<td>70.5</td>
<td>17</td>
<td>4.53</td>
<td>2.63</td>
<td>1.17</td>
<td>1.58</td>
</tr>
<tr>
<td>GC</td>
<td>38</td>
<td>173.1</td>
<td>69</td>
<td>20</td>
<td>5.52</td>
<td>3.24</td>
<td>1.13</td>
<td>2.53</td>
</tr>
<tr>
<td>AP</td>
<td>22</td>
<td>149.5</td>
<td>70</td>
<td>12</td>
<td>5.06</td>
<td>3.18</td>
<td>1.37</td>
<td>1.12</td>
</tr>
<tr>
<td>JQ</td>
<td>28</td>
<td>117.0</td>
<td>66.1</td>
<td>7</td>
<td>4.30</td>
<td>2.29</td>
<td>1.71</td>
<td>0.67</td>
</tr>
<tr>
<td>BM</td>
<td>23</td>
<td>164.0</td>
<td>70.5</td>
<td>18</td>
<td>4.38</td>
<td>0.95</td>
<td>1.31</td>
<td>0.95</td>
</tr>
<tr>
<td>SS</td>
<td>35</td>
<td>165.0</td>
<td>71</td>
<td>14</td>
<td>5.46</td>
<td>4.24</td>
<td>0.96</td>
<td>0.58</td>
</tr>
<tr>
<td>Mean =</td>
<td>32</td>
<td>159.3</td>
<td>69.5</td>
<td>15</td>
<td>5.03</td>
<td>3.21</td>
<td>1.26</td>
<td>1.15</td>
</tr>
<tr>
<td>± STD</td>
<td>8</td>
<td>22.0</td>
<td>1.6</td>
<td>4</td>
<td>0.52</td>
<td>0.52</td>
<td>0.24</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Volunteers were assessed before commencing each dietary protocol for above variable parameters.
consuming cholesterol or non-cholesterol capsules and kept three days of dietary records. The volunteers underwent dietary counselling on how to achieve a low cholesterol, low fat diet and were given literature and recipes before each of the dietary periods. A sample of the diet record instructions is available in Appendix two. Subjects were given seven days worth of capsules each week and were asked to take three capsules with breakfast and another three with supper over the entire three week study. In one day the volunteers would consume six capsules: two containing polyethylene glycol (a tracer to monitor volunteer compliance), and four containing either 550 mg of cholesterol or zero cholesterol (dried egg white). This was performed in a blind fashion so that the volunteers would not alter their eating habits at home. Body weights were monitored throughout each period and body fat and total body water was determined using bioelectrical impedance analysis (BIA) (BIA-101, RJL Systems, Inc.). Sample diets and dietary record analysis results are in Tables 4 and 5.

On day nineteen of each diet period, the volunteers reported to the Division of Human Nutrition metabolic kitchen for their meals (30 % fat, 55 % carbohydrate, 15 % protein, 160 mg cholesterol) which were designed by a dietician. For the remainder of the study, the volunteers were asked to refrain from snacking between meals and only drink the water supplied by the investigators. On day nineteen of each period the
Table 4 Meals prepared for volunteers during cholesterol synthesis measurement period

<table>
<thead>
<tr>
<th>Breakfast</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>bread (brown)</td>
<td>42.0 g</td>
</tr>
<tr>
<td>cereal (bran flakes)</td>
<td>66.0</td>
</tr>
<tr>
<td>2% milk</td>
<td>244.0 ml</td>
</tr>
<tr>
<td>egg white</td>
<td>110.0 g</td>
</tr>
<tr>
<td>margarine</td>
<td>25.0</td>
</tr>
<tr>
<td>orange juice (concentrate)</td>
<td>300.0 ml</td>
</tr>
<tr>
<td>jam</td>
<td>20.0 g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lunch</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>bread</td>
<td>75.0 g</td>
</tr>
<tr>
<td>chicken</td>
<td>75.0</td>
</tr>
<tr>
<td>lettuce</td>
<td>40.0</td>
</tr>
<tr>
<td>margarine (Fleischmans)</td>
<td>10.0</td>
</tr>
<tr>
<td>mayonnaise (light)</td>
<td>20.0</td>
</tr>
<tr>
<td>orange</td>
<td>140.0</td>
</tr>
<tr>
<td>apple juice (vit. C added)</td>
<td>300.0 ml</td>
</tr>
<tr>
<td>yogurt (low fat)</td>
<td>200.0 g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Supper</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>chicken</td>
<td>75.0 g</td>
</tr>
<tr>
<td>spaghetti</td>
<td>250.0</td>
</tr>
<tr>
<td>tomato sauce (with mushrooms)</td>
<td>140.0</td>
</tr>
<tr>
<td>margarine</td>
<td>20.0</td>
</tr>
<tr>
<td>broccoli (crowns and stems)</td>
<td>75.0</td>
</tr>
<tr>
<td>apple juice</td>
<td>300.0 ml</td>
</tr>
<tr>
<td>ice milk</td>
<td>100.0 g</td>
</tr>
<tr>
<td>chocolate syrup (hersheys)</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Daily caloric intake was 2997 kcal with each meal being isocaloric. Lunches were packed as take out but all other meals were eaten in the Division of Human Nutrition metabolic kitchen.
Table 5 Dietary record data over two separate three day periods as reported by volunteers

<table>
<thead>
<tr>
<th>Subject</th>
<th>Protein (% kcal)</th>
<th>Fat (% kcal)</th>
<th>CHO (% kcal)</th>
<th>Cholesterol (mg)</th>
<th>Total (kcal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS</td>
<td>17.0</td>
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<td>58.0</td>
<td>206</td>
<td>3025</td>
</tr>
<tr>
<td>MT</td>
<td>12.0</td>
<td>31.2</td>
<td>58.5</td>
<td>212</td>
<td>3056</td>
</tr>
<tr>
<td>LVP</td>
<td>12.8</td>
<td>21.1</td>
<td>67.3</td>
<td>250</td>
<td>5168</td>
</tr>
<tr>
<td>AH</td>
<td>11.3</td>
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<td>184</td>
<td>3730</td>
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<td>WH</td>
<td>13.2</td>
<td>32.0</td>
<td>56.4</td>
<td>140</td>
<td>2035</td>
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<td>CD</td>
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<td>2452</td>
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<td>PG</td>
<td>9.5</td>
<td>31.0</td>
<td>59.8</td>
<td>167</td>
<td>2525</td>
</tr>
<tr>
<td>Mean =</td>
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<td>28.0</td>
<td>60.4</td>
<td>211</td>
<td>3141</td>
</tr>
<tr>
<td>± STD</td>
<td>2.4</td>
<td>4.9</td>
<td>5.4</td>
<td>55</td>
<td>968</td>
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<tr>
<td>E4/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP</td>
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<td>207</td>
<td>3605</td>
</tr>
<tr>
<td>GC</td>
<td>13.7</td>
<td>29.0</td>
<td>56.7</td>
<td>279</td>
<td>3428</td>
</tr>
<tr>
<td>AP</td>
<td>17.7</td>
<td>34.5</td>
<td>46.7</td>
<td>312</td>
<td>2553</td>
</tr>
<tr>
<td>JQ</td>
<td>19.8</td>
<td>23.8</td>
<td>57.8</td>
<td>273</td>
<td>1745</td>
</tr>
<tr>
<td>BM</td>
<td>19.1</td>
<td>33.0</td>
<td>47.3</td>
<td>341</td>
<td>2363</td>
</tr>
<tr>
<td>SS</td>
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<td>28.3</td>
<td>56.2</td>
<td>261</td>
<td>2840</td>
</tr>
<tr>
<td>Mean</td>
<td>16.1</td>
<td>28.0</td>
<td>55.5</td>
<td>279*</td>
<td>2755</td>
</tr>
<tr>
<td>± STD</td>
<td>2.8</td>
<td>5.1</td>
<td>7.2</td>
<td>42</td>
<td>632</td>
</tr>
</tbody>
</table>

* Refers to significantly higher intake of cholesterol in the E4/- group (p=0.045). Computerized nutritional analysis were carried out using Nutricom V 2.0 program (1986, Smart Engineering Ltd., Vancouver, B.C.).
subjects received a priming dose of deuterated water, administered orally, at a level of 0.7 g D2O/kg body water (99.8 atom percent excess, ICN Biomedicals). Total body water was determined using bioelectrical impedance analysis (Kushner et al. 1990, Deurenberg et al. 1991). Throughout days nineteen to 21 the deuterated body water enrichment levels were maintained at a plateau by administering D2O at 1.4 g D2O/kg water on day nineteen and 0.7 g D2O/kg water on day twenty. On day nineteen a higher enrichment of D2O was administered to compensate for the expected amount of water consumed in the diet, therefore maintaining the body water enrichment at a plateau.

Blood was drawn from the subjects seven times over the course of each test diet. A base sample was drawn before the start of each protocol to determine the lipid profile of the volunteers before any dietary manipulations. The time interval between samplings was twelve hours, beginning with 0 hour at 7:30am on day nineteen and ending with 48 hours on day 21. Approximately 20 ml's of blood was drawn from each subject on each occasion by a trained phlebotomist.

The blood sample obtained at t=0 hour was analyzed for the overall lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides) and cholesterol synthesis rates from plasma water and cholesterol deuterium enrichments. Lipid profiles were determined at the Shaughnessy Research Centre, Vancouver, B.C. Triplicate samples
were analyzed for deuterium enrichment using a differential isotope ratio mass spectrometer (VG Isomass, 903D, Cheshire England).

3.2.3 Analytical Methods

Cholesterol for the capsules was obtained by lyophilyzing egg yolk and then mixing the dried egg yolk with free cholesterol (Sigma). Dried egg white (Albumen) was purchased from Alive, Vancouver, B.C. Finlandia Pharmaceuticals (Vancouver, B.C.) allowed the use of their laboratory to prepare cholesterol and placebo capsules.

Sample blood was drawn into seven ml tubes (containing no anticoagulating factors) which were allowed to clot on wet ice for a minimum of 25 minutes. The samples were then centrifuged at 1500 x g to separate out the serum. Serum was extracted and divided to allow for triplicate measurements of free cholesterol deuterium enrichment.

Lipid extraction from the serum was accomplished by adding 6 ml of methanol to 4 ml of serum and heating at 55°C. Hexane-chloroform (4:1, vol/vol) was added and shaken for 15 minutes followed by centrifugation at 1500 x g for 15 minutes. After centrifugation the upper phase was removed and the total procedure was repeated.

The upper phases of each extraction were then combined and dried under N2 gas at 55°C. The residue was then dissolved in several drops of chloroform and placed on thin layer chromatography (TLC) plates. The chromatography procedure separated free cholesterol, triglycerides, and cholesterol esters into distinct bands. The electrophoresis solvent
used was petroleum ether:ethyl ether:acetic acid (135:15:1.5 vol/vol/vol) and the plates were developed for approximately one hour. The plates were then air-dried and developed in iodine vapour to visualize the separated lipid bands. The free cholesterol fraction was scraped from the plates, eluted with chloroform-methanol (2:1, vol:vol), shaken for fifteen minutes and centrifuged at 1500 x g for fifteen minutes to remove the silica. The upper phase was removed, dried under N₂ gas, and then transferred into pre-annealed 20 cm Pyrex (Corning Glass Works, Corning, N.Y.) combustion tubes using chloroform as a solvent. The combustion tubes contained 500 mg cupric oxide wire (BDH Chemicals, Poole, England) and a two cm length of silver wire. The samples were evacuated to remove the chloroform, then sealed, and combusted at 550°C for four hours. The samples were air-cooled and then the combustion water was distilled into similar Pyrex tubes containing 60 mg zinc reagent (Biogeochemical Laboratories, Indiana University, Bloomington IN). Triplicate samples were flame sealed under vacuum and then heated at 520°C for 30 minutes to reduce the samples to hydrogen gas.

To determine plasma deuterium enrichments, plasma samples from 24 and 48 hour time points of each diet period were diluted sixfold with Vancouver tap water of known isotopic enrichment. This dilution was carried out to bring the isotopic enrichment of the samples into the range of the mass spectrometers working standards. It was not necessary to dilute baseline (t=0 hour) plasma samples. Tubes were flame sealed
under vacuum and heated at 520°C for 30 minutes to reduce the samples to hydrogen gas.

Free cholesterol and plasma water hydrogen samples were analyzed by differential isotope ratio mass spectrometry. The mass spectrometer was calibrated for each subject using water standards and the $H_3^+$ contribution was checked daily.

### 3.2.4 Data Analysis

The results from the isotope ratio mass spectrometer were expressed as parts per mil or

$\left(\frac{R_{\text{sample}}}{R_{\text{smow}}} - 1\right) \times 1000$

where $R$ is the ratio of heavy (2H) to light (1H) isotopic species (Jones et al. 1988). The reference standard was Standard Mean Ocean Water. Fractional synthetic rate (FSR) of cholesterol was determined as the fractional incorporation of precursor deuterium over time (Jones et al. 1988). The equation used to calculate the FSR was

$$FSR = \frac{D_{\text{cholesterol}}}{D_{\text{plasma water}}} \times 0.81 \times \frac{2H/C}{27} \times \frac{C/46}{H}$$

where $D_{\text{cholesterol}}$ and $D_{\text{plasma water}}$ express deuterium enrichment differences. The FSR of cholesterol was calculated over two twelve hour periods during each dietary period. The $2H/C$ ratio of 0.81 was derived from the assumption that deuterium incorporated into cholesterol come from water (seven $H$'s) and NADPH (fifteen $H$'s) and not from acetyl Co-A (Dietschy et al. 1984). One cholesterol molecule
contains 46 H atoms and 27 C atoms. The ratio (0.81) exists because theoretically NADPH and water contribute 22 hydrogens per cholesterol molecule (22D/27C = 0.81) (Dietschy et al. 1984).

3.2.5 Faecal Analysis

Subjects collected faecal samples on the days food records were kept during the third week of each dietary protocol. Polyethylene glycol (PEG) was administered twice daily over the three week dietary period to indicate compliance of the volunteers ingesting capsules. Polyethylene glycol is a quantitative faecal marker that has been used in the past to assess transit time through the gut of humans (Malawer et al. 1967). Subjects consumed 1.0 g of PEG daily and the faeces were subsequently analyzed using a variation of the turbidimetric method of Allen and coworkers (1979). In brief, containers with faecal samples were diluted with water to an equal, recorded, final weight. The assay required a one ml water blank, one ml PEG standard (0.5 mg - 10.0 mg), one ml faecal homogenate and to these samples two ml Albumin (150 mg/l), two ml 15% BaCl₂ (17.6 g/100 ml), six ml 0.3 N Ba(OH)₂(H₂O)₈, one ml 6.6% lead acetate, one ml ZnSO₄ (30 g/100 ml), one ml HgCl₂ (5.0 g/100 ml) was added. After each addition the solution was vortexed and then allowed to sit for ten minutes, filtered through Whatman #1 filter paper and at this point the filtrate was clear. To one ml of filtrate three ml gum arabic (12 mg/l) and four ml of TCA-BaCl₂ (30.0 g TCA, 0.5 g BaCl₂ in 100ml) were added and vortexed, then allowed to sit for 60
minutes. The spectrometer was set at a wavelength of 650 nm and samples optical density was determined in duplicate.

3.2.6 Statistical Analysis

The paired and unpaired student t-test was used to analyze the lipid profile data. A multi-sample ANOVA was used to test the FSR data for differences between genetic, diet, and feeding groups.
4.0 RESULTS

4.1 GENOTYPE SCREENING

4.1.1 PRIMER DESIGN

Initial oligonucleotide primers (Table 2, primers A-C) were designed with a single base pair mismatch at the 3' end of the sequence. Results indicated nonspecific amplification unless very stringent annealing temperatures were implemented. At this point various other buffers were tried and the dNTP concentration decreased, yielding inconsistent products. The shifting of the mismatch one nucleotide in from the 3' end yielded the same nonspecific results. These results indicated that a single mismatch was not sufficient to prevent amplification by the PCR. The introduction of an additional mismatch increases the specificity of the primers allowing for specific allele amplification.

4.1.2 Genotypic identification

Each PCR reaction contained the common primer (primer H) and one of the allele-specific primers described in Figure 2. Therefore, four reaction mixtures are required per subject for genotype diagnosis. Appropriate annealing conditions were determined using an empirical, temperature curve approach. The specific primers were 19 nucleotides in length and the common primer was 22 nucleotides. An attempt was made to combine two different allele-specific primers in one reaction mixture to
allow for genotype diagnosis in two reaction mixtures instead of four. The products could be differentiated easily because each set of primers produces a different sized product. Uneven yields of the two product concentrations were observed and may have been due to each primer competing for the same common primer; thus the smaller product always out-competed the larger product. Figure 3 depicts subjects possessing the apo E3/3 and E3/2 genotypes. Lane one is negative (primer D), lane two is positive (primer F), lane three is positive (primer E), and lane four is negative (primer G) indicating the homozygous E 3/3 genotype. Lanes five through eight are the reactions for subject two. Lane five (primer D) is negative indicating the absence of the \( \epsilon_4 \) allele; lanes six through eight are positive with primers E, F, and G, indicating the presence of E3 and E2 yielding a genotype of apo E3/2.

Initial experiments indicated that the internal control (Exon 13 of the human prothrombin gene) primers would not amplify under the stringent annealing conditions described. The annealing time was increased to three minutes and the number of cycles increased from 25 to 35. This gave positive results but increased the occurrence of false products due to nonspecific annealing. To eliminate false annealing, the reactions were seeded with a small aliquot, approximately five to ten ng, of cloned human prothrombin and conditions were returned to 25 cycles with a 30 second annealing time. Results indicated specific amplification of both the human prothrombin segment and the polymorphic sites of apo E.
Figure 3 Ethidium bromide stained polyacrylamide gel photograph depicting apo E genotypes
The fourteen subjects used to verify this procedure were previously phenotyped by using either the isoelectric focusing method or immunoblot technique. Genotypes identified were E4/4[1], E3/4[2], E3/3[7], E3/2[4]. The E3/3 genotype yielded a 145 base pair product with primer E, indicating the presence of Cys 112, and a 277 base pair product with primer F indicating the presence of Arg 158. The heterozygote E3/4 produced appropriately sized products with primers E and F identifying the E3 allele, but also produced a 145 base pair product with primer D indicating Arg 112. Homozygous E4/4 subjects reacted only with primers D and F marking the presence of Arg 112 and Arg 158, while E2/2 homozygotes react only with primers E and G indicating Cys 112 and Cys 158. In all reactions a 500 base pair fragment was observed indicating a successful amplification of the internal control.

4.2 DIET, GENOTYPE AND CHOLESTEROL SYNTHESIS

4.2.1 Individual physical characteristics

Subjects were initially screened for genotype and plasma lipid characteristics before commencing the dietary phase of the study. Physical characteristics and initial cholesterol and triglyceride profiles of each volunteer are located in Table 3. Volunteers ages ranged from 22 to 44 years with an average age of 34 ± 6 years for the apo E2/- group and 32 ± 8 years for the E4/- group. Weight, height, and percent body fat were determined for subjects of each genotype group.
indicating very similar results for each group with an average of 16 ± 6 percent body fat in apo E2 individuals and 15 ± 4 % in E4 possessing individuals.

Analysis of screening serum cholesterol and triglyceride levels indicated differences between the apo E4/- and E2/- groups with the E4 group having significantly higher total serum cholesterol levels than the E2 group (p ≤ 0.05). The triglyceride, LDL, and HDL values were not significantly different between the apo E2/- and E4/- group.

4.2.2 Dietary intake of volunteers while consuming experimental capsules

Three day food records were analyzed for subject compliance to the prescribed low fat, low cholesterol diet. Results of six days (three days from each diet period) of dietary records are present in Table 5. Each group consumed approximately 28 % of their total calories from fat. The apo E2/- group consumed 12.9 % and 60.4 % of protein and carbohydrate, respectively. The apo E4/- group ate 16.1 % and 55.5 % of protein and carbohydrate, respectively. Daily cholesterol intake was significantly lower in the E2/- group at 211 ± 55 mg/day than the E4/- group which consumed 279 ± 42 mg/day (p ≤ 0.05). Total caloric intake was approximately 3141 kcal in the E2 group and 2755 kcal in the E4 group. The range of reported total caloric intake was from 1745 kcal to 5168 kcal.
4.2.3 Volunteer compliance and PEG analysis

Results from the PEG analysis are presented in Table 6. The theoretical value expected was 3.0 g over a three day period. On average volunteers excreted $2.75 \pm 0.74$g of PEG over three days in the E2/- group and $2.67 \pm 0.34$g in the E4/- group indicating good overall compliance to the protocol. Low PEG excretion was usually the result of losses at collection time which were reported to the investigator and recorded.

4.2.4 Serum cholesterol response to dietary cholesterol fluctuations

The randomly assigned dietary groups consisted of five apo E2/- and three apo E4/- individuals consuming cholesterol in the first dietary phase, while the remaining subjects consumed the placebo capsules first. The time sequence, total cholesterol, and genotype are listed in Table 7. The baseline values represent fasting cholesterol levels of the subjects after a minimum of five weeks of unaltered, habitual diet consumption.

Serum cholesterol levels were determined before and 19 days after cholesterol or placebo treatment. Values for the cholesterol treatment period are in Table 8 and the placebo treatment in Table 9. After 19 days of consuming a high cholesterol diet, neither of the apo E groups showed a significant fluctuation in cholesterol or triglyceride levels.
Table 6 Subject compliance measured by PEG analysis of faecal samples

<table>
<thead>
<tr>
<th>Subject</th>
<th>PEG (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/-</td>
<td></td>
</tr>
<tr>
<td>BS</td>
<td>3.20</td>
</tr>
<tr>
<td>MT</td>
<td>3.55</td>
</tr>
<tr>
<td>LVP</td>
<td>3.35</td>
</tr>
<tr>
<td>AH</td>
<td>1.80</td>
</tr>
<tr>
<td>WH</td>
<td>2.45</td>
</tr>
<tr>
<td>CD</td>
<td>1.60</td>
</tr>
<tr>
<td>PG</td>
<td>3.30</td>
</tr>
<tr>
<td>Mean =</td>
<td>2.75</td>
</tr>
<tr>
<td>± STD</td>
<td>0.74</td>
</tr>
<tr>
<td>E4/-</td>
<td></td>
</tr>
<tr>
<td>MP</td>
<td>2.80</td>
</tr>
<tr>
<td>GC</td>
<td>2.55</td>
</tr>
<tr>
<td>AP</td>
<td>3.30</td>
</tr>
<tr>
<td>JQ</td>
<td>2.73</td>
</tr>
<tr>
<td>BM</td>
<td>2.55</td>
</tr>
<tr>
<td>SS</td>
<td>2.10</td>
</tr>
<tr>
<td>Mean =</td>
<td>2.67</td>
</tr>
<tr>
<td>± STD</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Values represent grams of PEG in faeces collected over a 3 day period. Volunteers were requested to consume 3.0 g of PEG capsules over the 3 day collection period.
Table 7 Apolipoprotein E genotype and serum cholesterol response with respect to time sequence of cholesterol-placebo treatment

<table>
<thead>
<tr>
<th>Subject</th>
<th>Genotype</th>
<th>Base 1</th>
<th>Day 19</th>
<th>Base 2</th>
<th>Day 19</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TChol (mmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS</td>
<td>E3/2</td>
<td>4.87</td>
<td>4.30(^{H})</td>
<td>4.29</td>
<td>3.78</td>
</tr>
<tr>
<td>MT</td>
<td>E3/2</td>
<td>3.96</td>
<td>3.96(^{H})</td>
<td>3.71</td>
<td>3.43</td>
</tr>
<tr>
<td>LVP</td>
<td>E3/2</td>
<td>3.63</td>
<td>3.35(^{H})</td>
<td>3.49</td>
<td>3.46</td>
</tr>
<tr>
<td>AH</td>
<td>E3/2</td>
<td>3.42</td>
<td>4.60(^{H})</td>
<td>3.72</td>
<td>3.53</td>
</tr>
<tr>
<td>WH</td>
<td>E3/2</td>
<td>4.57</td>
<td>4.47(^{H})</td>
<td>5.96</td>
<td>4.78</td>
</tr>
<tr>
<td>CD</td>
<td>E2/2</td>
<td>3.78</td>
<td>3.85</td>
<td>3.60</td>
<td>3.37(^{H})</td>
</tr>
<tr>
<td>PG</td>
<td>E3/2</td>
<td>3.48</td>
<td>4.15</td>
<td>3.86</td>
<td>3.41(^{H})</td>
</tr>
<tr>
<td>E4/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP</td>
<td>E3/4</td>
<td>4.53</td>
<td>4.48(^{H})</td>
<td>4.77</td>
<td>4.84(^{H})</td>
</tr>
<tr>
<td>GC</td>
<td>E3/4</td>
<td>5.52</td>
<td>5.06(^{H})</td>
<td>6.68</td>
<td>5.57</td>
</tr>
<tr>
<td>AP</td>
<td>E3/4</td>
<td>5.06</td>
<td>5.41(^{H})</td>
<td>5.68</td>
<td>5.95</td>
</tr>
<tr>
<td>JQ</td>
<td>E3/4</td>
<td>4.30</td>
<td>5.08(^{H})</td>
<td>5.36</td>
<td>5.06</td>
</tr>
<tr>
<td>BM</td>
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<td>4.58</td>
<td>4.72</td>
<td>4.47(^{H})</td>
</tr>
<tr>
<td>SS</td>
<td>E3/4</td>
<td>5.46</td>
<td>5.77</td>
<td>5.86</td>
<td>5.64(^{H})</td>
</tr>
</tbody>
</table>

Day 19 values with \(^{H}\) denotes the values obtained during the cholesterol supplemented dietary period (550 mg/day). Day 19 values without \(^{H}\) denotes the placebo dietary phase.
Table 8 Serum total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, and triglyceride levels before and after cholesterol treatment

<table>
<thead>
<tr>
<th>Base</th>
<th>TChol</th>
<th>TGL</th>
<th>HDL</th>
<th>LDL</th>
<th>19 days of high cholesterol</th>
<th>TChol</th>
<th>TGL</th>
<th>HDL</th>
<th>LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS</td>
<td>4.87</td>
<td>0.94</td>
<td>1.65</td>
<td>2.79</td>
<td>4.30</td>
<td>0.80</td>
<td>1.53</td>
<td>2.41</td>
<td></td>
</tr>
<tr>
<td>MT</td>
<td>3.96</td>
<td>1.11</td>
<td>1.20</td>
<td>2.26</td>
<td>3.96</td>
<td>0.80</td>
<td>1.28</td>
<td>2.32</td>
<td></td>
</tr>
<tr>
<td>LVP</td>
<td>3.63</td>
<td>0.79</td>
<td>1.42</td>
<td>1.85</td>
<td>3.35</td>
<td>0.85</td>
<td>1.38</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>AH</td>
<td>3.42</td>
<td>0.57</td>
<td>0.84</td>
<td>2.32</td>
<td>4.60</td>
<td>0.74</td>
<td>1.05</td>
<td>3.21</td>
<td></td>
</tr>
<tr>
<td>WH</td>
<td>4.57</td>
<td>0.83</td>
<td>1.22</td>
<td>2.97</td>
<td>4.47</td>
<td>0.99</td>
<td>1.04</td>
<td>2.98</td>
<td></td>
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<tr>
<td>CD</td>
<td>3.78</td>
<td>0.63</td>
<td>1.60</td>
<td>2.01</td>
<td>3.85</td>
<td>0.70</td>
<td>1.41</td>
<td>2.12</td>
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<tr>
<td>PG</td>
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<td>1.12</td>
<td>0.62</td>
<td>2.73</td>
<td>3.41</td>
<td>1.14</td>
<td>0.50</td>
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<tr>
<td>Mean</td>
<td>4.01</td>
<td>0.86</td>
<td>1.22</td>
<td>2.42</td>
<td>3.99</td>
<td>0.86</td>
<td>1.17</td>
<td>2.50</td>
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<tr>
<td>± STD</td>
<td>0.48</td>
<td>0.20</td>
<td>0.35</td>
<td>0.39</td>
<td>0.45</td>
<td>0.14</td>
<td>0.32</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>E4/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MP</td>
<td>4.77</td>
<td>1.20</td>
<td>1.19</td>
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<td>0.87</td>
<td>1.11</td>
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<td>1.00</td>
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<td>1.32</td>
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<td>0.89</td>
<td>1.19</td>
<td>3.82</td>
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</tr>
<tr>
<td>JQ</td>
<td>4.30</td>
<td>0.67</td>
<td>1.71</td>
<td>2.29</td>
<td>5.08</td>
<td>1.22</td>
<td>1.47</td>
<td>3.06</td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td>4.72</td>
<td>0.71</td>
<td>0.90</td>
<td>3.50</td>
<td>4.47</td>
<td>0.77</td>
<td>0.81</td>
<td>3.21</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>5.86</td>
<td>0.68</td>
<td>1.32</td>
<td>4.03</td>
<td>5.64</td>
<td>1.16</td>
<td>1.28</td>
<td>3.83</td>
<td></td>
</tr>
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<td>5.04</td>
<td>1.15</td>
<td>1.26</td>
<td>3.21</td>
<td>5.08</td>
<td>0.97</td>
<td>1.14</td>
<td>3.48</td>
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</tr>
<tr>
<td>± STD</td>
<td>0.52</td>
<td>0.65</td>
<td>0.24</td>
<td>0.52</td>
<td>0.37</td>
<td>0.16</td>
<td>0.21</td>
<td>0.30</td>
<td></td>
</tr>
</tbody>
</table>
Table 9 Serum total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, and triglyceride levels before and after placebo treatment

<table>
<thead>
<tr>
<th></th>
<th>Placebo Base</th>
<th>19 days of placebo</th>
<th>Mean ± STD Base</th>
<th>19 days of placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TChol</td>
<td>TGL</td>
<td>HDL</td>
<td>LDL</td>
</tr>
<tr>
<td>E2/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS</td>
<td>4.29</td>
<td>0.81</td>
<td>1.60</td>
<td>2.32</td>
</tr>
<tr>
<td>MT</td>
<td>3.71</td>
<td>0.92</td>
<td>1.31</td>
<td>2.09</td>
</tr>
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<td>1.44</td>
<td>1.36</td>
<td>1.48</td>
</tr>
<tr>
<td>AH</td>
<td>3.72</td>
<td>0.77</td>
<td>0.93</td>
<td>2.44</td>
</tr>
<tr>
<td>WH</td>
<td>5.96</td>
<td>1.23</td>
<td>1.22</td>
<td>4.18</td>
</tr>
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<td>3.78</td>
<td>0.63</td>
<td>1.60</td>
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</tr>
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<td>3.48</td>
<td>0.84</td>
<td>0.93</td>
<td>2.17</td>
</tr>
<tr>
<td>Mean =</td>
<td>4.06</td>
<td>0.95</td>
<td>1.27</td>
<td>2.38</td>
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<td>± STD</td>
<td>0.81</td>
<td>0.26</td>
<td>0.25</td>
<td>0.78</td>
</tr>
</tbody>
</table>

|                |       |     |     |     |       |     |     |     |
| E4/-           |       |     |     |     |       |     |     |     |
| MP             | 4.53  | 1.58| 1.17| 2.63| 4.48  | 1.41| 0.86| 2.98|
| GC             | 6.68  | 2.87| 1.08| 4.30| 5.57  | 1.99| 1.00| 3.67|
| AP             | 5.68  | 1.4 | 1.4 | 3.64| 5.95  | 1.07| 1.37| 4.09|
| JQ             | 5.36  | 0.86| 1.57| 3.40| 5.06  | 1.39| 1.40| 3.03|
| BM             | 4.38  | 0.95| 1.31| 2.64| 4.58  | 0.81| 0.78| 3.43|
| SS             | 5.46  | 0.58| 0.96| 4.24| 5.77  | 1.27| 1.28| 3.91|
| Mean =         | 5.35  | 1.37| 1.25| 3.48| 5.24  | 1.32| 1.12| 3.52|
| ± STD          | 0.76  | 0.75| 0.20| 0.67| 0.57  | 0.36| 0.25| 0.42|

All values are (mmol) and were taken in the morning before any meals were consumed.
Figure 4 Serum total cholesterol (Tchol), triglyceride (TG1), high and low density lipoprotein cholesterol (HDL, LDL) before and after placebo treatment.
Figure 5 Serum total cholesterol (Tchol), triglyceride (TG1), high and low density lipoprotein cholesterol (HDL, LDL) before and after cholesterol treatment.
Figures 4 and 5 represent the before and after treatment values for each group and lipid characteristic, indicating the non-responsiveness of serum cholesterol to dietary cholesterol fluctuations. While consuming the placebo capsules the groups showed no significant changes in cholesterol or triglyceride levels although there was a trend towards lower cholesterol levels after consuming a low cholesterol diet for 19 days (Table 9). The mean of each group's lipid characteristics are shown in Figures 4 and 5. These graphs illustrate the unaltered cholesterol levels in response to varying dietary cholesterol intakes.

4.2.5 Effect of feeding state on cholesterol synthesis rate

Cholesterol FSR data for each apo E group are shown in Table 10 and Figure 6. Data from the first day represent cholesterol FSR in the fed state (0-24 hours) and the second day's data represent a fasting cholesterol FSR. The apo E2/- group showed a significant decrease in cholesterol FSR when going from a fed to fasted state during both the placebo and high cholesterol treatment. The apo E4/- group showed a similar decrease in FSR during the cholesterol treatment period but did not show a significant decrease in fed to fasted cholesterol FSR during the placebo phase.

4.2.6 Effect of dietary cholesterol and apo E genotype on cholesterol synthesis

FSR data from the volunteers were analyzed to investigate the effect that diet and apo E genotype have on short term cholesterol
synthesis. The twelve and 24 hour time points were tested for any dietary or

Table 10 Cholesterol FSR in volunteers possessing either the E2 or E4 allele on low and high cholesterol diets

<table>
<thead>
<tr>
<th>Subject</th>
<th>0-24 hr Fed (FSR)</th>
<th>0-24 hr Fasted (FSR)</th>
<th>24-48 hr Fed (FSR)</th>
<th>24-48 hr Fasted (FSR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT</td>
<td>0.0768</td>
<td>-0.0163</td>
<td>0.0663</td>
<td>-0.0157</td>
</tr>
<tr>
<td>LVP</td>
<td>0.0564</td>
<td>0.0052</td>
<td>0.0425</td>
<td>-0.0058</td>
</tr>
<tr>
<td>AH</td>
<td>-0.0119</td>
<td>-0.0159</td>
<td>0.0021</td>
<td>0.0037</td>
</tr>
<tr>
<td>BS</td>
<td>0.0550</td>
<td>0.0063</td>
<td>0.0527</td>
<td>0.0068</td>
</tr>
<tr>
<td>WH</td>
<td>0.0663</td>
<td>0.0045</td>
<td>0.1009</td>
<td>-0.0036</td>
</tr>
<tr>
<td>PG</td>
<td>0.1063</td>
<td>-0.0298</td>
<td>0.0982</td>
<td>0.0248</td>
</tr>
<tr>
<td>CD</td>
<td>0.0352</td>
<td>0.0040</td>
<td>0.0482</td>
<td>0.0028</td>
</tr>
<tr>
<td>Mean</td>
<td>0.0549</td>
<td>-0.0078*</td>
<td>0.0587</td>
<td>-0.0072*</td>
</tr>
<tr>
<td>+ STD</td>
<td>0.0340</td>
<td>0.0124</td>
<td>0.0316</td>
<td>0.0094</td>
</tr>
</tbody>
</table>

| E4/-    |                   |                      |                    |                      |
| MP      | 0.0516            | 0.0410               | 0.0669             | 0.0105               |
| GC      | 0.1028            | 0.0289               | 0.1056             | 0.0348               |
| AP      | 0.0385            | 0.0028               | 0.0426             | 0.0004               |
| JQ      | 0.0127            | -0.0027              | 0.0103             | 0.0180               |
| SS      | 0.0526            | -0.0188              | 0.0163             | 0.0297               |
| BM      | 0.0454            | 0.0053               | 0.0532             | 0.0598               |
| Mean    | 0.0506            | 0.0094*              | 0.0492             | 0.0255               |
| + STD   | 0.0269            | 0.0199               | 0.0320             | 0.0191               |

* denotes the fasted values are significantly different (p=0.05) from the fed values.
Figure 6 Cholesterol FSR during fed (24) versus fasted (48) periods of each apo E group after the treatment.
genetic effects. Although Figure 7 shows a distinct difference between the genetic group's FSR at the 24 hour time point, statistical tests indicate there is no significant difference between the means seen on Tables 11 and 12. The change in FSR data from the twelve to 24 hour time point on each treatment was analyzed indicating no statistical difference between genetic and dietary groups.
Figure 7 Cholesterol FSR at 12 hour intervals after high and low cholesterol treatment.
Table 11  Cholesterol FSR data at 12 hour intervals in volunteers possessing the E4 allele on low and high cholesterol diets

<table>
<thead>
<tr>
<th>Subject</th>
<th>Cholesterol (FSR)</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP</td>
<td>0.0431</td>
<td>0.0041</td>
<td>0.0314</td>
<td>0.0048</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>0.0392</td>
<td>0.0636</td>
<td>-0.0095</td>
<td>0.0381</td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>0.0133</td>
<td>0.0252</td>
<td>-0.0007</td>
<td>0.0036</td>
<td></td>
</tr>
<tr>
<td>JQ</td>
<td>0.0089</td>
<td>0.0038</td>
<td>-0.0014</td>
<td>-0.0012</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>0.0086</td>
<td>0.0440</td>
<td>0.0012</td>
<td>-0.0200</td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td>0.0020</td>
<td>0.0435</td>
<td>-0.0067</td>
<td>0.0149</td>
<td></td>
</tr>
<tr>
<td>Mean =</td>
<td>0.0191</td>
<td>0.0307</td>
<td>0.0024</td>
<td>0.0067</td>
<td></td>
</tr>
<tr>
<td>+ STD</td>
<td>0.0159</td>
<td>0.0219</td>
<td>0.0135</td>
<td>0.0175</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject</th>
<th>Placebo (FSR)</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP</td>
<td>0.0243</td>
<td>0.0368</td>
<td>0.0000</td>
<td>0.0074</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>0.0365</td>
<td>0.0692</td>
<td>-0.0066</td>
<td>0.0415</td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>0.0171</td>
<td>0.0255</td>
<td>-0.0013</td>
<td>0.0018</td>
<td></td>
</tr>
<tr>
<td>JQ</td>
<td>0.0143</td>
<td>-0.0041</td>
<td>0.0007</td>
<td>0.0173</td>
<td></td>
</tr>
<tr>
<td>SS</td>
<td>-0.0075</td>
<td>0.0238</td>
<td>-0.0124</td>
<td>0.0421</td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td>0.0226</td>
<td>0.0306</td>
<td>0.0001</td>
<td>0.0597</td>
<td></td>
</tr>
<tr>
<td>Mean =</td>
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<td>0.0303</td>
<td>-0.0033</td>
<td>0.0283</td>
<td></td>
</tr>
<tr>
<td>+ STD</td>
<td>0.0133</td>
<td>0.0216</td>
<td>0.0048</td>
<td>0.0209</td>
<td></td>
</tr>
</tbody>
</table>
Table 12  Cholesterol FSR data at 12 hour intervals in volunteers possessing the E2 allele on low and high cholesterol diets

<table>
<thead>
<tr>
<th>Subject</th>
<th>12 (FSR)</th>
<th>24 (FSR)</th>
<th>36 (FSR)</th>
<th>48 (FSR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT</td>
<td>-0.0061</td>
<td>0.0830</td>
<td>-0.0124</td>
<td>-0.0031</td>
</tr>
<tr>
<td>LVP</td>
<td>0.0886</td>
<td>-0.0443</td>
<td>0.0351</td>
<td>-0.0304</td>
</tr>
<tr>
<td>AH</td>
<td>0.0149</td>
<td>-0.0268</td>
<td>0.0198</td>
<td>-0.0246</td>
</tr>
<tr>
<td>BS</td>
<td>0.0469</td>
<td>0.0081</td>
<td>0.0106</td>
<td>-0.0164</td>
</tr>
<tr>
<td>WH</td>
<td>0.0380</td>
<td>0.0283</td>
<td>-0.0013</td>
<td>0.0058</td>
</tr>
<tr>
<td>PG</td>
<td>0.0673</td>
<td>0.0390</td>
<td>-0.0307</td>
<td>-0.0016</td>
</tr>
<tr>
<td>CD</td>
<td>0.0103</td>
<td>0.0248</td>
<td>-0.0068</td>
<td>0.0105</td>
</tr>
<tr>
<td>Mean</td>
<td>0.0371</td>
<td>0.0160</td>
<td>0.0020</td>
<td>-0.0085</td>
</tr>
<tr>
<td>± STD</td>
<td>0.0310</td>
<td>0.0392</td>
<td>0.0202</td>
<td>0.0143</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject</th>
<th>12 (FSR)</th>
<th>24 (FSR)</th>
<th>36 (FSR)</th>
<th>48 (FSR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT</td>
<td>0.0617</td>
<td>0.0043</td>
<td>0.0039</td>
<td>-0.0195</td>
</tr>
<tr>
<td>LVP</td>
<td>0.0234</td>
<td>0.0191</td>
<td>-0.0312</td>
<td>0.0233</td>
</tr>
<tr>
<td>AH</td>
<td>-0.0315</td>
<td>0.0337</td>
<td>-0.0302</td>
<td>0.0342</td>
</tr>
<tr>
<td>BS</td>
<td>0.0222</td>
<td>0.0306</td>
<td>-0.0105</td>
<td>0.0037</td>
</tr>
<tr>
<td>WH</td>
<td>0.0427</td>
<td>0.0582</td>
<td>0.0072</td>
<td>-0.0094</td>
</tr>
<tr>
<td>PG</td>
<td>0.0544</td>
<td>0.0437</td>
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<td>-0.0124</td>
</tr>
<tr>
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<td>0.0204</td>
<td>0.0278</td>
<td>-0.0036</td>
<td>0.0065</td>
</tr>
<tr>
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<td>0.0276</td>
<td>0.0311</td>
<td>-0.0111</td>
<td>0.0038</td>
</tr>
<tr>
<td>± STD</td>
<td>0.0285</td>
<td>0.0159</td>
<td>0.0141</td>
<td>0.0181</td>
</tr>
</tbody>
</table>
5.0 DISCUSSION

5.1 APOLIPOPROTEIN E GENOTYPE IDENTIFICATION

Despite the identification of apo E as a genetic factor contributing significantly to within-population variation in plasma cholesterol level, methods for apo E identification remain time-consuming and labour-intensive. Isoelectric focusing (IEF) on a single polyacrylamide gel has been the method of choice in apo E phenotyping in the past (Warnick et al. 1979). A more recent alternative approach to identifying apo E phenotypes is use of a combination of isoelectric focusing of delipidated serum and immunoblotting (Havekes et al. 1987, Kamboh et al. 1988, Hill et al. 1990). Even more recently, a DNA amplification technique has been developed using PCR with radiolabelled probes or restriction enzymes. In this thesis an improvement of the PCR-probe method is described which eliminates the need for radioactive probes, incubation, hybridization, and washing procedures. This new technique requires only an agarose gel and can produce apo E genotype results within three hours after DNA isolation.

Diagnosis of individuals with alpha 1-antitrypsin deficiency or cystic fibrosis has used similar approaches to identify point mutations (Ballabio et al. 1990, Newton et al. 1989, Okayama et al. 1989). In each application the same principal is used; oligonucleotides with a 3'
mismatched base pair will not function as primers in the PCR mixture. In some cases, a single mismatch is not sufficient to prevent nonspecific amplification and a second deliberate mismatch must be incorporated into the oligonucleotide (Ballabio et al. 1990). Investigators have used this technique to detect different mutations in an allele selectively.

For instance, Neubauer and coworkers (1990) used the differential PCR method to detect allelic loss of the beta-interferon gene. Human immunodeficiency virus studies have investigated the effects of different primer-template mismatches on DNA amplification efficiency (Kwok et al. 1990). Results indicated some mismatches (A:G, G:A, and C:C) reduced PCR product yield drastically, while mismatches involving a T amplified efficiently.

Specificity of a primer for the template may be modulated by at least three means: incorporation of a deliberate base-pair mismatch, altering annealing temperature, or by decreasing dNTP concentration. We originally tried to differentiate the apo E alleles using primers with only a single mismatch at the 3' end. Results from the agarose gel indicated a large amount of nonspecific amplification. New oligonucleotides were synthesized with the mismatch one nucleotide from the 3' end; however, a significant amount of nonspecific amplification was observed. The addition of a further deliberate mismatch three nucleotides from the 3' end of the primer enhanced the specificity greatly. Optimal results were obtained by using a more stringent
annealing temperature of 58°C because lower temperatures also resulted in nonspecific amplification. To allow for amplification of the larger human prothrombin segment, the reactions were seeded with a cloned DNA sequence of the human prothrombin gene, allowing amplification of the internal control indicating each PCR mixture contained the appropriate reagents.

Initially, amplification of the various apo E alleles yielded inconsistent amounts of product. A primer was synthesized in an attempt to alleviate this problem by increasing the length to 29 nucleotides. Aspecific amplification was observed at a variety of different annealing temperatures and buffer conditions. Winship and coworkers (1989) observed that G-C rich sequences were difficult to amplify and added DMSO to the PCR mixture. This is thought to reduce the secondary structure of DNA resulting in amplification of G-C rich sequences. The addition of ten percent DMSO to our PCR mixtures increased the yield of certain apo E segments specifically even though DMSO is thought to reduce Taq polymerase activity by approximately 50 percent (Lawyer et al. 1989).

In summary, the PCR-allele-specific primer method of apo E genotyping offers advantages of simplicity and cost compared with other methods. Obtaining DNA from a mouthwash can obviate the need for venipuncture (Lench et al. 1988), but may be impractical due to small and inconsistent DNA yields. Because the method is based on nucleotide sequence variation, less common structural mutants of the apo E allele
could be identified. This new procedure may enable large populations to be screened rapidly and accurately, thus contributing to our understanding of apo E's allelic distribution and its regulation of cholesterol synthesis.

5.2 Influence of Dietary Cholesterol and Apolipoprotein E Genotype on Cholesterol Synthesis

5.2.1 Physical characteristics and Apolipoprotein E Genotype

The three pool model of human cholesterol turnover is influenced by parameters such as age and body weight. Therefore, it was necessary to match the two experimental groups as closely as possible (Goodman et al. 1980). Physical data were obtained to ensure any differences identified in the study could be attributed to apo E genotype and not another characteristic. The E2/- and E4/- groups were not significantly different with respect to age, body weight, and percent body fat. Body weight and percent body fat were determined before and after each dietary treatment to check that volunteers body composition did not change significantly over the three week protocol. Screening cholesterol levels were significantly higher in the E4/- group before each experimental period.
5.2.2 Effect of dietary challenge on serum lipids in the study groups

The serum cholesterol response to a change in dietary cholesterol quantity has been investigated extensively over the past decade (Beynen et al. 1987, McNamara et al. 1987, Miettinen et al. 1988, Mantarri et al. 1991). Results are highly variable, but the general consensus from the literature is that increasing dietary intake of cholesterol from 200 mg/day to 600 mg/day for a minimum of two weeks results in a significant increase in serum cholesterol levels. Volunteers in the present investigation from each apo E group, while on the high cholesterol diet, consumed 550 mg of cholesterol over and above their normal daily intake for 21 days. There were no significant changes in serum cholesterol levels in either of the apo E groups. This is in agreement with the findings of McNamara et al. (1987) who found that most subjects (69%) are able to compensate for an increased dietary cholesterol consumption. This group (McNamara et al. 1987) concluded that individuals were able to maintain consistent plasma cholesterol levels by altering their endogenous cholesterol synthesis and/or by changing excretion rates of already absorbed cholesterol.

5.2.3 Diet intake of study groups in the final week of each experimental phase

The objective of the dietary records was to ensure the groups consumed a low fat, low cholesterol diet and to determine if the two groups had similar nutrient intakes. The goal dietary intake was 15,
30, and 55% of total calories from protein, fat, and carbohydrate, respectively. Each group consumed approximately 28% of their total calories as fat and the average cholesterol intake was 245 mg/day (Table 4). These results indicate that the volunteers consumed meals that were within the recommended guidelines of this investigation, however, the results of the record analysis should be regarded as only a guideline of the volunteers true dietary intake. Some subjects consumed meals at restaurants or cafeterias where they had little knowledge of the types of oils and ingredients used in preparation of their meals. Calculated total caloric intake is also an indication that some volunteers were recording their food consumption inaccurately because their energy requirements were far more than their reported intake (Table 4 - Subjects AH and JQ). Although the diet records have several weaknesses they are still a reasonable representation of what a volunteer consumes outside the metabolic kitchen.

5.2.4 Apolipoprotein E polymorphism and cholesterol synthesis

Several investigators have postulated mechanisms on how apo E polymorphism effects serum cholesterol levels (Utermann 1986, Miettinen et al. 1988, Mantarri et al. 1991). A difference in cholesterol synthesis rates could result in different serum cholesterol levels, therefore the measurement of cholesterol FSR was carried out in individuals of the apo E2/- and E4/- genotypes. Results indicated no
difference in cholesterol FSR between the different apo E genotypes although they exhibited significantly different baseline serum cholesterol levels. Mantarri and coworkers (1991) found opposing results suggesting that apo E polymorphism influences serum cholesterol response to dietary intervention. The investigators found E4/- individuals to respond to a decrease in saturated fat intake by decreasing serum cholesterol levels. It may be concluded that apo E polymorphism is sensitive to dietary fat intake but not dietary cholesterol intake.

Figure 7 displays that the genetic groups did not react similarly to the high and low cholesterol diets. While consuming the high cholesterol diet the E2/- group decreased cholesterol synthesis at night. The E4/- group showed an increase in cholesterol synthesis at night in both treatment groups. This trend was not significantly different and may be due to the large amount of inter-individual variability present in each of the apo E groups. Investigations showing positive apo E polymorphism effects on serum cholesterol use a large number of homozygous E4/4 individuals, therefore the heterozygous individuals in this study may have contributed to the variable findings.
5.2.5 *Cholesterol FSR in fed versus fasted state*

Previous studies have shown short term food restriction to have an effect on endogenous cholesterol synthesis (Jones et al. 1988, Roe et al. 1991). In this investigation, subjects were fed for the initial twelve hours of the study and fasted for the remaining 36 hours. Except for one group, all groups showed a significant decrease in the FSR of cholesterol when going from a fed to fasted state (Figure 6). The E4/- group, while consuming the placebo capsules, showed no significant decrease in cholesterol FSR. This may indicate that E4/- possessing individuals do not have a sensitive endogenous cholesterol synthesis feedback mechanism while consuming low cholesterol diets.
6.0 SUMMARY AND CONCLUSIONS

This study investigated two aspects of human apo E. The first component of study was the examination of structural variants of apo E using molecular biology techniques. The second aspect was the interaction of apo E and dietary cholesterol level on cholesterol synthesis rates. It has become necessary to identify apo E genotypes and understand their role in cholesterol metabolism because of the relationship between apo E polymorphism and atherosclerosis.

Volunteers were recruited from previous apo E studies and the DNA samples from these individuals used in the development of a new technique for identifying apo E genotypes. This new technique, which enables rapid, accurate detection of apo E genotypes, was subsequently used to screen volunteers for the second portion of the study. Several metabolic and physical characteristics were monitored in each volunteer over two dietary phases. At the end of each dietary phase volunteers were administered deuterium to determine the incorporation rate of body water deuterium into newly synthesized free cholesterol. Subjects’ cholesterol synthesis rates were determined after a high and low cholesterol diet and also after fasting.

The level of cholesterol consumed in the diet did not effect cholesterol FSR and apo E did not influence cholesterol FSR. Feeding state did have an effect on cholesterol FSR indicating that fasting generally causes a reduction in cholesterol FSR. Individuals possessing
the E4 allele and consuming a low cholesterol diet did not show a reduction in cholesterol after fasting and may indicate these individuals are insensitive to feeding state after consuming a low cholesterol diet.

Results of this study may have an important role in clinical applications. Firstly, the new genotyping technique could be implemented for rapid, accurate determination of apo E genotypes. Second, E4 possessing individuals are not affected by a therapeutic diet consisting solely of a reduced dietary cholesterol intake and may require further alterations such as a change in fat consumption. Third, E4 possessing subjects do not respond to feeding state manipulations as E2 individuals do. These findings strongly suggest apo E polymorphism does not effect cholesterol metabolism during a low cholesterol diet.
6.0 REFERENCES


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APPENDIX ONE TESTING CONSENT FORM

Consent by Subject of Research Protocol

Protocol#__________Patient Name:______________________


Doctors Directing Research: Jones PJ, MacGillivray RT, Frohlich JJ.
Phone: 822-6253

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 examine how hereditary factors influence a person's response to changing levels of cholesterol in the diet. The study will be of 12 weeks duration during which time you will undergo 2 dietary treatment periods of 3 weeks each. In each period, with guidance from consultant dieticians, you will consume diets which are either (i) low in fat and high in cholesterol or (ii) low in both fat and cholesterol. Food items will be selected by you using an instruction pamphlet describing types of foods and oils which you are permitted to eat over each dietary period. Target intakes for each period will be 55, 30, and 15% of calories as carbohydrate, fat and protein, respectively. Alcohol and caffeine consumption will be prohibited over each dietary period.

On the final day of each study period, you will consume meals which are prepared in the metabolic kitchen at U.B.C.. The test diets will be comprised of solid foods, designed from food tables to typify average North American intake patterns. This diet will be fed as 3 similar sized meals at a level which should maintain your body weight. Meals will be prepared and served in the metabolic kitchen.

The rate of production of lipids in your body will be measured at the end of each of the 2 feeding periods. You will fast from the evening meal on study day 4 and report to the metabolic testing area at the Division of Human Nutrition at 7:30 am on day 5. Twenty ml's of blood will be collected for determination of the natural enrichments of deuterium in cholesterol and plasma water. You will then drink 40 ml's of water labelled with a special, non-radioactive tag. Further blood
samples will be collected at 12, 24, 36 and 48 hr after you take the labelled water dose by trained phlebotomists.

II. Potential Risks and/or Benefits:

There are no known side effects of risks associated with the ingestion of labelled water as indicated in the protocol. There are no risks associated with other aspects of these procedures other than that of 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 to anyone as to results to be obtained. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. I understand that I will receive $100 upon completion of the study. If I decide to withdraw before completion, I will receive an appropriate pro-rated fraction of this amount.

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

Doctor:__________________________
Witness:__________________________ Signature of Subject
Date:__________________________
APPENDIX TWO  DIETARY RECORDS

Effect of Human Apolipoprotein E Genotype on Cholesterol Synthesis Rates as Measured by Deuterium Incorporation

Instructions on keeping a 3-day food record

Please keep a record of everything you eat and drink on the attached forms for the following 3 days:

1. Write down everything you eat and drink. 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:

   A) Use volume measures (cups, Tbsp, or mls) for cereals, rice, pasta, vegetables, canned fruit, peanut butter, mayonnaise, salad dressings, butter, margarine, sauces, gravies, soups, jam, beverages, etc.

   B) Use cooked weights (oz or grams) for meat, fish, and poultry. Note: the weight of meat and poultry and fish decreases by about 25% during cooking. For example:
      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 or specify small, medium, or large serving based on the attached diagrams.

   D) Be specific about the type of food, brand name, how the food was prepared, and content of mixed dishes.

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

   F) If the food is prepared by someone other than youself 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!! eg. sugar on cereal or in coffee, dressing on salad, candy, soft drinks, alcohol.
### Example:

<table>
<thead>
<tr>
<th>Time</th>
<th>Food</th>
<th>Amount</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:00am</td>
<td>2% milk</td>
<td>1/2 cup</td>
<td>UBC Caf</td>
</tr>
<tr>
<td></td>
<td>whole wheat bread</td>
<td>2 slices</td>
<td></td>
</tr>
<tr>
<td></td>
<td>margarine</td>
<td>2 tsp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>omelette: eggs</td>
<td>2 large</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cheddar</td>
<td>1/2 oz.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>orange juice</td>
<td>6 oz.</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX THREE INFORMATION SHEETS
INTERACTIVE INFLUENCE OF DIETARY CHOLESTEROL AND APOLIPOPROTEIN E GENOTYPES ON CHOLESTEROL SYNTHESIS

Investigators: Blair Main and Dr. Jones

A BASIC DESCRIPTION OF PHASE 2: DIETARY INFLUENCES

Specific aim: To test the interactive effects of i) diet cholesterol levels and ii) apo E genotype on the fractional synthetic rate of cholesterol.

Significance: This investigation will improve our understanding of how cholesterol synthesis is influenced by dietary cholesterol changes (800 mg/day compared to 300 mg/day) in apo E2/- and E4/- individuals. This study will more specifically address why individuals possessing the E4 allele have elevated plasma cholesterol levels - ie. Do E4's have a higher rate of cholesterol synthesis?

Protocol

3 week dietary period
6 week vacation (this can be changed)
3 week dietary period
Total of 12 weeks
At the end of the 12 weeks a $100 honorarium will be mailed to you.

What do the dietary periods consist of?

1. During both dietary periods you will be asked to consume a low fat, low cholesterol diet - this will be described to you before the study starts.
2. You will be asked to consume capsules on a daily basis (3 in the morning and 3 in the evening). The capsules will contain freeze dried egg yolk (high in cholesterol, 6 capsules = 550 mg cholesterol) or freeze dried egg white (zero cholesterol). The capsules were prepared for our study at Finlandia Pharmaceuticals.
3. In the third week of each of the dietary periods you will be asked to keep dietary records for 3 consecutive days (basically this means you write down everything you eat and drink). On these 3 same days you will also be asked to collect your faecal samples. This is a precaution to ensure all of the capsules are being taken.
4. On day 19 of the study you will be given breakfast (cereal and scrambled eggs), lunch (yogurt, chicken sandwich, and an orange) and supper (spaghetti with tomato sauce and chicken, veggies, ice milk).
5. Before breakfast on day 19 you will be given an oral dose of deuterium which is a stable, non-radioactive isotope of water. This has been used in metabolic studies since the early 1930's with no known harmful side effects. Deuterium is used in this study to determine the rate of cholesterol synthesis.

6. 2 blood samples will be drawn on day 19, 2 on day 20, and 1 on day 21.

7. Day 20 is a fasting day to determine if apo E effects fasting cholesterol synthesis. There are flavour packs which will be made available to mix with your water - it keeps the hunger down!!

8. On day 21 the last blood sample will be drawn and you have an all request breakfast at Chez Blair's in the metabolic kitchen.

Transportation can be arranged if it is a problem.
Reading material is free if you are interested.
Lipid profile results will be available at the end of the study.

If you have any questions at all please call me, Blair Main, at home (875-0502) or at work (822-8634).
FAX# 822-5143

Thank you in advance for your cooperation in this investigation.
APPENDIX FOUR  SCHEDULE FOR DIET PERIOD

Apolipoprotein E Dietary Cholesterol Study

Schedule of important times:

October 4-6 - Introduction, weigh in, body fat determination, baseline cholesterol if not known.

October 7 - Start of low cholesterol, low fat diet. Capsules to be taken with breakfast and supper.

October 9-12 - The second weigh in to determine average body weight.

October 27 - 7:30 am  Breakfast and blood sample #1.
          7:30 pm  Supper and blood sample #2.

October 28 - 7:30 am  Fast, blood sample #3.
          7:30 pm  Fast, blood sample #4.

October 29 - 7:30 am  Request breakfast, blood sample #5.

NOTE: Starting Oct. 7 no caffeine should be consumed and alcohol consumption should be no greater than 1 drink per day. After Oct. 29 you will have a 4 week vacation and then this similar procedure will be repeated.

If you have any questions please call me or come by my office.
Blair Main  875-0502
          822-8634  Office #323
Dr. Peter Jones  822-6253

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APPENDIX FIVE  CHOLESTEROL ENRICHMENT BEFORE FSR CALCULATIONS OF EACH APO E GROUP ON BOTH TREATMENTS
APPENDIX SIX  INDIVIDUAL APO E4/- DATA POINTS FOR FED VERSUS FASTED STATE ON BOTH TREATMENTS

![Graph showing FSR/day vs Cholesterol and Placebo with different markers for treatments MP, GC, AP, JQ, SS, BM]
APPENDIX SEVEN  INDIVIDUAL APO E2/- DATA POINTS FOR FED VERSUS FASTED STATE ON BOTH TREATMENTS