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UNIVERSITY OF ALBERTA

NIACIN, ASPIRIN AND HOMOCYSTEINE INTERRELATIONSHIPS: A CLINICAL STUDY

by

ANDREA FISHER

A thesis submitted to the Faculty of Graduate studies in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

NUTRITION AND METABOLISM

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **NIACIN, ASPIRIN AND HOMOCYSTEINE INTERRELATIONSHIPS: A CLINICAL STUDY** submitted by **ANDREA FISHER** in partial fulfillment of the requirements for the degree of **Master of Science in Nutrition and Metabolism**.

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Dr. G. Mathison

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Date Approved: September 29, 1999

DEDICATION

To my parents, my sister and Christopher.
Your love, support and encouragement have made this achievement possible.

ABSTRACT

Niacin (1.5 -6g per day) is an effective broad-spectrum hypolipidemic agent; however, it may potentially elevate plasma levels of homocysteine, a risk factor for cardiovascular disease. Niacin-induced hyperhomocysteinemia has occurred in experimental animals. The present study was undertaken to investigate if niacin also modifies homocysteine status in humans. A total of 15 subjects (7-male; 8-female),with an average age of 60.2 ± 7.8y and plasma cholesterol levels >5.8 mmol/L, were recruited. These subjects were treated with aspirin alone (325 mg) for one week followed by niacin (plus aspirin) for seven weeks. The dose level of niacin was gradually increased from 500 mg/d to a maximum dose level of 1500 mg/day that was maintained for the final three weeks of the study. Following this study, subjects continued to receive 1500 mg of niacin/d (plus aspirin) for an additional three weeks; but, supplements of folate (1mg/d), B6 (100μg/d) and B12 (250μg/week) were also administered. Blood samples were collected before treatment, following aspirin alone (1 wk), post niacin therapy (7wks) and post B-vitamin intervention (10 wks). Lipid profiles, homocysteine and vitamin levels (eg. B12, folate and B6) were determined in the separated plasma and a MANOVA analysis determined significant changes in the parameters tested.

Treatment with niacin resulted in significant decreases in plasma total cholesterol, triglycerides and low-density lipoprotein levels, while high-density lipoprotein levels significantly increased ($p < 0.05$). More than one-half of the study subjects (53%) experienced elevations in plasma homocysteine levels. The raised mean homocysteine

level ($p=0.4615$) was accompanied by a significant reduction in the mean plasma folate concentration ($p= 0.0115$). The administration of the supplemental B-vitamins (folate, vitamin B6 and vitamin B12) for three weeks resulted in a reduction of the mean plasma homocysteine level by 21.2%. In the presence of B-vitamin supplementation, niacin's hypolipidemic effects remained unchanged. Hence, the addition of B-vitamin supplementation to the niacin therapy is recommended as the treatment regimen for the control of cardiovascular disease.

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ABBREVIATIONS:

ALKP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMI	Acute myocardial infarction
AMP	Adenosine monophosphate
Apo	Apoprotein
ASA	Acetylsalicylic acid
ATP	Adenosine triphosphate
BHMT	Betaine-homocysteine methyltransferase
BHT	Bis[3, 5-diterbutyl-4-hydroxyphenylether] propane
BUN	Blood urea nitrogen
CAD	Coronary artery disease
cAMP	Cyclic 3', 5'-adenosine monophosphate
CARDIA	Coronary Artery Risk Development in Young Adults
CBD	Cerebrovascular disease
CBS	Cystathionine β-synthase
CHD	Coronary heart disease
CVD	Cardiovascular disease
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
ECAP	European Concerted Action Project
FFA	Free fatty acids
FFQ	Food-frequency questionnaire
G_s	Stimulatory guanine nucleotide linked G-protein
G_i	Inhibitory guanine nucleotide linked G-protein
GLC	Glucose
HDL-C	High density lipoprotein cholesterol
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	High Performance Liquid Chromatography
HSL	Hormone sensitive lipase
IDL	Intermediate low density lipoprotein
IHD	Ischemic heart disease
INT	2-[p-iodophenyl]-3-p-nitrophenyl-5-phenyltetrazolium chloride
INTH	formazan
IR	Immediate release
LDL-C	Low density lipoprotein cholesterol
LRC-CPPT	The Lipid Research Clinics Coronary Primary Prevention Trial
Lp(a)	Lipoprotein (a)
MAGL	Monoacylglycerol lipase
MANOVA	Multivariate Analysis of Variance
MANCOVA	Multivariate Analysis of Covariance

ABBREVIATIONS: (Continued)

MI	Myocardial infarction
MRFIT	Multiple Risk Factor Intervention Trial
MTHF	5,10-methylene tetrahydrofolate
MTHFR	5,10-methylene tetrahydrofolate reductase
NAD	Nicotinamide adenine dinucleotide
NADH + H	Nicotinamide adenine dinucleotide (reduced form)
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH + H	Nicotinamide adenine dinucleotide phosphate (reduced form)
NCEP	National Cholesterol Education Program
NO	Nitric Oxide
PA	Pyridoxic acid
PG	Prostaglandin
PGA	Polyglutamic acid
PLP	Pyridoxal-5'-phosphate
PRPP	5-phosphoribosyl-1-pyrophosphate
PVD	Peripheral vascular disease
RNI	Recommended Nutrient Intake
TBIL	Total bilirubin
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SEM	Standard error of the mean
SR	Sustained release
RNA	Ribonucleic acid
R_s	Lipotic β_1-receptors
R_i	Antilipotic α_2-receptors
RBC	Red blood cell
TC	Total Cholesterol
TG	Triglyceride
THF	Tetrahydrofolate
US	United States
VLDL	Very low density lipoprotein

CHAPTER ONE: LITERATURE REVIEW

1. INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of death in North America. According to Health Canada (1997), ischemic heart disease (IHD) caused the highest percentage of all deaths in Canada (males 21.8%, females 19.9%), followed by acute myocardial infarction (AMI) and stroke. In the province of Alberta, the mortality rate associated with CVD is approximately 240 per 100000 individuals. Since CVD is a major cause of morbidity and mortality in North America, considerable research has been carried out to identify the major risk factors associated with the disease.

The longitudinal Framingham Study (Gordon et al., 1977), conducted in the US, identified many of the risk factors associated with CVD. According to this study a significant inverse relationship exists between high density lipoprotein (HDL) levels and the incidence of coronary heart disease (CHD). This hallmark study (Castelli, 1984; Castelli et al., 1986) and others (Stamler et al., 1986) found that serum total cholesterol (TC) levels are strongly related to CHD. Other factors such as blood pressure, weight, smoking, activity level, blood glucose levels, stress, increasing age and family history could also play a causal role in CHD (Castelli, 1984; Consensus Conference, 1985).

In recent years, elevated low density lipoprotein cholesterol (LDL-C) levels have been implicated in the development of artheroclerotic lesions that facilitate the development of atherosclerosis (Jialal and Devaraj, 1996; Lamarche et al.,1997; Gardner et al., 1996) Hypertriglyceridemia has been identified as an independent risk factor for CVD (Austin et al., 1998; Sprecher et al., 1998). Triglycerides (TG) are important lipid components of both LDL and very low density lipoproteins (VLDL) which are also involved in the pathogenesis of atherosclerosis, thus, adversely affecting CVD risk (Krauss, 1998; Jeppesen et al., 1998). Recently, other factors such as plasma lipoprotein (a) and

homocysteine concentrations have also been implicated in positive association with CVD risk (See Section 1.44 and Section 1.6).

Seventy-five percent of the Canadian population between the ages of 18-74 years have one or more risk factors for CVD and 44% suffer from elevated blood cholesterol (Health Canada, 1997). The Lipid Research Clinic Coronary Primary Prevention Trial (LRC-CPPT) found that a reduction in plasma TC levels and a reduction of LDL levels diminished the mobidity and mortality of CHD (Lipid Research Clinic Program, 1984a; Lipid Research Clinic Program, 1984b). The data suggested that a 1% drop in plasma total cholesterol levels facilitates a 2% drop in CAD risk. The Helinski Heart Study has also demonstrated that modifying lipid profiles to increase high density lipoprotein cholesterol (HDL-C) and to reduce TC, TG and LDL levels subsequently decreases the incidence of CHD (Frick et al., 1987)

Since major risk factors such as genetic predisposition, gender and age are not modifiable, it is of paramount importance for high risk individuals to modify life-style risk factors such as smoking, excessive body weight, and physical inactivity. Dietary intervention is recommended as the first line of treatment for high-risk individuals with elevated total cholesterol levels (Consensus Conference, 1985; NCEP, 1988; NCEP, 1993).

1.1 DIETARY INTERVENTION

The National Cholesterol Education Program (NCEP, 1988) provided guidelines for the treatment of high cholesterol in adults 20 years of age and older. NCEP defined categories of CHD risk according to three categories of plasma TC levels and LDL levels (Table 1.0). The report identified LDL levels as the primary target of cholesterol lowering therapy. In 1993, NCEP acknowledged plasma HDL levels as a negative risk factor for CHD and added initial HDL levels to the CHD risk classification scheme. The second report of the expert panel (NCEP, 1994) suggested an initial complete lipid

analysis and provided guidelines to assess CHD risk associated with fasting plasma TG levels.

Table 1.0: Classification of CHD risk based on initial lipoprotein levels

Classification	TC (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	TG (mmol/L)
Desirable	< 5.2	> 0.9	< 3.4	< 2.3
Borderline High	5.2 - 6.2		3.4 - 4.1	2.3 - 4.5
High	> 6.2		> 4.1	> 11.3

(NCEP, 1993; NCEP, 1994)

The NCEP Adult Treatment Panel (1993) recommends dietary intervention for high-risk individuals or borderline high-risk individuals with two or more of the following risk factors: male ≥ 45 years; female ≥ 55 years or premature menopause without estrogen therapy; family history of premature CHD; current cigarette smoking; hypertension; low HDL levels and diabetes mellitus. Dietary therapy occurs in two steps that are designed to progressively reduce intakes of saturated fatty acids and cholesterol, and to promote weight loss in overweight patients by eliminating excess calories. The STEP-ONE DIET calls for the reduction of total fat to less than 30% of calories, saturated fatty acids less than 10% of calories and cholesterol intake of less than 300 mg/day (NCEP, 1988). Serum cholesterol should be measured and adherence to the diet assessed at four to six weeks and three months after diet therapy. If cholesterol and LDL goals have not been met, a STEP-TWO DIET should be followed. Since the STEP-TWO DIET further reduces saturated fatty acid intake to less than 7% of total calories and dietary cholesterol to less than 200 mg/day, a registered dietitian should be consulted.

The Interim Report (Frohlich et al, 1998) proposed a strategy similar to NCEP for the diagnosis and treatment of dyslipidemia (Table 1.1). The Working Group recommended that cardiovascular risk assessment include an account of personal history, a physical examination and a fasting plasma lipoprotein analysis. Like NCEP, the report calls for a modification of life-style factors and dietary intervention as the first step to treat

dyslipidemia. The Interim report is the only report to recommend the assessment of LDL levels and the TC:HDL ratio to determine the course of treatment . The TC:HDL ratio is considered as accurate as NCEP guidelines as a risk assessment tool for CHD (Grover et al., 1995). Other research has indicated that a simple measure of non-HDL cholesterol (serum cholesterol minus serum HDL cholesterol) has considerable potential as a risk assessment tool due to it's simplicity and ability to consider all potentially atherogenic lipoproteins (LDL, intermediate-density lipoprotein (IDL), VLDL, Lp (a)) and not simply LDL (Frost and Havel, 1998).

Table 1.1: The Interim report - criteria for treatment

Patient's Risk Category		*Initiate Drug Therapy if:*		
*Risk Factors	Risk Level	LDL (mmol/L)		TC: HDL ratio
≥ 4	Very High	≥ 3.5	or	> 5
≥ 3	High	≥ 4.5	or	> 6
≥ 2	Moderate	≥5.0	or	> 7
≥ 1	Low	≥6.0	or	> 8

*Risk factors: advancing age, family history of premature CAD, cigarette smoking, hypertension (Frohlich et al., 1998).

Investigations into methods to improve CVD risk assessment are concurrent and may change in the future. However, the recommendations of NCEP and the Interim report are the most often used. It is important that intensive dietary intervention be given the opportunity to work before considering pharmacological measures. If lipid goals are not met after six months of diet and lifestyle changes, then an appropriate drug therapy should be implemented without delay (NCEP, 1988; NCEP, 1993; Braunwald, 1997; Frohlich et al, 1998).

1.2 HYPOLIPIDEMIC AGENTS

Several lipid-lowering agents are currently available for those persons where dietary treatment has failed. Five categories of agents have become the mainstays of pharmacological therapy: 3-Hydroxy-3-Methylglutaryl Coenzyme A (HMG-CoA)

Reductase Inhibitors; bile acid sequestrants; fibric acid derivatives; probucol; and niacin (nicotinic acid). Each group of hypolipidemic agents has a different mode of action targeting different lipoproteins. As with most drugs, the benefits to therapy are not without side effects; therefore, possible side effects and an individuals lipid profile must be considered before choosing a drug regimen.

1.21 HMG-COA Reductase Inhibitors:

HMG-CoA reductase inhibitors are a group of hypolipidemic agents known as *The statins*: fluvastatin (lescol), lovastatin (mevacor), pravastatin (pravacol), simvastatin (zocor) and atorvastatin (lipitor) (Yeshurun and Gotto, 1995; Braunwald, 1997). Despite their structural differences, the above agents act primarily on the liver with a similar mode of action. The statins have an inhibitory effect on HMG-CoA reductase- the rate limiting enzyme in cholesterol biosynthesis (Alexander et al., 1998; Braunwald, 1997; Illingworth, 1988). Inhibiting HMG-CoA reductase inhibits the conversion of HMG-CoA to mevalonic acid (Illingworth, 1988) thereby reducing the endogenous production of cholesterol. The result is a compensatory increase in high affinity hepatic LDL receptors that remove LDL and smaller amounts of VLDL and IDL from the circulation (Alexander et al., 1998). Hepatic synthesis of LDL, VLDL (Braunwald, 1997; Illingworth, 1988; Blum and Levy, 1989) and the secretion of TG may also be reduced by statin therapy (Grundy, 1998).

HMG-CoA reductase inhibitors have the most potent LDL lowering ability of the lipid lowering drugs (Davignon et al., 1992; Endo, 1992; Blum and Levy, 1989). The statins also moderately reduce plasma TG levels and moderately increase plasma HDL levels. HMG-CoA reductase inhibitors may induce biochemical and systemic side effects. Persons using this drug therapy may experience headaches, nausea, fatigue, skin rashes and gastrointestinal side effects (Illingworth, 1988); however, these hypolipidemic agents are generally well tolerated (Shils et al., 1994). Hepatotoxicity due to an elevation of

liver transaminases and myopathy are of the clinical manifestations that may be associated with the use of the statins (Alexander et al., 1998; Shils et al.,1994).

1.22 Bile Acid Sequestrants:

Bile acid sequestrants, also known as bile acid resins, are quartenary ammonium salts that interrupt enterohepatic circulation of bile acids (Alexander et al., 1998; Yeshurun et al., 1995). Bile acid sequestrants act as polycationic exchange resins in the intestinal lumen where they bind bile acids resulting in increased fecal loss (Alexander et al.,1998). This increase in bile acid loss causes a corresponding increase in 7-alpha-hydroxylase, the rate limiting enzyme for bile acid synthesis. The increase in the conversion of cholesterol into bile acids stimulates a compensatory increase in hepatic LDL receptors thus reducing serum LDL levels (Illingworth 1988; Illingworth, 1987; Blum and Levy, 1989).

Cholestyramine (questran) and cholestipol (colestid) are the two agents in the category of lipid-lowering drugs. Treatment with the resins may lower LDL serum levels (15-35%), raise serum HDL levels (3-5%) (Yeshurun and Gotto., 1995); however, resin therapy may raise serum TG levels due to an inhibition of lipoprotein lipase (Illingworth, 1987). The most common side effects that one could experience are: constipation, abdominal pain, nausea, bloating, and flatulence (intestinal obstruction is a rare occurrence). Liver function abnormalities and mucoskeletal aches and pains are also associated with resin use (Pearson, 1996). Since bile acids sequestrants are not absorbed, they may facilitate lipid and fat-soluble vitamin malabsorption (Illingworth, 1987). Folate absorption may also be affected with prolonged therapy; thus vitamin supplementation is recommended (Schachter, 1996).

1.23 Fibric Acid Derivatives:

Fibric acid derivatives include clofibrate (atromid-s), gemfibrozil (lopid), fenofibrate and benzafibrate (Pearson, 1996; Alexander et al., 1998). The mechanism of action for these

derivatives is complex and not yet completely defined. However, it is known that fibric acid derivatives decrease serum TG (Braunwald, 1997) and VLDL synthesis (Alexander et al., 1998). This is secondary to an increase in lipoprotein lipase, an enhanced rate of LDL catabolism and a reduction in hepatic VLDL synthesis (Illingworth, 1988; Alexander et al., 1998; Yeshurun and Getto, 1995; Grundy and Vega, 1987). Fibric acid derivatives may increase HDL levels 10-20% (Illingworth, 1988) and they may alter the composition of LDL particles to a less dense, less atherogenic phenotype (Alexander et al., 1998).

As with other lipid-lowering drugs, persons taking this hypolipidemic therapy may experience side effects. Common side effects are gastrointestinal disturbances such as nausea, abdominal pain, flatulence and dyspepsia (Alexander et al., 1998). Fibric acid derivatives may facilitate weight gain, decrease libido and promote the development of gallstones due to increased cholesterol secretion into bile (Shils et al., 1994; Braunwald, 1997). Mild myopathy has been reported and mild hepatotoxicity has occurred in some instances; however, liver dysfunction is reversible upon discontinuation of treatment (Yeshurun and Gotto, 1995).

1.24 Probucol:

Probucol, also known as Lorelco (Pearson, 1996), is a bis-phenol derivative (Blum and Levy, 1989; Alexander et al., 1998). It is a highly non-polar antioxidant substance (Parthasarathy, 1992) that reduces plasma total cholesterol levels by reducing the plasma LDL and HDL fractions (Pearson, 1996). Treatment with probucol can reduce LDL levels by 10-15% and decrease HDL levels by 20-30%; however, plasma TG levels remain unaffected (Yeshurun and Gotto, 1995). It's primary lipid lowering mechanism is not completely elucidated; however, research has shown that probucol increases plasma LDL clearance independent of the LDL receptor pathway (Alexander et al., 1998). This increased rate of LDL catabolism has been linked to an increase in fecal bile acids (Pearson, 1996).

Probucol is lipophillic and is transported by LDL and VLDL in plasma (Marshall, 1992). Studies have shown that probucol may also play an inhibitory role in the progression of atherogensis by limiting oxidative LDL modification and foam cell formation/accumulation in the arterial wall (Steinberg, 1986; Parthasarthy et al., 1986). Probucol may prevent the generation of lipoperoxidases that have been implicated in arterial LDL oxidation due to it's structural similarity to a very powerful antioxidant, bis[3, 5-diterbutyl-4-hydroxyphenylether] propane (BHT) (Parthasarthy, 1992; Fruebis et al., 1994). Although it has been suggested that BHT may also be antiatherogenic, it does not share the same cholesterol-lowering ability as probucol (Fruebis et al.,1994).

Less than 5% of persons using this lipid-lowering therapy experience mild gastrointestinal side effects such as diarrhea, nausea, abdominal pain and flatulence (Shils et al., 1994). The main concern with probucol therapy is the possible prolongation of the QT interval and the potentiation of rhythm disorders associated with prolonged repolarization (Alexander et al., 1998). Other dermatological and ophthalmic side effects such as rash and blurred vision may also occur (Pearson, 1996). Since probucol lowers HDL cholesterol, it's use may only benefit those who have normal HDL levels.

A person's lipid profile, medical history and the side effects association with the therapy must be considered before implementing a drug regimen. It is also important to consider the cost effectiveness of the therapy (Table 1.2) since these agents may have to be taken over the course of a lifetime.

It is not uncommon for patients with hyperlipidemia to use one or more of the available agents in combination to achieve lipid goals. Nicotinic acid is a hypolipidemic vitamin that is recommended by the NCEP as a first-line treatment for hyperlipidemia due to it's cost-effectiveness and it's ability to modify all lipoproteins with the exception of chylomicrons (NCEP, 1993; Alexander et al., 1998).

Table 1.2: The annual cost of hypolipidemic therapy

Hypolipidemic Agent	Daily Regimen	Cost per year (US $)
HMG-CoA Reductase Inhibitors		
• Simvastatin	5 mg tablet - 1/day	$622.54
• Fluvastatin Sodium	20 mg capsule -1/day	$372.30
• Lovastatin	20 mg capsule -1/day	$728.72
• Pravastatin	10 mg tablet- 1/day	$625.03
Bile Acid Resins		
• Cholestyramine	4 - 9 g powder- 1/day	$483.00
• Cholestipol Hypochloride	5 g granule- 1/day	$483.00
Fibric Acid Derivatives		
• Gemfibrozil	600 mg tablet- 2/day	$399.16
• Clofibrate	500 mg tablet- 4/day	$258.27
Probucol	500mg tablet- 2/day	$814.68
Niacin (Nicotinic Acid)	500 mg tablet- 6/day	$62.20

(Pearson, 1996)

1.3 NIACIN (NICOTINIC ACID)

Niacin is considered a generic description for pyridine-3-carboxy acid and its derivatives (Van Der Berg, 1997). Nicotinic acid (pyridine-3-carboxy acid) and nicotinamide (pyridine-3-carboxyamine) are the main representatives (Figure 1.0). In North America, niacin is referred to exclusively as nicotinic acid (Basu and Dickerson, 1996).

Figure 1.0: The structure of nicotinic acid and nicotinamide

Nicotinic Acid Nicotinamide

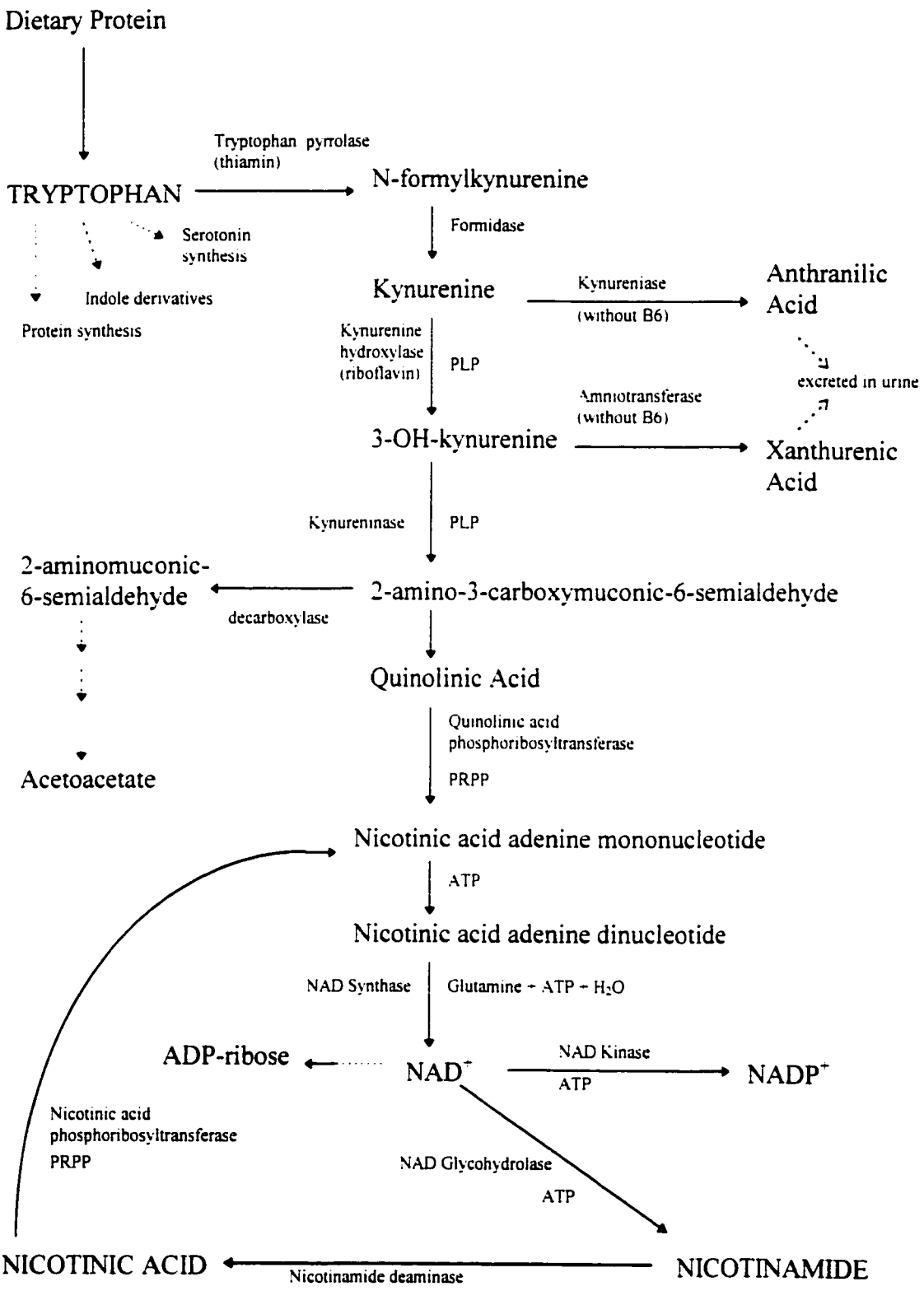
1.31 Niacin : The Nutrient

Niacin is a water soluble vitamin which is rich in dietary sources such as tuna, halibut, beef, chicken, pork and other meats, and enriched cereals (Groff et al., 1995). In addition to it's dietary sources, niacin can be synthesized in the body from the essential amino acid tryptophan (Bender and Bender, 1997; Groff et al., 1995). It's daily recommended nutrient intake (RNI) (Health Canada, 1990) is expressed as niacin equivalents (NE) (60 mg tryptophan = 1 mg niacin = 1 NE) to account for *in vivo* synthesis and dietary intake (RNI for adults = 14 to 16 NE/day). Niacin is considered a dietary essential when *in vivo* synthesis from tryptophan is interrupted (Bender and Bender, 1997).

1.32 Niacin Biosynthesis

Tryptophan is an essential amino acid that is primarily used for protein synthesis; however, 3% of dietary tryptophan is used in the synthesis of nicotinamide adenine dinucleotide (NAD) (Figure 1.1) (Groff et al., 1995). The first step in the process converts tryptophan to N-formylkynurenine by the thiamine dependent enzyme, tryptophan pyrrolase (Bender and Bender, 1997). This holo-enzyme controls the entrance of tryptophan into this irreversible oxidative pathway. The enzyme is sensitive to induction by cortisol and glucagon and is repressed by the production of NAD. N-formylkynurenine undergoes a series of reactions requiring riboflavin or vitamin B6 to form quinolinic acid. Quinolinic acid is the intermediate precursor in the synthesis of (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) from tryptophan (Henderson, 1983; DiPalma and Thayer, 1991). Niacinamide is the end product of NAD catabolism (Dietrich, 1971) and is the biologically active form since it is a constituent of the above coenzymes (Basu and Dickerson, 1996). In mammals, hepatic deaminase activity allows for the irreversible conversion of nicotinamide to nicotinic acid since no known enzyme can reverse this reaction (DiPalma and Thayer, 1991). Both nicotinic acid and nicotinamide are equally adequate precursors for NAD(P) synthesis.

Figure 1.1: The *in vivo* synthesis of NAD from tryptophan



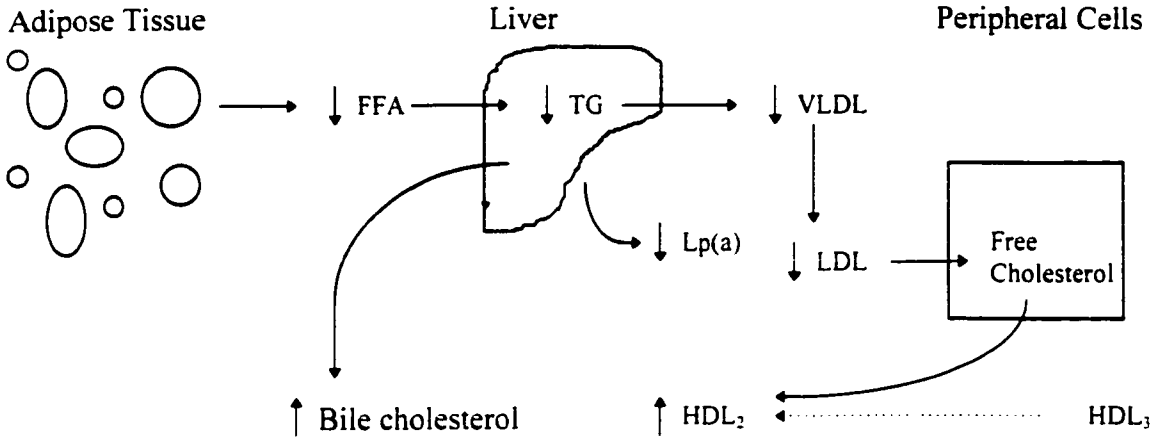
Adapted from: (Basu and Dickerson, 1996; Groff et al., 1995; Bender and Bender, 1997)

NAD and NADP serve numerous functions *in vivo* and are required primarily by dehydrogenases in oxidation reactions such as in glycolysis and the beta-oxidation of fatty acids (Groff et al., 1995). NAD(H) and NADPH+H (reduced forms) donate electrons in reduction reactions in cellular respiration and many biosynthetic pathways such as fatty acid synthesis.

1.4 NIACIN (NICOTINIC ACID): THE HYPOLIPIDEMIC AGENT

Nicotinic acid (*niacin*), but not nicotinamide, has been used as an effective broad spectrum lipid-lowering agent for decades (Yeshurun and Gotto, 1995). In pharmacological doses of 1.5 - 6g per day, niacin reduces serum LDL levels by 10-25%, concurrently raises serum HDL levels by 15-35% and lowers TG levels by 20-50% (Farmer and Gotto, 1996). Niacin is considered the most effective agent for raising serum HDL levels (Braunwald, 1997). Although the exact mechanism by which niacin normalizes lipoprotein levels has not been completely elucidated, research suggests a combination of actions to be responsible for it's lipid lowering effects (DiPalma and Thayer, 1991). These include: an inhibition of lipolysis in adipose tissue, an inhibition of hepatic VLDL production and secretion, increased serum HDL_2 levels, the lowering of serum lipoprotein (a), and increased biliary cholesterol output. An outline of the possible effects of niacin in therapeutic dose levels on lipoprotein metabolism is outlined in Figure 1.2.

Figure 1.2: An overview of the effect of pharmacological doses of niacin on lipoprotein metabolism.

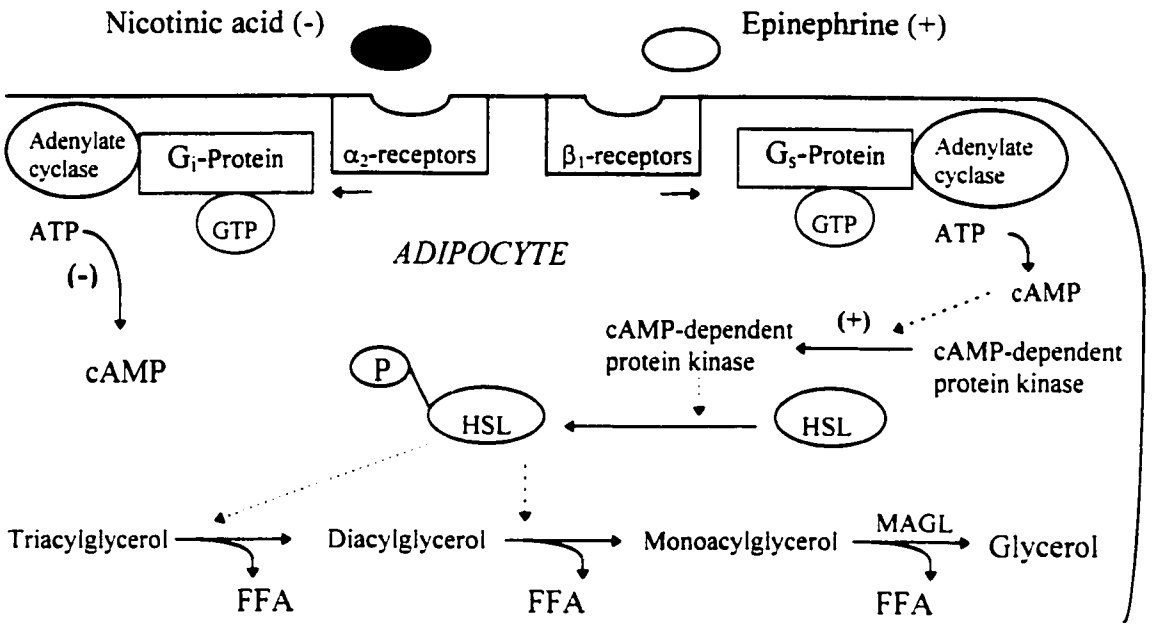


Modified from: (Knopp et al., 1985)

1.41 Inhibition of lipolysis in adipose tissue

Pharmacological doses of niacin reduce serum free fatty acid (FFA) levels through partial inhibition of lipolysis (Alexander et al., 1998; Marcus et al., 1989; Hans Jurgen; 1983; Capurso, 1991) and enhanced re-esterfication of free fatty acids FFA in adipocytes (Vick-Mo and Mjos, 1978; Wahlberg and Walldius, 1993) (Figure 1.3). Inhibition of lipolysis is mediated by adenylate cyclase coupled receptors (Marcus et al., 1989). Glucagon, epinephrine and β-corticotrophin bind to lipotic-β_1-receptors (Rs) that activate adenylate cyclase by stimulating guanine nucleotide-linked G-proteins (G_s) (Matthews and Van Holde, 1990). Adenylate cyclase increases intracellular cyclic adenosine monophosphate (cAMP) levels resulting in a cascade of events that activates cAMP-dependent protein kinase thereby activating and phosphorylating hormone sensitive lipase (HSL). HSL catalyzes that hydrolytic release of FFA from the 1- and 3- position of the glycerol backbone. Monoacylglycerol lipase (MAGL) cleaves the FFA from the 2-position thereby completing the process. The following diagram simplifies the above cascade of events:

Figure 1.3: An overview of lipolysis in the adipocyte and the proposed inhibitory action of nicotinic acid



Modified from: (Matthews and Van Holde, 1990)

Nicotinic acid inhibits lipolysis by binding to antilipolytic-α_2-receptors (Ri) on the plasma membrane (Marcus et al., 1989; Davies and Saggerson., 1981). This promotes the interaction of inhibitory G-proteins (G_i) with adenylate cyclase thus reducing intracellular cAMP levels (Matthews and Van Holde, 1990). The reduction in cAMP levels by niacin reduces mobilization of FFA from adipose tissue (DiPalma and Thayer, 1991; Groff et al., 1995; D'Costa et al., 1979).

1.42 Inhibition of hepatic VLDL production and secretion

Niacin's ability to decrease the production and release of hepatic VLDL is considered to be it's major lipid-lowering mode of action (Farmer and Gotto, 1996). The antilipolytic effect of niacin on adipose tissue reduces FFA in the blood; thus, reducing the substrates available for hepatic triglyceride synthesis (Knopp et al., 1998; Knopp et al., 1985; Groff et al., 1995; Grundy et al., 1981). This decrease in hepatic re-esterfication of FFA

reduces TG available for subsequent incorporation and release in the form of VLDL (Capurso et al., 1991; DiPalma and Thayer, 1991). Since LDL is a catabolic product of VLDL (DiPalma and Thayer, 1991), niacin's ability to reduce the number of VLDL particles released (Tornvall et al., 1990) also reduces the generation of serum LDL particles (Alexander et al., 1998; Groff et al., 1995; DiPalma and Thayer, 1991). Direct inhibition on LDL secretion has also been suggested (Knopp et al., 1998). Niacin's ability to reduce apo-protein B (apo-B), a major structural lipoprotein of VLDL and LDL, has been indicated as another possible mode of action (Tornvall et al., 1990).

1.43 Increased serum HDL_2 levels

Nicotinic acid is considered the most potent HDL raising agent available (Braunwald, 1997). Furthermore, nicotinic acid therapy results in concomitant changes in HDL class distribution (Knopp et al., 1998; Tornvall et al., 1990; Knopp et al., 1985). Niacin increases HDL_2-subclass (Tornvall et al., 1990; Knopp et al., 1998), a larger, less dense HDL sub-class formed from precursor HDL_3 (Tornvall et al., 1990; Johansson and Carlson, 1990). The exact mechanism of niacin's action is unclear; however, research suggests that niacin reduces HDL catabolism (Alexander et al., 1998; Kashyap, 1998). It is proposed that in reverse cholesterol transport, HDL binds with a hepatic receptor that removes cholesterol esters from the HDL particle (Acton et al., 1996). The HDL particle subsequently unbinds and recirculates to pick up more cholesterol from peripheral tissues. Another separate receptor mechanism removes whole HDL particles from circulation (Steinburg, 1996). It is postulated that niacin down regulates the catabolism pathway without affecting reverse cholesterol transport thus increasing serum HDL levels and apoprotein-A (apo-A) concentrations (a major structural protein of HDL) (Kashyap, 1998).

1.44 A Reduction of Serum Lipoprotein (a) Levels

Lipoprotein (a) is an abnormal genetic variant of LDL (Zeeman, 1991) that may exist in more than 90% of the human population (Albers et al., 1977). The population based Coronary Aretery Risk Development in Young Adults (CARDIA) study revealed that elevated Lp(a) levels may be race dependent and more prevalent in black populations (Marcovina et al., 1993). Lp(a) is similar to LDL in both lipid composition and the presence of apoprotein (apo) B-100 (Marcovina and Koschinsky, 1998). However, Lp(a) contains an apo-A component, a large glycosylated protein synthesized primarily by the liver, that is covalently linked to the apo-B component by one or more disulfide bridges (Gaubatz et al., 1983; Gotto 1998; Zeeman, 1991; Scannu et al., 1991). Recently elevated Lp(a) levels have been indicated in association with increased CAD-related morbidity and mortality (Yeshurun and Gotto, 1995).

Lp(a) has been indicated as a dominant genetic risk factor for CHD (Rhodes et al., 1986), especially if serum levels are elevated above 25-30 mg/dL in the presence of elevated serum LDL levels (Gotto, 1998; Armstrong et al., 1986). Since apo-A is structurally homologous to plasminogen, Lp(a) may affect the fibrinolytic pathway by binding to various matrix components including fibrin clots thus producing a prothrombic effect (Gotto, 1998; Zeeman, 1991; Marcovina and Koshinisky, 1998). Lp(a) may also undergo oxdization in the arterial wall; therefore, contributing to the formation of foam cells and the progression of atherosclerosis (Gotto, 1998).

Nicotinic acid is the only antihyperlipidemic agent that lowers Lp(a) levels (Gotto, 1998; DiPalma and Thayer, 1991). The ability of nicotinic acid to reduce serum in Lp(a) levels has been shown in strong linear correlation ($r = 0.88$) with the concurrent reduction in serum LDL levels (Carlson et al., 1989). Since Lp(a) is not a by-product of VLDL or LDL metabolism, niacin's ability to reduce the common structural lipoprotein, apo-B (Tornvall, 1990), has been suggested as niacin's mode of action.

1.45 Increased biliary cholesterol output

Nicotinic acid therapy induces a small, yet significant increase, of hepatic secretion of cholesterol into bile (Grundy et al., 1981; Angelin et al., 1979). The biliary cholesterol pool consists of pre-formed hepatic cholesterol with a small contribution from *de novo* synthesis (Holland et al., 1993). Since niacin lowers serum TC levels it does not stimulate HMG-CoA reductase and it decreases hepatic neosynthesis (Holland et al., 1993), it has been postulated that the slight increase in cholesterol output is due to increased mobilization of cholesterol from peripheral tissue (Okoliscanyi et al.,1986; Grundy et al., 1981). Holland et al (1993) suggest that increases in hepatic cholesterol secretion may be linked to niacin's ability to reduce cAMP concentrations in adipose tissue (Marcus et al., 1989).

Elevated intracellular hepatic concentrations of cAMP have been shown to inhibit biliary lipid output (Hamlin et al., 1990). Since niacin reduces adipocyte cAMP levels, it may also reduce hepatic cAMP concentrations and hence increases biliary pre-formed cholesterol output (Holland et al., 1993). This small increase in bile cholesterol output does not alter the physical composition of bile (Grundy et al., 1981: Holland et al., 1993; Mok and Grundy, 1977); thus, it is doubtful that niacin enhances gallstone development. The Coronary Drug Project (1975) found modest increases of cholelithiasis with niacin treatment; but, the findings were not statistically significant. Despite this, gall bladder disease is contraindicated with pharmacological doses of niacin since gall stone formation is still considered a possible side effect of niacin therapy.

1.46 Potential adverse effects of niacin therapy

Niacin is a broad spectrum hypolipidemic agent (Yeshurun and Gotto, 1995) that has the unique ability to reduce all atherogenic lipoproteins (Gotto, 1998). However, like all lipid-lowering agents, therapeutic doses of niacin may cause several untoward effects. The primary side effects associated with niacin therapy are cutaneous flushing (trunical

and facial) and gastrointestinal disturbances (nausea, vomiting, diarrhea, anorexia) (Illingworth et al., 1994; Illingworth. 1997; Farmer and Gotto, 1995; Knopp 1998). Flushing is secondary to the release of endogenous prostacyclin (PGI_2), an eicosanoid synthesized in the vascular endothelium by transmembrane transferase of prostaglandin endoperoxides from platelets or from intracellular membrane-bound arachidonic acid (Luria, 1990; Gryclewski et al., 1988). Pre-administration of acetyl salicylic acid (ASA), a prostaglandin inhibitor, deters the vasodilation and flushing response to niacin (Alexander et al., 1998; Farmer and Gotto, 1996; Yeshurun and Gotto. 1995). Cutaneous flushing is dose dependent; may occur 15 minutes to two hours after the hyperlipidemic dose; and diminishes within weeks of treatment (Illingworth. 1987; Gibbons et al., 1995; CPS, 1997). It is recommended that individuals commence niacin treatment by taking 100-250 mg of niacin twice per day with meals followed by dose increases of 250 mg per day at 7-14 day intervals (CPS, 1997; Illingworth. 1987). This titration up to the lipid lowering dose of 1.5g-6g nicotinic acid per day reduces non-compliance due to gastrointestinal and dermatological side effects.

Therapeutic doses of nicotinic acid may induce other dermatological side effects such as puritis, hyperpigmentation, itchiness, dry skin, rash, and acanthosis nigricans (Yeshurun and Gotto. 1995). Other metabolic and ophthalmological side effects may occur. The Coronary Drug Research Project (1975) reported significant increases in cardiac arrhythmia with niacin therapy. Niacin may also induce mild and occasionally moderate/severe hyperglycemia (Schwartz. 1993); therefore. diabetes mellitus is contraindicated to the use of niacin (CPS. 1997; Etchason et al., 1991). Research has demonstrated that niacin induces insulin resistance without adversely affecting pancreatic β-cell function (Kahn et al., 1989) or stimulating insulin secretion (Rizza et al., 1985). It is postulated that niacin may stimulate hepatic glucose output that outweighs peripheral glucose utilization (Wahlberg et al., 1992; Garg and Grundy. 1990), or niacin may act directly on peripheral tissues to induce glucose resistance (Kahn et al., 1989). The cellular mechanisms have yet to be identified.

Pharmacological doses of nicotinic acid may also exacerbate peptic ulcers and may cause dose-dependent hyperuricemia and gouty arthritis (Etchason et al., 1991; Capurso, 1991; CPS, 1997; Farmer and Gotto, 1996; Illingworth et al., 1994). Nicotinic acid may induce reversible ocular symptoms such as maculopathy, characterized by retinal cysts and blurred vision (Millay, 1988), dry eyes and swollen discoloured eyelids (Fraunfelder et al., 1995). Research suggests that an accumulation of intracellular fluid is responsible for adverse rare ophthalmic complications (Jampol, 1988).

1.47 Hepatotoxicity

The less common, but potentially more serious untoward effect of niacin therapy, is a dose-related hepatotoxicity (Gray et al., 1994). Liver manifestations are often asymptomatic; however, elevations in alkaline phosphatase (ALKP), alanine transaminase (ALT) and bilirubin concentrations are reported to occur with prolonged treatment at high doses (Alexander et al., 1998; Yeshurun and Gotto, 1995; Illingworth, 1988; Etchason et al., 1991). Niacin-induced hepatitis is usually reversible after discontinuation of niacin therapy and its occurrence is most prevalent with the use of sustained-release (SR) preparations of nicotinic acid (Yeshurun and Gotto, 1995). SR-niacin preparations are compressed nicotinic acid formulations with an inert resinous base that releases nicotinic acid over a period of several hours (Rader et al., 1992). SR-niacin affects liver enzyme systems longer than crystalline or immediate-release (IR) preparations of niacin. (Heudebert et al., 1993; Knopp et al.,1998). Differences in the excretory mechanisms provide the rationale for the more toxic effects of SR preparations (See Section 1.48). SR-preparations reduce cutaneous flushing and exhibit more potent LDL lowering effects than crystalline preparations (McKenny et al., 1994). However, SR-niacin does not effectively increase serum HDL levels (Knopp et al., 1985), may induce hepatitis at low doses of < 2g/day (Etchason et al., 1991) and may attenuate more severe gastrointestinal side effects (Mckenny et al., 1994).

Crystalline niacin is a better alternative to SR-niacin preparations since it is less likely to cause hepatotoxicity, especially if taken in lipid lowering doses of < 3g/day (Gray et al., 1993; Figge et al., 1993). Recent studies have demonstrated that the long-term use of IR-nicotinic acid (2-3g/day) for hyperlipidemia does not induce any changes in liver function (Henkin et al., 1991; Mckenny et al., 1994). Other research has shown that in cases where SR-niacin induces hepatic dysfunction, rechallenging individuals with IR-niacin returns hepatic enzymes to normal levels (McKenny et al., 1994; Henkin et al., 1990). However, switching from high doses of IR-nicotinic acid (6g/day) to SR-preparations (3g/day) in an effort to improve flushing side effects, has been shown to cause fulminant hepatic failure (Etchason et al., 1991); thus there is great concern about the unmonitored use of readily available non-prescription SR-niacin preparations (Henkin et al., 1991; Rader et al., 1992). As with all hypolipidemic agents, it is recommended thatl individuals taking hypolipidemic doses of niacin be monitored for changes in liver enzyme levels at regular intervals (Farmer and Gotto, 1996; Gray et al., 1994).

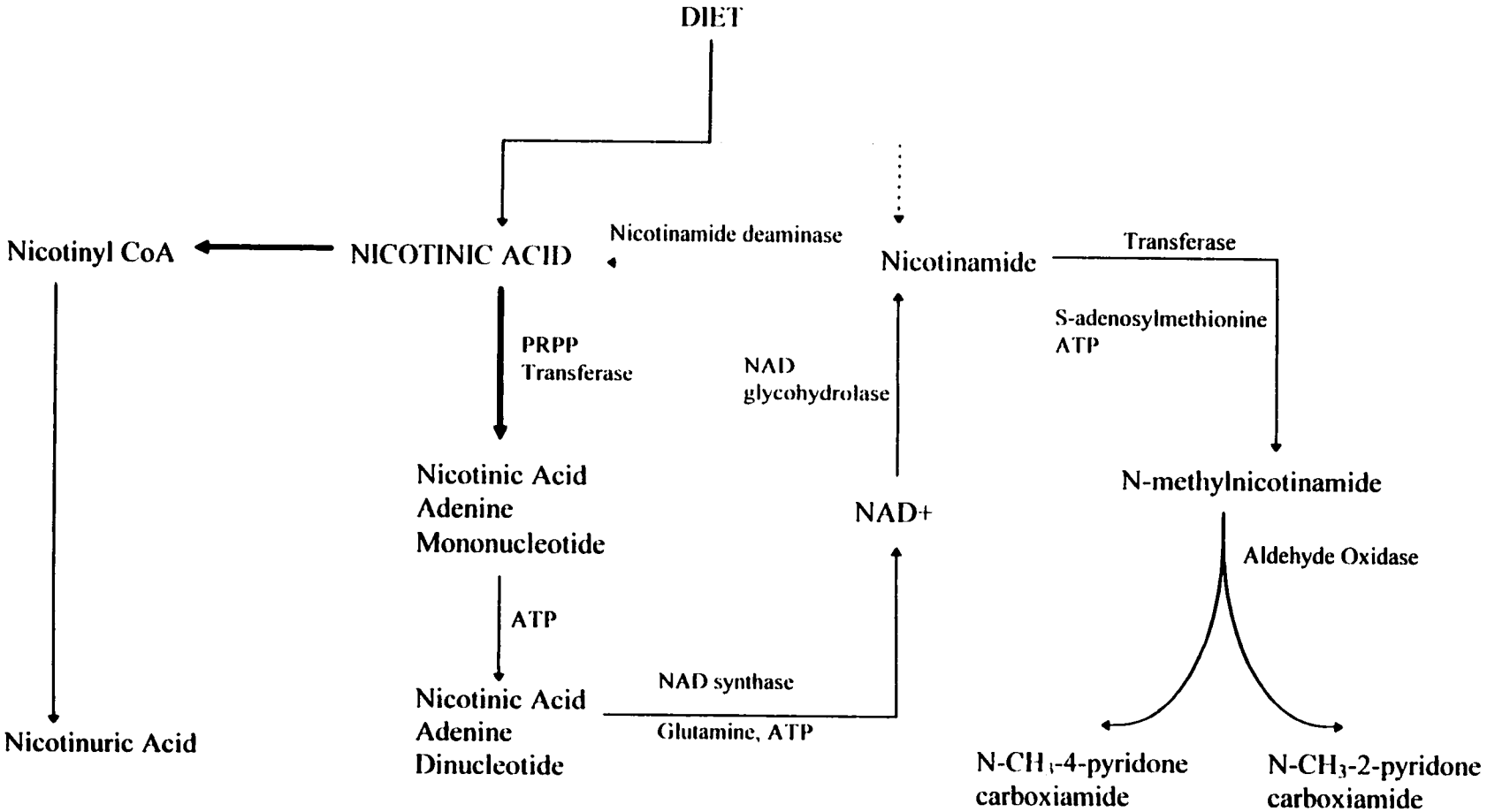
1.48 Nicotinic Acid Metabolism

Niacin, a water soluble vitamin, is not stored in the body beyond it's tissue saturation level (Groff et al., 1995). It is absorbed from the small intestine by a sodium-dependent saturable carrier (Bender and Bender, 1997); but at pharmacological doses, there may be a significant amount of passive diffusion. Nicotinic acid can be metabolized in two pathways: (1) the conversion of nicotinic acid to NAD (through sequential reactions with 5-phosphoribosyl-1-pyrophosphate (PPRP), adenosine triphosphate (ATP) and glutamine); subsequent degradation of NAD to nicotinamide at the level of the nucleotide; methylation and/or oxidation to yield N-methylnicotinamide or methylated-pyridones as excretory products or (2) the formation of nicotinyl coenzyme A (CoA) and subsequent conjugation with glycine to produce nicotinuric acid (Figure 1.4) (Mrochek et al., 1976; Jacobson et al., 1995; Stern et al., 1992). The primary metabolites produced are N-methylnicotinamide (20-30%) and N-methyl-2-pyridone-5-carboxyamide (40-60%) (Groff et al., 1995).

Pharmacological doses of IR-niacin significantly increases N-methyl-2-pyridone-5-carboxyamide excretion more than SR-release preparations (Stern et al., 1992). However, at higher doses approaching 3g per day, N-methyl-nicotinamide concentrations may increase (Henderson, 1983) and drug administration rates may exceed the capacity of the non-conjugated pathway (Morgan et al., 1998; Stern et al., 1992). Therefore, when saturation occurs nicotinuric acid concentrations increase. It is postulated that increased hepatotoxicity occurs more with SR-preparations because fewer conjugated metabolites are produced; the delivery rate to the liver is slower and recovery periods between doses are poorer (Morgan et al., 1998).

The non-conjugated pathway of excretion involves the methylation of nicotinamide to N-methylnicotinamide and subsequent oxidation to pyridones in the presence of aldehyde oxidase (Shibata and Matsuo, 1989). The methyl group required is donated by S-adenosylmethionine (SAM), an intermediate in the production of homocysteine from methionine. By theory, it can be proposed that increased doses of IR-nicotinic acid may increase the demand for SAM and adversely affect homocysteine metabolism. A recent study by Basu and Mann (1997) found significant increases in urinary and plasma homocysteine levels in Sprague-Dawley rats after three weeks of dietary supplementation of pharmacological doses (1g /kg diet) of niacin. Serum elevations of homocysteine have been implicated as an independent risk factor for the development of arterial and venous disease (Boers, 1997a). Thus, it is important to determine if hypolipidemic doses of niacin renders similar effects in humans.

Figure 1.4: An overview of nicotinic acid metabolism



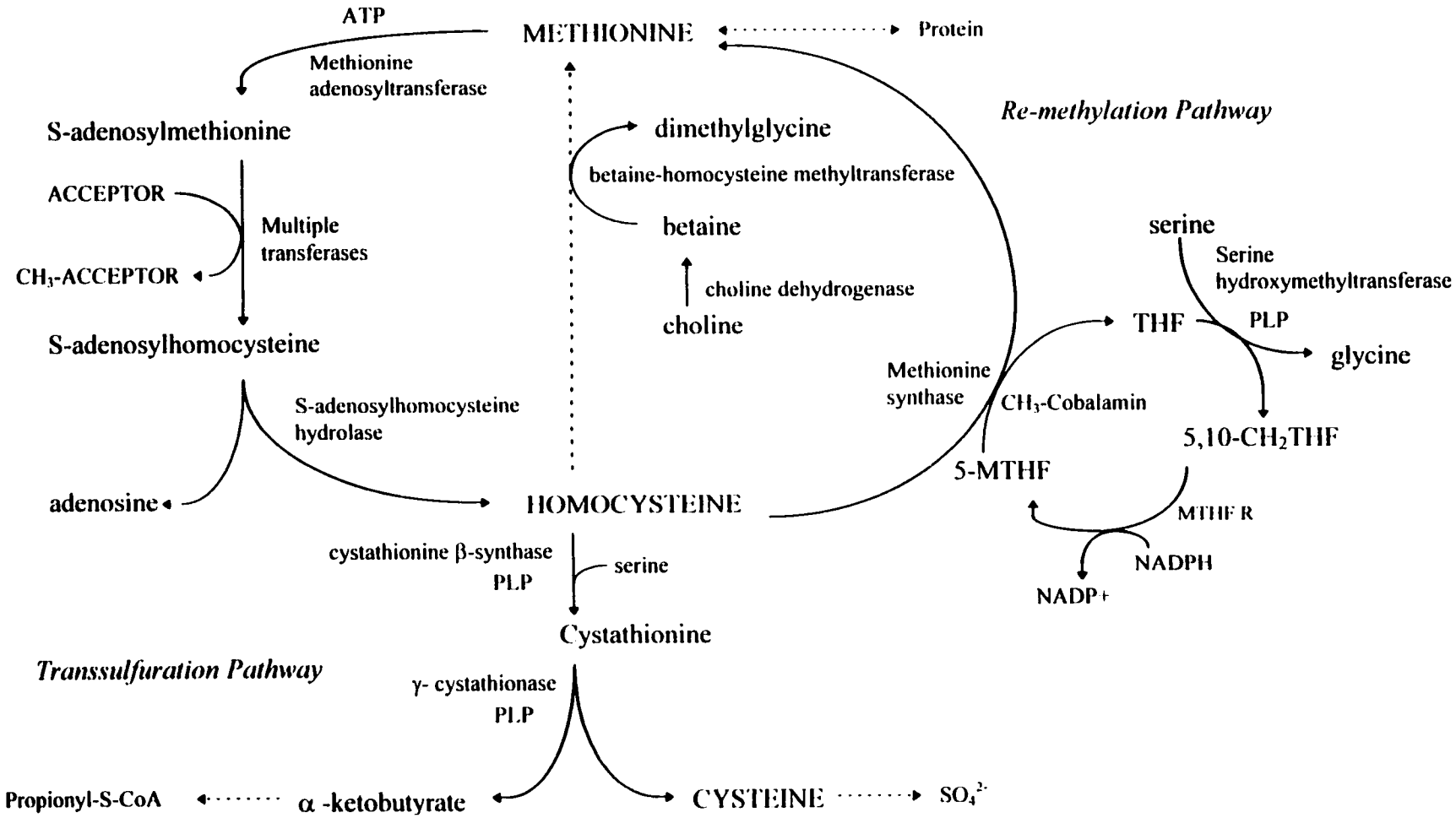
(Adapted from: Bender and Bender, 1997; Stern et al., 1992)

1.5 HOMOCYSTEINE

Homocysteine is a sulfhydryl amino acid derived from the metabolism of methionine (Cattaneo, 1997; Welch et al., 1997). This essential amino acid is incorporated into protein or is catalyzed by the ATP dependent enzyme, S-adenosyltransferase, to yield S-adenosylmethionine, SAM (Figure 1.5) (Ueland et al., 1992; Fowler, 1997). A small fraction (<10%) of SAM undergoes decarboxylation for polyamine synthesis (Ueland et al., 1992). SAM primarily functions as a methyl donor for methyltransferase in many physiological reactions (e.g. the methylation of DNA, RNA, protein) (Finkelstein, 1998; Allen et al., 1998) including the methylation of nicotinamide for urinary excretion (Shibata and Matsuo, 1989). The de-methylated product, S-adenosylhomocysteine (SAH), is hydrolyzed to yield adenosine and homocysteine (Carson, 1982).

Methionine metabolism is the only source of homocysteine in humans since this metabolite is not found the diet (Finkelstein, 1988). Homocysteine can be metabolized in two ways: (1) it is degraded in an irreversible transsulfuration pathway and (2) it is re-methylated to methionine by either betaine-homocysteine methyltransferase (BHMT) or methionine synthase (Verhoef et al., 1996). Homocysteine metabolism, however, is regulated by cellular methionine and SAM concentrations. When methionine is in excess, the transsulfuration pathway is favoured due to an increase in SAM production (Fowler et al., 1998; Fowler, 1997; Allen et al., 1998). SAM, in a subsequent reaction, directly stimulates cystathionine β-synthase (CBS), the enzyme that catalyzes the first step in the transsulfuration sequence. However, SAM can also inactivate BHMT and methylenetetrahydrofolate reductase (MTHFR) thereby inhibiting re-methylation pathways. Cystathionine β- synthase, a pyridoxal -5' - phosphate (PLP) dependent enzyme, catalyzes the condensation of homocysteine with serine to form cystathionine (Ubbink, 1997). Subsequently, cystathionine is hydrolysed by another PLP-dependent enzyme, γ-cystathionase, to form cysteine and α-ketobutyrate. The irreversibility of this transsulfuration pathway offers an explanation as to why cysteine is not a precursor for methionine synthesis (Finkelstein, 1990).

Figure 1.5: Homocysteine metabolism



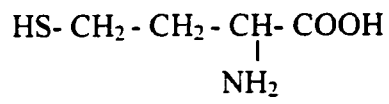
Adapted from: (Selhub and Miller, 1992; Allen et al., 1998)

When methionine concentrations fall and SAM production is low, homocysteine can be re-methylated via two pathways to conserve methionine concentrations (Allen et al., 1998; Fowler, 1998). Betaine (trimethylglycine), an oxidized product of choline, can donate a methyl group to homocysteine in the presence of BHMT to form methionine. Homocysteine may also undergo re-methylation in a B12-dependent reaction that requires the transfer of a methyl group from N^5 -methyltetrahydrofolate (5-MTHF) to homocysteine (Ubbink, 1997). Tetrahydrofolate (THF) is converted to N^5N^{10}-methyleneTHF (5,10-CH_2 THF) that is further methylated to 5-MTHF by MTHFR. Cobalamin (B12) binds with the enzyme methionine synthase and transfers the methyl group from 5-MTHF to homocysteine thus resulting in methionine formation (Groff et al., 1995) and THF regeneration (Finkelstein, 1990). This dominant re-methylation pathway occurs in all mammalian tissues (Finkelstein, 1998; Boers, 1994), whereas the BHMT pathway is confined only to the liver (Refsum et al., 1998).

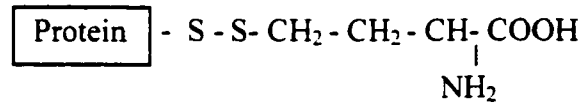
Homocysteine can be found in many forms *in vivo*. The most dominant form in plasma (70%) is bound to the protein albumin via a disulfide bond (Still and McDowell, 1998; Refsum et al., 1997). Approximately 30% of plasma homocysteine exists as free-oxidized low molecular weight disulfide fractions (Ueland, 1995; Refsum et al., 1998). Free-homocysteine combines with other homocysteine species to form dimers (homocystine) or with other thiols, predominately cysteine, to yield homocysteine-cysteine mixed disulfides (Figure 1.6). The free-reduced sulfhydryl form exists only in trace amounts. Since homocysteine steadily fluctuates between all forms (Still and McDowell, 1998), it is recommended that total plasma homocysteine concentrations be measured to determine it's meaningful status (Boers, 1994).

Figure 1.6: Structures of homocysteine species *in vivo*

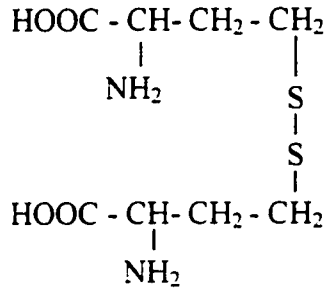
Homocysteine



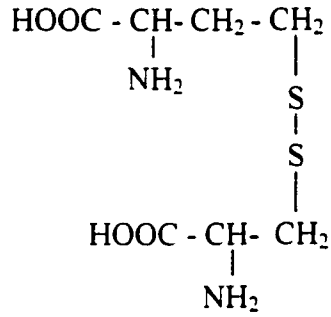
Protein- bound homocysteine



Homocystine (Homocysteine-homocysteine disulfide)



Homocysteine -Cysteine mixed disulfide



** The term *homocysteine* encompasses all forms found *in vivo*. Adapted from: (Ueland 1995; Malinow et al., 1999)

Plasma total homocysteine (*homocysteine*) is a sensitive marker of intracellular homocysteine metabolism (Boers, 1994; Townend, 1998; Ueland, 1993). The normal reference range for the adult population is 5 -15μmol/L with a mean concentration of 10μmol/L (Bellamy and McDowell, 1997; Refsum et al., 1997). Since increasing age has been associated with slight increases in homocysteine concentrations (towards the upper limit of the normal reference range), age related normal values have also been suggested (Kang et al., 1986; Andersson et al., 1992). Plasma homocysteine levels may also be linked to gender and menopausal status (Andersson et al., 1992). Other researchers suggest that normal homocysteine reference ranges should be based on subjects replete in folate (Rossi et al., 1999) since the upper limit of the normal range may be as low as 12 μmol/L in individuals with adequate B-vitamin status (Ubbink, 1995). Although reported reference levels vary slightly, hyperhomocysteinemia is generally considered when the concentrations exceed 15μmol/L (Kang, 1996; Kang et al, 1992). Hyperhomocysteinemia is defined as moderate if plasma concentrations are between 16-30μmol/L, intermediate if

concentrations are between 30-100μmol/L and severe when concentrations are >100μmol/L.

1.6 HYPERHOMOCYSTEINEMIA

Hyperhomocysteinemia, a condition associated with the development of thromboembolism and vascular disease, may be an acquired condition or may develop as a result of rare genetic enzymatic deficiencies. There are three autosomal recessive inborn errors of metabolism that cause an accumulation of homocysteine in plasma and subsequently homocystinuria: (1) Cystathionine -β-synthase (CBS) deficiency (2) Methylenetetrahydrofolate reductase (MTHFR) deficiency and (3) methionine synthase deficiency (Mudd et al., 1995).

1.61 CBS Deficiency

CBS deficiency is the most prevalent inherited enzymatic disorder of transsulfuration homocysteine metabolism causing homocystinuria (Mudd et al., 1995). This metabolic disorder has a prevalence of 1:335000 worldwide occurring mostly in populations of Irish and Italian descent (Naughten et al., 1998). CBS is a heme protein (Kery et al., 1994) which is genetically linked to chromosome 21 and is allosterically regulated by SAM (Krauss, 1998). Mutations of this important enzyme reduce the affinity of the enzyme for PLP, serine and homocysteine (Guba et al., 1996). Cysteine production is reduced and hence it becomes a dietary essential (Walter at al., 1998). Since CBS defects are the most common cause of severe hyperhomocysteinemia (Cattaneo, 1999), persons suffering from this condition may exhibit fasting plasma homocysteine levels as high as 500μmol/L (Mudd et al., 1995) accompanied by increased levels of methionine. The latter is the reflection of an increased rate of homocysteine re-methylation (Pollitt, 1987).

Research has elucidated that *homozygous* recessive CBS mutations are associated with the development of vascular occlusions, premature arteriosclerosis and thrombosis (Mudd

et al., 1995). The probability of thromboembolic events occurring before the age of 20 years is between 30% and 60% before the age of 40 years (Mudd et al., 1995). Other clinical manifestations associated with homozygous CBS defects include: ocular defects (Gerding, 1998), skeletal abnormalities, psychiatric disorders and CNS (central nervous system) problems (Guba et al., 1998; Franchis, 1998; Pollitt, 1987; Mudd et al., 1985). *Heterozygous* CBS mutations occur in 0.3% of the population (Malinow, 1994) where the CBS enzymatic is reduced by 50%, however, these mutations do not exhibit any phenotypical symptoms (Mudd et al., 1995). Heterozygosity was originally thought to be a mild risk factor for hyperhomocysteinemia (Boers, 1985), but recent studies have shown otherwise (Kluijtmans et al., 1996; Engbersen et al., 1995). Research has also shown that heterozygous CBS mutations are not associated with increased risk of cardiovascular disease (Kluijtmans et al., 1996; Wilcken et al., 1983).

1.62 MTHFR Deficiency

Inherited defects of re-methylation account for 5-10% of cases of severe hyperhomocysteinemia leading to homocystinuria (Cattaneo, 1997; De Stefano et al., 1996). These rare MTHFR mutations decrease enzymatic activity and the availability of 5-MTHF for the major homocysteine re-methylation pathway (Townend et al., 1998; Pollitt, 1987) leading to hyperhomocysteinemia and low to normal methionine levels (Goyette et al., 1995). Generally, observed homocysteine levels are slightly lower than those observed with CBS mutations (Kang et al., 1993). Persons with severe MTHFR deficiency have extremely low MTHFR activity (0-20%) and may exhibit a variety of phenotypical symptoms including developmental delay, motor abnormalities and psychiatric disturbances (Goyettte et al., 1995). However, a less severe but more common C667T genetic variant of MTHFR (resulting in the substitution of alanine resides with valine residues) (Frosst et al., 1995; Kang, 1991) that is characterized by the absence of neurological abnormalities has been identified (Guba et al., 1998). This *thermolabile* variant occurs in both homozygous and heterozygous forms and is characterized by decreased enzymatic activity ($\leq$ 50%) at 37°C exhibiting decreased

thermostability at 46°C in comparison to the normal enzyme *in vitro* (Frosst et al., 1995; Engbersen et al., 1995; Kang et al., 1993; Kang et al., 1988). This *homozygous thermolabile* variant is the most common inherited disorder of folate metabolism (Verhoef et al., 1998; Guba et al., 1998) occurring in approximately 5% of the general population (Malinow et al., 1994; Kang, 1991). Individuals of European and Japanese descent as well as French Canadians have a prevalence rate as high as 14 -15% (Motulsky, 1996; Christensen et al., 1997) whereas Dutch (5.2%) and African Americans (1.4%) have lower frequencies of occurrence (Kluijtmans et al., 1996).

Homozygous thermolabile MTHFR individuals develop higher homocysteine elevations than individuals with normal or heterozygote mutations (Verheof et al., 1998; Frosst et al., 1995; De Franchis et al., 1996). However, elevated homocysteine levels are not found in all individuals with ≤ 50% MTHFR activity (Cattaneo, 1999). Recent investigations have found that hyperhomocysteinemia occurs only with homozygous individuals with low folate status (Verhoef et al., 1997; Guttermsen et al., 1996; Ma et al., 1996), suggesting that affected individuals may have higher folate requirements (Townend et al., 1998). Homozygous thermolabile C667T mutations have also been found in positive association with premature vascular disease and a higher incidence of CAD (Kang et al., 1993; Kang, 1991; Gallagher et al., 1996). Kluijtmans et al. (1996) demonstrated that homozygosity may be associated with a threefold increase of CVD risk over normal control subjects; however, these finding are not consistent. New evidence suggests that these homozygous mutations are not significant risk factors for CAD and do not constitute risk for transient ischemic attack or stroke (Ma et al., 1996; Deloughery, 1996; Lalouschek et al., 1999; Van Bockxmeer et al., 1997).

1.63 Deficiency of methionine synthase

Methionine synthase is an important B12 dependent enzyme that catalyzes the re-methylation of homocysteine to methionine (Dixon et al., 1996). Functional deficiencies of methionine synthase are secondary to inherited disorders of cobalamin metabolism,

characterised by defects in the formation of methyl-cobalamin (De Baulny et al., 1998; Ueland, 1992; Surtees, 1998). Cobalamin mutations involving homocysteine re-methylation are accompanied by hyperhomocysteinemia and clinical symptoms such as psychomotor impairment, lethargy, failure to thrive, megaloblastic anemia and thromboembolic disease (Gulati et al., 1996; Ueland et al., 1992). Disturbed function of methionine synthase may also result from malabsorption disorders, decreased intrinsic factor (IF) associated with old age, or poor nutrition (Fowler, 1998; Fowler 1997).

1.64 Other determinants of hyperhomocysteinemia:

Nutritional deficiencies of folate, vitamin B6 and B12, modulators of homocysteine metabolism, are the primary causes of acquired mild to moderate hyperhomocysteinemia (Cattaneo, 1999). A deficiency of vitamin B6 impairs the transsulfuration pathway, the only degradation pathway available for homocysteine (Townend, 1998). Deficiencies of folate and vitamin B12 impair intracellular homocysteine re-methylation. Research has consistently identified compromised folate and B12 status as strong determinants of hyperhomocysteinemia (Shelub et al., 1996; Shelub et al., 1993; Ubbink et al., 1997; Dalery et al., 1995; Guttermsen et al., 1996). Other research has indicated that sub-optimal circulating levels of vitamin B6, vitamin B12 and folate, alone or in combination, are inversely correlated with elevated homocysteine levels and the development of CAD (Robinson et al., 1995; Shelub et al., 1995; Dalery et al., 1995; Ubbink et al., 1993; Kang et al., 1987). Supplementation of these vitamin co-factors is a cost-effective means to lower plasma homocysteine concentrations and reduce CAD risk (Ubbink et al., 1993a) (See Section 1.72).

Hyperhomocysteinemia is associated with clinical disease states such hypothyroidism, psoriasis, and renal disease (Ueland et al., 1992). Hypothyroidism has been shown to be a strong predictor of homocysteine elevations possibly due to increased creatinine methylation or reduced MTHFR activity (Nedrebo et al., 1998). It is postulated that individuals suffering from psoriasis exhibit elevated homocysteine levels due to an

increased folate demand for rapid cell proliferation (Ueland et al., 1992). In the case of chronic renal failure, the extent of the condition correlates with homocysteine elevations (Friedman et al., 1995). Levels may escalate as high as two to four times higher than normal and may only be lowered slightly with dialysis treatment (Chauveau et al., 1993; Bostom et al., 1995b). Research has implicated reduced renal clearance of homocysteine and impaired renal homocysteine metabolism as the possible mechanisms behind homocysteine elevations (Chauveau et al., 1993; Boston et al., 1995a).

Hyperhomocysteinemia has been found in association with life-style factors (summarized in Table 1.3) such as smoking, alcoholism and excessive coffee consumption. Research suggests a dose-dependent relationship between elevated homocysteine levels and the number of cigarettes smoked per day. (Nygard et al., 1995). Heavy smokers (>20 cigarettes per day) may exhibit levels 20% higher than persons who have never smoked. Chronic alcohol consumption may induce homocysteine levels twice as high as non-drinkers due to a poor diet and subsequent compromised folate and PLP status (Cravo et al., 1996). Elevations of homocysteine have also been observed in cases of high coffee consumption (>9 cups per day) (Nygard et al., 1998). A positive dose-dependent relationship between coffee consumption and homocysteine concentrations has been identified (Nygard et al., 1998; Stolzenberg-Solomon et al., 1999).

Table 1.3: Causes of Hyperhomocysteinemia

Genetic:	Disease:
• CBS deficiency	• renal failure
• MTHFR deficiency	• hypothyroidism
• methionine synthase deficiency	• psoriasis
Acquired:	Drug induced:
• sub-optimal intakes of folate, vitamin B6 and/or vitamin B12	• oral contraceptive
• alcoholism	• methotrexate
• heavy cigarette smoking	• nitrous oxide
• excessive coffee consumption	• niacin ???

Adapted from: (Van Den Berg and Boers, 1996; Townend, 1998)

Other factors influencing homocysteine levels include drugs interfering with folate metabolism (e.g. methotrexate), agents interfering with B12 metabolism (e.g. nitrous oxide) (Malinow, 1994) and agents influencing B6 metabolism (e.g. oral contraceptives) (Ueland et al., 1992). Since pharmacological doses of niacin may increase the demand of SAM for nicotinamide methylation and excretion, niacin may be another agent that facilitates homocysteine accumulation. It is important to identify agents that induce hyperhomocysteinemia since homocysteine has been shown to be an independent risk factor in the development of atherosclerotic vascular disease.

1.7 HYPERHOMOCYSTEINEMIA: A RISK FACTOR FOR CVD

In recent years, mild hyperhomocysteinemia has attracted much attention. Many clinical investigations have been performed in an effort to determine the relationship between homocysteine and the risk of CVD. Evidence of hyperhomocysteinemia as an atherosclerotic risk factor is derived from the collective consideration of recent case-control, cross-sectional and prospective studies.

A case-control based meta-analysis of 27 studies by Boushey et al. (1995) identified hyperhomocysteinemia as a strong independent risk factor for CAD, cerebrovascular disease (CBD) and peripheral vascular disease (PVD). The results of this study suggest that the risk distribution between hyperhomocysteinemia and CVD may be graded, much like that of hypercholesterolemia. It has been found that a 5μmol/L increment increase in homocysteine levels elevates an individuals risk of CAD by 40%, a risk comparable to that of a 0.5 mmol/L increase in TC concentrations (Boushey et al., 1995). The European Concerted Action Project (ECAP), a multi-centre case-control investigation of 700 individuals with vascular disease and matched controls, also identified mild hyperhomocysteinemia as a graded independent risk-factor for vascular disease (Graham et al., 1997). The ECAP determined that the risk associated with mild hyperhomocysteinemia is equivalent in strength to hypercholesterolemia and smoking. A follow-up logistic regression analysis of the ECAP further confirmed that increased risk

is associated with fasting homocysteine levels as low as 12 μmol/L (Robinson et al., 1998). Other case-control studies have also found supportive evidence of the positive association between homocysteine and atherosclerosis and vascular thrombosis (Den Heijer et al., 1998; Den Heijer et al., 1996; Malinow, 1996; Falcon et al., 1994; Cheng et al, 1997).

Several cross-sectional studies have identified hyperhomocysteinemia as a significant risk factor for various atherosclerotic diseases. Verhoef et al. (1997) demonstrated a positive linear relationship between elevated homocysteine concentrations and atherosclerosis over a wide range of plasma concentrations. Mean fasting homocysteine levels were 9% higher in individuals with coronary occlusions than in the control group. A study by Shelub et al. (1995) found a graded risk response between homocysteine concentrations and the development of extracranial carotid-artery stenosis in the elderly participants of the Framingham Heart Study. Clarke et al. (1991) also found evidence in support of the for the 'homocysteine theory of atherosclerosis'. Homocysteine elevations, up to three times higher than controls, were detected in approximately 30% of individuals with vascular disease. Research has indicated that the plasma homocysteine elevations not only increase vascular disease risk but facilitate disease progression (Taylor et al., 1996). In summary, the above cross-sectional studies and others (Van den berg et al., 1996; Von Eckardstein et al., 1994; Shelub et al., 1996) provide evidence that hyperhomocysteinemia is possibly an independent graded risk-factor for cardiovascular disease. The strongest supportive evidence, however, originates from recent prospective investigations (Bellamy and McDowell, 1997).

Few prospective investigations have failed to identify significant associations between homocysteine and CVD (Evans et al. 1997; Verhoef et al., 1997). The Physicians Health Study, a large prospective study involving >14000 male physicians with no prior vascular disease, determined that individuals who developed myocardial infarction (MI) (n=271) had significantly higher mean baseline plasma homocysteine levels than matched controls who remained free from MI (Stampfer et al., 1992). A threefold increase in risk for MI

was associated with homocysteine concentrations in the top 5% of the homocysteine distribution (>15.8μmol/L). The Norwegian Tromso Study involving >20000 individuals found that the mean plasma homocysteine level (12.7 μmol/L) of the subjects that developed MI (n=271) was also significantly higher than controls (11.3 μmol/L) (Arnesen et al., 1995). The logistic regression analysis of this study identified homocysteine as an independent risk-factor of greater strength than diabetes, TG levels and hypertension.

The relationship between homocysteine and CVD mortality has also been a topic of clinical investigations. The Homocysteine and Progression of Atherosclerosis Trial followed 351 individuals for a period up to 78 months (Taylor et al., 1999). After adjustment for a variety of risk factors, it was determined that a 1μmol/L increase in plasma homocysteine increases the risk of death from cardiovascular disease by 5.6%. Hyperhomocysteinemia was also associated with the clinical symptomatic progression of CHD, CBD and lower extremity occlusive disease. Nygard et al. (1997) also found a strong graded relationship between homocysteine levels and mortality in 587 men followed over a period of 4.6 years. The mortality estimate for homocysteine levels >15μmol/L was 24.7%, whereas the risk associated with levels < 9μmol/L was only 3.8%. According to Malinow et al. (1999), the best estimate of CAD mortality associated with mild hyperhomocysteinemia comes from a meta-analysis by Omenn et al. (1998). Researchers concluded that the relative risk of mortality with homocysteine levels > 15μmol/L and levels < 10μmol/L is similar to the difference in risk associated with plasma TC levels between 4.6 and 7.1 mmol/L.

Collective consideration of the above studies and others (Wald et al., 1998; Motulsky et al., 1998) provides substantial evidence that hyperhomocysteinemia is an independent graded risk-factor for various forms of CVD and it's related mortality (Boers et al., 1997a; Boers, 1997b). Therefore, the treatment of hyperhomocysteinemia (Section 1.72) and proposed pathological mechanisms of action have become the focus of recent investigations.

1.71 Proposed pathological mechanisms of homocysteine

The exact pathophysiological mechanism responsible for the association between hyperhomocysteinemia and atherosclerotic vascular disease is not completely understood (Cattaneo et al., 1999; Van den Berg and Boers, 1996; Lentz et al., 1996). Observed vascular changes associated with the condition may be multi-factorial (Guba et al., 1996). Recent *in vitro* and *in vivo* studies have identified several plausible mechanisms. Homocysteine may promote thrombosis by exhibiting adverse effects on the endothelium, LDL-C, coagulation factors and smooth muscle cells (Ueland et al., 1992).

Studies have provided evidence that homocysteine has a cytotoxic effect on endothelial cells. It's copper-catalyzed oxidation leads to a reduction of O_2 and the generation of hydrogen peroxide (H_2O_2) by it's sulfhyrl group (Starkebaum and Harlan, 1986). Superoxide anions are also generated through homocysteine's oxidation (Bellamy and McDowell, 1997; Loscalzo, 1996). The perpetuation of free radicals is a recognized mechanism contributing to vascular disease. To maintain cellular integrity, H_2O_2 is normally converted to H_2O via endothelial catalyse or glutathione peroxidase. It has been shown that homocysteine inhibits glutathione peroxidase activity directly, thus it potentiates peroxide-mediated cellular damage (Upchurch et al., 1995). Results from a recent clinical investigation offer support for the homocysteine-induced oxidative stress hypothesis (Chambers et al., 1999).

Several *in vitro* studies of cultured endothelial cells suggest that homocysteine may induce cellular injury by facilitating LDL oxidation (Stamler and Silvka, 1996) and by impairing regulatory and anti-thrombotic properties of the endothelium (Stehouwer and Jakobs, 1998). Nitric oxide (NO) or endothelial-derived relaxing factor (EDRF), produced in the endothelium, exerts protective effects against homocysteine-mediated damage (Stamler et al., 1993). Under normal physiological conditions, homocysteine reacts with NO to form S-nitrosohomocysteine, a potent vasodilator. It has been shown that prolonged exposure of cultured cells to homocysteine impairs NO formation, thus,

reducing NO mediated platelet fibrinolysis (Stamler et al., 1993). The findings of a recent clinical investigation (n=13) supports this concept (Chambers et al., 1998). The endothelial dysfunction, due to impaired NO activity, has been found to be related inversely to plasma homocysteine concentrations (Chambers et al., 1998). NO may act alone or synergistically with prostacyclin in a negative feed-back fashion to inhibit platelet aggregation and adhesion to the endothelium (Ueland et al., 1992). Prostaglandin synthesis and release may be impaired in homocysteine-damaged tissue. *In vitro* studies implicate that homocysteine inhibits cyclo-oxygenase activity directly, in a dose-dependent manor, thereby inhibiting prostacyclin liberation (Quere et al., 1995; Wang et al., 1993).

Homocysteine may induce a pro-coagulatory state by activating the coagulation cascade or by decreasing pro-fibrinolytic defences (Stehouwer and Jakobs, 1998). The binding of thrombin to thrombomodulin activates protein C, a regulatory component that inhibits thrombin activation of fibrinogen and subsequent fibrin clot formation. (Guba et al., 1996). Homocysteine has been shown to decrease thrombomodulin anticoagulant activity (Mercie et al., 1999; Harpel et al., 1992; Roger and Conn. 1990). Lentz et al. (1991) found that it decreased thrombomodulin activity by an average of 34% in cultured aortic endothelial cells. Homocysteine also activates Factor XII and Factor V in the pro-coagulation pathway (Roger and Conn. 1990). *In vitro*, it decreases the binding of another fibrinolytic protein, plasminogen activator (Hajjar, 1993), and reduces anti-thombin expression (Nishinga et al., 1993). It also modifies platelet arachidonic acid metabolism to increase the production of a potent platelet aggregator, thromboxane A_2 (Di Minno et al., 1993). Furthermore, it stimulates smooth muscle proliferation (Tsai et al., 1994) and collagen formation/accumulation in the vasculature (Majors et al., 1997). Other research has implicated that homocysteine, in concentrations as low as 8μmol/L, may augment the affinity of Lp(a) for fibrin, therefore further contributing to homocysteine pro-thrombotic effects (Harpel et al., 1992).

1.72 Treatment of Hyperhomocysteinemia

Since B-vitamin deficiencies are the most common causes of mild to moderate hyperhomocysteinemia (Cattaneo, 1999), recent investigations have focused on nutritional intervention strategies to ameliorate elevated plasma homocysteine concentrations. Vitamin supplementation is a potential cost-effective strategy since several studies have indicated that supplemental folic acid, alone or in combination with vitamin B12 and vitamin B6, effectively reduces plasma homocysteine levels.

A recent meta-analysis of 12 clinical randomised controlled-trails that implemented folate supplementation, alone or in combination with B12 and B6, identified folate as the most potent homocysteine-lowering vitamin (Clarke, 1998). Supplemental folate, in doses of 0.5-5 mg/day, significantly reduced plasma homocysteine levels by 25%; however, supplements >1mg did not provide any additional lowering effects. The addition of B12 (0.02mg - 1mg/day) further reduced levels by an additional 3-7%, however, no significant effect was found due to B6 supplementation. A double-blind randomised-control trial (n=172) supplemented various levels of folate (100μg, 200μg, or 400μg) (Daley et al., 1997). All doses significantly improved folate status but 400μg/day proved to be slightly more effective. Other studies have also shown that folate supplementation renders the greatest homocysteine-lowering effects (Ubbink et al., 1997; Rasmussen et al., 1996; Fenech et al., 1998; Ubbink et al., 1994); however, it is important to recognise that vitamin B12 and vitamin B6 supplementation should not be omitted. Full response to folate supplementation cannot be achieved if B12 status is low (Landren, 1995) and the ability of supplemental folate to mask an existing B12 deficiency must also be considered (Zittoun and Zittoun, 1999). Folate (400μg) when taken in combination with vitamin B12 (6μg) has been shown to reduce homocysteine (Bronstrup et al., 1998). Since vitamin B6 supplemental therapy has been found to be significantly effective in some instances (Franken et al., 1994; Guba et al., 1994), vitamin B6 should also be included in the supplemental regimen.

Several recent investigations have found that B-vitamin combination therapy has superior homocysteine-lowering ability. A placebo-controlled, single blind, dose-ranging study evaluated the effect of daily supplementation of folate (400μg, 1mg, or 5mg) in combination with B12 (500μg) and B6 (12.5mg) in 95 subjects with documented CAD (Lobo et al., 1999). The combination therapy with 400μg of folate significantly reduced homocysteine concentrations by an average of 13.8%. The observed decrease was similar in all treatment groups thus suggesting that combination therapies with folate doses >1mg may not achieve enhanced homocysteine-lowering effects. A double-blind, randomised factorial-design study found that a combination therapy of 1mg folate, 7.2 mg B6, and 0.02 mg of B12 rendered a 27% reduction in plasma homocysteine levels over an eight week intervention period (Woodside et al., 1998). Other researchers have also found promising results with supplementation of 1mg folate, 50μg B12 and 10mg B6 per day (Ubbink et al., 1993b). Although dietary counselling may assist individuals with choosing foods richer in these necessary B-vitamins, research has shown that individuals may not achieve homocysteine-lowering levels of B-vitamins through diet alone (Ubbink et al., 1993b). Since research has demonstrated the effectiveness of combination supplementation, it suggested as the best approach to lowering escalating homocysteine levels (Stein and McBride, 1998).

1.8 OBJECTIVES OF THE PRESENT STUDY

Intermediate-release niacin preparations are available without a prescription and are gaining popularity with physicians as the hypolipidemic agent of choice. Since niacin has been available for decades, the untoward effects of taking large doses of this lipid-lowering agent are well established. However, very little research has focused on the effect of pharmacological doses (1.5 -6g per day) on homocysteine metabolism. Recent investigations involving rats have demonstrated that lipid-lowering doses of niacin increases plasma homocysteine concentrations in normolipidemic rats (Basu and Mann, 1996; Makhani, 1998). The results of these studies suggest that the niacin -induced hyperhomocysteinemia was caused by an increased demand for SAM and a subsequent

reduction in the re-methylation and transsulfuration of homocysteine due to B-vitamin depletion.

A clinical trial (n=101) by Blankenhorn et al. (1991) investigated the effect of an aggressive combination hyperlipidemic therapy of niacin (4.2 g/d) and colestipol (a bile acid resin) on plasma homocysteine concentrations over a four year period. This mode of therapy resulted in a decrease in plasma cholesterol levels and a concurrent increase in plasma homocysteine concentrations. However, it was uncertain if the homocysteine elevations were due to an impaired folate absorption induced by colestipol therapy or an increased demand of SAM for urinary excretion of niacin. Since, homocysteine is an established risk factor for atherosclerotic vascular disease, it is of significant importance to determine if pharmacological doses of niacin induce the accumualtion of this cytotoxic risk factor in humans.

It is hypothesised that niacin, in its pharmacological dose levels, adversely affects homocysteine metabolism causing an accumualtion of homocysteine and subsequent reductions in plasma folate, vitamin B6 and vitamin B12 which are required for the re-methylation and transsulfuration of the sulfur amino acid. A clinical trial was designed to test this hypothesis with the following objectives:

1. To determine if acetylsalicylic acid (ASA) alone affects plasma cholesterol and homocysteine status.

Rationale: IR-niacin induces undesirable cutaneous symptoms (flushing and itchiness) within 15-60 minutes post-treatment. Since these effects are caused by niacin-induced prostaglandin (PG) synthesis, it is expected that the administration of ASA (a PG inhibitor) 30-minutes prior to niacin therapy will ameliorate niacin's cutaneous symptoms and will improve an individual's tolerance to it's daily regimen. Therefore, ASA was first tested alone and then in combination with niacin for effects on lipid status.

2. To determine if a pharmacological lipid-lowering niacin regimen (1.5g/d) in combination with ASA (325mg/d) modifies plasma homocysteine status.

3. To examine if the addition of folate, vitamin B6 and vitamin B12 to the niacin (plus ASA) therapy has a beneficial effect on plasma homocysteine levels without reversing the hypolipidemic action of niacin.

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CHAPTER TWO: THE NIACIN (PLUS ASPIRIN) ASSOCIATED CHANGES IN CHOLESTEROL AND HOMOCYSTEINE STATUS

2. INTRODUCTION

Nicotinic acid (*niacin*) is an effective, inexpensive, hypolipidemic agent which is readily available without a prescription (Yeshurun and Gotto, 1995). Niacin, in doses of 1.5- 6g per day, facilitates significant reductions in all atherogenic lipoproteins accompanied by significant increases in HDL-C levels (Farmer and Gotto, 1996). Some of it's untoward side effects include flushing and itching, which are secondary to the transient release of endogenous prostaglandins in the skin (Luria, 1990; Gryclewski et al., 1988; Morrow et al., 1992). The pre-administration of aspirin or acetylsalicylic acid (ASA), a prostaglandin inhibitor, has been shown to improve one's tolerance to niacin's cutaneous symptoms (Jungnickel, 1997). Gradually tapering upward to a lipid-lowering dose of niacin has also been reported to improve one's tolerance to the daily regimen (Illingworth, 1988; CPS, 1997).

Excess niacin is primarily excreted as either N-methylnicotinamide and/or methylated-pyridones in the urine (Jacobsen et al., 1995; Groff et al., 1995). Since S-adenosylmethionine (SAM) provides the methyl groups for this excretory process it is feasible that pharmacological dose levels (100-200X's physiological requirements) of niacin may facilitate an accumualtion of homocysteine, a product of SAM following it's de-methylation (Verhoef et al., 1996; Fowler et al., 1998, Fowler et al., 1997). A recent investigation by Basu and Mann (1996) demonstrated that after only three weeks of treatment with niacin (1g/kg diet), plasma and urinary homocysteine concentrations were significantly increased in rats. This hyperhomocysteinemia was accompanied by decreased plasma B6 concentrations. This investigation, however, measured only the free-forms of homocysteine in plasma. Although it has been suggested that the free-fraction, which accounts for approximately 30% of total homocysteine in circulation (Ueland, 1995), is primarily responsible for homocysteine's atherothrombogenicity,

(Mansoor et al., 1995), it is important to measure total homocysteine status (Boers, 1994). This is particularly important since homocysteine readily fluctuates between all forms *in vivo* (e.g. free and protein-bound forms) and plasma storage may cause a redistribution of thiols resulting in an increase in the protein-bound fraction (Ueland and Refsum, 1989). In view of this fact, a subsequent longitudinal investigation was carried out in rats measuring total homocysteine levels after hypolipidemic treatment with niacin (1g/kg diet) for twelve weeks (Makhani, 1998). The results from this study revealed that lipid-lowering doses of niacin elevated total homocysteine (*designated as homocysteine*). This increase was accompanied by reduced plasma concentrations of vitamin B6 and vitamin B12. A recent clinical investigation that assessed plasma homocysteine levels after a combination therapy of cholestipol and niacin (4g/d) also found significant increases in plasma homocysteine concentrations (Blankenhorn et al., 1991). However, whether or not niacin was responsible for the observed accumualtion of this cytotoxic sulfhydryl amino acid was uncertain due to the concomitant use of the bile acid sequestrant.

The present study was undertaken to examine if the experimental data collected from earlier work in rats was relevant to humans. A clinical study, involving a select group of hypercholesterolemic subjects, was designed to investigate the effect of niacin therapy on total plasma homocysteine levels in parallel with lipid status. Since homocysteine metabolism is modulated by vitamin B12, vitamin B6 and folic acid, the effect of niacin on these vitamins was also investigated.

2.1 METHODS

The Department of Agricultural. Food and Nutritional Science Human Ethics Committee at the University of Alberta approved the experimental protocol. Recruitment strategies involved newspaper advertisements and television announcements focused on individuals in the Edmonton area. More than 200 individuals were screened over a three-month recruitment period. Eighteen individuals who met inclusion/exclusion criteria (Table 1.4) were recruited for the study. The trail was 7 weeks in length consisting of three experimental periods (Figure 1.7). A total of 15 subjects (8 women and 7 men) completed the study. A food-frequency questionnaire (FFQ) was used as a screening tool to render general information about the diets of the participants. All subjects followed the same experimental protocol, thus, each person acted as their own control.

Table 2.0: Inclusion and exclusion criteria for the present study

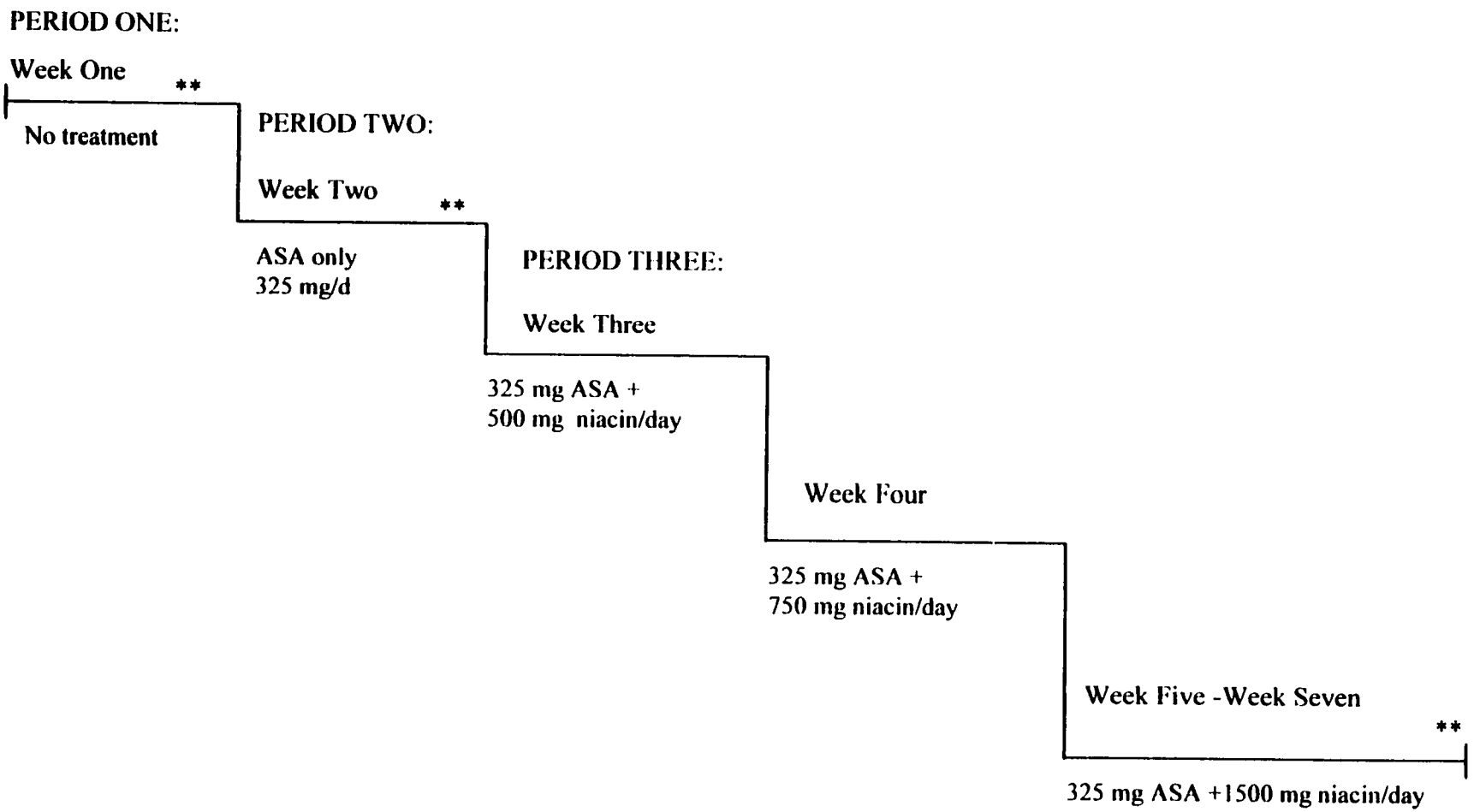
Inclusion Criteria:

- individuals with elevated TC levels (> 5.8 mmol/L)
- men: 40 -70 years
- women: 50-70 years (post-menopausal)
- non-smokers
- low-alcohol consumers (< 3 oz. alcohol per week)
- English speaking individuals who live in the Edmonton Area

Exclusion Criteria:

- individuals taking vitamins or amino acid supplements or medications affecting lipid status/contraindicated with aspirin
- individuals who have taken hypolipidemic drugs within the last six months
- individuals with diabetes, thyroid dysfunction, liver problems (e.g. jaundice, glycogen storage diseases etc.) or any other medical condition affecting lipid status (e.g. gall bladder disease)
- individuals with history of peptic ulcer disease or gout
- women taking estrogen replacement therapy
- persons exercising/dieting to lose weight or persons who have lost significant weight within two-three months prior to the start of the study (≥ 10 lbs.)

Figure 2.0: The experimental protocol of the present clinical investigation



Note: ** Blood and urine samples were collected

During the first week, subjects were counselled on the experimental protocol, anthropometric measurements were taken, written consent was attained, and the necessary materials and information were provided. No treatment was implemented during the first week. Fasting baseline blood and urine samples were collected by healthcare professionals at Dynacare Kasper Medical Laboratory in Southwest Edmonton.

Niacin, in large doses, results in cutaneous symptoms which are caused by prostaglandin synthesis (Luria, 1990; Gryclewski et al., 1988). In order to counteract this effect two measures were taken: (1) niacin was given 30 minutes after one-half of an ASA tablet (½ of 325mg), an inhibitor of prostaglandin synthesis, and (2) the final dose of niacin was achieved by gradually increasing it's dose level. Therefore, during the second week, subjects were pre-treated with ASA. One-half of an ASA tablet (½ of 325mg) was taken in the morning and evening with food/milk and the second blood and urine samples were collected. Over the next two weeks subjects implemented the niacin (plus ASA) regimen. Subjects started by taking one-half of an ASA tablet (½ of 325mg) 30 minutes prior to a 250 mg dose of niacin (twice per day) and gradually increased to a lipid-lowering regimen of one-half of an ASA tablet (½ of 325mg) + 750 mg niacin (twice per day). Niacin was taken with food to avoid stomach upset; hot beverages were avoided to further diminish cutaneous effects. The hypolipidemic therapy was continued for three weeks before final blood and urine samples were collected. All blood samples were wrapped in foil to protect light sensitive compounds such as vitamin B12 (Basu and Dickerson, 1996). Samples were placed on ice and centrifuged within one hour of collection to prevent homocysteine elevations due to synthesis by erythrocytes (Ubbink et al., 1992; Malinow et al., 1994). Plasma and urine samples were stored frozen (-70°C and -25°C respectively) until thawed for laboratory analysis.

2.11 Determination of plasma total cholesterol

Plasma samples were analyzed using Sigma Diagnostics Cholesterol Procedure No. 352 (St. Louis, MO). This procedure is based on the enzymatic assay of total cholesterol by

Allain et al. (1974). This method is more specific for total cholesterol analysis than non-enzymatic assays, uses only one reagent and requires no prior plasma treatment.

Cholesterol esters in plasma are initially hydrolyzed by cholesterol esterase to form free cholesterol (FC) (Figure 2.1). The FC formed from this rate-determining reaction is subsequently oxidized by cholesterol oxidase, an enzyme specific for 3-β-sterols (Allain et al., 1974), to form cholest-4-en-3-one. The hydrogen peroxide simultaneously produced by this reaction oxidatively couples with 4-aminoantipyrine, *p*-hydroxybenzensulfonate and chromagens in the presence of peroxidase. The result is the production of quionineimine dye, a compound that has with a maximum absorbance at a wave-length of 500 ηm.

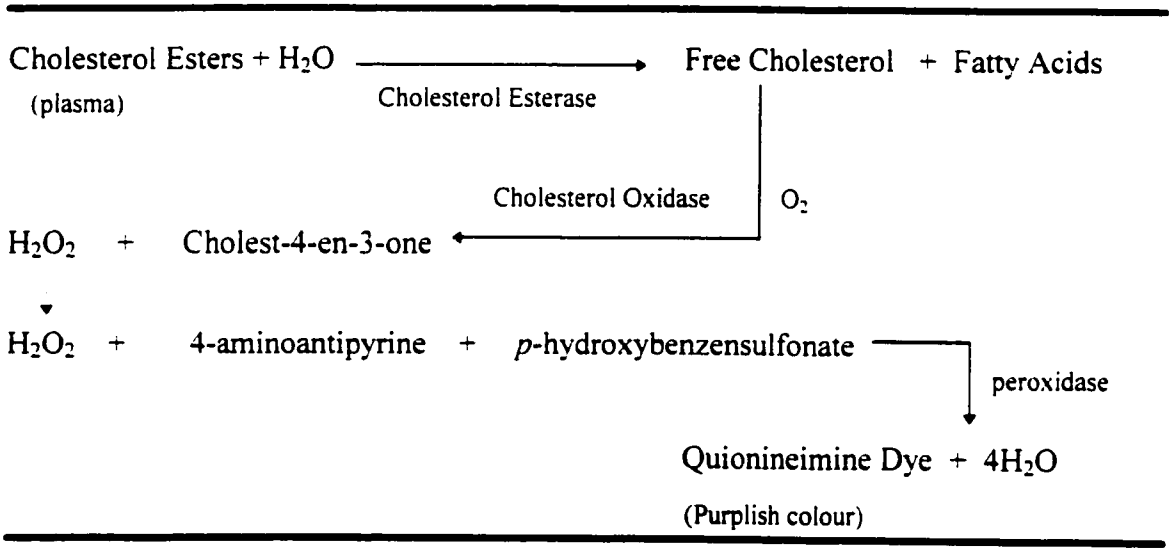


Figure 2.1: An overview of the enzymatic analysis of plasma total cholesterol
Adapted from: (Sigma Diagnostic Cholesterol Procedure No. 352,-St.Louis. MO)

2.12 Determination of plasma HDL

Plasma samples were analyzed using the Sigma Diagnostic Procedure No. 352-3 (St. Louis, MO). Various methods have been developed to separate HDL from other lipoproteins such as centrifugation and electrophoresis, however, this precipitation

procedure is considered simple and effective (Warnick et al. 1982). Several precipitating agents have been used to separate the HDL fraction including heparin -$MnCL_2$ (Warnick and Albers, 1978), phosphotungstate- $MgCL_2$ (Assman et al., 1983) and dextran-sulfate-Mg^{2+} (Warnick et al., 1978). Warnick et al. (1982) modified the dextran-sulfate-$MgCL_2$ method to ensure complete precipitation of LDL and VLDL by adjusting pH. Since heparin- $MnCl_2$ interferes with the enzymatic analysis of cholesterol and Mg^{2+} ions do not, this method is deemed the method of choice. It is suggested that the negatively charged groups on the lipoproteins interact with the charged Mg^{2+} and precipitate out in solution. Therefore, the higher lipid containing lipoproteins (VLDL and LDL) form insoluble complexes more readily than smaller protein-rich HDL particles. The HDL fraction remains in solution and is separated from the insoluble complexes by centrifugation. The remaining cholesterol is considered representative of the concentration of HDL in solution thus, the enzymatic assay for cholesterol (Section 2.12) determines HDL concentration.

2.13 Determination of plasma triglyceride

Plasma samples were analyzed using the Sigma Diagnostics Procedure No. 336 (St.Louis, MO). This procedure was modified from the method of Bucolo and David (1973) that links the enzymatic hydrolysis of TG to the simultaneous enzymatic determination of glycerol (Figure 2.2).

Plasma triglycerides are first hydrolyzed by lipoprotein lipase to yield glycerol and free fatty acids (FFA). Glycerol is phosphorylated by ATP in the presence of glycerol kinase. The glycerol-1-phosphate produced is oxidized by NAD in the presence of glycerol-1-phosphate dehydrogenase to yield NADH and dihydroxyacetone phosphate. NADH is oxidized with the simultaneous reduction of 2-[p-iodophenyl]-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) to yield formazan (INTH), a coloured compound, with a maximum absorbance at 500 ηm.

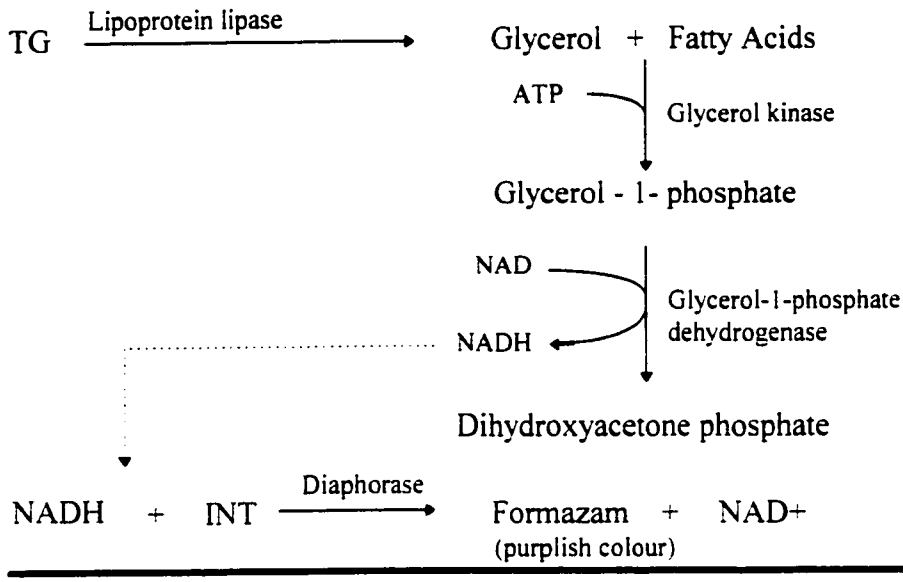


Figure 2.2: The enzymatic determination of plasma triglyceride
Adapted from: (Sigma Diagnostics Procedure No. 336 St.Louis. MO).

2.14 Determination of plasma LDL

Plasma LDL may be calculated accurately from enzymatically measured TC. HDL and TG. Since research has shown that the mass of plasma TG is related to the VLDL in a constant proportion of 5:1 in normal subjects and with most all types of hyperlipoproteinemia (Fredrickerson et al., 1967; Fredrickerson et al., 1972). Friedwald et al. (1972) devised a method of calculating LDL concentration (mg/dL) from plasma concentrations of total cholesterol. HDL and TG:

$$\text{LDL (mg/dL)} = \text{Total Cholesterol (mg/dL)} - \text{HDL (mg/dL)} - \frac{\text{TG (mg/dL)}}{5}$$

The above equation may not accurately estimate plasma LDL concentration if TG levels are above 400 mg/dL. Due to it's simplicity, the Friedwald equation was employed to calculate LDL concentration (mg/dL) and the results were converted to SI units by a conversion factor of 0.0295.

2.15 Determination of plasma and urinary sulfur amino acid concentrations

Homocysteine and cysteine concentrations in both plasma and urine were analyzed by a high performance liquid chromatography (HPLC) procedure (Still and McDowell, 1998). HPLC is the most widely used method for homocysteine and cysteine determination Thiols, mixed-disulfides and protein-bound thiols were reduced and released with tri-η-butylphosphine. Proteins were precipitated with perchloric acid, thiol groups were derivatized with ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBDF) and the resulting mixture was centrifuged. HPLC with a fluorometric detector identified SBDF-labelled thiols remaining in the supernatant. SBDF was detected at a wavelength of 515 ηm. SBDF offers several advantages over monobromobiane, another derivatizing agent commonly used. Monobromobiane has a higher reactivity than SBDF; however, SBDF is more specific for homocysteine/cysteine and does not require the use of an elution gradient to eliminate impurities. SBDF allows for the incorporation of internal standards and is considered the straight forward method of choice.

2.16 Determination of PLP and PA

PLP and pyridoxic acid (PA) were analyzed by a high performance liquid chromatography (HPLC). In order to extract the above isomers from plasma, perchloric acid was used as the precipitating agent. The mixture was vortexed extensively and centrifuged at 25000g. Sodium bisulfite was used as the derivatizing agent. PLP and PA concentrations remaining in the supernatant were identified by HPLC fluorescence detection at 400 ηm. This highly sensitive method is similar to that of Kimuria et al. (1996).

2.17 Plasma B12, plasma folate and RBC folate determination

EDTA-blood samples were analyzed by a Dualcount Solid Phase No Boil Assay from the Diagnostic products Corporation (Los Angela's, CA). The radioassay was designed for

the measurement of whole blood folic acid or the simultaneous measurement of B12 in serum or plasma and folate in serum or plasma.

First, hemolysates were prepared to stabilise methylenetetrahydrofolate (MTHF) (the primary form of folate in circulation) and the hematocrit was determined. The hemolysates and plasma samples were thawed only once at room temperature for the radioassay procedure. The dualcount procedure first separated vitamin B12 and folate from their carrier proteins by heat activated alkaline denaturation in the presence of dithiothreitol and potassium cyanide. The denaturation step inactivated anti-intrinsic factor antibodies and extreme levels of vitamin B12 transport proteins. The dithiothretiol protected the folate from oxidization (Gutchc S and Mansbach L, 1977) and ensured that the released B12 did not become complexed during prolonged heating thus remaining available for the radioassay (Lee-own V et al.. 1979).

The second step of the assay introduced purified intrinsic factor and folate binding protein immobilised on microcrystalline cellulose particles as binders for B12 and folate respectively. The high pH of 9.3 ensured that IF had a high activity where the folic acid binder had equal affinity for MTHF and polyglutamic acid (PGA) (the folate form used in the calibrators). The unlabelled and labelled (^{125}I-folate & ^{57}Co-B12) species competed for a limited number of binding sites on their specific binder. The B12 analogues did not interfere since the binder was free of R-protein. Finally, the removal of the supernatant via aspiration allowed for the isolation of bound-fractions. The bound labelled vitamins were analyzed using a gamma counter. The radioactivity of ^{125}I-folate and ^{57}Co-B12 were inversely related to the concentrations in the sample. From the results of the whole blood folate, RBC folate was calculated using the following equation:

$$\underline{\text{RBC Folate (ng/ml)}} \approx 21R\,(100/H)$$

H= hematocrit (%)
R= whole blood folate (ng/mL)

2.18 Determination of liver enzymes and plasma clinical chemistry

The Pathology Department at the University of Alberta performed all clinical chemistry tests on plasma. Plasma levels of total bilirubin (TBIL), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), blood urea nitrogen (BUN), creatinine and glucose (GLC) were assessed. Urinary samples were analyzed for creatinine. A clinical analyzer compatible for human plasma and urine samples, the KODAK 700XR, analyzed all samples. Commercially available human controls were tested daily to ensure accuracy. None of the samples were run unless control measures fell within manufacturer specified ranges. The results were reviewed by a Certified Pathologist to identify any pathological concerns.

2.19 Statistical Analysis

Food frequency data were analyzed with Food Processor 7.02 Software. Pearson correlation coefficients and linear regressions were calculated with SAS 7.0 Software. The lsmeans and standard error of the mean (SEM) for all parameters measured were determined. Lipoprotein, B-vitamin, sulfur amino acid and blood chemistry data were analyzed using a Multivariate Analysis of Variance (MANOVA) procedure with SAS 7.0 software. Differences in plasma or urinary concentrations were significant if $p< 0.05$. Spearman correlation coefficients (R) were determined to identify significant correlations between changes in lipoprotein concentrations, B-vitamins and sulfur amino acid concentrations. R-values were also significant if $p< 0.05$. A Multivariate Analysis of Covariance (MANCOVA) was also performed to determine if age-related effects on lipoprotein, B-vitamins and sulfur amino acids were significant. Age was considered a significant covariate if $p < 0.05$.

2.2 RESULTS

During the first week of this pharmacological intervention trial, individuals were counselled and anthropometric measures were taken(Table 2.1)

Table 2.1: Description of volunteers that participated in the present study

Gender	N	Age (years)	Height (m)	Weight (kg)	BMI
Male	7	59.4 ± 10.6*	1.69 ± 0.12	83.6 ± 12.3	29.3 ± 4.4
Female	8	60.9 ± 4.8	1.61 ± 6.1	72.6 ± 22.5	27.8 ± 3.5
Total	15	60.2 ± 7.8	1.65 ± 9.8	77.7 ± 12.8	29.3 ± 4.3

*Mean ± SEM, N = number of subjects, BMI = kg/m^2

The average age and body mass index (BMI) of the 15 participants were 60.2 ± 7.8 and 29.3 ± 4.3, respectively. Subjects agreed to maintain their current exercise and dietary habits in order to prevent weight loss or gain throughout the trial. The estimated dietary intakes based on the completed food- frequency questionnaire are summarized in Table 2.12. The data indicate that the mean intake of carbohydrates, protein and fat fall within the recommended percent of dietary calories for Canadians (Health Canada, 1990).

A closer look at the estimated dietary intakes of folate, vitamin B6 and B12, modulators of homocysteine metabolism, determined that the study participants achieved dietary intakes that were greater then the RNI for adults (Table 2.13). The FFQ revealed that subjects consumed within the range of the recommended daily servings from each dietary food group (Canada's Food Guide, 1999), thus, it is unlikely that the study participants were at risk of deficiency. The estimated intakes of vitamin B6 ($r= 0.66$, $p=0.0076$) and folate ($r=0.58$, $p=0.0458$) were significantly correlated with baseline plasma values. Thus, a linear regression procedure was performed to further define the relationship between the independent intake variable and the dependent plasma values (Figure 2.3 and Figure 2.4). However, the significant results should be interpreted with caution as the Food Processor Software did not account for the bioavailability of folate from food.

Table 2.12: A summary of estimated daily nutrient intakes from a food-frequency questionnaire.

Nutrient	Males (n=7)	Females (n=8)	Total (n=15)
Energy (C)	2514 ± 568	2146 ± 490	2318 ± 547
Protein (g)	108 ± 15	112 ± 29.9	110 ± 23
% Energy	17 ± 3	20 ± 2	19 ± 3
Carbohydrate (g)	328 ± 121	280 ± 77	302 ± 96
% Energy	50 ± 11	50 ± 4	51 ± 7
Fat (g)	88 ± 44	67.19 ± 14	77 ± 26
% Energy	29 ± 10	27 ± 3	28 ± 7
Saturated Fat (g)	23 ± 6	22 ± 5	22 ± 5
Monounsaturated Fat (g)	26 ± 10	24 ± 6	25 ± 7
Polyunsaturated Fat (g)	15 ± 6	13 ± 3	14 ± 5
Dietary Fibre (g)	37 ± 20	30 ± 7	34 ± 13
Cholesterol (mg)	245 ± 65	265 ± 95	256 ± 85
Thiamine (mg)	2 ± 1	2 ± 1	2 ± 1
Riboflavin (mg)	2 ± 1	2 ± 0	2 ± 1
Niacin (NE)	83± 9	69± 24	75 ± 18
Vitamin B6 (mg)	3 ± 1	3 ± 1	3± 1
Vitamin B12 (μg)	11 ± 6	8 ± 4	9 ± 5
Folate (μg)	316 ± 95	326 ± 72	322 ± 83
Vitamin A (RE)	2145 ± 906	2734 ± 1076	2495 ± 971
Vitamin D (μg)	6 ± 1	8 ± 3	7 ± 3
Vitamin C (mg)	178 ± 105	176 ± 21	177 ± 60
Calcium (mg)	732 ± 914	1075 ± 148	928 ± 201
Iron (mg)	19 ± 4	18 ± 6	18 ± 5
Zinc (mg)	16 ± 3	13 ± 4	14 ± 3

* Mean ± SEM

Table 2.13: Percentage of the subjects with estimated B-vitamin intakes less than 67% of the RNI at the onset of the study

Nutrient	Estimated Dietary Intake	> 100 % RNI *	< 67% RNI
Folate (μg/d)			
men (n=7)	316 ± 95	100%	0%
women (n=8)	326 ± 72	100%	0%
total (n=15)	321 ± 83	100%	0%
Vitamin B12 (μg/d)			
men (n=7)	11 ± 6	100%	0%
woman (n=8)	8 ± 4	100%	0%
total (n=15)	9 ± 5	100%	0%
Vitamin B6 (mg/d)			
men (n=7)	3 ± 1	100%	0%
woman (n=8)	3 ± 1	100%	0%
total (n=15)	3 ± 1	100%	0%

*Intakes are expressed as Mean ± SEM: Reference: (Health Canada, 1990)

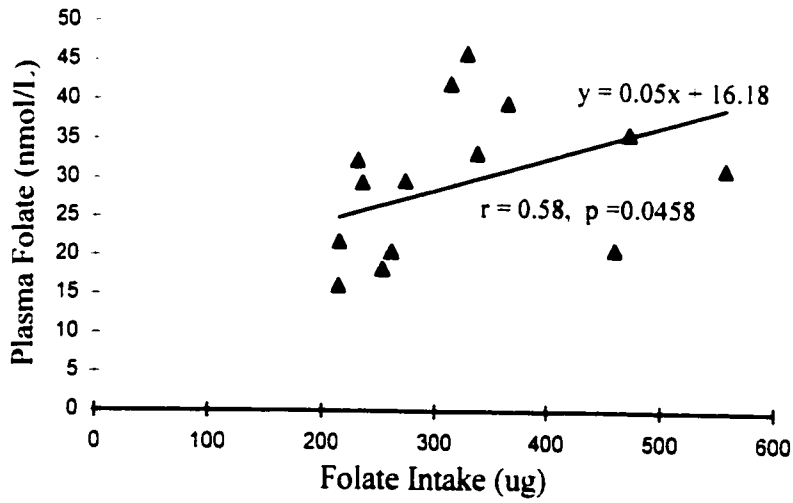


Figure 2.3: Linear regression analysis of plasma folate and estimated folate intake.
Note: y = plasma folate; x= estimated folate intake

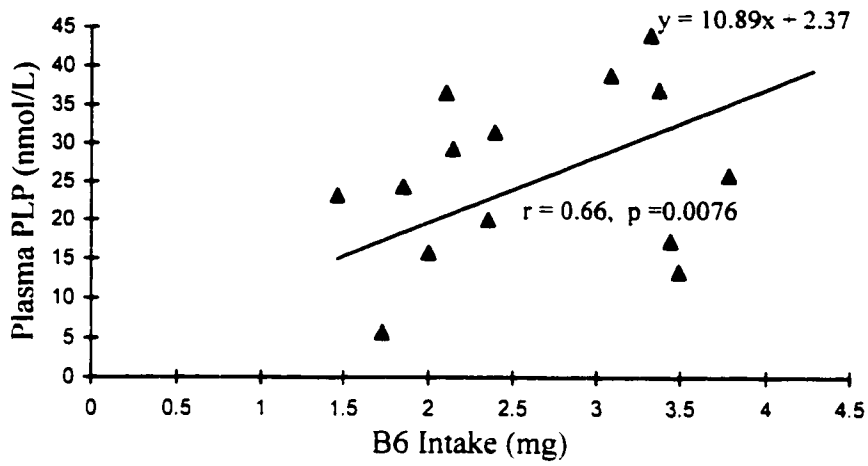


Figure 2.4: Linear regression analysis of plasma PLP levels and estimated vitamin B6 intake. Note: y= plasma PLP; x= estimated vitamin B6 intake

The biochemical status of folate, vitamin B6 and vitamin B12, at the onset of the study, is shown in Table 2.14. The mean values for men and women participants fell within normal ranges with the exception of vitamin B6. However, only 27% (4/15 individuals) had slightly lower than defined normal PLP levels (>20 ηmol/L) despite adequate estimated dietary intakes of vitamin B6. The mean plasma PLP concentration, however, fell within the normal range for an all groups of study.

Table 2.14: The biochemical status of folate, vitamin B12 and vitamin B6 at the onset of the study

Vitamin	Men (n=7)	Women (n=8)	Total (n=15)	Normal Value **
Folate				
ηmol/L plasma	28.0 ± 6* (0%)	31.0 ± 8 (0%)	29.6 ± 2 (0%)	> 4
ηmol/L RBC	1204.2± 404 (0%)	1311.5 ± 420 (0%)	1261.4 ± 507 (0%)	> 550
Vitamin B12				
ρmol/L plasma	260.2 ± 77 (0%)	347.9± 118 (0%)	306.9 ± 110 (0%)	> 150
Vitamin B6 (PLP)				
ηmol/L	37.3 ± 9 (7%)	29.3 ± 11 (20%)	33.1 ± 4 (27%)	> 20

* Mean ± SEM ; Numbers in parentheses present the proportion of subjects below the normal clinical value
** Reference: (Zeeman, 1991; Fandek et al., 1995; Lee and Nieman, 1996; Robinson et al., 1995)

Subjects provided blood and urine samples at baseline (no treatment), after one week of pre-treatment with ASA (325mg/d) and following the niacin (plus ASA) regimen. Plasma samples were analyzed to determine lipoproteins, B-vitamins, homocysteine and cysteine levels. Since cholesterol and homocysteine levels may be partially age-related, a MANCOVA was performed to determine if the parameters tested would have to be adjusted for age effects (Table 2.15). Since this procedure determined that age did not significantly influence any of the parameters tested, no adjustments to the data were made.

Table 2.15 A MANCOVA analysis of lipoprotein, B-vitamin and sulfur amino acid data collected to determine the significance of age- related effects

Parameter	p-value	Significance
TC	0.1085	NS**
HDL	0.3345	NS
TG	0.0729	NS
LDL	0.4815	NS
RBC folate	0.3707	NS
Folate	0.4847	NS
B12	0.2358	NS
PLP	0.2907	NS
PA	0.1117	NS
Homocysteine	0.3855	NS
Cysteine	0.5018	NS
Homocysteine (urine)	0.4273	NS
Cysteine (urine)	0.5020	NS

*Each parameter tested is listed with it's corresponding p-value in the same row. A p-value < 0.05 is considered significant.
**NS = non-significant

A significant reduction in TC and TG levels was observed after only one week of daily aspirin administration (Figure 2.5). Mean HDL concentrations were increased (1.15 vs. 1.21 mmol/L) while LDL concentrations decreased (4.92 vs. 4.62. mmol/L), however, these changes were not statistically significant. An individual analysis of the data found that 73% of the participants (11/15 individuals) responded to the ASA intervention with a decrease in plasma cholesterol concentrations (Figure 2.6). Eighty-six percent of the participants (13/15 individuals) achieved lower TG levels with ASA treatment alone

(Figure 2.7). This change was significantly correlated with the change in TC concentrations ($r=0.9392$, $p=0.001$). After the implementation of the niacin (plus ASA) therapy a significant reduction in all atherogenic plasma lipoproteins was observed with a concurrent increase in plasma HDL concentrations in parallel with the HDL: LDL ratio.

The pre-treatment with ASA and the gradual upward tapering of niacin to it's hypolipidemic dose levels was effective in minimizing the cutaneous symptoms experienced in this study (Table 2.16). There were only two individuals (13%) who dropped out of the study due to a cutaneous intolerance to niacin. Forty-seven percent of the participants reported that they did not experience any adverse side effects throughout the course of the aspirin and the niacin (plus ASA) intervention. Dermatological effects were reported throughout the first week of treatment (only one individual experienced a mild degree of flushing at the hyperlipidemic dose of 1.5g niacin/d). Gastrointestinal effects were experienced periodically throughout the study by those who reported symptoms; however, the nausea experienced by one individual after the ASA treatment sub-sided after a few days and no further side effects were reported from this individual.

Aside from an assessment of clinical symptoms, selected plasma profiles were determined to identify any underlying problems that could be indicative of hepatotoxicity, renal dysfunction or glucose intolerance (Table 2.17). These parameters included ALT, ALKP, TBIL, GLC, BUN and creatinine. Mean and individual plasma values for each of the parameters tested fell within normal ranges and did not significantly change after the pharmacological intervention trial.

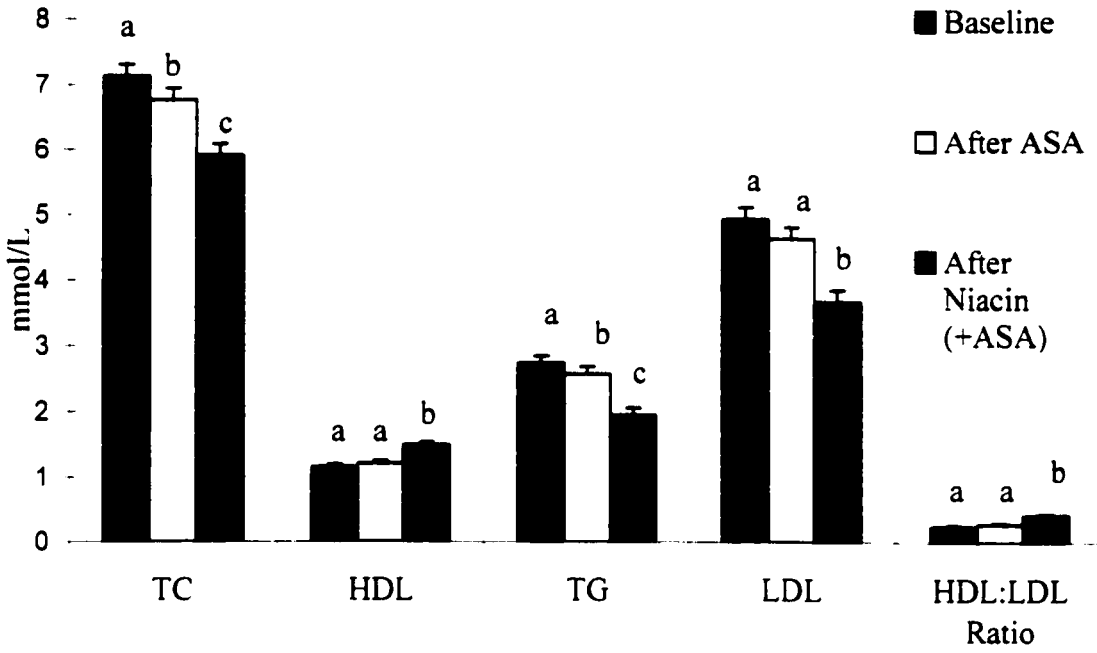


Figure 2.5: The effect of ASA alone (325mg/d) and the proceeding niacin (+ASA) regimen on plasma lipoprotein concentrations

*Each lipoprotein value is expressed as the Lsmean ± SEM of 15 subjects. Lipoprotein values not sharing the same letter in each period are significantly different ($p<0.05$).

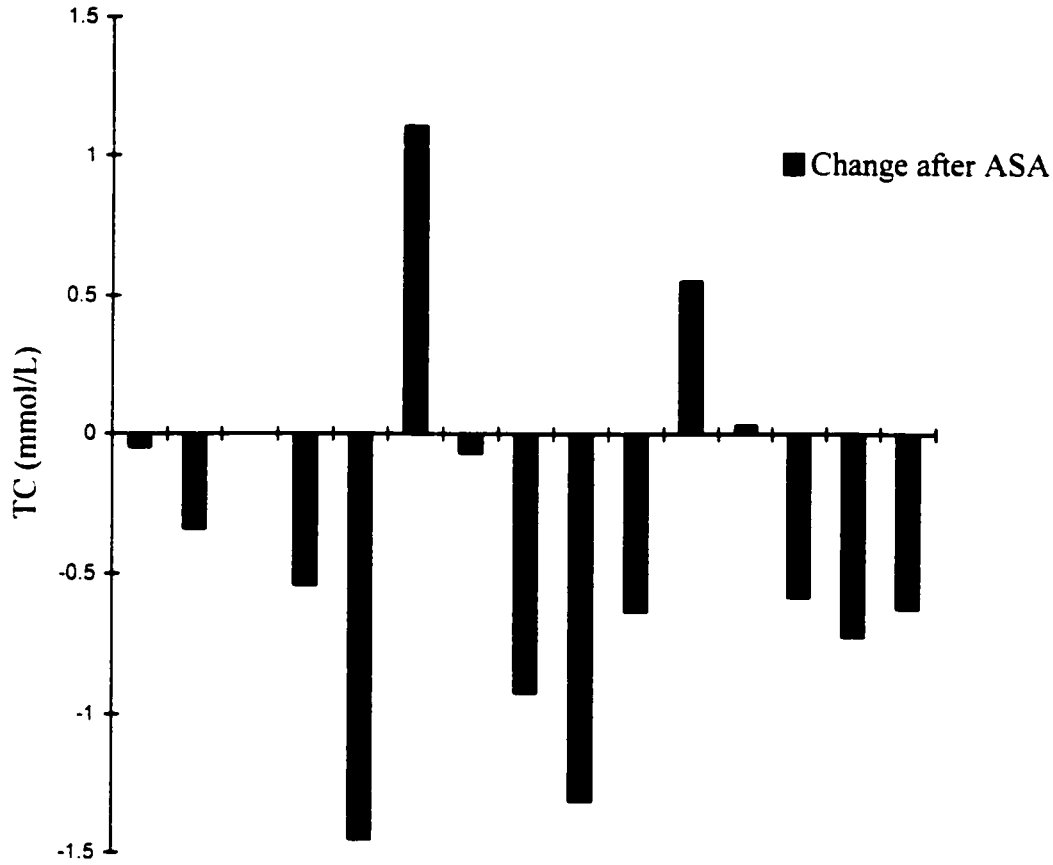


Figure 2.6: An individual analysis of the effect of ASA alone (325mg/d) on plasma total cholesterol levels

*Each line represents the change in an individuals plasma TC levels (n=15) after ASA administration (1 wk) in the present study.

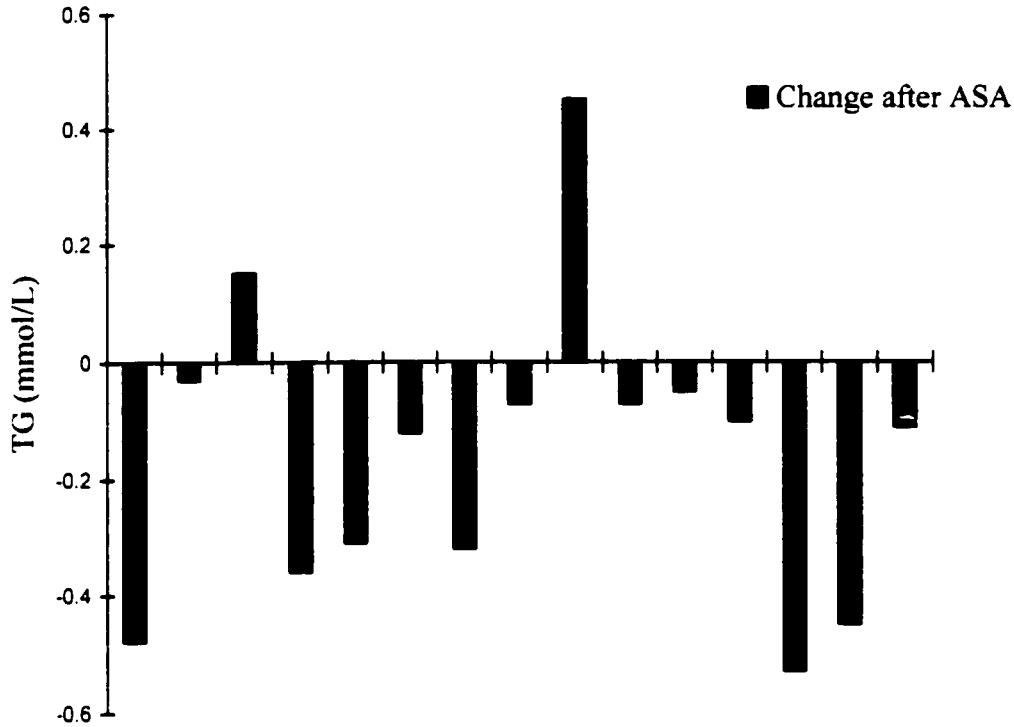


Figure 2.7: An individual analysis of the effect of ASA alone (325mg/d) on plasma triglyceride levels

*Each bar represents the change in an individuals plasma TC levels (n=15) after ASA administration (1 wk) in the present study.

Table 2.16: Frequency of common clinical symptoms associated with niacin (+ASA) therapy in subjects (n=15) during the 5 week trial period.

Common Side Effects:	Pre-treatment with ASA (325 mg/d)		Niacin (+ ASA) Therapy	
	n	%	n	%
Dermatological:				
flushing	-	-	3	20
itchiness	-	-	2	13
tingling	-	-	1	7
rash	-	-	-	-
Gastrointestinal:				
nausea	1	7	1	7
vomiting	-	-	-	-
diarrhea	-	-	-	-
constipation	-	-	-	-
heart burn	-	-	1	7
Other:				
headache	-	-	1	7
dyspepsia	-	-	1	7
fatigue	-	-	1	7
dry eyes	-	-	1	7
blurred vision	-	-	-	-
dizziness	-	-	-	-
None	14	93	7	47

Table 2.17: Blood chemistry results over the present clinical trial (7 wks.)

Test	Normal Values**	Baseline	After ASA	After Niacin (plus ASA)	P-value
Plasma					
ALT (units/L)	30 - 110	23.53 ± 1.3[a]	25.00 ± 1.3[a]	26.93 ± 1.3[a]	> 0.05
ALKP (units/L)	10 - 32	10 ± 0.0[a]	10 ± 0.0[a]	10 ± 0.0[a]	-
TBIL (µmol/L)	3 - 22	11.90 ± 1.1[a]	11.88 ± 1.1[a]	11.64 ± 1.1[a]	> 0.05
GLC (mmol/L)	3.9 - 5.6	5.42 ± 0.4[a]	5.60 ± 0.4[a]	5.67 ± 0.4[a]	> 0.05
Creatinine (µmol/L)	70 - 110	84.42 ± 2.0[a]	83.71 ± 2.0[a]	84.49 ± 2.0[a]	> 0.05
BUN (mmol/L)	3 - 7	5.71 ± 0.2[a]	5.70 ± 0.2[a]	5.17 ± 0.2[a]	> 0.05

*Each test value is expressed as the Lsmean ± SEM of 15 subjects. Values not sharing the same letter are significantly different ($P<0.05$).

** References: (Fandek et al., 1995; Tilkian et al., 1987)

The effect of the pre-treatment with ASA (325 mg/d) and proceeding niacin (plus ASA) therapy on sulfur amino acid status is presented in Figure 2.8. The administration of aspirin for one week prior to the niacin (plus ASA) therapy reduced the mean plasma homocysteine concentration (15.6 vs. 13.57 μmol/L, p=0.3486) and facillitated a rise in the mean plasma cysteine concentration (50.29 vs. 45.83 μmol/L, p=0.8405); however, these changes were not statistically significant. These mild changes in homocysteine status were paralleled by a changed plasma homocysteine to cysteine ratio (0.38 vs. 0.33 μmol/L, p=0.5937) and increased urinary homocysteine excretion (Table 2.18). Although these changes were not statistically significant, a large portion of the study subjects (73% = 11/15 individuals) responded favourably to the ASA treatment with a drop in plasma homocysteine concentrations (Figure 2.9). This lowering effect was not related to the observed TC (r=0.16, p=0.56) and TG (r=0.02, p=0.94) lowering effect of ASA.

The reduction in plasma levels of homocysteine in the presence of ASA, was also accompanied by non-significant decreases in levels of plasma vitamin B12, folate as well as RBC folate (Table 2.19). A significant direct correlation (r= 0.82, p=0.0002) between the observed decrease in plasma B12 concentrations and observed increase in plasma PLP concentrations was also found.

Although ASA had a modest lowering effect on plasma homocysteine concentrations, 53% of subjects experienced an increase in homocysteine concentrations that resulted in a mean increase (13.57 vs. 15.38 μmol/L) after only five weeks of niacin (plus ASA) intervention (Figure 2.8). This change was paralleled with a higher plasma homocysteine: cysteine ratio as well as increased urinary homocysteine levels (Table 2.18); however, plasma and urinary data were not statistically significant.

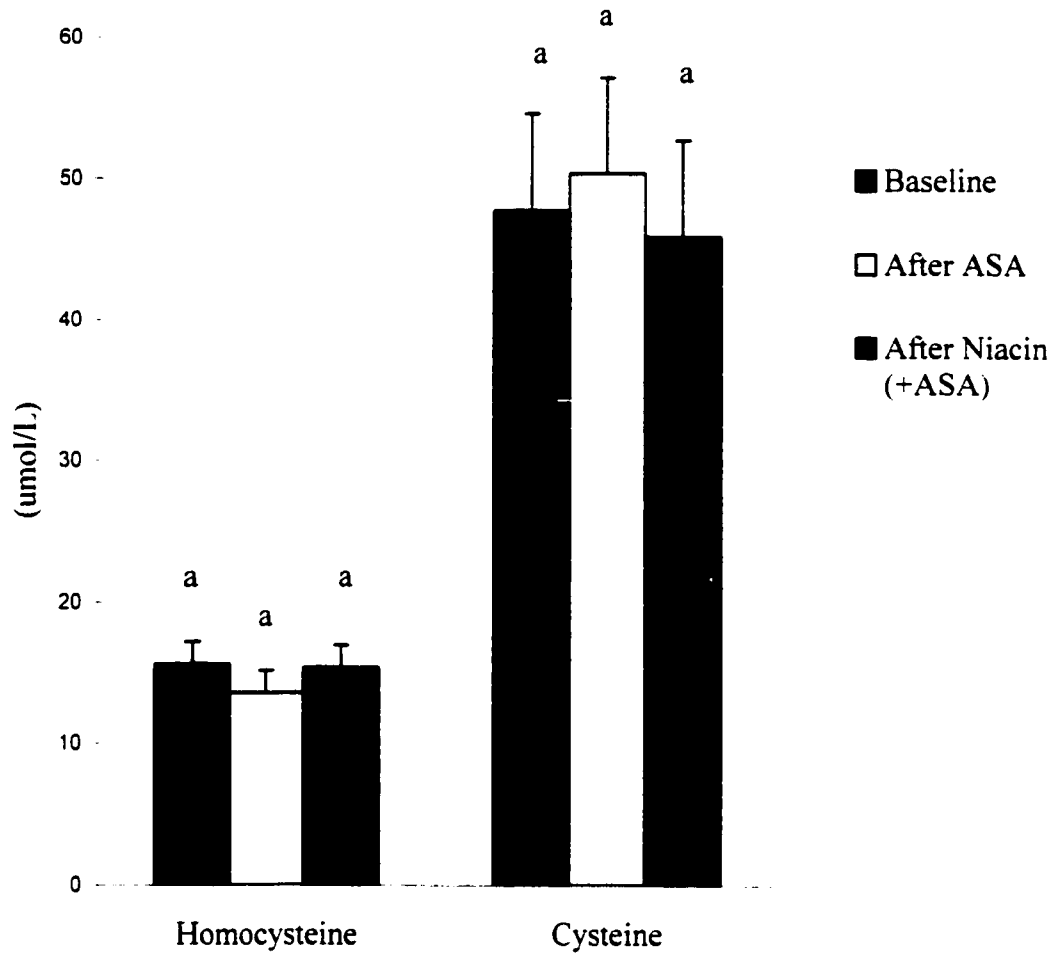


Figure 2.8: The effect of ASA alone (325mg/d) and the proceeding niacin (+ASA) regimen on plasma homocysteine and cysteine levels

*Each sulfur amino acid bar value is expressed as the Lsmean ± SEM of 15 subjects. Sulfur amino acid bars not sharing the same letter in each period are significantly different ($p<0.05$).

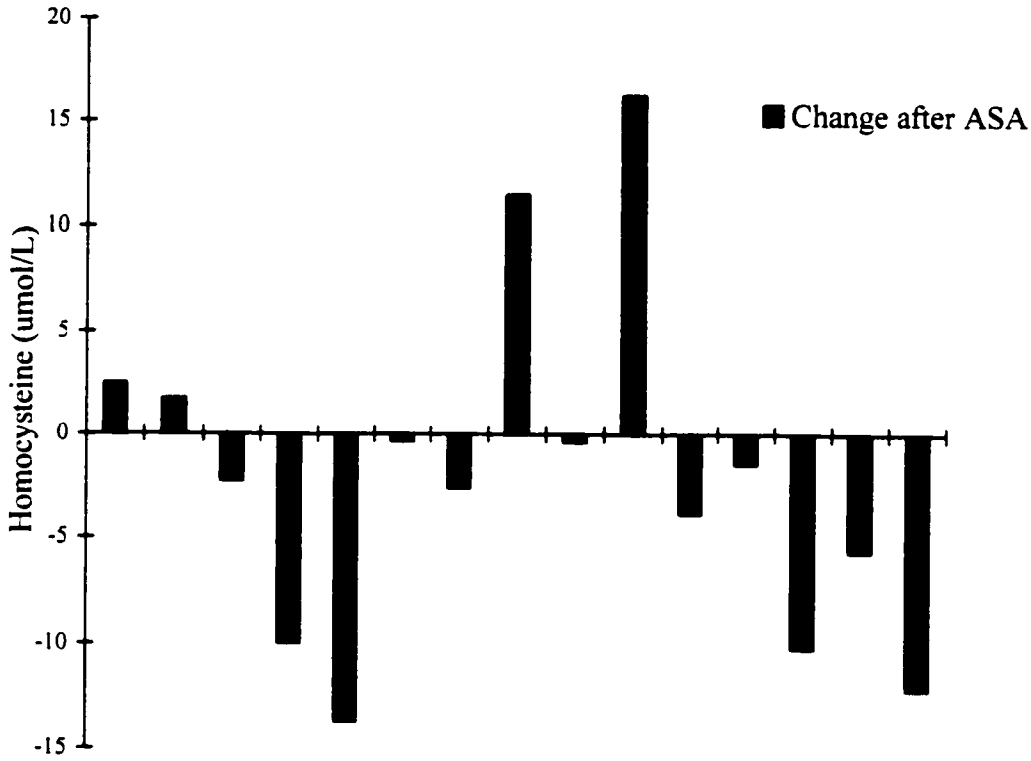


Figure 2.9: An individual analysis of the effect of ASA alone (325mg/d) on plasma homocysteine levels

*Each bar represents the change in an individuals plasma homocysteine levels (n=15) after administering ASA (1 wk) in the present study.

Table 2.18: The effect of ASA alone (325mg/d) and a niacin (+ ASA) regimen on urinary homocysteine and cysteine excretion

Amino acid	Baseline	After ASA (ηmol)	After Niacin (+ASA) (ηmol)	P-value
Homocysteine	3.63 ± 0.52[a]	4.11 ± 0.50[a]	4.38 ± 0.52[a]	> 0.05
Cysteine	57.5 ± 5.21[a]	53.16 ± 5.01[a]	51.12 ± 5.45[a]	> 0.05

* Each amino acid value is expressed as the Lsmean ± SEM of 15 subjects. In each row, values not sharing the same letter are significantly different (P<0.05).
** Amino acid values are expressed as ηmol/g creatinine.

Table 2.19: The effect of ASA (325mg/d) on baseline plasma B-vitamin levels

B - vitamin	Baseline	After Aspirin Treatment	P-value
RBC Folate (ηmol/L)	1261.44 ± 115.02[a]	1179.84 ± 126.29[a]	0.2321
Plasma Folate (ηmol/L)	29.56 ± 2.12[a]	27.42 ± 2.33[a]	0.3963
Plasma B12 (ρmol/L)	306.87 ± 20.50[a]	239.02 ± 22.50[a]	0.0688
Plasma PLP (ηmol/L)	33.01 ± 4.27[a]	34.12 ± 4.69[a]	0.6413
Plasma PA (ηmol/L)	2.98 ± 0.17[a]	2.90 ± 0.20[a]	0.8046

* Each B-vitamin value is expressed as the Lsmean ± SEM of 15 subjects. In each row, values not sharing the same letter are significantly different (P<0.05).

An analysis of plasma B-vitamin levels determined that mean plasma folate concentrations significantly decreased (27.42 vs. 19.3 ηmol/L, p=0.0115) after the niacin (+ ASA) therapy (Table 2.2). All other changes in the mean B-vitamin levels were not statistically significant; however, the observed increase in the mean plasma PLP concentration was strongly significantly correlated with the elevation in the mean plasma homocysteine level (r=0.69, p=0.0045) and the reduction in the mean plasma cysteine concentration (r =0.75, p=0.0014).

Table 2.2 : The effect of niacin (+ASA) therapy on plasma B-vitamin levels

B -vitamin	After Aspirin Treatment	After Niacin plus Aspirin Treatment	P-value
RBC Folate (ηmol/L)	1179.84 ± 115.02[a]	1242.00 ± 115.02[a]	0.8159
Plasma Folate (ηmol/L)	27.42 ± 2.33[a]	19.32 ± 2.21[b]	0.0115
Plasma B12 (ρmol/L)	239.02 ± 22.50[a]	248.33 ± 21.45[a]	0.7265
Plasma PLP (ηmol/L)	34.12 ± 4.69[a]	38.20 ± 4.47[a]	0.5471
Plasma PA (ηmol/L)	2.90 ± 0.20[a]	2.68 ± 0.24[a]	0.5334

* Each B-vitamin value is expressed as the Lsmean ± SEM of 15 subjects. In each row, values not sharing the same letter are significantly different (P<0.05)

2.3 DISCUSSION

Niacin is a vitamin that facilitates a lipid-lowering effect when taken in doses 100-200X's higher than the RNI (Farmer and Gotto, 1996). The present study investigated the relationship between a niacin (plus ASA) regimen and homocysteine status in 15 hypercholesterolemic subjects. Since ASA requires at least three days for gastric mucosa adaptation (Fischer, 1999; Graham et al., 1983), ASA was pre-administered (325mg/d) for one week prior to the traditional regimen. This pre-treatment also helped to deter negative cutaneous responses to niacin. Furthermore, the results of the present study suggest that ASA may possess hypolipidemic properties that work synergistically with niacin to normalize hyperlipidemia.

After only one week of ASA administration, the mean plasma TC and TG levels were significantly decreased. Previous research has found that ASA increases basal insulin levels and reduces serum glucose levels in normal subjects (Giugliano et al., 1978; Boron, 1992); thus, it decreases the release of FFA from adipose tissue and increases FFA removal from circulation (Carlson and Ostman, 1961). Micossi et al. (1978) reported that ASA reduced serum FFA concentrations after four days of supplementation (10g/d); however, no changes in serum TC were observed. Other research has shown that daily ASA administration (≥ 5g/d) reduced serum TC levels (Alexander et al., 1959; Hetzel et al., 1959). A significant TC lowering effect with a lower dose of ASA (1.5 g/d) has also been reported (Eidlitz, 1969). According to the present investigation, significant reductions in TC and TG levels were achieved with a much lower dose of ASA (325 mg/d). These reductions may have resulted from the ability of salicylates to reduce FFA and glycerol mobilization from adipose tissue (Vik-Mo, 1977) and to increase FFA re-esterfication in the adipocyte (Vik-Mo and Mjos, 1978). A more recent investigation involving rats suggests an alternative mechanism (Prigge and Gebhard, 1997). ASA administration may significantly increase bile cholesterol secretion; however, in rats, serum cholesterol levels and bile flow rate remained unaffected.

The present study also found that ASA may have a positive influence on homocysteine status. Oral ASA administration (325mg/d) lowered homocysteine levels in 73% of the participants. The mechanism behind the lowering of this atherosclerotic risk factor is unknown. Since a primary function of ASA is to inhibit prostaglandin synthesis (McEvoy et al., 1998), it can be speculated that prostaglandin inhibition may be related to it's observed homocysteine lowering action; but there is no scientific evidence to support or dispute this concept. A recent investigation found that ASA did not affect folate status or the SAM/SAH ratio in rat liver (Varela-Moreiras et al., 1993); thus ASA did not affect this regulatory mechanism of homocysteine metabolism (Verhoef et al., 1996). The results of the present study suggest that an increase in the activity of the folate-dependent re-methylation pathway may be responsible since decreases in the mean plasma folate and vitamin B12 level were paralleled with an increase in plasma PLP concentrations. This view is further supported by the subsequent increase in urinary homocysteine and decrease in urinary cysteine excretion; however, the exact mechanism is not elucidated.

The high response rate to ASA suggests that a relationship may exist between ASA and homocysteine. As far as it could be determined, no clinical trials have focused on the relationship between ASA and homocysteine. However, from the research available it can be postulated that ASA may help to counteract the cytotoxic effect of homocysteine in the vasculature. Since ASA influences nitric oxide (NO) formation (Nisho and Watanabe, 1998; Fisher et al., 1999), ASA may help to ameliorate the impairment of NO production and subsequent endothelial dysfunction induced by escalating homocysteine levels (Chambers, 1998). Thus, ASA may favourably alter homocysteine levels and help to neutralize it's negative effect on NO production in the endothelium. These are potential therapeutic benefits of ASA that warrant further clinical investigation.

The results of the present study revealed that the niacin (plus ASA) effectively lowered lipid levels with minimal side effects. The therapy did not facilitate a significant mean plasma homocysteine increase; however, the short-term intervention did increase the mean level by 13.3%. Significant reductions in the mean plasma folate level suggests

that the hypolipidemic regimen may have stressed the primary cellular re-methylation pathway of homocysteine. Since niacin, a water soluble vitamin, cannot be stored in the body beyond it's tissue saturation, it is not surprising that pharmacological dose levels would increase homocysteine levels since SAM is required for niacin's methylated excretion. The rise in the plasma PLP level was accompanied by increased homocysteine and decreased urinary excretion of cysteine. These results suggest that the transsulfuration pathway was also affected. Significant changes in B12 status were not observed. This was not surprising since the biological half life of B12 in the body is more than 480 days (Basu and Dickerson, 1996).

The results of the present study were in agreement with previous investigations in rats (Basu and Mann, 1997; Makhani, 1998) since homocysteine elevations were observed in more than one-half of the study subjects after treatment with pharmacological dose levels of niacin. Basu and Mann (1996) attributed significant increases in free-homocysteine levels to decreased plasma PLP levels in the presence of niacin. A longitudinal investigation also found that supplemental niacin (1000mg/kg diet) increased plasma total homocysteine levels in rats (Makhani, 1998). This increase was also attributed to reductions in plasma PLP as well as reductions in vitamin B12 levels since folate status remained unaffected. However, unlike previous research, the results of the present study indicate that niacin stressed the folate-dependent re-methylation pathway, not the transsulfuration pathway, of homocysteine. The increased demand for folate in the re-methylation process resulted in a depletion of plasma folate levels and an increase in the plasma homocysteine to cysteine ratio (0.33 vs. 0.65 mmol/L) after a short intervention period.

Homocysteine is an independent risk factor for atherosclerotic disease (Nygard et al., 1997). According to one prospective investigation (n>20000), as levels approach 12μmol/L or higher, the risk of CVD increases (Arresen et a., 1995). An increase in plasma levels as low as 1μmol/L has been reported to elevate the risk of CVD related mortality by 5.6% (Taylor et al., 1999). Therefore, the mean plasma homocysteine

elevation of 1.81 μmol/L, in the present study, is an important observation that holds clinical significance. Since the niacin (+ASA) regimen did adversely affect the mean plasma homocysteine level despite the possible homocysteine-lowering effect of ASA, it is important to determine if the observed increases can be ameliorated by the supplementation of B-vitamin cofactors without impeding niacin's hypolipidemic action.

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CHAPTER THREE: THE EFFECT OF B-VITAMIN SUPPLEMENTATION ON HOMOCYSTEINE STATUS IN HYPERCHOLESTEROLEMIC INDIVIDUALS ON A NIACIN (PLUS ASPIRIN) REGIMEN

3. INTRODUCTION

The daily implementation of a niacin (plus ASA) regimen effectively lowers atherogenic lipoproteins; however, this regimen may also facilitate mild hyperhomocysteinemia, a risk factor for CVD (Chapter Two). Although the observed mean plasma homocysteine elevations of the previous study were not statistically significant, 53% of study subjects experienced homocysteine elevations after only 3 weeks of treatment with niacin at it's hypolipidemic dose level (1.5g/d). Is important to consider that mild elevations as low as 1 μmol/L are associated with an increased risk of CVD mortality (Taylor et al., 1999). Homocysteine concentrations as low as 12 μmol/L have been associated with an increased risk for atherosclerotic thromboembolic disease (Arnesen et al., 1995). A recent meta-analysis concluded that the relative risk of mortality with homocysteine concentrations >15μmol/L and < 10 μmol/L is similar to the risk associated with plasma TC levels between 4.6 and 7.2 mmol/L (Omenn et al., 1998). Collective consideration of case-control studies (Boushey et al., 1995; Graham et al., 1997; Den Heijer et al., 1998), prospective investigations (Stampfer et al., 1992) and clinical investigations (Nygard et al., 1997; Wald et al., 1998) has provided substantial support for homocysteine as an independent risk factor for CVD. Thus, it is of paramount importance to determine possible causes of hyperhomocysteinemia and efficient ways to ameliorate homocysteine elevations.

Since folate, vitamin B12 and vitamin B6 are modulators of homocysteine metabolism, recent studies have focused on oral supplementation of these vitamins and homocysteine status. Homocysteine is primarily re-methylated to methionine via methionine synthase, and enzyme that requires folate and vitamin B12, or is degraded by CBS and γ-

cystathionase, enzymes that are dependent on vitamin B6 in the form of PLP (Finkelstein, 1990). Supplemental folate (1mg/d) has the most potent lowering effect on homocysteine status (Ubbink, 1997; Ramussen et al., 1996; Fenech et al., 1998). However, a combination therapy that also includes vitamin B6 and B12 is considered the best approach since full response to folate cannot be achieved if B12 status is low (Landren, 1995). Since IR- niacin in doses of <3 g/d is primarily excreted as CH_3- nicotinamide and methylated pyridones (Henderson, 1983), high doses of niacin may deplete B-vitamin co-factors of homocysteine metabolism due to an increased demand for SAM. Since the previous study (Chapter Two) found significant decreases in plasma folate levels after short term use of the traditional niacin (+ASA) regimen, the following study was designed to examine if dietary supplementation of folate, vitamin B6 and vitamin B12 has a positive effect on homocysteine status without compromising niacin's hypolipidemic action.

3.1 METHODS

The following experimental protocol was approved by the Department of Agricultural, Food and Nutritional Science Human Ethics Committee at the University of Alberta. Fifteen subjects (8 women and 7 men) who previously followed a traditional niacin (+ASA) regimen previously for five weeks (Section 2.1) participated in the three week supplementation study. Subjects continued the hypolipidemic therapy of one-half of an ASA tablet (½ of 325mg) + 750mg niacin (twice per day) with the concomitant daily oral-administration of folate (1mg folate+ 20mg vitamin C) and B6 (100mg pyridoxine). Vitamin B12 (250μg cyanocobalamin) capsules were taken weekly since individuals had normal plasma levels (Chapter Two). At the end of the niacin (+ASA) therapy and supplemental intervention, final blood and urine samples were collected. Subjects were provided with their personal lipoprotein history and were advised to consult a physician if they chose to continue the hypolipidemic regimen. Samples were placed on ice and centrifuged within one hour of collection to prevent homocysteine elevations due to continued synthesis by erythrocytes (Ubbink et al., 1992; Malinow et al., 1994). Plasma

and urine samples were stored frozen (-70°C and -25°C respectively) until thawed for laboratory analysis.

3.11 Determination of plasma total cholesterol

Plasma samples were analyzed using Sigma Diagnostics Cholesterol Procedure No. 325 (St. Louis, MO). The enzymatic assay, modified from a procedure developed by Allain et al. (1974), is more specific than non-enzymatic assays, uses only one reagent and requires no prior treatment of plasma (Section 2.11).

3.12 Determination of plasma HDL

Plasma samples were analyzed using Sigma Diagnostics Procedure No. 352-3 (St. Louis, MO). This precipitation separation procedure separated was modified from a method developed from Warnick et al. (1982). Required two-steps where the remaining total cholesterol concentration was representative of the total HDL remaining in solution (Section 2.12).

3.13 Determination of plasma triglyceride

Plasma samples were analyzed using the Sigma Diagnostic Procedure No. 336 (St. Louis, MO). This enzymatic procedure was modified from that of Bucolo and David (1973) that links the enzymatic hydrolysis of TG to the enzymatic determination of glycerol (Section 2.13).

3.14 Determination of plasma LDL concentration

Plasma LDL was determined using the 'Friedwald Equation'. Friedwald et al. (1972) devised a method of calculating LDL concentration (mg/dL) from plasma concentrations of total cholesterol, HDL and TG:

$$\text{LDL (mg/dL)} = \text{Total Cholesterol (mg/dL)} - \text{HDL (mg/dL)} - \frac{\text{TG (mg/dL)}}{5}$$

The above equation may not accurately estimate plasma LDL concentration if TG levels are above 400 mg/dL. The results were converted to SI units by a conversion factor of 0.0295.

3.15 Determination of plasma and urinary sulfur amino acid concentrations

Homocysteine and cysteine concentrations in both plasma and urine were analyzed by a high performance liquid chromatography (HPLC) (Still and McDowell, 1998). This widely used HPLC method precipitated proteins with perchloric acid. Thiol groups were derivatized with ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBDF) and the resulting mixture was centrifuged. HPLC with a fluorometric detector identified SBDF-labelled thiols remaining in the supernatant. SBDF was detected at a wavelength of 515 ηm (Section 2.15).

3.16 Determination of liver enzymes and plasma clinical chemistry

The Pathology Department at the University of Alberta performed all clinical chemistry tests on plasma with a Kodak 700XR analyzer. Plasma levels of total bilirubin (TBIL), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), blood urea nitrogen (BUN), creatinine and glucose (GLC) were assessed. Urinary samples were analyzed for creatinine. Commercially available human controls were tested daily to ensure accuracy. The results were reviewed by a Certified Pathologist to identify any pathological concerns.

3.17 Statistical Analysis

The lsmeans and standard error of the mean (SEM) for all parameters measured were determined. Lipoprotein, B-vitamin, sulfur amino acid and blood chemistry data were analyzed using a Multivariate Analysis of Variance (MANOVA) procedure with SAS 7.0

software. Differences in plasma or urinary concentrations were significant if $p < 0.05$. Spearman correlation coefficients (R) were determined to identify significant correlation's between changes in lipoprotein concentrations, B-vitamins and sulfur amino acid concentrations. R-values were also significant if $p < 0.05$.

3.2 RESULTS

The administration of the hypolipidemic regimen with B-vitamin supplementation resulted in non-significant changes in mean plasma levels of TC (5.9 vs. 5.8 mmol/L) and LDL (3.66 vs. 3.55 mmol/L) (Figure 3.0). The decrease in the mean plasma TC concentration was significantly correlated with the mean decreases of the plasma LDL level ($r=0.9509$, $p=0.0001$). The slight decreases in atherogenic lipoproteins were paralleled by an increase in the HDL to LDL ratio (0.42 vs. 0.44, $p=0.5995$). After the completion of the hypolipidemic regimen and B-vitamin supplementation intervention, blood chemistry tests were performed at the end of the trail to identify possible indicators of hepatotoxicity and renal problems (Table 3.0). No significant differences were found with all of the parameters tested. All clinical tests remained within the normal clinical range with the exception of mean glucose levels (5.6 vs. 6.27 mmol/L); however, other sources report that normal fasting glucose levels may be as high as 6.4 mmol/L (Lee and Nieman, 1996).

The effect of supplemental folate, vitamin B12 and vitamin B6 on plasma sulfur amino acid status in individuals on the niacin (+ASA) regimen is presented in Figure 3.1. The supplementation regimen resulted in statistically non-significant reductions in the mean plasma homocysteine concentration (15.38 vs. 12.37 μmol/L, $p=0.2058$) and cysteine level (45.83 vs. 36.95 μmol/L, $p=0.2212$) and a paralleled decrease in the homocysteine to cysteine ratio (0.65 vs. 0.37 mmol/L). Although the reductions in mean plasma levels were non-significant, a direct significant relationship between the reduction in homocysteine and the reduction in cysteine levels was found ($r=0.6500$, $p=00087$).

Eighty-eight percent of the subjects who responded to the B-vitamin supplemental regimen had experienced plasma homocysteine elevations previously with the short term use (5 weeks) of the niacin (+ASA) regimen (Chapter Two). The B-vitamin supplemental regimen also facilitated non-significant increases in the urinary excretion of homocysteine and cysteine (Table 3.1). The increase in homocysteine excretion was directly correlated with the decrease in plasma homocysteine concentrations ($r=0.6000$, $p=0.0233$).

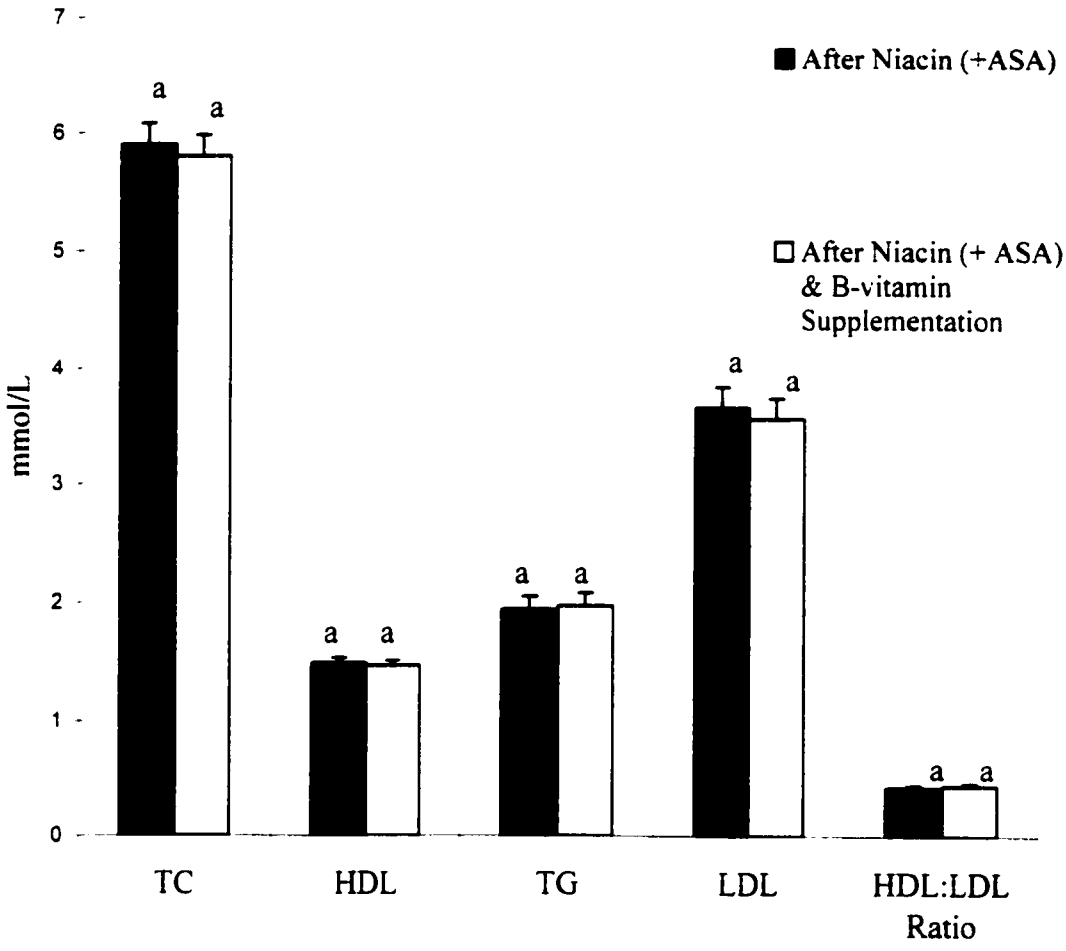

Figure 3.0: The effect of a niacin (+ASA) regimen followed by B-vitamin supplementation on plasma lipoprotein concentrations

* Each lipoprotein value is expressed as the Lsmean ± SEM of 15 subjects. Lipoprotein values not sharing the same letter in each period are significantly different ($p<0.05$).

Table 3.0: Blood chemistry results over the 3 week niacin (+ ASA) intervention & B-vitamin supplementation trial

Test	Normal Values**	After Niacin (plus ASA)	After Niacin (plus ASA) & B-vitamin supplementation	P-value
Plasma				
ALT (units/L)	30 - 110	26.93 ± 1.3[a]	26.90 ± 1.3[a]	1.0000
ALKP (units/L)	10 - 32	10 ± 0.0[a]	10 ± 0.0	-
TBIL (μmol/L)	3 - 22	11.64 ± 1.1[a]	14.13 ± 1.1[a]	0.9214
GLC (mmol/L)	3.9 - 5.6	5.67 ± 0.4[a]	6.27 ± 0.4[a]	0.3542
Creatinine (μmol/L)	70 - 110	82.49 ± 2.0[a]	84.47 ± 2.0[a]	0.5188
BUN (mmol/L)	3 - 7	5.17 ± 0.2[a]	5.72 ± 0.2[a]	0.1514

*Each test value is expressed as the Lsmean ± SEM of 15 subjects. Values not sharing the same letter are significantly different ($P<0.05$).

** References: (Fandek et al., 1995; Tilkian et al., 1987)

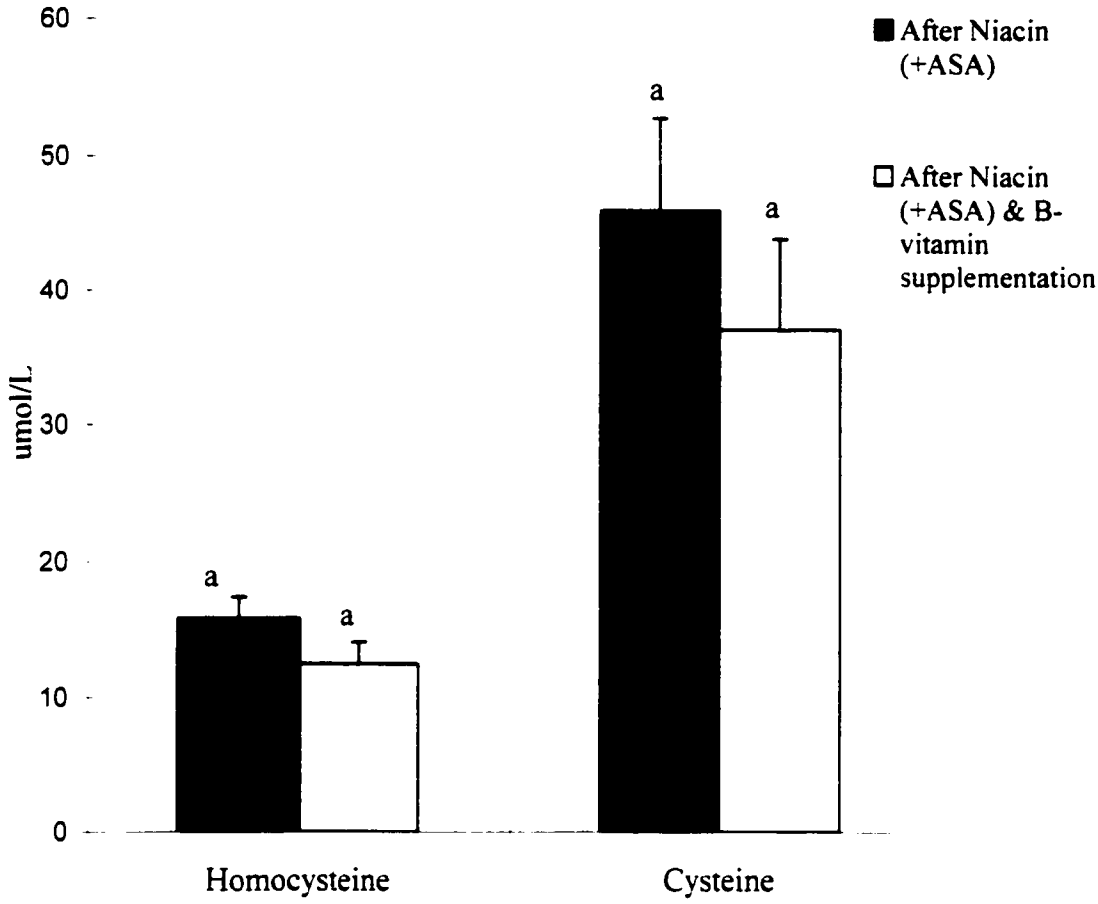

Figure 3.1: The effect of B-vitamin supplementation on plasma homocysteine and cysteine levels in individuals on a niacin (plus ASA) regimen

*Each amino acid value is expressed as the Lsmean ± SEM of 15 subjects. Values not sharing the same letter are significantly different ($P<0.05$).

Table 3.1: The effect of niacin (+ ASA) regimen followed by 3-weeks of B-vitamin supplementation on urinary homocysteine and cysteine excretion

Amino acid	After Niacin (+ASA) (ηmol)	After Niacin (+ASA) & B-vitamin supplementation (ηmol)	P-value
Homocysteine	4.38 ± 0.52^{a}	5.27 ± 0.50^{a}	0.1769
Cysteine	51.12 ± 5.45^{a}	58.37 ± 0.52^{a}	0.3410

* Each amino acid value is expressed as the Lsmean ± SEM of 15 subjects. In each row, values not sharing the same letter are significantly different ($P<0.05$).
** Amino acid values are expressed as ηmol/g creatinine.

3.3 DISCUSSION

Niacin is an effective hypolipidemic agent that facilitates the lowering of atherogenic lipoproteins when taken in doses of 1.5-6g per day (Farmer and Gotto, 1996). To deter cutaneous symptoms, it is common practice to administer ASA 30 minutes prior to IR-preparations or crystalline niacin (Alexander et al., 1998; Jungnickel, 1997). This therapeutic regimen (1.5g niacin + 325mg ASA per day) effectively lowered mean plasma TC, TG and LDL concentrations in 15 hypercholesterolemic subjects (Chapter Two). The results of the present study indicate that the addition of supplemental folate, vitamin B6 and vitamin B12 to the therapeutic regimen did not adversely effect niacin's hypolipidemic action (Figure 3.0). This finding is of considerable importance since the addition of a combination B-vitamin supplemental therapy had a positive effect on plasma homocysteine levels.

Niacin, a water soluble vitamin, is linked to homocysteine metabolism since it requires a methyl group from SAM for it's urinary excretion (Jacobsen et al., 1995). Homocysteine is readily re-methylated to methionine via methionine synthase, an enzyme requiring folate and B12, and/or is irreversibly degraded to cysteine, a transsulfuration process requiring vitamin B6 in it's PLP form (Verhoef et al., 1996). The results of the previous clinical study suggest that pharmacological dose levels of niacin may stress the re-methylation pathway since significant reductions in plasma folate levels were found in combination with a mean increase in plasma homocysteine levels (Chapter Two). The present study administered a combination supplemental regimen of folate, vitamin B12 and vitamin B6 in an effort to normalize homocysteine levels. The supplemental regimen achieved a 2.91μmol/L mean reduction in homocysteine levels. This mean level was lower than the initial level (13.7μmol/L) prior to the onset of the niacin (+ASA) regimen (Chapter Two).

Research has shown that supplemental folate has the most potent effect on plasma homocysteine since it increases the activity of the re-methylation pathway by increasing

the amount of THF available (Ubbink, 1997; Clarke, 1998). The present study used a combination of B-vitamin cofactors (folate, vitamin B12 and vitamin B6) in an effort to ameliorate slightly elevated homocysteine concentrations. Plasma B12 levels were within the normal range (Chapter Two), but a weekly supplement of 250μg of cobalamin was administered to ensure that adequate B12 would be available for the re-cycling of folates. If vitamin B12 is not available in sufficient amounts, the re-methylation process cannot proceed (Basu and Dickerson, 1997). MTHF transfers a methyl group to homocysteine via a B12 dependent enzyme, methionine synthase, resulting in the re-generation of methionine and THF (Matthews, 1998). The transfer of the CH_3 group to cobalamin is the only outlet for cellular THF re-cycling; thus, inadequate levels in vitamin B12 impairs folate metabolism and 'traps' folate in it's methylene form (Allen et al., 1998; Basu and Dickerson, 1997). It has been proposed that if folate and B12 levels are compromised, then elevations of homocysteine cannot be alleviated by B6 alone since elevated SAM levels are necessary to trigger an increase in CBS activity (Selhub and Miller, 1992).

In the present study, the daily supplementation of 1mg of folate, which included the weekly administration of B12, helped to alleviate homocysteine's accumulation by maintaining adequate re-methylation. This is supported by the findings of Woodside et al. (1998) and Lobo et al (1999), where a combination regimen that also included folate (1 mg/d) lowered mean plasma homocysteine levels. Woodside et al. (1998) found significant homocysteine reductions in men of approximately 27% after eight weeks of treatment. However, it is important to recognise that in the present study, a 21.2% decrease in the mean plasma homocysteine level occurred after only after three weeks of supplemental intervention. Thus, to determine the long term benefits of the supplemental regimen, a longitudinal clinical trial is needed.

Homocysteine is considered an independent graded risk factor for various forms of CVD (Boushey et al., 1995; Graham et al., 1997) due to adverse effects on the vascular endothelium (Guba et al., 1996; Ueland et al., 1992; Upchurch et al., 1995). The supplementation of B-vitamin co-factors is a cost effective way to ameliorate potentially

adverse homocysteine elevations. The present investigation found that supplemental B-vitamin therapy reduced mild homocysteine elevations induced by a short-term hypolipidemic niacin (+ASA) regimen without adversely affecting lipid status. Therefore, in order to help to prevent plasma homocysteine elevations with the therapeutic use of IR-niacin, the addition of B-vitamin supplementation (folate, vitamin B6 and vitamin B12) to the hypolipidemic regimen is recommended.

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CHAPTER FOUR: GENERAL DISCUSSION AND CONCLUSIONS

4. DISCUSSION AND CONCLUSIONS

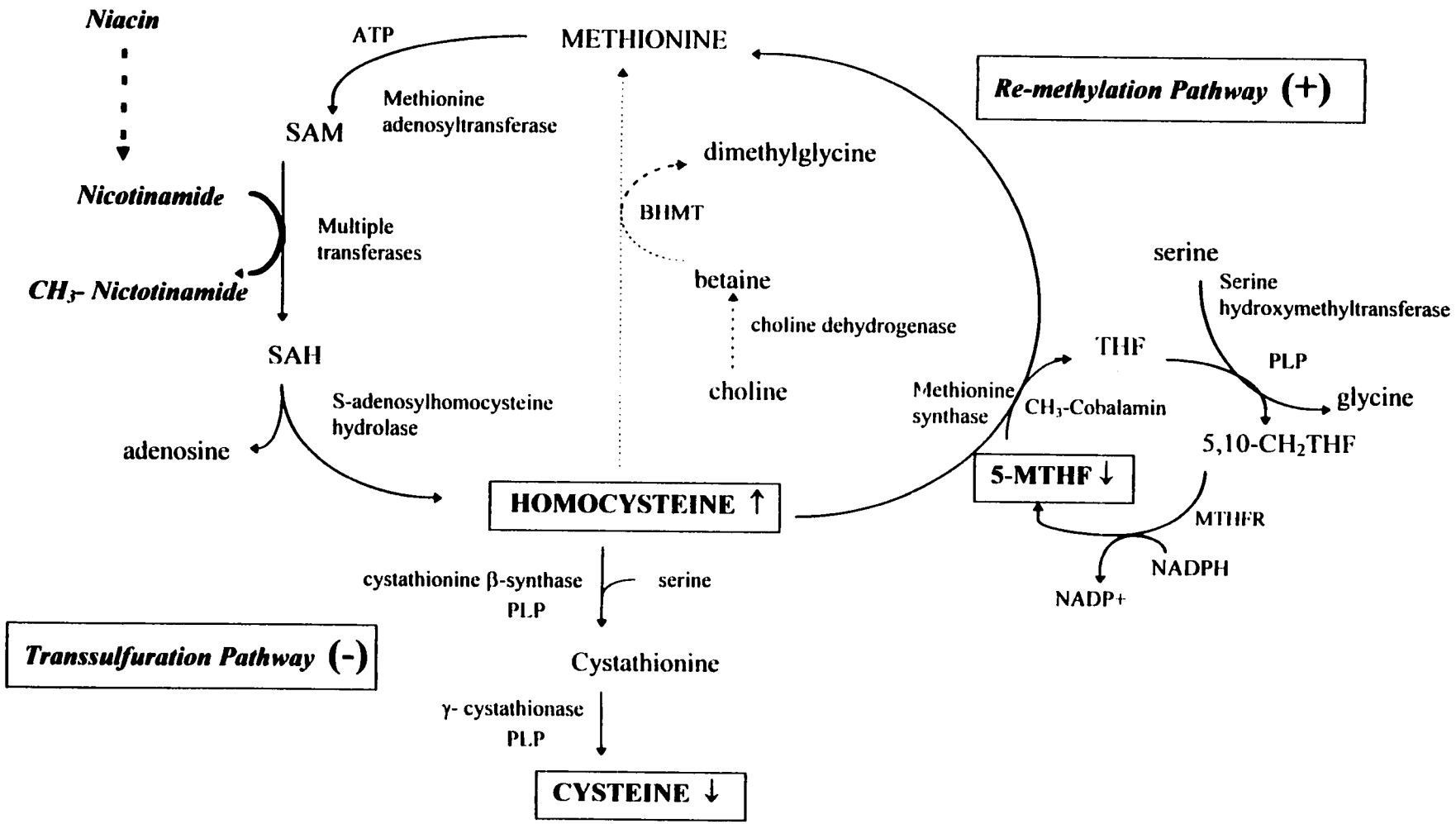
Nicotinic acid (niacin) is a broad spectrum hypolipidemic agent that lowers all atherogenic lipoproteins (Yeshurun and Gotto, 1995; Farmer and Gotto, 1996) while concurrently raising circulating HDL-C concentrations (Braunwald, 1997). This lipid-lowering agent is gaining popularity since it is relatively inexpensive in comparison to other hypolipidemic agents (Pearson, 1996) and it is readily available without a prescription. The most common side effects experienced with IR-preparations include cutaneous symptoms (flushing, itchiness, dry skin) and gastrointestinal disturbances (Illingworth, 1988; Illingworth, 1987). Since flushing is secondary to the endogenous production of prostaglandins, the pre-administration of aspirin or ASA (a prostaglandin inhibitor) minimizes the cutaneous response to niacin (Luria, 1990; Jungnickel, 1997). In the present study, only 20% of the participants reported cutaneous flushing upon initiation of the niacin (+ASA) regimen (Chapter Two); therefore, this study provides further evidence in support of ASA's ability to deter cutaneous reactions to niacin.

Homocysteine, an intermediate of methionine metabolism (Cattaneo, 1997; Finkelstein, 1988; Finkelstein, 1990), is considered an independent risk factor for atherosclerotic thromboembolic disease (Boushey et al., 1995; Graham et al., 1997; Shelub et al., 1995). Homocysteine is metabolized in two ways: (1) it is re-methylated to methionine via methionine synthase or BHMT (in the liver) (Refsum et al., 1998; Finkelstein, 1990) or (2) it condenses with serine in an irreversible transsulfuration reaction to generate cysteine (Verhoef, 1996). Homocysteine metabolism is primarily regulated by cellular SAM concentrations and the availability of B-vitamin cofactors needed for re-methylation (folate and B12) and transsulfuration (vitamin B6) (Fowler et al., 1997). Since SAM is the methyl donor for niacin's methylated excretion (Shibata and Matsuo, 1989), it is feasible that pharmacological dose levels of niacin would influence homocysteine status.

Recent investigations involving rats were the first to report that IR-niacin, in pharmacological dose levels, facilitated the development of hyperhomocysteinemia (Basu and Mann, 1996; Makhani, 1998). These studies indicated that the niacin-induced hyperhomocysteinemia resulted from reduced plasma PLP levels (Basu and Mann, 1996) and/or reduced availability of vitamin B12 (Makhani, 1998). These findings suggest that the benefits of niacin's hypolipidemic action may be negated by its hyperhomocysteinemic effects. Thus, it is of considerable importance to the determine the extrapolative significance of the above observations in human subjects.

The present investigation was the first clinical trial of it's kind to investigate if a traditional therapeutic niacin (+ASA) regimen modifies homocysteine status. This therapeutic regimen facilitated a mean increase in plasma homocysteine of 13.3% after only three weeks of treatment at niacin's hypolipidemic dose level (1.5g/d). Although the mean increase was not statistically significant, over one-half of the study participants experienced higher homocysteine levels after this short term intervention period;. Thus, the results support previous findings that niacin adversely affects homocysteine metabolism (Basu and Mann, 1996; Makhani, 1998). Since folate levels were significantly reduced and the PLP levels increased, it can be deduced that a stressed re-methylation pathway was responsible for the rise in homocysteine concentrations (Figure 4.0). It is important to recognise that the study participants had normal plasma B-vitamin levels (folate, B12 and B6) prior to the therapeutic treatment and their estimated daily intakes of these vitamins were greater than 100% of the RNI. In this case it was clear that dietary intakes achieved through diet alone could not meet the demand for folate necessary to provide for niacin's methylated excretion. Thus, a B-vitamin supplemental regimen was initiated.

Figure 4.0: The effect of a pharmacological niacin (1.5g/d) plus ASA (325 mg/d) regimen on homocysteine metabolism



Adapted from: (Selhub and Miller, 1992; Allen et al., 1998)

A noteworthy limitation of the present investigation was it's small sample size (n=15; men =7; women =8). It was difficult to recruit individuals who met all of the necessary criteria (Chapter 2-Table 2.0). The criteria controlled for many confounding variables that would otherwise have interfered with the niacin therapy and/or influenced homocysteine status. Therefore, the sample population may not be an accurate representation of the general population. The participants were middle-aged, free-living moderately active individuals who did not smoke, drink, or take medications and they achieved normal B-vitamin status through adequate dietary intake (>100% RNI). In the general population, borderline intakes of the necessary B-vitamins may be more common, especially in individuals who consume vegetarian diets. Since other factors such as smoking, drinking, excessive coffee consumption, and medications and disease may negatively influence homocysteine status (Townend, 1998; Section 1.64), it is most likely that the therapeutic niacin (+ASA) regimen would attenuate hyperhomocysteinemia in individuals at higher risk. The elderly population (>70 years) may be in a higher risk category since intrinsic factor required for B12 absorption is often compromised and renal problems are common. Since fibric acid derivatives and HMG-CoA reductase inhibitors, were found not to have an effect on homocysteine levels (Lorgeril et al. 1999), these agents may be a better alternative to niacin for higher risk individuals. Bile acid resins may also adversely affect homocysteine status since 'the resins' impair folate absorption (Schachter, 1996; Blackenhorn et al., 1991).

Recent investigations have focused on nutritional intervention strategies to lower plasma homocysteine concentrations. Although folate supplements render the most significant reductions (Clarke et al., 1998; Ubbink et al., 1997), combination supplemental therapies that include all B-vitamin cofactors involved in homocysteine metabolism (folate, vitamin B6 and B12) are considered the best approach (Stein and McBride, 1998). The present study examined if the supplementation of the B-vitamins would positively influence homocysteine status without impairing the hypolipidemic action of the niacin therapy. Although the changes in homocysteine levels were not significant, the present study (Chapter Three) achieved lower homocysteine concentrations (21.2%) after only

three weeks of supplemental therapy. Since the hypolipidemic ability of the niacin (+ASA) regimen was not adversely affected, B-vitamin supplementation is recommended for all users of IR-niacin.

The supplementation of B-vitamin cofactors has been thoroughly investigated and is considered the main-stay of homocysteine-lowering intervention therapy (Cattaneo, 1999). However, betaine is another alternative. Since the betaine dependent pathway is confined to the liver and is not involved in the primary cellular re-methylation pathway (Finkelstein, 1998), betaine supplements were not administered in the present study. The use of betaine has not been thoroughly investigated, perhaps because the use of B-vitamin cofactors is a cost effective strategy with minimal side effects (Boers, 1994). Betaine is also expensive and poorly tolerated (Stein and McBride, 1998; Still and McDowell, 1998). It was reported that betaine decreased homocysteine levels in individuals who were non-responsive to a monotherapy of supplemental B6 (Wilken et al., 1983). Post-methionine loading homocysteine levels have been lowered with betaine in doses well above physiological requirements (6g/d); however, the subjects had normal fasting levels of homocysteine prior to betaine intervention (Franken et al., 1994).

Betaine or trimethylglycine, an intermediate formed from the irreversible oxidation of choline (Finkelstein, 1998), supplies the methyl group to homocysteine independent of B-vitamin cofactors (Townend et al., 1998). However, research has shown that choline is indirectly linked to folate and B12 metabolism (Finkelstein, 1998; Finkelstein, 1988; Kim et al., 1994). Although highly dependent on dietary intake, choline can be synthesized from phosphatidylethanolamine (Finkelstein 1990; Zeisel et al., 1991; Basu and Dickerson, 1997). This *de novo* synthesis requires the triplemethylation of phosphatidylethanolamine by SAM to render phosphatidylcholine, from which choline can be liberated (Finkelstein, 1990; Kim et al., 1994). This process yields three homocysteinyl moieties and only one methionine moiety, therefore to maintain homeostasis, homocysteine is re-methylated via methionine synthase (Kim et al., 1994). Recent research has shown that folate is a critical limiting factor for endogenous choline

synthesis despite adequate methionine intakes required for phosphatidylethanolamine methylation (Jacob et al., 1999). Since the traditional niacin (+ASA) intervention in the present study significantly decreased the mean plasma folate level, it can be postulated that the re-methylation of homocysteine via the BHMT pathway may have been impeded as well.

Choline is essential for the synthesis of phospholipids and acetylcholine as well as the generation of labile methyl groups (Zeisel et al., 1991). Choline is considered a lipotrophic factor required for the secretion of hepatic triglyceride (Endoh et al., 1996). A recent rat investigation implicated that pharmacological doses of niacin may induce hepatic hyperglycemia or 'fatty liver' (Makhani, 1998) that is generally associated with low levels of choline or methionine (Endoh et al., 1996). In the present study niacin was administered concomitantly with ASA. Although ASA is not a lipotrophic factor, recent research has shown that ASA lowers oxidative stress and plays an inhibitory role in the pathogenesis of cirrhosis, fibrosis and hepatocarginogenesis (Dendra et al., 1994; Endoh et al., 1996). Thus, the daily administration of ASA may be beneficial benefit to niacin users by exhorting a protective effect on liver function.

The present investigation found that ASA may have several beneficial effects when taken concomitantly with niacin. After only one week of low-dose ASA administration (325mg/d), significant reductions in mean plasma TC and TG concentrations were found. The results implicate that ASA may possess hypolipidemic properties that work synergistically with niacin to facilitate the lowering of atherogenic lipoproteins. Previous research has indicated that ASA lowers FFA mobilization from adipose tissue (Vik-Mo, 1977), increases FFA re-esterfication in the adipocyte and increases bile cholesterol secretions (Prigge and Gebhard, 1997). ASA may also attenuate niacin's hypolipidemic action by affecting the rate of niacin's excretion. Although niacin is primarily excreted as CH_3-nicotinamide and methylated pyridones in doses < 3g/d, a small portion of IR- niacin may conjugate with glycine to yield a minor excretory product, nicotinuric acid (Henderson 1983). Since ASA is primarily conjugated with glycine to form salicyluric

acid and/or glucuronic acid for urinary elimination, ASA competes with niacin for excretion (CPS, 1997; Dipalma and Thayer, 1991). Thus, concomitant administration of ASA with niacin increases niacin's bioavailability and sustains higher plasma levels to attenuate niacin's hypolipidemic action (Ding et al., 1989).

The results of the present study also suggest that ASA may have a positive effect on plasma homocysteine levels since 73% of the participants experienced lower homocysteine levels with only one week of daily ASA supplementation. The mechanism is not elucidated. After an evaluation of the literature, it is clear that more research is needed to determine if indeed ASA reduces homocysteine levels or offers other therapeutic benefits. A longitudinal study that examines the effect of a monotherapy of ASA on plasma homocysteine levels is recommended.

The present study has set the framework for future clinical investigations. The hypolipidemic regimen was initiated to lower lipoprotein risk factors for CVD, but the results suggest that it may potentially adversely affect another risk factor, plasma homocysteine. More than one-half of the study subjects responded to the niacin (+ASA) regimen with a rise in plasma homocysteine concentrations despite the short intervention period (3 weeks at niacin's hypolipidemic dose) and the use of niacin at it's minimum lipid-lowering dose level. Although changes in the mean homocysteine level did not achieve statistical significance, the result are of clinical importance since elevations as low as 1μmol/L significantly increases CVD-related mortality (Taylor et al., 1998). Niacin therapy is generally a life-long commitment; therefore, the next step is to implement a large longitudinal investigation to determine the effect of niacin at various dose levels (1.5 -3g/d) in combination with ASA (325 mg/d) on homocysteine levels in a hypercholesterolemic population. Since SR-preparations prolong the demand for SAM due to a slower delivery rate of niacin to the liver (Morgan et al., 1998), it is also necessary to determine if these preparations play a role in the development hyperhomocysteinemia.

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APPENDICIES:

APPENDIX A:

A food-frequency questionnaire.

Food Group:	Average Use Last Year								
Dairy Foods	**Never or less than once per month**	**1 - 3 per month**	**1 per week**	**2-4 per week**	**5-6 per week**	**1 per day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Skim Milk (8 oz. Glass)									
1 % Milk (8 oz. Glass)									
2 % Milk (8oz. Glass)									
Whole Milk (8 oz. Glass)									
Cream: e.g. Coffee, Whipped (Tbs.)									
Sour cream: Light 1% milk fat (Tbs.)									
Sour cream: regular (Tbs.)									
Non-dairy Coffee Whitener (tsp.)									
Ice - Cream (1/2 cup) Flavour:									
Sherbet or Ice milk (1/2 cup)									
Yoghurt (1 cup)									
Cottage Cheese (1 oz.)									
Cream Cheese (1 oz.)									
Mozzarella Cheese (1 oz.)									
Cheddar Cheese (1 oz.)									
Margarine (pat) added to food or bread or use in cooking Type: stick low-fat stick tub low fat tub									
Butter (pat) added to food or bread or use in cooking Type: stick low-fat stick tub low fat tub									
Other Cheeses: **e.g.** Ricotta Cheese (1 oz.) Blue Cheese (1 oz.) Gouda Cheese (1 oz.) Swiss Cheese (1 oz.)									
Fruit:									
Orange (1)									
Raisons (1 oz. or small pack) or grape (1/2 cup)s									
Prunes (1/2 cup)									
Cantaloupe (1/4 melon)									
Grapefruit (1)									
Grapefruit Juice (small glass 4 oz.)									
Plum, (1 medium)									
Bananas (1 medium)									

Food Group:	**Average Use Last Year**								
Fruit	**Never or less than once per month**	**1 - 3 per month**	**1 per week**	**2-4 per week**	**5-6 per week**	**1 per day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Apricot (1 fresh or ½ cup canned)									
Strawberries, fresh, frozen, canned (1/2 cup)									
Blueberries, fresh, frozen, canned (1/2 cup)									
Pears, (1 medium or ½ cup canned)									
Apple (1 medium)									
Apple Juice (small glass 4 oz.)									
Orange Juice (small glass 4 oz.)									
Peach (1 medium or ½ cup canned)									
Other Fruit juices: (1 small glass 4 oz) e.g. fruit punch five-alive fruit juice pineapple juice									
Other Berries:									
Other Fruit:									
Vegetables									
Tomatoes (1)									
Tomato Juice (4 oz. Glass)									
Tomato Sauce (1/2 cup) e.g. Spaghetti sauce									
Mushrooms fresh or canned (1/2 cup)									
String Beans (1/2 cup)									
Tofu or soybeans (3-4 oz.)									
Broccoli (1/2 cup)									
Corn (1 ear, or1/2 cup frozen or canned)									
Cabbage or Cole slaw (1/2 cup)									
Carrots, raw (1/2 carrot or 2 sticks)									
Carrots, cooked (1/2 cup)									
Brussels Sprouts (1/2 cup)									
Peas or Lima Beans (1/2 cup fresh canned or frozen)									
Mixed Vegetables (1/2 cup)									
Beans/lentils, baked or dried (1/2 cup)									
Yellow (winter) squash (1/2 cup)									
Eggplant, zucchini, or other summer squash (1/2 cup)									
Yams or sweet potatoes (1/2 cup)									
Spinach, cooked (1/2 cup)									

Food Group:	**Average Use Last Year**								
Vegetables	**Never or less than once per month**	**1 - 3 per month**	**1 per week**	**2-4 per week**	**5-6 per week**	**1 per day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Spinach (raw as in salad) (1/2 cup)									
Celery (2-4"sticks)									
Turnip, cooked (1/2 cup)									
Turnip Greens, cooked (1/2 cup)									
Kale, mustard or chard greens (1/2 cup)									
Iceberg or head lettuce (1/2 cup)									
Romaine lettuce (1/2 cup)									
Cauliflower (1/2 cup)									
Beets, cooked (1/2 cup)									
Alfalfa Sprouts (1/2 cup)									
Garlic, fresh or powder (1 clove ot tsp.)									
Other Vegetables:									
Meat & Alternatives									
Eggs (1)									
Chicken with skin (6 oz.)									
Chicken without skin (6 oz.)									
Turkey with skin (6 oz.)									
Turkey without skin (6 oz.)									
Frankfurter (1)									
Hot Dog Wiener, pork/ beef (1)									
Hot dog wiener, chicken (1)									
Bacon (2 slices)									
Liver, beef (4 oz.)									
Sausage (slice, 1 oz.)									
Salami (slice, 1 oz.)									
Balogna (slice, 3 oz.)									
Tuna fish (3 oz.)									
Pork (6 oz)									
Lamb (6 oz.)									
Beef (6 oz.)									
Mackerel (6 oz.)									
Salmon (6 oz.)									
Sardines (1 can, 3 oz.)									
Shrimp (10 medium)									
Lobster (1 medium)									
Hamburger, lean (e.g. 1 patty)									
Hamburger, medium (e.g. 1 patty)									
Hamburger, regular (e.g. 1 patty)									
peanuts, salted (3 oz.)									

Food Group:	**Average Use Last Year**								
Vegetables	**Never or less than once per month**	**1 - 3 per month**	**1 per week**	**2-4 per week**	**5-6 per week**	**1 per day**	**2-3 per day**	**4-5 per day**	**6+ per day**
peanut butter, smooth									
sunflower seeds, 1 oz.									
Other meats, fish, organ meats, nuts, beans or seeds etc.									
Bread & Cereals									
Cooked Oatmeal (1 cup)									
White Bread (1 slice)									
Brown Bread (100%) Whole Wheat (1 slice)									
Brown Bread (70%) Whole Wheat (1 slice)									
Bagel (1) white or whole wheat									
English Muffin (1)									
Brown Rice (1 cup)									
White Rice (1 cup)									
Wheat Germ (1 Tbs.)									
Oat Bran (1 Tbs.)									
Pasta (spaghetti noodles , etc.) (1 cup)									
Bulgar (1 cup)									
Kasha (1 cup)									
Cousous (1 cup)									
Pancakes (3)									
Waffles, plain (3)									
Potatoes: Baked (1), Boiled (1) or mashed (1 cup)									
Potato Chips/Corn Chips (80g Bag)									
Crackers (2)									
Cold Breakfast Cereal (1 Cup) Type:_____									
French Fried Potatoes (1)									
Other Breads and Cereals:									
Beverages:									
Diet Cola with Caffeine (e.g. coke) (1 can/glass)									
Diet Cola Caffeine Free (e.g. coke)									
Other Diet Carbonated Beverage (e.g. 7-up, gingerale, Fresca) (1 can/glass)									
Other Regular Carbonated Beverage (e.g. Sprite) (1 can/glass)									
Regular Cola Caffeine Free (1 can/glass)									

Food Group:	**Average Use Last Year**								
Beverages:	**Never or less than once per month**	**1 - 3 per month**	**1 per week**	**2-4 per week**	**5-6 per week**	**1 per day**	**2-3 per day**	**4-5 per day**	**6+ per day**
Regular Cola with Sugar (e.g. Coke (1 can/glass)									
Other Regular Cola Caffeine Free (1 can/glass)									
Non- Carbonated Fruit Drinks (1 can/glass)									
Decaffeinated Coffee (1 cup)									
Coffee (1 cup)									
Tea (1 cup) - not herbal									
Beer, light (1 glass/bottle)									
Beer (1 glass/bottle)									
White Wine (4 oz. Glass)									
Liquor (e.g. whiskey, gin etc.) (1 oz.)									
Red Wine (4 oz. Glass)									
Other Beverages, Juices etc.									
Sweets, Baked Goods, Miscellaneous									
Chocolate Bar (1) Type:									
Brownies (1)									
Doughnuts (1)									
Sweet Roll (1)									
Pastry (e.g. croissant)									
Popcorn (1 cup) with/wthout butter									
Jams, jellies, preserves or honey (1Tbs.)									
Chowder or Cream Soup (1 cup)									
Mayonnaise Dressing (1 Tbs.)									
Oil & Vinegar Dressing (1 Tbs.)									
Mustard, dry/prepared (1 Tbs.)									
Other Cookies									
Other Spreads:									
Other Soups (1 cup) Canned or Home-made: **e.g** campbells chicken & rice soup (1 can)									

Food Group:	Average Use Last Year								
Miscellaneous: *attach recipe	Never or less than once per month	1 - 3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2-3 per day	4-5 per day	6+ per day
Main Dishes: e.g. cod us gratin (250 g)									
Traditional Foods: e.g cabbage rolls (2 medium)									
Take Out Food Items: (e.g. Big Mac)									
Other Desserts: e.g. apple crisp (1/2 cup)									
Other Miscellaneous:									

The above food-frequency questionnaire was modified from: (Willett, 1988).

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