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# The Effect Of Proalgazyme And Its Subfractions On Lipid Metabolism In Diet Induced Hypercholesterolemic Hamsters: Correlation With Plasma Metabolomic Profile

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# THE EFFECT OF PROALGAZYME AND ITS SUBFRACTIONS ON LIPID METABOLISM IN DIET INDUCED HYPERCHOLESTEROLEMIC HAMSTERS: CORRELATION WITH PLASMA METABOLOMIC PROFILE

by

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Approved by:

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Advisor                                    Date

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**DEDICATION**

I would like to dedicate this thesis to my husband, Daniel Geamanu, who provided all the strength and power needed to pursue my dream of becoming a research doctor. It's been a long and difficult journey at times, but he never gave up on his support and always encouraged me to dream big. I want to thank him for his love, support, trust, and more than anything, his patience over the past few years.

This manuscript is also dedicated in loving admiration to my parents, Alexandra and Cristian Mateescu, whose solid support and continued encouragement gave me the foundation and strength to pursue my studies. I learned from my father that is never too late to follow your dreams, and I am thankful for his constant motivation in my endeavors.

My dedication to research and community work has brought difficult times in the family life, but with the support and love received, nothing seemed impossible to achieve. I am deeply thankful for everything I am today because of the nourishment received at home.

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# LIST OF ABBREVIATIONS

ABC A1: ATP-binding cassette transporter, sub-family A, member 1

Apo A1: Apolipoprotein A1

ATP: Adult Treatment Panel

BAF: Biologically active fraction

BASA: Bile acid sequestrant agents

BSH: Bile salt hydrolase

CETP: Cholesteryl ester transfer protein

CHD:  Coronary heart disease

CPMG: Carr-Purcell-Meiboom-Gill

CVD: Cardiovascular disease

DASH: Dietary Approaches to Stop Hypertension

DGATA2: Diacylglycerol acyltransferase-2

EDTA: Ethylenediaminetetraacetic acid

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

HDL: High density lipoprotein

HMG CoA: Hydroxyl-methylglutaryl coenzyme A

LCAT: Lecithin: cholesterol acyltransferase

LDL: Low density lipoprotein

LPL: Lipoprotein lipase

MS: Mass spectroscopy

NCEP: National Cholesterol Education Program

NMR: Nuclear magnetic resonance

OPLS: Orthogonal projections to latent structures

PAZ: ProAlgaZyme

PCA: Principal component analysis

PLS: Partial least squares

PLS-DA: Partial least square- discriminant analysis

RCT: Reverse cholesterol transport

RT-PCR: Reverse transcriptase polymerase chain reaction

SRB 1: Scavenger receptor class B1

SREBP: Sterol response element binding protein

TC: Total cholesterol

TG: Triglycerides

TMAO: Trimethylamine N-oxide

TOF: Time of flight

TQ: Tandem quadrupole

VLDL:  Very low density lipoproteins

# CHAPTER 1

# INTRODUCTION

## 1.1 Cardiovascular Disease

Despite significant progress in medical therapeutics, cardiovascular disease (CVD) remains the leading cause of mortality in the United States and other industrialized  nations [1]. Currently, CVD is responsible for more than 2200 American deaths/day, an average of one death every 39s [2] and it is predicted that one in two healthy men and one in three healthy women will develop some form of CVD during their lifetime [3].

Cardiovascular disease is described as a health state that affects the cardiovascular system, and includes cardiac disease, vascular disease of the brain and kidney, and peripheral arterial disease. Coronary heart disease (CHD) is the most common form of heart disease. It occurs when the arteries supplying blood to the heart become narrower due to the buildup of plaque, also known as atherosclerosis. The site where the plaque is located determines the type of CHD, such as coronary artery disease (plaque in the arteries supplying blood to the heart), peripheral artery disease (plaque in the arteries supplying blood to the arms and legs), and carotid artery disease (plaque in the arteries that supply blood to the brain). More serious consequences of plaque formation are myocardial infarction and stroke, which can occur when blood flow to the heart, or brain, respectively, is severely reduced or discontinued due to the hardening and narrowing of the arteries.

Other conditions such as arrhythmia, cardiomyopathy, congenital heart defects, and hypertensive heart disease are also considered part of CVD, but they are not

caused by plaque formation, which is mainly associated with an imbalanced dietary regimen.

The prevalence of CVD is expected to increase in the next decade, mainly due to increase in sedentary lifestyle, obesity, and aging of the population, which will increase the incidence of atherosclerosis, stroke, acute myocardial infarction, and other CVD related diseases. In 2008, the approximate total cost of CVD treatments was nearly US$ 500 billion, placing CVD as a top expensive disease to both human lives and finances [1]. Moreover, it is predicted by the American Heart Association that by 2030, 40.5 % of the US population will be diagnosed with some form of CVD and the total direct medical costs of CVD are projected to triple, from $273 billion/year to $818 billion, while the indirect costs will increase by 61% [4].Therefore, it is imperative that effective measures need to be applied in prevention, early detection, and management of CVD, as this is a very costly disease in terms of both lives and economics.

## 1.2 Lipoprotein Metabolism

Cholesterol molecule was first isolated from gallstones in 1789, and since then, it became one of the most extensive topic for research [5].  Found in all cells of the body, cholesterol is a waxy steroid metabolite that is responsible for the structure, permeability and fluidity of the cell membrane of higher eukaryotic systems. In addition, cholesterol serves as a precursor for the biosynthesis of vitamin D, bile acids, and steroid hormones [6]. Cholesterol, itself, is scattered amongst cellular membranes, accounting for  20-25% of the lipid molecules found in the plasma [5].

Lipids in general, including cholesterol, are not soluble in blood; therefore they are associated to proteins for transport in an aqueous media, becoming complex aggregates known as lipoproteins. Cholesterol, TG, and phospholipids are the major lipids that are used for hormone synthesis, formation of the cell membrane, and production of bile acid. Within the circulation, these aggregates are in a state of constant flux, changing in composition and physical structure as the peripheral tissue takes up the various components before the remnants return to the liver.

The most abundant lipid constituents are TG, phospholipids (mostly phosphatidylcholine and sphingomyelin), cholesterol esters, free cholesterol, and proteins known as apolipoproteins (Apo). The different protein components found in lipids determine the overall structure and metabolism, and the interactions with the receptor molecules in liver and peripheral tissues. However, the nomenclature is based on the relative densities of the aggregates following ultracentrifugation, taking into account the diameter of the broadly spherical particles. Hence, the main groups are classified as chylomicron, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) (Figure 1.1) [1, 7]. Furthermore, based on advanced separation procedures, intermediate density lipoprotein (IDL) and subclasses of HDL ($HDL_1$, $HDL_2$, $HDL_3$, etc.) are often defined.
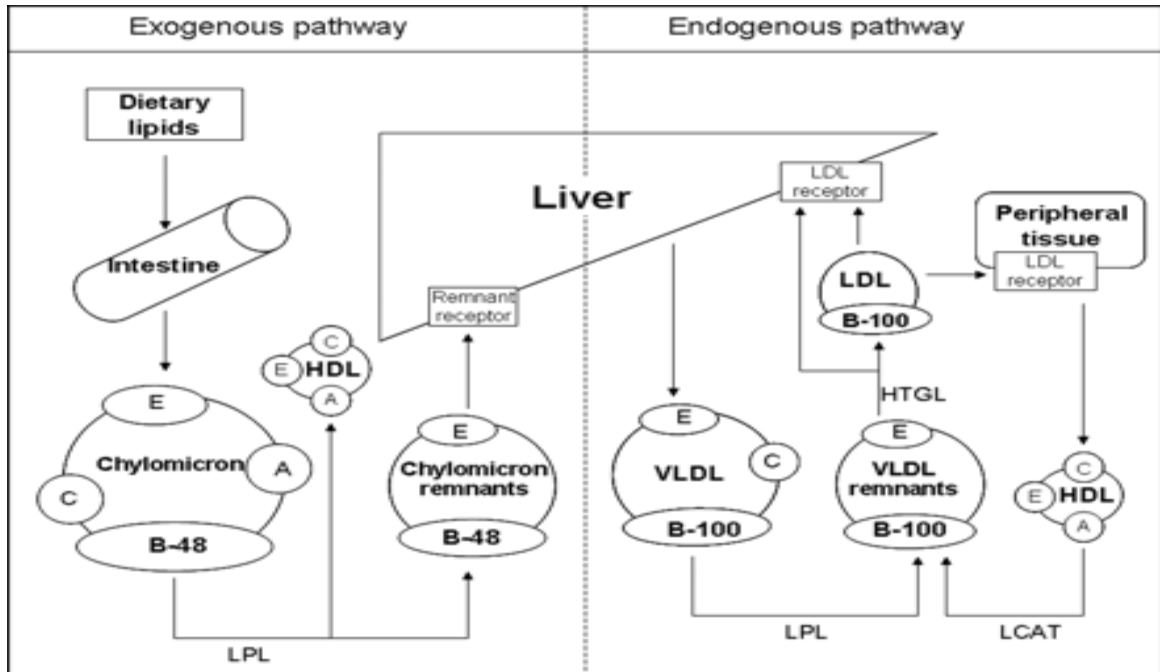
**Figure 1.1** Metabolic Pathways of Endogenous and Exogenous Lipoproteins.

**Abbreviations:** Apolipoprotein A, A; Apolipoprotein B, B-48, B-100; Apolipoprotein C, C; Apolipoprotein E, E; Hepatic triglyceride lipase, HTGL; High density lipoprotein, HDL; Lipoprotein lipase, LPL; Lecithin-cholesterol acyltransferase, LCAT; Low density lipoprotein, LDL; Very low density lipoprotein, VLDL [7]

**A. Chylomicrons** are the largest particles amongst all lipoproteins. Dietary cholesterol and TG are transported to different parts of the body from the intestine by chylomicrons, which are mediated by the enzyme lipoprotein lipase (LPL). Chylomicrons remain present in the plasma for three to six hours after consuming a meal, after which they are recycled. The remnant chylomicrons, containing the dietary cholesterol, Apo E and B 48 are cleared from the circulation by the liver via a receptor- mediated process.

**B. VLDL** molecule is secreted into circulation by the liver and contains mostly TG of the remnant chylomicrons, cholesterol, cholesterol esters and specific apolipoproteins (B100, C, and E). As the transport of VLDL molecules is progressing, the core of TG is reduced and phospholipids on the surface are transferred to HDL. Skeletal, cardiac, or adipose tissue receive TG from VLDL and use it for energy, or in the case of adipose tissue, for storage. Further, a large portion of the VLDL remnants are converted to LDL with additional loss of TG. Apo B100 and E are the remaining proteins required for recognition of the VLDL remnants, and LDL by the LDL receptors in the liver.

**C. LDL** molecules are the major carriers of cholesterol from the liver to the peripheral tissues, where the receptors recognizing Apo B100 capture the particles. Within these tissues, the cholesterol esters are hydrolyzed to release free cholesterol, which is further integrated into the plasma membrane and utilized as desired. It is widely known that high levels of LDL cholesterol increase CVD risk due to the atherosclerotic plaques deposited in the arterial wall.

**D. HDL** molecules contain higher amounts of proteins and a lower concentration of cholesterol and lipids, as compared with the other lipoproteins. Their primary function is to enable secretion of cholesterol from cells, esterification of cholesterol in plasma and relocating it to other lipoproteins, and the return of cholesterol to the liver from peripheral tissues for excretion. This process is known as 'reverse cholesterol transport' (RCT) and has the ability to protect against excess cholesterol accumulation and oxidative damage (Figure 1.2) [8]. Moreover, HDL cholesterol is thought to have antioxidative, anti-inflammatory, anticoagulation, platelet anti-aggregatory, and profibrolytic effects [9], which can alleviate the harm generated by increased levels of LDL cholesterol.

The nascent HDL particles are synthesized in the extracellular space of the small intestine and liver as protein-rich molecules. Apo A1 is the major protein component of HDL in plasma. The protein promotes cholesterol efflux from tissues to the liver for excretion, and it is a cofactor for lecithin: cholesterol acyltransferase (LCAT), which is responsible for the formation of most plasma cholesteryl esters. A specific transporter molecule, ATP-binding cassette transporter, sub-family A, member 1 (ABCA1) facilitates the transfer of phospholipids and cholesterol to lipid-poor apolipoproteins, especially Apo A, in the nascent HDL particles. Increased levels of ABCA1 imply that an organism may resourcefully collect cholesterol deposits from arterial walls and transport it back to the liver.
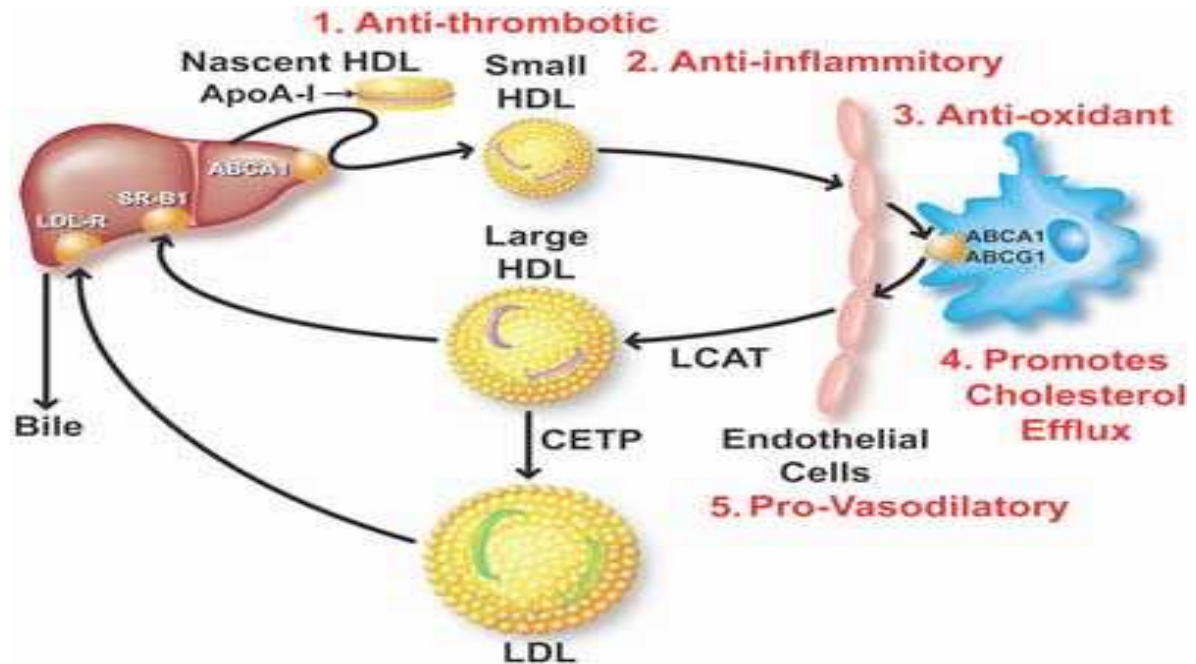
**Figure 1.2** HDL/ Reverse Cholesterol Transport Pathway

**Abbreviations:** ATP-binding cassette transporter, sub-family A, member 1, ABCA1; ATP-binding cassette sub-family G member 1,ABCG1; Cholesteryl ester transfer protein, CETP; High density lipoprotein, HDL; Lecithin-cholesterol acyltransferase, LCAT; Low density lipoprotein, LDL; Low density lipoprotein receptor, LDL-R; Scavenger receptor class B1, SRB1 [8]

In order for RCT mechanism to function properly, HDL has to bind to the receptors on the extra-hepatic and hepatic cells. Through its Apo E component, HDL is able to attach to LDL receptors in addition to HDL receptors. Hence, at its receptor site, HDL is able to compete with LDL. Another key molecule involved in RCT is scavenger receptor class B1 (SRB1), which has the ability to increase HDL-mediated cholesterol efflux. SRB1 functions in hepatocytes to cleave cholesterol esters off transporter molecules; hence they can be re-metabolized by the liver.

In addition, cholesterol esters of HDL can be transferred to VLDL and LDL by the action of cholesterol ester transfer protein (CETP). This enzyme allows for excess cellular cholesterol to be returned to the liver by the LDL-receptor pathway [10], as cholesterol esters can be exchanged for TG by CETP from Apo B containing lipoproteins to HDL and vice versa. Overall, in this process, cholesterol is removed from the peripheral tissues and carried to the liver. About 30% of the serum cholesterol is carried during the RCT process [1]. Thus, within the vascular endothelium, if the quantity of cholesterol deposited is reduced, the risk of atherosclerosis and formation of fatty plaque is also reduced.

**1.3 Risk Factors**

With respect to heart disease, as with any chronic illness, there are several risk factors-modifiable and non-modifiable- linked to the development and progression of CVD. The major non-modifiable factors, which cannot be changed, include genetic alterations, family history, increasing age, gender (men have higher risk), post menopausal, and race (African Americans, Hispanics, native Indians and Hawaiians, and some Asians have increased risk when compared with Caucasians). The major

risk factors that one can modify, treat or control by lifestyle modifications and/or medicine include diabetes, high blood pressure, poor diet and sedentary routine, obesity, smoking, stress, and abnormal levels of blood lipids (partially modifiable, not related to genetic aberrations). A combination of any two or more of the risk factors highly increases the development of atherosclerosis and other related coronary disease.

**1.3.A Diet**

Diet is an important determinant of chronic disease risk, particularly heart disease. It represents a significant component of any strategy to achieve population level reductions in the burden of CVD. Although drug treatment, such as lipid-lowering statin drugs, may be necessary among individuals at high risk of CVD or with genetic alterations, adoption of a healthy diet is the preferred method in the general population in order to prevent or delay the onset of the disease.

The primary dietary determinants of hypercholesterolemia are fats, in particular, saturated and trans fatty acids, and dietary cholesterol. Over the last few decades, various studies have confirmed that the level of saturated versus unsaturated fatty acids in one's diet can play a direct role in CVD [11]. Valuable results were published from the epidemiological studies of the seven countries [12], the Framingham studies [13], as well as the Japanese individuals migrating from Japan to Hawaii and California [14]. All of these investigations demonstrated the environment's influence on plasma cholesterol levels, in particular the impact of the relative levels of saturated versus unsaturated fatty acids in one's diet. Several other studies have also experimented with the substitution of various fats to observe the

outcome on plasma cholesterol levels. It was noted that when polyunsaturated fatty acids were substituted for saturated fatty acids, patients experienced reduced plasma cholesterol levels, and had a decreased rate of death and repeat myocardial infarctions [15, 16].

In addition, while Brown and Goldstein [17] are mostly famous for their work on mechanistic action of LDL receptors, they have also researched the mechanisms behind the effects of unsaturated fatty acids to lower plasma cholesterol levels. Transcription factors, such as sterol response element binding proteins (SREBP) raise cholesterol, fatty acid, and LDL receptor biosynthesis. SREBP transcription and SREBP precursor proteins are reduced when unsaturated fatty acids are present in the diet. Additionally, another mechanistic process of certain unsaturated fatty acids is to control peroxisome proliferator activated receptor alpha and carbohydrate regulatory element binding protein/Max-like factor X function, which can result in decreased concentration of plasma lipids [18-20], and may also influence lipoprotein catabolism independently of the LDL receptor [21].

Like the role of dietary fat in regulating plasma cholesterol levels, the importance of dietary cholesterol absorption in regulating plasma and body cholesterol homeostasis has been shown in numerous epidemiological and clinical studies. Populations consuming dietary cholesterol less than 100 mg per day along with low-fat diets have low LDL cholesterol levels and low incidence of coronary disease [22, 23]. The National Cholesterol Education Program guidelines recommend dietary cholesterol to not exceed more than 300 mg per day.

### 1.3.B Obesity

Excess adiposity and obesity are the root cause of numerous diseases, which contribute to a considerable lifelong morbidity and are highly linked to cardiovascular mortality. The rapid increase in obesity prevalence is most of the time not due to genetic modifications, but rather is a societal disparity between physiology and environment, due to abundance of food and a sedentary lifestyle. Obesity, mainly central obesity, represents a major culprit of the metabolic syndrome, which includes insulin resistance, hypertension, diabetes mellitus, non-alcoholic fatty liver disease, and dyslipidemia, all risk factors for development of CVD [24, 25].

The risk relationship between obesity and atherosclerosis was seen in young men (15 to 34 years old) in the PDAY study [26], as well as in an older population (35 to 54 years old) [27], suggesting that risk factors such as obesity, may operate continuously as atherosclerosis progresses. Among the traditional risk factors, the dyslipidemia may to some extent be attributable to the increased release of free fatty acids from adipose tissue, which may consecutively increase very low density lipoprotein (VLDL) production and plasma triglycerides (TG). The release of free fatty acids may also contribute to insulin resistance, which has been highly associated with atherogenic pro-inflammatory and pro-oxidant vascular alterations [28, 29].

Apart from atherosclerosis, heart failure is also more prevalent among obese patients, possibly due to the expansion of the blood volume associated with expanded adipose tissue mass. Frequently, when hypertension is associated with obesity, it can contribute to left ventricular hypertrophy and ultimately heart failure.

These correlations warrant future research to learn about the impact of the increased adipose tissue accumulation on cardiovascular pathophysiology.

### 1.3.C Hypertension

Hypertension is one of the most common disorders worldwide and it ranks as the leading chronic risk factor for mortality, accounting for 13.5% of all deaths [30]. High blood pressure is responsible for half of all strokes and ischemic heart disease, and can affect many other CVD, including atherosclerosis, CHD, and renal disease. Moreover, in overweight patients the negative impact of hypertension on CVD is more significant as compared with normal weight individuals [31].

A diet high in sodium and low potassium represents the most important environmental factor affecting hypertension. The American population consumes an average of more than 6 g of sodium daily and more than one third of the population has hypertension [17]. In contrast, in communities were the sodium intake is less than one gram per day, the prevalence of hypertension is about 1 % of that in industrialized societies.

The American Heart Association set the recommendations for sodium and potassium consumption based on the DASH study (Dietary Approaches to Stop Hypertension), which represents a diet based on grains, low-fat dairy, fish and poultry, and rich in fruits and vegetables. The DASH study showed that the blood pressure was reduced in subjects following this diet, as compared with the average American diet [32].

### 1.2.D Dyslipidemia

Abnormal lipoprotein metabolism is often observed as a secondary effect of diabetes mellitus, obesity, hypothyroidism, or kidney disease, while only few individuals carry inhered defects in lipoprotein metabolism, such as hyper- or hypocholesterolemia. The term dyslipidemia refers to abnormal plasma lipid levels, including hypercholesterolemia (increased levels of serum LDL cholesterol), hypertriglyceridemia (increased levels of TG), and low levels of high-density lipoprotein (HDL) cholesterol. The combination of low HDL cholesterol, high LDL cholesterol, and hypertriglyceridemia is referred to as the lipid triad, and along with other non-lipid risk factors comprise the metabolic syndrome, which is highly correlated to increased risk for CVD [33]. Accordingly, the TC to HDL cholesterol ratio is a more significant predictor of risk than TC alone [34].

The role of lipoproteins in the metabolism of TG and cholesterol in relationship to CVD has been highly discussed over the last decades. Numerous epidemiological studies have shown that high concentrations of plasma TC and LDL cholesterol are associated with an increased risk for atherosclerosis [35]. A meta-analysis conducted on 90,000 patients showed that 1 mmol (39mg/dL) reduction in the concentration of LDL cholesterol resulted in a 23% reduction in cardiovascular events [36].

The Framingham Heart Study was a significant project that revealed the association between development of CVD risk and plasma lipid abnormality. This study began in 1948 and monitored over ten thousand subjects that had several risk factors for CVD, in particular an abnormal blood lipid profile [34]. Since the first results of the Framingham Heart Study were published, various trials have been

conducted to analyze the impact of these factors on occurrence of CVD, with an emphasis on serum cholesterol levels [1].

Over the last decade, there have been various advancements to control and minimize the risk and incidence of CVD, particularly enhancing and monitoring plasma lipid profiles. The Adult Treatment Panel (ATP) has been set up by the National Cholesterol Education Program (NCEP) to provide guidelines for maintaining optimal cholesterol levels. ATP III, originally published in 2002 and updated in 2004, is the most current panel as warranted by advances in the science of cholesterol management [1].

Apart from TC and LDL cholesterol, numerous epidemiological studies suggest that low levels of HDL cholesterol (<1.0 mmol/L or 40mg/dL) represent a significant risk factor for cardiovascular events, independent of LDL cholesterol levels [37], whereas high levels of HDL cholesterol act as a protective agent against CVD [38]. A decrease in HDL cholesterol levels can be due to one or a combination of the known non-modifiable risk factors (*e.g.* genetic aberrations) or the modifiable risk factors, such as obesity, malnutrition, and different drugs, like anabolic steroids, beta-blockers, progestins, and isotretinoin [1]. It has also been shown that often a low HDL cholesterol level is associated with an increase in TG level. This high TG - low HDL cholesterol combination, in part also explains the risk generated by abdominal obesity. Due to the low-high state of HDL cholesterol and TG levels, respectively, this combination is characteristic of insulin resistance syndrome, and provides a link between obesity, diabetes, and dyslipidemia.

## 1.4 Current Therapies

### 1.4.A Statin Drugs

Since a major risk factor for CVD is elevated LDL cholesterol, numerous studies have been conducted to identify methods to lower this lipoprotein. Most common therapy used for lowering LDL cholesterol focuses on hydroxyl-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins [1]. This class of drugs was developed to compete with HMG-CoA for binding at the catalytic site of HMG-CoA reductase and therefore to decrease the synthesis of cholesterol [39]. There are several statins on the market that are widely prescribed for hypercholesterolemic patients to achieve guideline-recommended LDL cholesterol goals and can reduce the risk of initial and recurrent cardiovascular disease by 20-30 % [40].

However, statins are known to cause severe adverse effects such as heartburn, diarrhea, flatulence, nausea, vomiting, headache, rhabdomyolysis, myalgia, myositis, and raised level of liver enzymes [1]. Also statins are not suitable for patients with increased levels of serum transaminase, active liver disease, or if pregnant or lactating. Also, interaction with other medications needs to be closely monitored when therapy with statins occurs for extended periods of time [1].

### 1.4.B Bile Acid Sequestrants Agents (BASA)

Bile acid sequestrants agents (BASA) are another class of drugs for decreasing the levels of LDL cholesterol. They generally work by interfering with the enterohepatic recirculation. Bile acid sequestrants bind bile acids in the gut, forming insoluble complexes which are excreted. By blocking enterohepatic recirculation and

diminishing the liver of ready-made bile acids, the liver increases synthesis of cholesterol and up-regulates LDL receptors, which results in increased clearance of LDL cholesterol from the systemic circulation. As a new generation of BASA therapy, Colesevelam is considered to be a high-capacity agent, with four to six times increased potential as compared with older BASA. It can reduce LDL cholesterol in a dose-dependent manner [41]. Although the side effects are somewhat of a lesser degree as compared with the old generation of BASA, some gastrointestinal distress, such as abdominal pain, constipation, heartburn, and bloating does occur.

### 1.4.C Cholesterol Absorption Inhibitors (Ezetimibe)

Ezetimibe, similar to BASA, blocks the exogenous pathway of cholesterol metabolism and can lower LDL cholesterol to a similar extent as Colesevelam. It works by inhibiting the absorption of ingested cholesterol by about 50% [42]. However, because Ezetimibe is extensively metabolized in the liver and it is systemically absorbed, there are concerns with regards to the safety of administering the drug during pregnancy, to nursing mothers and children, and other patients with impaired liver function. Some of the noted side effects associated with Ezetimibe include gastrointestinal distress, headache, fatigue, and myalgia.

### 1.4.D Niacin

Niacin (Nicotinic acid) is a well known therapy to increase HDL cholesterol, while decreasing the LDL and TC in plasma. Niacin inhibits the release of free fatty acids from adipose tissue and the production of TG in the liver. In addition, niacin also directly inhibits the hepatocyte microsomal diacylglycerol acyltransferase-2 (DGAT2), the key enzyme involved in the conversion of diacylglycerol to

triglycerides. Decreased synthesis of TG results in intrahepatic degradation of Apo B, with less VLDL available to produce LDL cholesterol. Moreover, niacin can decrease the size of the VLDL particle, which affects the rate of exchange of TG for cholesterol mediated by CETP. The acquired excess triglycerides in the large LDL particles undergo rapid hydrolysis by hepatic lipase, which leads to the formation of small dense LDL particles.

Thus, the goal of niacin therapy is to reduce LDL and TC levels, as well as increase levels of HDL cholesterol, overall improving the lipoprotein plasma profile. However, multiple adverse effects, such as nausea, abdominal pain, flushing, and pruritus have been reported, and the poor patient adherence make this a non desirable drug. Furthermore, several clinical studies have advised that therapy involving nicotinic acid in high doses caused glucose intolerance in patients with abnormal glucose metabolism [43], as well as high toxicity levels and gastrointestinal disturbance [40]. Nicotinic acid therapy has also been found to decrease insulin sensitivity and increase plasma glucose levels [44, 45].

### 1.4.E ApoA1 Milano Complex

The importance of Apo A1, the main structural protein of HDL (70%) has been well established, as it plays a major role in RCT, removing cholesterol from macrophages and returning it to the liver for excretion in bile. Previous animal studies have shown that augmenting Apo A1 increases HDL cholesterol levels and decreases atherosclerosis and cardiovascular events [1]. The recent research studies investigated a recombinant Apo A1 Milano complex, which was shown to

increase HDL cholesterol levels in patients with acute coronary syndrome [46], but this is an invasive treatment requiring intravenous dosing.

Other Apo A1 targeting therapies are under investigation, including synthetic peptides such as L-5F, D-4F, and ETC-642, which are intended to imitate the favorable effect of Apo A1 in promoting cholesterol efflux from macrophages, decrease inflammation, and progression of atherosclerosis [47-50]. However, the application of these peptides is limited since the administration of the treatment is intravenous, and the peptides have a complex structure and are very expensive to produce. Therefore, a different approach to increase Apo A1 remains to be discovered.

Recently, several new therapies targeting lipoproteins have been applied in clinics, either as monotherapy or in combination with statins, but severe side effects and poor outcome warrant more research. A recent analysis showed that during 1999-2006 the prevalence of hypercholesterolemia and related factors remained stationary among the US population, despite the increase in usage of cholesterol lowering medications [51].

Thus there is an acute need for a new therapeutic approach that can reduce the circulating cholesterol levels and improve the HDL/LDL cholesterol ratio significantly, without the incurrence of adverse effects. It has been suggested that LDL and HDL cholesterol should be adjusted simultaneously in order to prevent CVD. The risk for a cardiovascular event can be decreased by nearly 1% for each1% reduction in LDL and by more than 1% for each 1% increase in HDL, suggesting that improvement in

both lipoprotein fractions has a synergistic effect as compared with either lipoprotein alone [52].

There is also a further need of replacing current statin therapy and regulating cholesterol at a gene signaling level, which the new emerging therapies are targeting. Many studies are underway to identify new choices for therapies that can decrease the LDL cholesterol, while increasing the HDL cholesterol with minimal side effects. One of the targeted therapies currently being studied is inhibition of CETP [40]. Promising results were obtained by partial inhibition of CETP [1], and if a suitable drug can be formulated, this therapy can be successful in clinical management of hypercholesterolemia.

### 1.4.F Omega-3 Polyunsaturated Fatty Acids

Over the last decade, several other treatments intended to improve the lipid profile have been developed, especially naturally derived as food supplements. Omega-3 polyunsaturated fatty acids can reduce the production and secretion of VLDL particles, and increase TG removal from VLDL and chylomicron particles via the upregulation of lipoprotein lipase. Several studies have shown that when doses higher than 3g/day of omega-3 fatty acids were administrated, TG were reduced by 32% [53, 54]. However, not enough statistics are known about the decrease in CVD risk, especially in woman, since most studies on fish consumption and omega-3 fatty acids were conducted in men. Also, the overall lipid profile was improved only when omega-3 fatty acids supplements were given in combination therapy with statin drugs, reducing some major coronary events in patients followed for 4.6 years [54].

### 1.4.G Other Natural Compounds

Since most synthetic drugs designed to treat hypercholesterolemia have so far, a low success rate in decreasing the rate of cardiovascular events, most therapeutic interventions include an aggressive change in diet and lifestyle, alone or in combination with drug therapy. Some approaches include consuming green tea [55], plant sterols [56], soluble fiber [57], cocoa and dark chocolate [58] have produced promising results and warrant further investigation.

More recent studies have found that the probiotic bacteria, especially bile salt hydrolase (BSH)-active probiotic bacteria, have demonstrated their ability to decrease cholesterol levels in several randomized clinical trials [59-62]. It was reported that an increased activity of BSH can increase intraluminal bile acid deconjugation, resulting in raised levels of circulating deconjugated bile salts, which in turn decrease cholesterol absorption by enterocytes [63, 64]. Various algal and seaweed extracts have also been studied to determine their ability to decrease hypercholesterolemia and avoid cardiovascular events. Several scientists have reported that seaweeds, isolated algal polysaccharides and their water-soluble fractions demonstrated hypocholesterolemic effects in experimental animals [65, 66]. Recent research conducted by Bocanegra *et al* showed that the lipoprotein profile of male Wistar rats was significantly improved when fed freeze-dried algae added to their diets for 3 weeks, reducing the TC, TG, phospholipids, and protein contents of the various lipoprotein fractions [67].

### 1.4.H ProAlgaZyme (PAZ)

PAZ algae infusion is the fermentation product of a blend of freshwater organisms, including red and green algae [68]. It contains less than 100 ppm of total

dissolved solids, consisting of a mixture of approximately 90% salts (free of heavy metals at a detection limit of < 0.1 ppm), while the remaining 10% is a proprietary mixture of organic constituents. In a preliminary study, Oben *et al* [68] evaluated the effects of PAZ on body weight, body mass index, blood lipids, fasting blood glucose levels, and markers of inflammation in individuals with the metabolic syndrome. The results showed a significant beneficial outcome on the various parameters analyzed, including the lipid profile, suggesting that PAZ could be an effective method for lowering the risk of developing CVD.

**1.5 Metabolomics**

As observed in recent reports regarding therapies for hypercholesterolemia and risk for CVD, it was concluded that the lipid profile alone does not provide a complete picture of the disease progression. Analysis of a multifactorial disease, such as atherosclerosis and other forms of CVD, using a limited number of biomarkers can lead to inaccurate diagnosis and treatment regimes, weakening the advancement of new therapies. In contrary, the omics-based approaches (metabolomics, in particular) have allowed scientists to characterize, at the molecular level, complex biological systems and their changes in pathological processes [69, 70] providing an excellent tool for examining phenotypes using hundreds of metabolite descriptors.

It has been determined that the human genome encodes approximately 30,000 genes, which are responsible for translating more than 100,000 distinct proteins [70]. The complete set of all chromosomes and genes is referred to as the genome, a term widely used in research for more than seven decades. However, the 'omic'

technology was not introduced until 1986 by Thomas Roderick [71]. The science of the genomes of organisms was implemented to provide information about the function of different genes and to compare them among multiple organisms.

Moreover, along with genomics, the science of proteomics signifies the comprehensive study of proteins produced by an organism during its lifetime based on its genome. However, the proteome of an organism might change due to several factors, including cellular environment, biochemical interactions, while the genome remains constant. Also, newly translated proteins might undergo various modifications (such as glycosylation, phosphorylation, degradation, alternative splicing) and might not be part of the cellular processes or the organism's metabolism. Thus, it is more accurate to study proteins or metabolites that have been part of biochemical and cellular processes, providing an accurate state about the changes within the system under different conditions, including environment, drugs, disease, and toxicity. These discoveries lead to the emerging field of metabolomics, the latest 'omic' approach, which measures changes in populations of low molecular weight metabolites under a given set of conditions [72].

Metabolomics is a comprehensive and quantitative study of the complete set of intracellular and extracellular metabolites produced as an end product of an organism's metabolism [70]. This technique can be utilized to investigate the effect of environmental stress or chemical composition of the organism, metabolite pattern, effect of a drug, or disease progression. The analysis can be performed using cells, tissues, body fluids, such as urine, saliva, plasma, and tears [73], having the option to perform it as a non-invasive procedure. One of the important attributes of

metabolomics is the possibility of identifying a molecule or a set of molecules responsible for changes in the physiology of an organism under a particular stress. Metabolite profiles can be regarded as key indicators of normal phenotype and pathology, providing novel information about new biomarkers, drug interactions, toxicological insult, nutritional status, and the effects of these factors on the genome.

The human metabolome is directly influenced by exogenous (such as diet, drugs, physical status, and stress) and endogenous factors (such as body composition, age, gender, and health status), which vary amongst individuals (Figure 1.3). Metabolomics technology can bring new insight into personalized medicine and might help answer questions related to specific metabolites by integrating or connecting different biochemical pathways. Qualitative and quantitative metabolomic analyses provide a view of the biochemical status of an organism at a definite time, under specific conditions. Thus, the extension of this technology to human medicine and human nutrition offers enormous potential, with the possibility of linking metabolomics with the wider elements of nutrigenomics.

| Extrinsic factors | | Intrinsic factors |
| --- | --- | --- |
| •Diet (nutrients and nonnutrients)<br><br>•Drugs<br><br>•Physical activity<br><br>•Colonic flora<br><br>•Stress | → **The Metabolome:**<br><br>The sum of all endogenous and exogenous metabolites ← | •Body composition<br><br>•Tissue turnover<br><br>•Resting metabolic rate<br><br>•Age<br><br>•Genotype<br><br>•Health status<br><br>•Reproductive status<br><br>•Diurnal cycle |
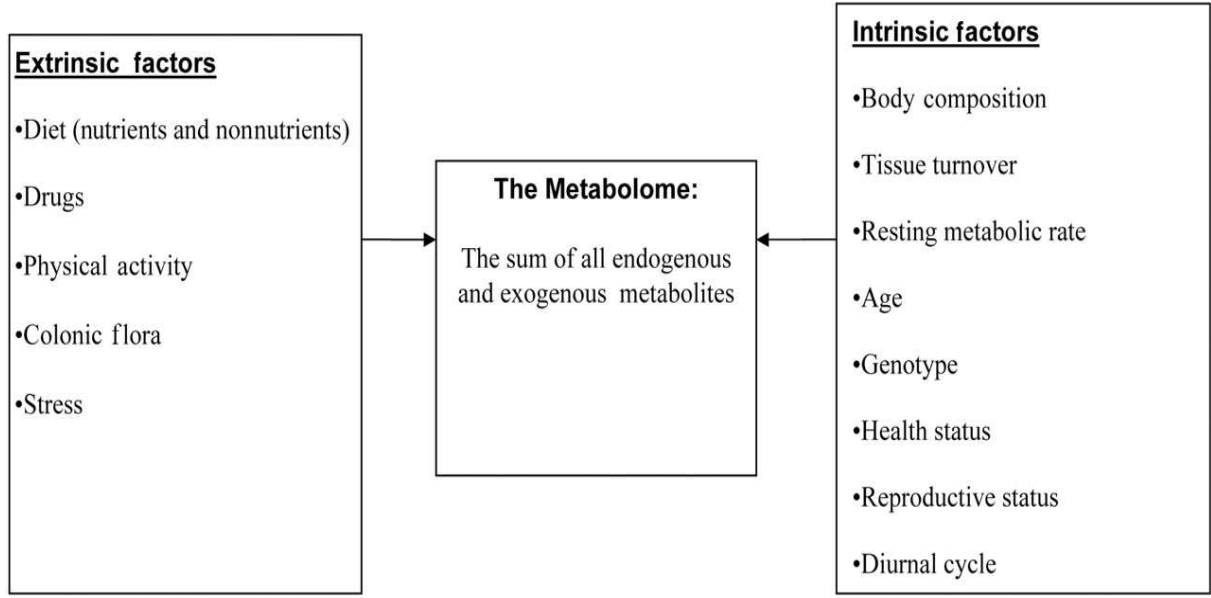
**Figure 1.3** Exogenous and Endogenous Factors Likely to Influence the Human Nutritional Metabolome [74]

## 1.6 Nuclear Magnetic Resonance and Mass Spectroscopy

Since the majority of the metabolites are present virtually in all tissues and fluids, the concentration of the metabolites is the major factor that distinguishes different phenotypes [75]. Therefore, the techniques to analyze the samples and process the acquired data must be highly specific and accurate. At present, with rapid advances in analytical chemistry technologies, including nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), the capacity of accurately identifying small metabolites has become easier.

There are different types of MS techniques that can be used, including time-of-flight (TOF) MS, tandem quadruple (TQ) MS, which can be combined with liquid chromatography, gas chromatography or capillary electrophoresis, as a useful analytical tool for metabolomics [76-78]. While MS technology is more sensitive as compared with NMR, the disadvantage is that MS does not confer a uniform detection, caused by variable ionization efficiency. Also, MS based techniques require extensive preparation of the sample before analysis, such as extraction and derivatization, which can alter the composition of the sample and can lead to invalid data and conclusions [79].

The NMR technique is based on the magnetic properties of atomic nuclei, usually hydrogen nuclei ($^1$H NMR), but $^{13}$C, $^{31}$P, $^{15}$N, and $^{19}$F are also commonly used. The identification of different atomic nuclei is based on their resonance frequencies, which are dependent on their locations or environment in the molecule [80]. The intensity of the NMR spectrum changes linearly with the concentration of the

compound present. [81]. Thus, signal intensities give direct information about the relative concentrations of different compounds in a complex mixture.

The major advantage of using NMR spectroscopy is its high reproducibility (> 98%), ability to analyze both liquid and solid samples, tissues and cell extracts [82], and it can help in the identification of unknown metabolites. NMR spectroscopy can fingerprint an entire biological sample, requiring minimum sample size and sample preparation time, while evaluating the entire metabolic status of an organism. $^1$H NMR metabolomics approach has been largely used with great success in identification of biomarkers and monitoring the effects of biological stressors [83], and more recently it has become an important tool for diagnosis and treatment of chronic diseases, such as obesity, diabetes and CVD [2].

An innovative study conducted by Brindle *et al* in patients with CVD identified characteristic plasma metabolites that were different in individuals with angiographically normal coronary arteries [84]. This discovery could displace the necessity to perform angiography, an invasive and time consuming procedure. Comparing patients with triple vessel disease with controls, this metabolomics technique was able to predict the presence of coronary disease with a specificity of 93% and a sensitivity of 92% [84]. However, when these data were reproduced, after normalization by gender and statin drug therapy, it was shown that it can only predict for 61% of the patients [85]. Still, while $^1$H NMR is considered a poor predictor on a per sample basis, perturbations in specific metabolites associated with the metabolic syndrome have been identified. Numerous studies have followed and acknowledged

changes in plasma profiles, in particular associated with alterations in the lipoprotein particles, insulin resistance and atherosclerosis [86-88].

Metabolomics, despite the smaller expected size of the metabolome, compared with the other 'omics' approaches, generates an enormous quantity of data. Analysis and interpretation is a critical step that could otherwise lead to erroneous conclusions. Thus, the choice of the most appropriate statistical analysis is crucial, in order to extract the maximum amount of accurate biological information from the data set. Although, the area of developing sophisticated statistical tool is still in progress, a number of techniques are available.

## 1.7 Multivariate Data Analysis (MVDA)

MVDA is a powerful tool for the analysis of data sets containing a large number of variables. It visualizes the correlation between variables in complex or large data sets (*e.g.*, thousands of signals in NMR spectra) in relation to a target variable such as disease status, diet intervention, or environmental influence.

### Principal Component Analysis (PCA)

PCA is a multivariate projection method designed to extract and display the systemic variation in the data matrix X (the table of integrals from NMR plasma samples). This is an unsupervised analysis, which implies that the software has no prior knowledge about the data matrix [89]. This method enables differentiation between samples based on their metabolites composition. In PCA, the data sets of related large variables are transformed into small uncorrelated variables known as principle components (PC). This transformation is defined so that the first PC has the largest possible variance (accounts for as much of the variability in the data as

possible), and each succeeding component has the highest variance possible under the constraint that is orthogonal to the preceding component.

The data set is visualized as a PCA score plot, where each score represents one observation, such as one NMR spectrum (one sample/animal). The observations will be grouped together or scattered based on the similarities and/or dissimilarities in their metabolomic profile. The contribution of each individual variable to PC can be calculated as a loading plot. The corresponding loading plot will provide information about the part of the spectrum that is responsible for the similarities and/or dissimilarities in the data set. The specific region of the spectrum that is responsible for the separation between the groups can be located using this method.

## Partial Least Square (PLS) and PLS- Discriminant Analysis (PLS-DA)

PLS is a regression extension of PCA, which is used to connect the information in two blocks of variables, X and Y. The data from the metabolomics analysis (X parameter) can be correlated with other independently measured factors (*e.g.* total cholesterol, HDL cholesterol, gene expression) keeping these factors as Y parameters. PLS helps to determines whether or not a correlation exists between the two data sets, which otherwise would be considered unrelated.

In PLS-DA the data set is distributed into classes and its objective is to find a model that separates the classes of observation on the basis of their X-variables, while using a hypothetical Y-variable. Both of these methods of analysis are supervised, which implies that some information about the data set is provided to the software prior to analysis [89]. Taken together, PCA, PLS and PLS-DA give valuable

information about the variability and/or similarity of large data sets, which otherwise will be an impossible task using standard statistical tools.

## 1.8 Metabolite Identification and Quantification (Chenomx)

Chenomx software represents a metabolite database that helps to identify and quantify the concentration of the metabolites by comparing the acquired data set or NMR signal intensities to pre-existing signals from a library of compounds with known concentration. While MVDA identifies the areas of the spectrum responsible for similarities and/or dissimilarities between the groups, it is important to identify the metabolites represented by the spectral regions or peaks in question.

The corresponding metabolites within that range can be easily identified using Chenomx NMR 7.6 software (Chenomx Suite, Alberta, Canada). This software uses targeted profiling to reduce analysis time, combining advanced analysis tools with a compound library of more than 300 common metabolites. Targeted profiling can be applied to any NMR spectra of virtually any complex mixture, including urine, blood plasma, saliva, and various cell extracts. Chenomx is MVDA independent, but it is faster and easier to use once the peaks responsible for the separation between groups are identified [81].

Profiling of the NMR spectra is accomplished using the Profiler module. Essentially, a Lorentzian peak-shaped model of each reference compound is generated from the database information and superimposed upon the actual spectrum. The linear combination of all modeled metabolites gives rise to the total spectral fit, which can be evaluated with a summation line. Once the changes in metabolite concentrations have been determined, the pathways affected by the

respective metabolites can be identified using online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg).

Therefore, the metabolite profiling technique has the potential to become an essential tool in medicine, which can be applied for diagnosis and treatment of any disease. Metabolites can be detected based on any of their physiochemical or biological properties, and they can be used as early biomarkers to distinguish between healthy and diseased state, toxicity levels, nutritional interaction or other factors. Metabolomics approach is a relatively new field in medicine and pharmacology, but reveals great promise, which potentially will allow for better understanding of human diseases, including CVD.

For decades, numerous scientists across the globe have been investigating the etiology of atherosclerosis and the occurrence of cardiovascular events, such as stroke and myocardial infarction. Despite considerable improvements in therapeutics of cardiovascular disease, epidemiological data reveal that the mortality rates associated with the disease are still on the rise. Recent developments have suggested that identifying the changes in the metabolite profiles will grant a higher degree of understating of the disease. Because of its excellent capability to analyze the metabolome, metabolomics provides a much more comprehensive assessment of patient's health status, in comparison with the measurement of particular metabolites. This approach makes metabolomics a great technique for the identification, quantification, and development of biomarkers.

## 1.9 Aims of Study

The aim of the present study is to document the potential effect of PAZ and its subfractions on plasma lipid concentrations in a hypercholesterolemic hamster model. In a preliminary study, Oben *et al* [68] evaluated the effects of PAZ on body weight, body mass index, blood lipids, fasting blood glucose levels, and markers of inflammation in individuals with the metabolic syndrome. The results showed a significant beneficial outcome on the various parameters analyzed, including the lipid profile, suggesting that PAZ could be an effective method for prevention of CVD. However, some inconclusive results were reported by Oben *et al* [68], such as exceptionally large changes observed between week 8 and 10, with some subjects experiencing about a two-fold increase in HDL cholesterol. In addition no measures of TG or LDL cholesterol were reported at 10 weeks.

Thus, this study evaluates the efficacy of PAZ as a preventative and therapeutic agent in an animal model. Male Golden Syrian hamsters were selected as the experimental model since they have been used extensively to study human lipoprotein metabolism [90, 91]. It has been noted that in both humans and hamsters, the liver has a low rate of cholesterol synthesis and it is the main site for plasma LDL cholesterol clearance [92].

**Hypothesis**

*The study hypothesizes that PAZ and/or its subfractions would effectively improve the plasma lipoprotein profile. In addition, it is hypothesized that the change in the plasma metabolomic profile due to PAZ intervention will reflect the improvement in the plasma lipid profile.*

The following specific aims were undertaken to test the hypothesis:

**AIM 1: To determine the biological activity of complete PAZ and its subfractions in a hypercholesterolemic animal model**

The objective of this aim was to test the effectiveness of PAZ and its subfractions as a preventative agent in a high fat induced hypercholesterolemic hamster model. For this, PAZ and its subfractions were administrated as part of drinking fluid along with the dietary (high fat) regimen. Eighty male Golden Syrian hamsters were randomized into control (n=20; control diet) and treatment groups (n=60; high fat diet, 30% calories from fat). Further, the control group was subdivided into water (CW) or PAZ (CP) groups (n=10), based on their drinking fluid. The treatment group was subdivided into 6 groups (n=10): HW (water), HP (complete PAZ), HF1, HF2, HF3, HF4 (4 different subfractions of PAZ obtained by sequential affinity gel chromatography). After 4 weeks of treatment, animals were sacrificed and blood was collected by cardiac puncture. Hepatic mRNA was extracted and analyzed to determine if there were changes in the gene expression related to HDL cholesterol metabolism.

**AIM 2: To investigate the change in plasma metabolomic profiles upon administration of PAZ and the biologically active fraction (BAF)**

Plasma metabolomic profile using 1D proton NMR was performed to determine the changes in metabolite concentrations among animals that received water (control), complete PAZ, and the fraction found to be biologically active in Aim1. Data were analyzed using multivariate data analysis SIMCA P+ software. Targeted profiling was applied to NMR spectra to determine the metabolites that are different between the groups of interest. Once these metabolites had been identified and

quantified (Chenomx), analysis of the pathways involving the specific metabolites was conducted. In addition, metabolomic profile was correlated with the plasma lipid profile.

## AIM 3: To determine the therapeutic effect of the biologically active fraction on hypercholesterolemia

The goal of this aim was to determine if plasma lipid profile of animals in a hypercholesterolemic state can be improved upon treatment with the fraction found to be biologically active in Aim 1. Secondly, this part of the study aimed to determine the time course required for the therapeutic effect on lipid parameters.

Fifty male Golden Syrian hamsters were fed a high fat diet for 4 weeks prior to receiving treatment with the biologically active fraction found in Aim 1. Group HW from Aim 1 was considered as group T0 (T = treatment) for this study and served as control, *i.e.* animals on high fat diet and water alone. The remaining 40 animals were randomized into 5 groups: T3, T7, T10, T14, and T21, and received the biologically active fraction for 3, 7, 10, 14, and 21 days, respectively, as their drinking fluid. Each group was sacrificed following the same procedure as in Aim 1 for blood and tissue collection. Further, hepatic mRNA was extracted and analyzed to determine if there were changes in the gene expression related to HDL cholesterol metabolism.

## AIM 4: To investigate the change in plasma metabolomic profiles upon therapy with the biologically active fraction

Plasma metabolomic profile using 1D proton NMR was performed to determine the changes in metabolite concentrations after 21 days of therapy, as compared with controls. Data were analyzed using multivariate data analysis SIMCA P+ software.

Targeted profiling was applied to NMR spectra to determine the metabolites that are different between the treatment group (T21) and the T0 group, which served as control for this aim. Once these metabolites were identified and quantified using Chenomx software, analysis of the pathways involving the specific metabolites was conducted. In addition, the metabolomic profile was correlated with the plasma lipid profile.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 AIM 1: To determine the biological activity of complete PAZ and its subfractions in a hypercholesterolemic animal model

**Rationale:** The objective was to test the effectiveness of PAZ and its subfractions as a preventative agent in a high fat diet induced hypercholesterolemic hamster model. For this, PAZ and its subfractions were administrated as part of the drinking fluid along with the dietary regimen. Plasma lipid profile was measured using enzymatic assays, while lipoprotein distribution was determined by density gradient ultracentrifugation. Further, hepatic mRNA expression was also analyzed for potential effects on genes involved in HDL/ reverse cholesterol transport.

### 2.1.A Animal Protocol and Experimental Design

Eighty male Golden Syrian hamsters (*Mesocricetus auratus*), LVG strain were purchased from Charles River Laboratories, Wilmington, MA. Upon arrival they were 8 weeks old and weighed approximately 80 g each. The animals were acclimatized and given water and laboratory rodent diet 5001(Lab Diet, Richmond, IN) *ad libitum* for one week prior to the initiation of the experimental regimen. They were housed individually in a temperature-controlled room (25℃) and maintained on a 12-h light/dark cycle. They were randomly distributed into control (n = 20, receiving control purified diet, Table 2.1) and treatment groups (n = 60, receiving high fat diet, 30% calories from fat, Table 2.2). Further, the control group was subdivided into water (CW) or PAZ (CP) groups (n=10/group), based on their drinking fluid.

**Table 2.1** Composition of the Control Purified Diet

| Ingredient | kcal/gm | grams/kg |
|---|---|---|
| Casein | 3.58 | 220 |
| Dextrose | 3.8 | 160 |
| Cornstarch | 3.6 | 460.9 |
| L-Arginine | 4 | 1 |
| L-Tryptophan | 4 | 1.1 |
| Cellulose | 0 | 50 |
| Corn Oil | 9 | 60 |
| Mineral Mix (#260001) | 0 | 35 |
| Vitamin Mix (# 360001) | 3.84 | 10 |
| Choline Bitartrate | 0 | 2 |

**Mineral Mix contained the following components:** 6,000 g/kg Calcium (Ca); 3,100 g/kg Phosphorus (P); 6,100 g/kg Potassium (K); 1,500 g/kg; Sodium (Na); 2,310 g/kg; Chlorine (Cl); 600g/kg Magnesium (Mg); 140 g/kg Iron (Fe); 5.8 g/kg Copper (Cu); 3.7 g/kg Manganese (Mn); 23.5 g/kg Zinc (Zn); 0.32 g/kg Chromium (Cr); 1.6 g/kg Iodine (I); 0.20 g/kg Selenium (Se); 0.20 g/kg Fluorine (F); 1.20 g/kg Cobalt (Co).

**Vitamin Mix contained the following components:** 2 g/kg Thaimin HCl; 1.5 g/kg Riboflavin; 0.7 g/kg Pyridoxine HCl; 9 g/kg Niacin; 4 g/kg Calcium Pantothenate; 0.2 g/kg Folic acid; 0.06 g/kg Biotin; 1 g/kg Vitamin B12 (0.1%); 0.4 g/kg Menadione Sodium Bisulfite; 1g/kg Vitamin A Palmitate; 10 g/kg Vitamin E Acetate; 0.6 g/kg Vitamin D3; 10 g/kg Inositol; 959.5 g/kg Sucrose. Vitamin Mix was used at rate of 10 g/kg of diet.

**Table 2.2** Composition of the experimental custom purified diet

| Ingredient | kcal/gm | grams/kg |
|---|---|---|
| Casein | 3.58 | 110 |
| Lactalbumin | 3.9 | 110 |
| Cornstarch | 3.6 | 370.2 |
| L-Arginine | 4 | 2.5 |
| L-Tryptophan | 4 | 0.3 |
| Dyetrose | 3.8 | 175 |
| Cellulose | 0 | 44 |
| Coconut Oil | 9 | 138.6 |
| Soybean Oil | 9 | 1.4 |
| TBHQ* | 0 | 0.028 |
| Cholesterol | 0 | 1 |
| Mineral Mix (#260001) | 0 | 35 |
| Vitamin Mix (# 360001) | 3.84 | 10 |
| Choline Bitartrate | 0 | 2 |

**Abbreviation:** * TBHQ: Tertiary butylhydroquinone

The treatment group was subdivided into 6 groups (n=10/group): HW (water), HP (complete PAZ), HF1, HF2, HF3, HF4 (4 different subfractions of PAZ obtained by sequential affinity gel chromatography). Figure 2.1 describes the study design for Aim 1. Drinking fluid was given at either 5% (HF1, HF2, and HF3) or 20% (HP, HF4) concentration (v/v) for 4 weeks. This dose was established based on the previously reported dose of 4 oz of PAZ per day in humans [68]. Modifications were made to account for body weight and amount of fluid intake per day in hamsters versus humans. High fat diet containing 30 % calories from fat (Dyets Inc., Bethlehem, PA) was used to induce the rapid hypercholesterolemic state in the hamsters. Body weight, food and water intake were recorded weekly.
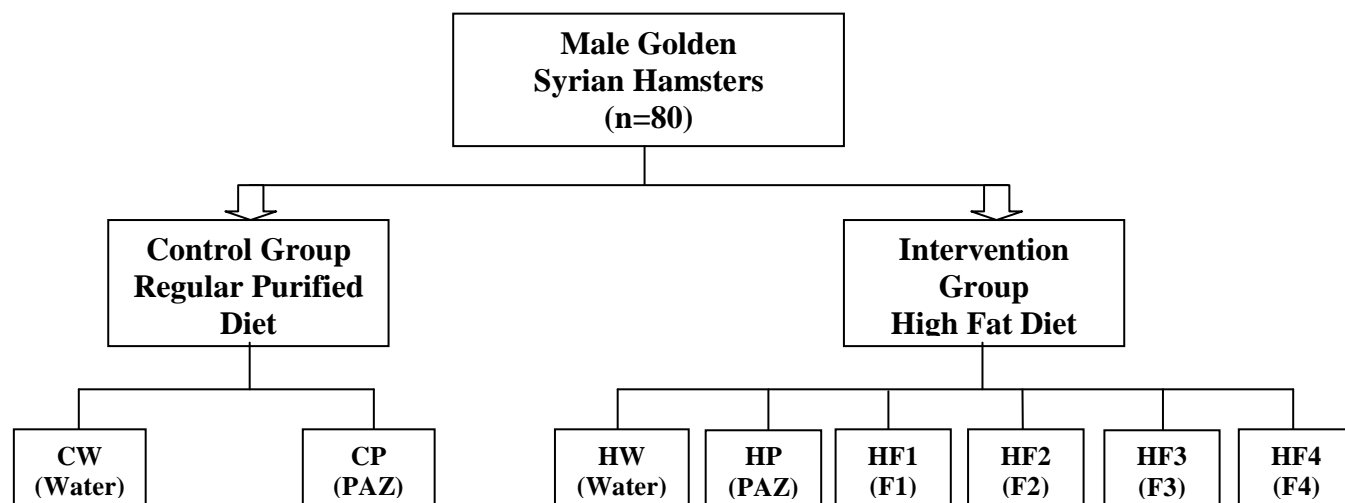
**Figure 2.1** Study Design for Aim 1

Prior to the beginning of the study, PAZ was fractionated by sequential affinity gel chromatography (Oxford Biomedical Research, Rochester Hills, MI). Complete PAZ was passed through four chromatography columns (2.7 cm x 23 cm; approximately 90 mL of resin at full capacity) at a flow rate of approximately 6 mL per minute using a peristaltic pump (Figure 2.2).

Column 1 containing a weak anion exchange resin (diethylaminoethyl cellulose) captured proteins and on elution resulted in the F1 fraction. Column 2 containing a strong anion exchange resin (BioRad AG 1-X8) was designed to capture molecules containing carboxyl groups and other negatively charged functionalities, as well as negatively charged ions, and the eluate from the column was designated as the F2 fraction. Column 3 was a strong cation exchange column (Dowex Monosphere 88) intended to capture molecules containing amino groups and other positively charged functionalities, as well as positively charged ions. Eluate from column 3 was designated as the F3 fraction.

Column 4 (silica gel 90 C18 reversed phase) was a C18 derivatized column that binds non polar organic molecules. The eluate from this column was not used in this study. Instead, the flow through (labeled as F4), which contains relatively few molecules, including polar but uncharged organic molecules, as well as molecules of low polarity that were not captured by columns 1, 2, 3, or 4, was assessed for its effect on hamster lipid profile. The pH of all fractions was measured and adjusted to an approximate value of 7.0, prior to being administered to the animals.
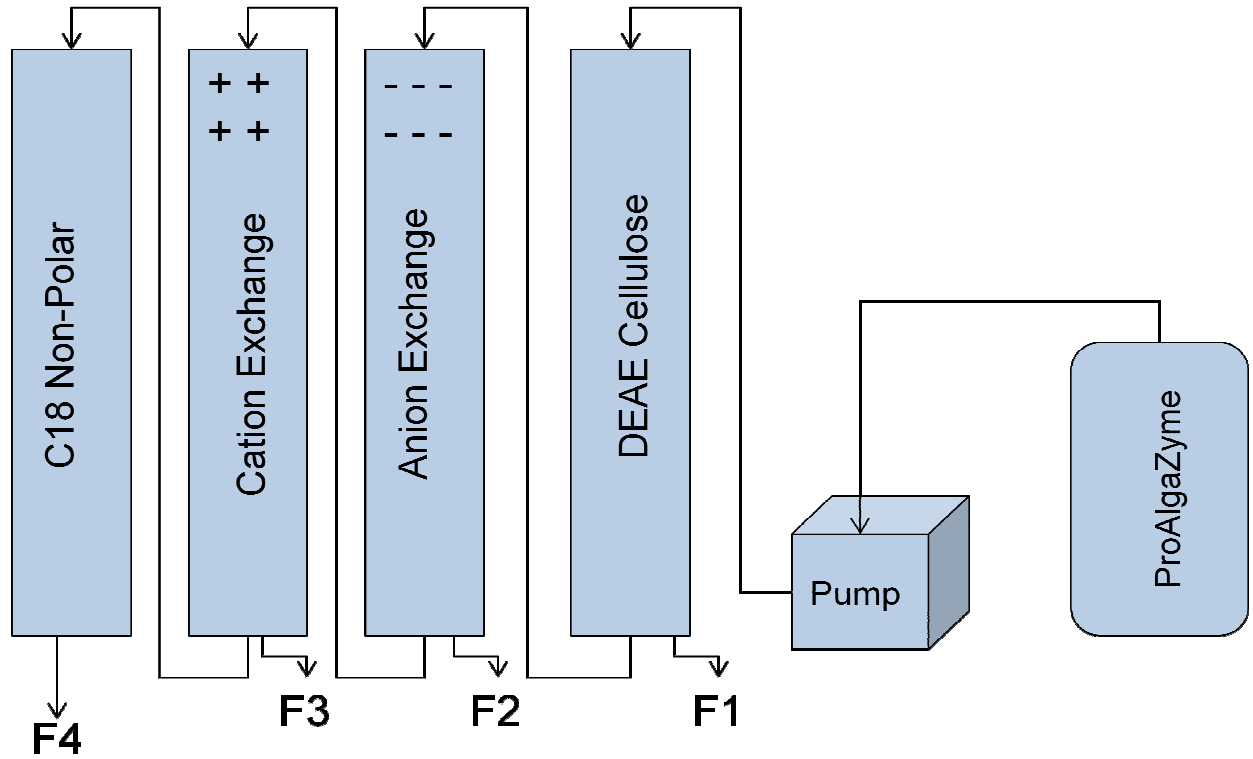
**Figure 2.2** Separation of PAZ into Fractions by Sequential Affinity Gel Chromatography

After 4 weeks of treatment, animals were sacrificed and blood, liver, and adipose tissue were collected. Hamsters were fasted for 8 hours and anesthetized under $CO_2$ gas (Metro Welding, Detroit, MI) prior to sacrifice. Blood was collected by cardiac puncture with syringes previously rinsed in potassium ethylenediamine tetraacetic acid (EDTA) solution (15% w/v) and kept on ice. Plasma was separated and collected after centrifugation at 1,000 x g for 15 minutes at 4°C. Liver and adipose tissue were collected and immediately flash-frozen in liquid nitrogen for subsequent analysis. The weight of the liver was recorded immediately after harvesting.

### 2.1.B Plasma Lipid Analysis

Various enzymatic assays were performed on the collected plasma to determine the changes in the lipoprotein concentrations as a result of PAZ and its subfractions administration. Plasma TC and TG concentrations were determined enzymatically, while HDL cholesterol was measured in the supernatant following precipitation with $Mg^{2+}$/dextran sulfate (Pointe Scientific, Canton, MI). The concentration of non-HDL cholesterol was calculated as the difference between the measured TC and HDL cholesterol, and included as the sum of VLDL, IDL, and LDL cholesterol. Choline-containing phospholipids and free cholesterol was also determined enzymatically (Wako Chemicals USA Inc., Richmond, VA). The cholesterol ester was determined by taking the difference between the total and free cholesterol values. The detailed procedures, including modifications to the manufacturer's protocol are described below.

### Total Cholesterol Assay

Briefly, 200 µl of 37℃ pre-incubated reagent mixed with 2 µl of plasma sample was incubated for 5 minutes at 37℃ and read at 490 nm using KC4 software (EL x 800 microplate absorbance reader, Bio-Tek, Winooski, VT). The reaction was carried out in a 96-well assay microplate. Calculations were done according to the formula provided in the manufacturer's protocol and plasma total cholesterol concentrations reported as mmol/L.

### Triglyceride Assay

Briefly, 200 µl of 37℃ pre-incubated reagent mixed with 2 µl of plasma sample was incubated for 5 minutes at 37℃ and read at 490 nm using KC4 software, EL x 800 microplate absorbance reader. The reaction was carried out in a 96-well assay microplate. Calculations were done according to the formula provided in the manufacturer's protocol and triglyceride concentrations reported as mmol/L.

### HDL Cholesterol Assay

For separation of HDL cholesterol, a 100 µl of plasma sample was mixed with 10 µl of reagent, and after 5 minutes incubation at room temperature, it was centrifuged at 2,000 x g for 5 minutes. For determination of the HDL cholesterol concentration, a 96-well assay microplate was used to mix 200 µl of reagent with 10 µl of supernatant, followed by incubation for 10 minutes at 37℃. The plate was read at a wavelength of 490 nm using KC4 software, EL x 800 microplate absorbance reader. Calculations were done according to the formula provided in the manufacturer's protocol and plasma HDL cholesterol concentrations reported as mmol/L.

## Ultracentrifugation

The lipoproteins were isolated by density gradient ultracentrifugation, essentially as stated by Chapman *et al* [93]. For each group, lipoprotein isolations (x 2) were carried out using plasma pooled from 4-5 hamsters in 16 x 93 mm ultracentrifuge tubes. Lipoproteins were isolated using a Beckman SW-40 rotor (Beckman Coulter, Brea, CA) at 35,000 rpm and 15°C for 46 hours [94]. Follo wing ultracentrifugation, 26 x 500 µl fractions per tube were collected by sequentially pipetting from the top. The total cholesterol, triglyceride, choline-containing phospholipids, and free cholesterol concentration in each fraction were measured using enzymatic reagents as described. The lipoprotein profile and lipoprotein particle size (diameter) for LDL and HDL cholesterol molecules were determined, using the following formula [95] :

$$R = (nm) = \frac{1.093 \, (TG) + 1.044 \, (CE)}{0.968 \, (FC) + 0.97 \, (PL) + 0.705 \, (PR)} \times (3 \times 2.15)$$

$$D \, (nm) = 2R + 2(2.15)$$

In the above formula, TG , triglyceride mass; CE, Cholesterol Ester mass; FC, Free Cholesterol mass; PL, phospholipids mass; PR, protein mass; R, radius; D, diameter.

## Phospholipids Assay

Briefly, the color reagent was mixed with the buffer, and 250 µl of the reconstituted reagent mixed with 5µl of sample was incubated at 37°C for 5 minutes. The reaction was carried out in a 96-well assay microplate and read at a wavelength of 570 nm using KC4 software, EL x 800 microplate absorbance reader. Calculations were done according to the formula provided in the manufacturer's protocol.

## Free Cholesterol Assay

Briefly, the content of one bottle of color reagent was dissolved in the buffer solution. 200 µl of the reconstituted color reagent mixed with 10 µl of sample was incubated at 37℃ for 5 minutes. The reaction was carried out in a 96-well assay microplate and read at a wavelength of 570 nm using KC4 software, EL x 800 microplate absorbance reader. Calculations were done according to the formula provided in the manufacturer's protocol. Further, amount of cholesterol ester was calculated by subtracting the free cholesterol from the total cholesterol amount.

## 2.1.C Real Time Polymerase Chain Reaction (PCR) for Gene Expression Analysis

## Total RNA Extraction

Total RNA was extracted from liver tissues using miRNeasy Mini Kit (Qiagen, Valencia, CA) to determine the changes in the gene expression related to HDL cholesterol metabolism. Briefly, 25 mg of previously flash frozen liver tissue was homogenized in 700 µl lysis reagent provided by the kit. After 5 minutes, 140 µl of chloroform was mixed in the tube, following by centrifugation at 12,000 x g for 15 minutes at 4℃. The upper aqueous phase was removed, and 525 µl of 100% ethanol was added to the tube. The mixture was then transferred into the RNeasy Mini columns. RWT and RPE buffers (provided by the kit) were added to the column and eluted via centrifugation at 8,000 x g. The total RNA was eluted with 40 µl RNAase free $H_2O$ and centrifuged at 1,000 x g for 1 minute. It was stored at -80℃ until further use.

## Total RNA to cDNA Preparation

In summary, 0.4 µg of total RNA was further subjected to reverse transcription using the High-Capacity RNA-to- cDNA Master Mix kit (Applied Biosystems, Carlsbad, CA). The reaction was carried in a total of 20 µl mixture (4ul of Complete Master Mix, 8 µl of total RNA, and 8 µl of nuclease-free $H_2O$) in Master cycler (Eppendorf, Hauppauge, NY). The program was set for 5 minutes at 25℃, 30 minutes at 42℃, 5 minutes at 85℃, and 1 hour at 4 ℃. The obtained cDNA was further used to perform quantitative PCR to determine the alteration in the gene expression due to the diet and treatment intervention.

## Real time RT-PCR Analysis

A total of 2 µl of  cDNA was used for each real-time RT-PCR reaction using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and MX3005P instrument (Strategene, Santa Clara, CA)  to determine the relative transcription levels of specific genes (ABCA1, ApoA1, CETP, and SRB1) involved in HDL/reverse cholesterol transport metabolism.   The cycle conditions were: 10 min at 95℃ followed by 40 cycles of incubation at 95℃ for 15s  each, then 60℃ for 1 min. Optimization of primer concentration was performed prior to the experiment to avoid accumulation of nonspecific products or primer-dimers. Likewise, non-template control (NTC) wells were added to the plate for the same reason. Data were analyzed according to the comparative threshold ($C_t$) cycle method and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in each sample. Levels of gene expression were reported as fold differences compared with hamsters fed the high fat diet and water.

**Table 2.3** Real time RT-PCR Primers

| Gene | Primers | Reference |
|------|---------|-----------|
| CETP | F:5'-AAGGGTGTCGTGGTCAGTTCT-3'<br>R: 5'-ACTGATGATCTCGGGGTTGAT-3' | [96] |
| Apo A1 | F: 5'-ACC-GTT-CAG-GAT-GAA-AAC-TGT-AG-3'<br>R:5'-GTG-ACT-CAG-GAG-TTC-TGG-GAT-AAC-3' | [97] |
| SRB-1 | F:5'- AAG-CCT-GCA-GGT-CTA-TGA-AGC-3'<br>R:5'- AGA-AAC-CTT-CAT-TGG-CTC-CCT-A-3' | [97] |
| ABCA1 | F:5'-ATA-GCA-GGC-TCC-AAC-CCT-GAC-3'<br>R: 5'-GGT-ACT-GAA-GCA-TGT-TTC-GAT-GTT-3' | [98] |

**Abbreviations:** CETP, Cholesteryl ester transfer protein; Apo A1, Apolipoprotein A-1; SRB1, Scavenger receptor class B member 1; ABCA1, ATP-binding cassette transporter A1

### 2.1.D Statistical Analysis

All data were expressed as the mean ± standard error (SE). Differences between the control and treatment groups were determined using one-way analysis of variance tests (IBM SPSS Inc, Chicago, IL). The data were analyzed to determine the effect of the algae infusion relative to distilled water, while the animals were fed the high fat diet. Statistical significance was defined as $P < 0.05$.

### 2.2 AIM 2: To investigate the change in plasma metabolomic profiles upon administration of PAZ and the biologically active fraction

**Rationale:** Plasma metabolomic profile using 1D proton NMR was performed to determine changes in metabolite concentrations among animals that received water (HW), complete PAZ (HP), and the biologically active fraction found in Aim 1. Data were analyzed using multivariate data analysis SIMCA P+ software. Targeted profiling was applied to NMR spectra to determine the metabolites that are present in different concentrations between the analyzed groups. Once these metabolites had been identified and quantified using Chenomx software, analysis of the pathways involving the specific metabolites was conducted.

### 2.2.A Sample Preparation

Plasma samples (from Aim 1) previously stored at -80℃ were thawed and centrifuged at 9,000 X g for 3 minutes. Samples were diluted with deuterium oxide ($D_2O$) for proper optimization of the concentration. To these diluted samples a reference buffer (NMR solvent) solution of 5 mmol/L disodium-2,2-dimethyl 2 – silapentane-5-sulphonate (DSS) and 10 mmol/L imidazole in $D_2O$ (Sigma-Aldrish, Mississauga, ON) was added in a 9 : 1 ratio (9 parts of diluted plasma sample : 1

part NMR solvent). DSS was used as spectral reference and imidazole as pH indicator for the NMR spectra. After preparation, samples were transferred to 5mm NMR tubes (Sigma-Aldrich, St. Louis, MO).

## 2.2.B 1D ^{1}H-NMR Spectroscopy

The 1D ^{1}H-NMR technique was conducted on hamster plasma samples collected at the terminal point of the study (Aim 1) on a 600 MHz Agilent instrument, operating at 599.773MHz frequency and a temperature of 300 K. Prior work by Martin *et al* [99] was used as a starting point for the protocol, but further optimization was conducted. One-dimensional NMR spectra were acquired using the Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence [99] with presaturation to attenuate broad signals from proteins and lipoproteins. Application of the named pulse sequence resulted in spectra with signals only from the small metabolites, due to their longer transverse relaxation time. These spectra were measured using a spin-echo loop time of 0.16 s and a recycling time of 14 s. A total of 64 scans were collected using a spectral width of 10 parts-per-million (ppm) and an acquisition time of 4 s.

The NMR data were processed using ACD/ Spec Manager 7.00 software (Advanced Chemistry Development Inc., Toronto, Canada). The principal of the NMR spectrophotometer is that when an external magnetic field is applied, the magnetic moment of the nucleus aligns itself with the applied field to create different energy levels. Transitions of atomic nuclei between these energy levels over a period of time are recorded as free induction decay (FID) files.

Since these FID files are time domain signal files, it is very difficult to differentiate between the spectra of different compounds. Therefore, the FID files are further

stacked together as a group and then transformed by Fourier transformation (Ft, mathematical algorithm) to a frequency domain. After Ft, the acquired spectrum can be resolved into different peaks arising from different compounds or metabolites. Each metabolite is represented by single or multiple peaks in the spectra and the height of the peak represents the intensity/concentration of the metabolite. Spectra were processed by editing, auto-phasing, and auto-baseline correction using the ACD software. Intelligent binning was used to divide the edited spectra into 1000 bins. The spectra was further digitized to a table of common integrals and exported as a non-negative value text file for multivariate data analysis.

### 2.2.C Multivariate Data Analysis (MVDA)

Once $^1$H-NMR spectra were converted to numeric values (digitized) using ACD software, MVDA was used for pattern recognition in the data set. This analysis was accomplished using SIMCA P+13.0 statistical software (Umetrics, Kinnelon, NJ).

PCA is a multivariate projection method designed to extract and display the systemic variation in the data (the table of integrals from NMR plasma samples). This unsupervised analysis was applied to the spectra of animals that received water, complete PAZ, and the biologically active fraction, and any differences between the metabolites were investigated. The corresponding loading plot provided information about the parts of the spectrum that are responsible for the similarities and/or dissimilarities in the data set. PLS is a regression extension of PCA and was used to correlate the plasma metabolomic profile with other measured factors, such as HDL cholesterol. In PLS-DA the data set was distributed into classes and its objective was

to find a model that separates the classes of observation on the basis of their X-variables, while using a hypothetical Y-variable.

## 2.2.D Metabolite Identification and Quantification

MVDA was used to determine the regions of the spectra responsible for similarities and/or dissimilarities between the groups. Further, the focus was on the peaks of spectrum that differentiate the groups, and the corresponding metabolites within that range, which were identified using Chenomx NMR 7.6. Profiling of the NMR spectra was accomplished using the Profiler module. Metabolites from the data base were identified and quantified, and the pathways affected by the respective metabolites were identified using online KEGG database.

## 2.3 AIM 3) To determine the therapeutic effect of the biologically active fraction on hypercholesterolemia

**Rationale:** The aim was to determine the therapeutic effect of the biologically active fraction on animals which had already achieved a hypercholesterolemic state. Secondly, this part of the study aimed to determine the time course required for the effect of the biologically active fraction on lipid parameters.

In order to achieve this aim, the animals were fed high fat diet for the first four weeks of the study. This was followed by administration of the biologically active fraction via their drinking fluid for 3, 7, 10, 14, and 21 days, respectively. All groups were sacrificed following the same procedure as in Aim 1 for blood and tissue collection. Further, mRNA was extracted from liver tissues to determine the changes in the gene expression related to HDL cholesterol metabolism.

## 2.3.A Animal Protocol and Experimental Design

Forty male Golden Syrian hamsters (*Mesocricetus auratus*), LVG strain were purchased from Charles River Laboratories, Wilmington, MA. Upon arrival they were 8 weeks old and weighed approximately 80 g each. The animals were acclimatized and given water and laboratory rodent diet 5001(Lab Diet, Richmond, IN) *ad libitum* for one week prior to the initiation of the experimental treatment. They were housed individually in a temperature-controlled room (25℃) and maintained on a 12-h light/dark cycle.

Animals were randomly distributed into 5 groups (n=8; T3, T7, T10, T14, and T21) and fed HF diet for 4 weeks prior to the treatment regimen (Figure 2.3). The biologically active fraction, (20% v/v) was administrated as their drinking fluid for 3 days (to T3 group), 7 days (to T7 group), 10 days (to T10 group), 14 days (T14 group), and 21 days (T21 group), respectively, as their treatment. HW group from Aim 1 was considered as T0 groups in this study and served as control, as the animals in this group received HF diet for 4 weeks and water as their drinking fluid. At each end point of the study, same procedure described in Aim 1A was used to sacrifice the animals and collect blood and tissues.
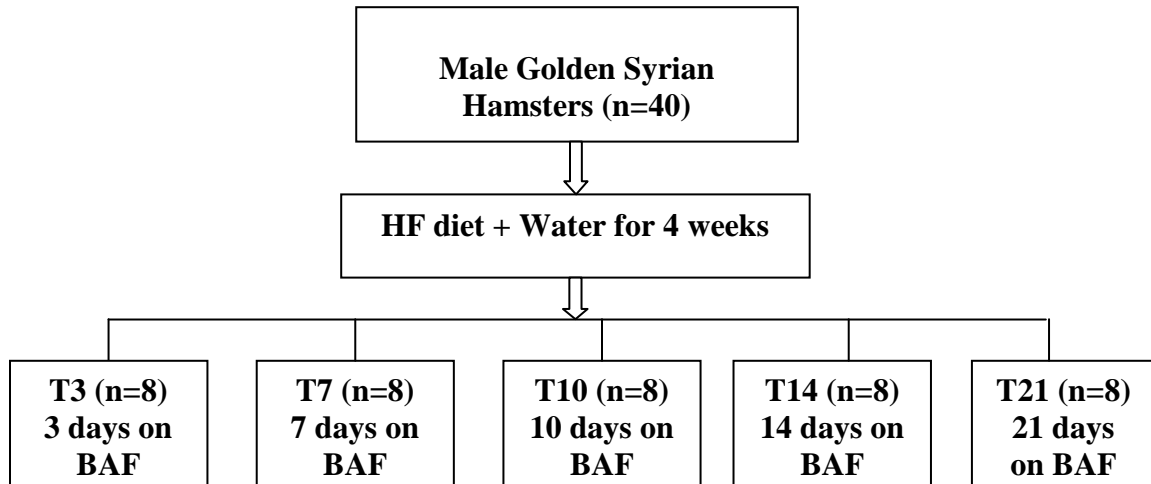
**Figure 2.3** Study Design for Aim 3

**2.3.B Plasma Lipid Analysis**

Various lipid assays were performed on the collected plasma to determine the changes in the lipoprotein concentrations as a result of the therapeutic intervention with the biologically active fraction of PAZ. Plasma TC and TG concentrations were determined enzymatically, while HDL cholesterol was measured enzymatically in the supernatant following precipitation with $Mg^{2+}$/dextran sulfate as previously described (Pointe Scientific, Canton, MI). The concentration of non-HDL cholesterol was calculated as the difference between the measured TC and HDL cholesterol, and included the sum of VLDL, IDL, and LDL cholesterol.

**<u>Total Cholesterol Assay</u>**

As previously discussed under Aim 2.1.B

**<u>Triglyceride Assay</u>**

As previously discussed under Aim 2.1.B

**<u>HDL Cholesterol Assay</u>**

As previously discussed under Aim 2.1.B

**2.3.C Real Time Polymerase Chain Reaction for Gene Expression Analysis**

**<u>Total RNA Extraction</u>**

 As previously discussed under Aim 2.1.C

**<u>Total RNA to cDNA Preparation</u>**

As previously discussed under Aim 2.1.C

 **<u>Real time RT-PCR Analysis</u>**

 As previously discussed under Aim 2.1.C (relative transcription levels of Apo A1)

**2.3.D Statistical Analysis**

All data were expressed as the mean ± standard error (SE). Differences between the control (HW/T0) and treatment groups (T3, T7, T10, T14, and T21) were determined using one-way analysis of variance tests. The data were analyzed to determine the therapeutic effect of the biologically active fraction of PAZ relative to distilled water, after the animals were fed a high fat diet for 4 weeks. Statistical significance was defined as $P < 0.05$.

**2.4 AIM 4) To investigate the change in plasma metabolomic profiles upon therapy with the biologically active fraction**

**Rationale:** Plasma metabolomic profile using one-dimensional proton NMR was performed to determine the changes in the concentration of metabolites present in the control group (T0) and the treatment group representing the final time point, T21. Data were analyzed using multivariate data analysis SIMCA P+ software. Targeted profiling was applied to NMR spectra to determine the metabolites that have a significantly different concentration between the groups. Once these metabolites have been identified and quantified using CHENOMX software, analysis of the pathways involving the specific metabolites was conducted.

**2.4.A Sample Preparation**

As previously discussed under Aim 2.2.A

**2.4.B 1D $^1$H-NMR Spectroscopy**

As previously discussed under Aim 2.2.B

**2.4.C Multivariate Data Analysis**

As previously discussed under Aim 2.2.C

PCA and PLS-DA were applied to T0 and T21 groups to look for any differences between the metabolites. PLS was used to correlate plasma lipid profile with the metabolomic data.

**2.4.D Metabolite Identification and Quantification**

As previously discussed under Aim 2.2.D

**CHAPTER 3**

**RESULTS**

**3.1 AIM 1: To determine the biological activity of complete PAZ and its subfractions in a hypercholesterolemic animal model**

**3.1.A Metabolic Effects of High Fat Diet and PAZ Supplementation**

All hamsters survived the entire duration of the study. Animals in all experimental groups consumed similar amounts of food (grams) and fluid (ml). The final body weight and weight gained over the duration of the study was not significantly different among high fat groups or the control; however CP had a significantly lower body weight and weight gain as compared with the groups that received the high fat diet. Liver weights were also not significantly different between groups (Table 3.1).

The change in body weight over time is shown in Figure 3.1. Week 0 represents the body weight (grams) that the animals had upon arrival, whereas week 0' represents the body weight after the first week of acclimatization, when animals were fed regular diet. Week 0' was the starting point on the high fat diet, and week 4 was the final time point of the study. There was a similar trend in body weight gain for all animals; however the animals receiving the high fat diet had a faster growth rate as compared with the animals receiving regular purified diet.

**Table 3.1** Anthropometrics of Male Hamsters Fed High Fat Diet and PAZ or its Fractions for 4 Weeks as a Potential Preventative Agent for Hypercholesterolemia

|  | CW | CP | HW | HP | HF1 | HF2 | HF3 | HF4 |
|---|---|---|---|---|---|---|---|---|
| **Body weight, g** | 106.9±2 | 104.9±3* | 119 ±3 | 117±3 | 117±3 | 118±3 | 122±2 | 119±3 |
| **Body weight gain, g/4 wk** | 21.6±1 | 19.7±2.5* | 32.7± 2 | 29.7±2 | 30.3±2.6 | 33.1±3 | 29.9±3.1 | 31.1±1 |
| **Food Intake, g/d** | 6.2±0.1 | 6.2±0.2 | 7.1±0.1 | 6.8±0.1 | 6.7±.0.2 | 6.8±0.2 | 7.0±0.2 | 6.6±0.2 |
| **Energy Intake, Kcal/day** | 22.6±0.4 | 22.6±0.7 | 29.3±0.4 | 28.1±0.4 | 27.7±0.8 | 28.1±0.8 | 28.9±0.8 | 27.3±0.8 |
| **Fluid intake, ml/d** | 8.8±0.4 | 8.7±0.5 | 8.3 ±0.2 | 8.2±0.6 | 8.3±0.7 | 7.7±0.5 | 7.7±0.4 | 7.2±2.1 |
| **Liver weight, g** | 3.8 ±0.1** | 3.7 ±0.1** | 5.4 ± 0.2 | 5.8±0.3 | 5.5±0.2 | 5.4±0.3 | 5.5±0.3 | 5.4±0.2 |

**Notes:** Values are mean ± SE, n = 10/group. * $P < 0.05$, CP vs. all treatment groups (high fat diet); **$P < 0.001$, CW and CP vs. all treatment groups (high fat diet). Statistical program ANOVA with Tukey's procedure was used, SPSS software.
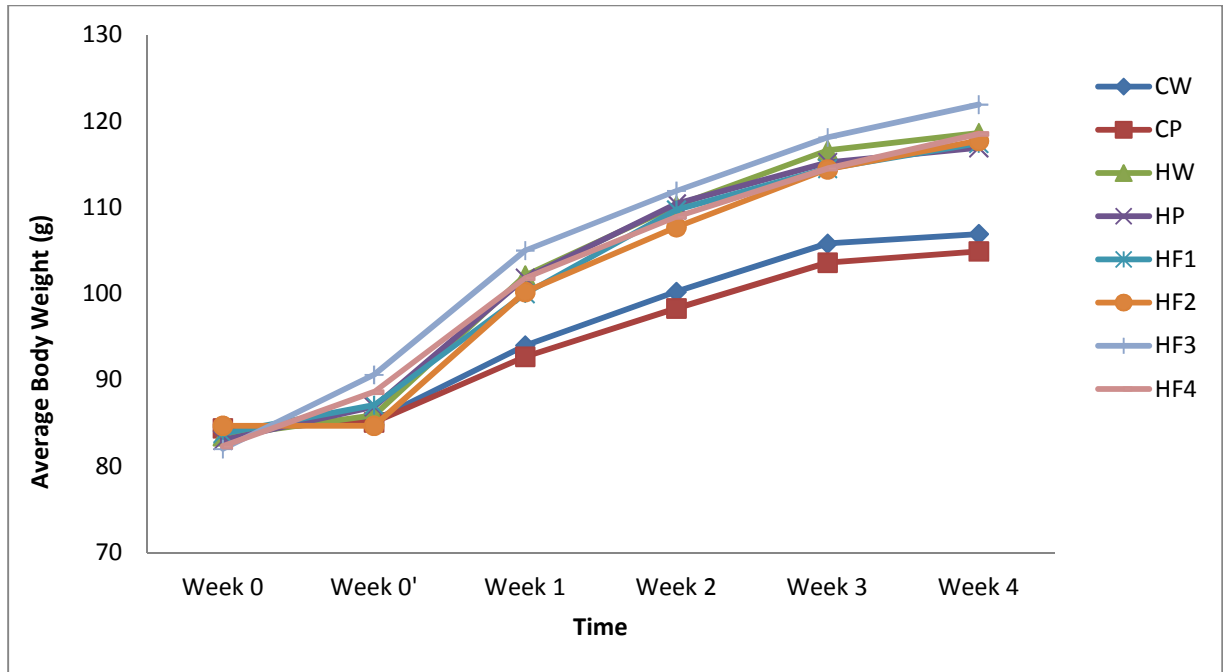
**Figure 3.1** Change in Body Weight over 4 Weeks of the Study. Male hamsters received high fat diet and PAZ or one of the fractions for the duration of the study, starting with week 0'. Values are mean of the body weight of all animals from the group. N = 10/group

**3.1.B Plasma Lipid Analysis**

In order to determine that the hypercholesterolemic state was induced in the animals that received a high fat diet, plasma TC, TG, and non-HDL cholesterol concentrations were measured in animals that received regular diet and water (CW) and high fat diet and water (HW). Results showed a significant increase in plasma TC and non-HDL cholesterol. The TG in HW group were also increased, but did not reach significance (Figure 3.2). Therefore, we concluded that we successfully induced a hypercholesterolemic state in the animal model.

When comparing the groups on high fat diet alone, plasma total cholesterol (Figure 3.3) and triglycerides (Figure 3.4) were not significantly different in the treatment groups as compared with the high fat controls, HW. However, the TC/HDL cholesterol ratio was significantly lower in all experimental animals when compared with controls ($P < 0.05$ in the HP, HP1, and HP2 groups; $P < 0.001$ in the HP3 and HP4 groups, Figure 3.5). Consumption of PAZ or its fractions lowered non-HDL cholesterol concentrations in the HP group ($P < 0.05$) and in the HP3 and HP4 ($P < 0.001$) groups, as compared with the controls (Figure 3.6). Moreover, the concentration of plasma HDL cholesterol was significantly increased in the HP, HP3, and HP4 groups ($P < 0.001$), when compared with controls (Figure 3.7).
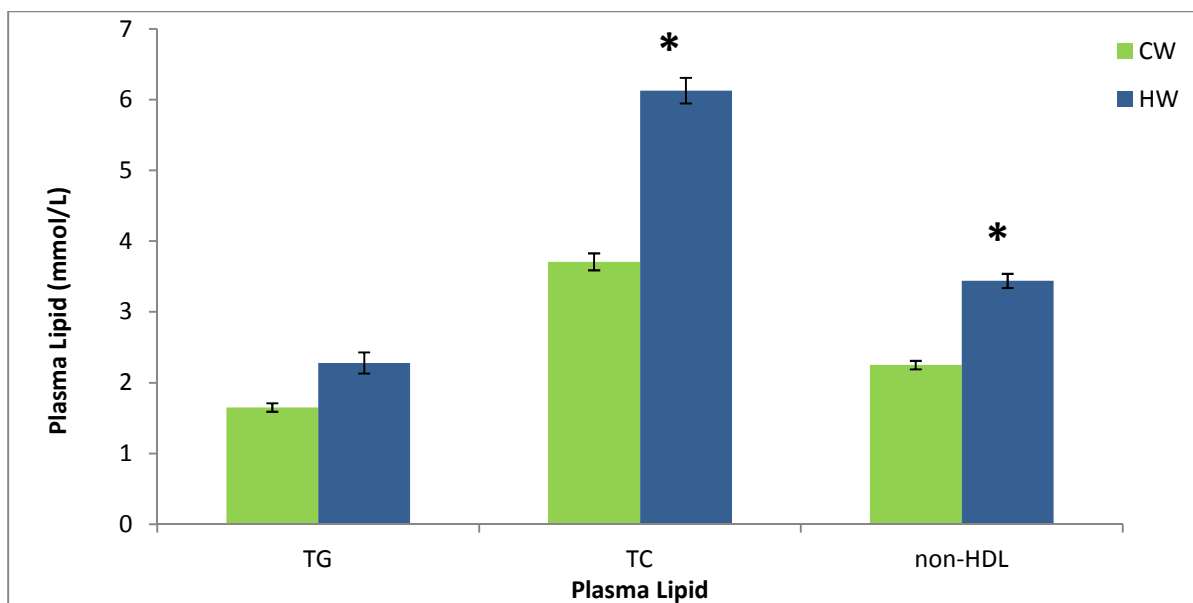
**Figure 3.2** Effect of High Fat Diet on Lipid Profile

**Notes:** Lipid parameters (TG, TC, and non-HDL cholesterol) of CW and HW groups.

Values are mean ± SE. * $P < 0.001$ as compared with CW group.

**Abbreviations:** TG, triglycerides; TC, total cholesterol; HDL, high lipoprotein

concentration; CW, control diet + water group; HW, high fat diet +water group

**Figure 3.3** Effect of PAZ and its fractions on Total Cholesterol Concentration

**Notes:** Values of total cholesterol measured in groups that received high fat diet along with water (HW), complete PAZ (HP), PAZ fraction 1 (HF1), PAZ fraction 2 (HF2), PAZ fraction 3 (HF3), and PAZ fraction 4 (HF4) for 4 weeks. Values are mean ± SE. No sig. difference as compared with HW group.
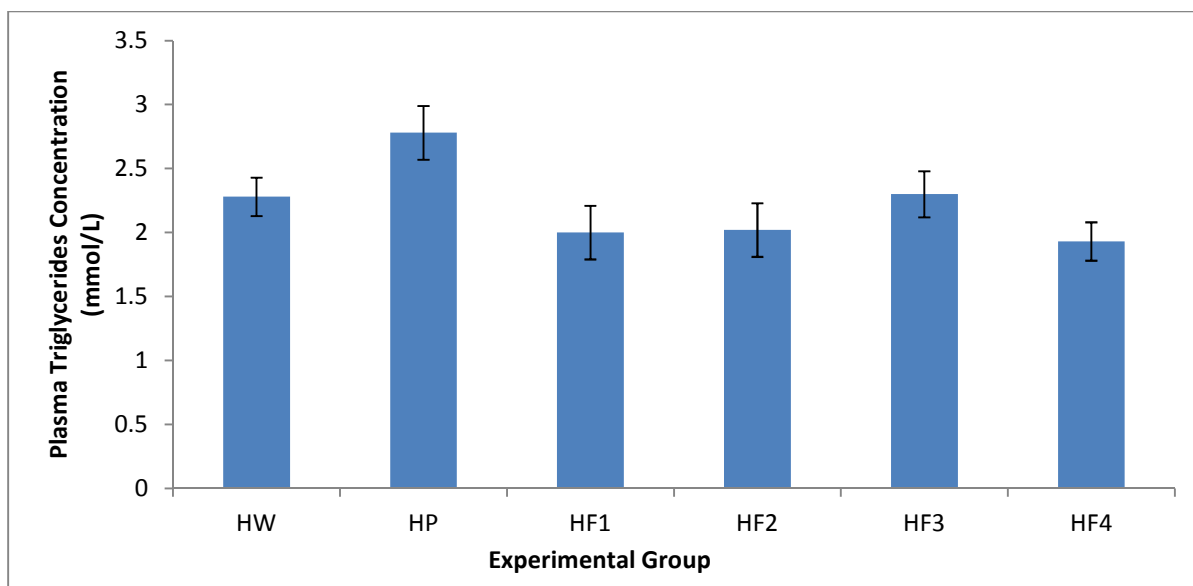
**Figure 3.4** Effect of PAZ and its fractions on Triglycerides Concentration

**Notes:** Values of triglycerides measured in groups that received high fat diet along with water (HW), complete PAZ (HP), PAZ fraction 1 (HF1), PAZ fraction 2 (HF2), PAZ fraction 3 (HF3), and PAZ fraction 4 (HF4) for 4 weeks. Values are mean ± SE. No sig. difference as compared with HW group.
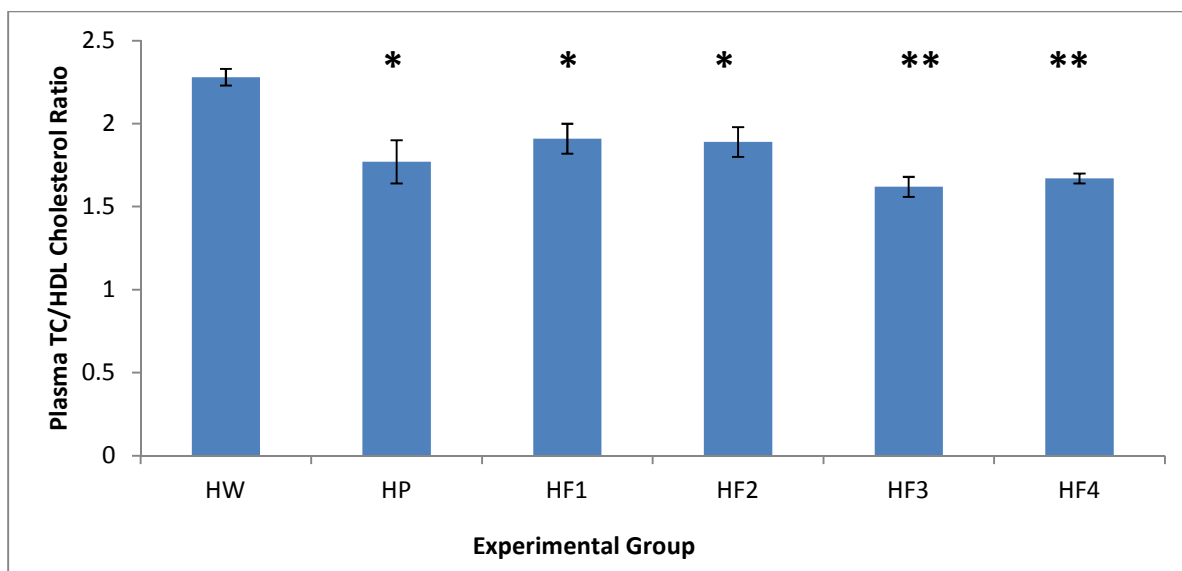
**Figure 3.5** Effect of PAZ and its fractions on TC/ HDL Ratio

**Notes:** The ratio of TC/HDL cholesterol was measured in groups that received high fat diet along with water (HW), complete PAZ (HP), PAZ fraction 1 (HF1), PAZ fraction 2 (HF2), PAZ fraction 3 (HF3), and PAZ fraction 4 (HF4) for 4 weeks. Values are mean ± SE. * $P < 0.05$, ** $P < 0.001$ as compared with HW group.
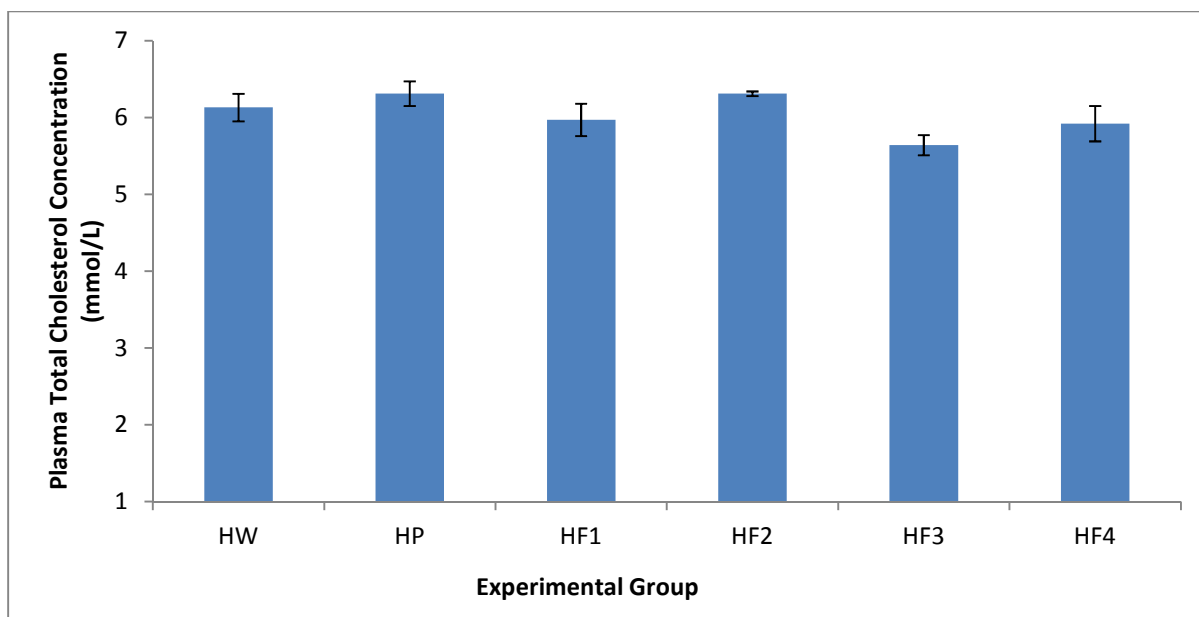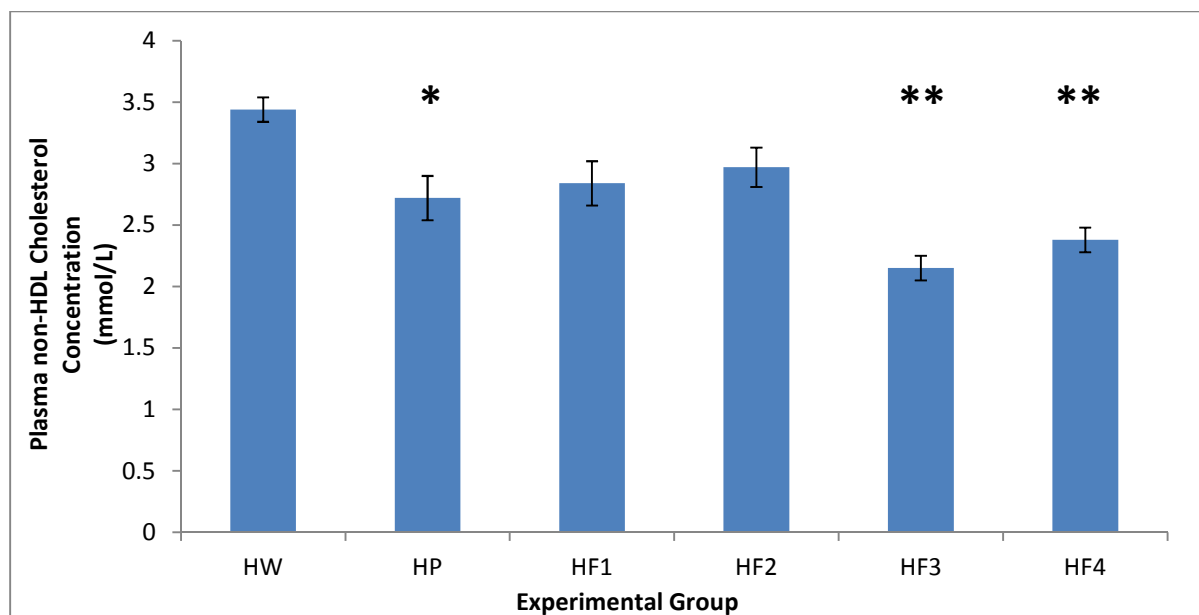
**Figure 3.6** Effect of PAZ and its fractions on non-HDL Cholesterol Concentration

**Notes:** Values of non-HDL cholesterol measured in groups that received high fat diet along with water (HW), complete PAZ (HP), PAZ fraction 1 (HF1), PAZ fraction 2 (HF2), PAZ fraction 3 (HF3), and PAZ fraction 4 (HF4) for 4 weeks. Values are mean ± SE. *$P < 0.05$, ** $P < 0.001$ as compared with HW group.
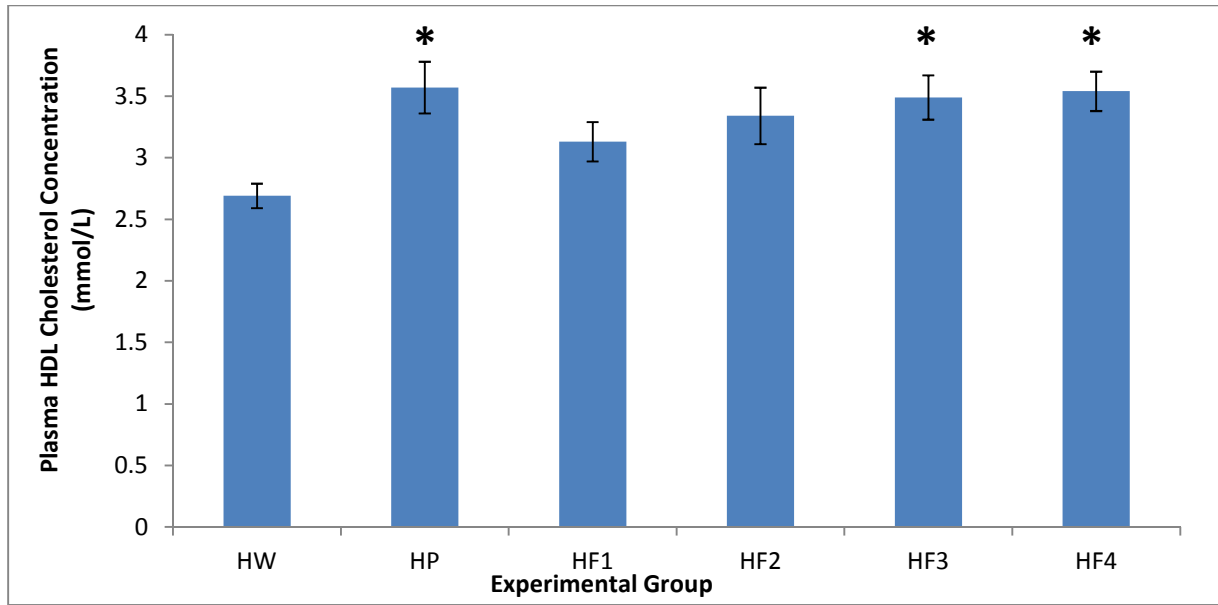
**Figure 3.7** Effect of PAZ and its fractions on HDL Cholesterol Concentration

**Notes:** Values of plasma HDL cholesterol measured in groups that received high fat diet along with water (HW), complete PAZ (HP), PAZ fraction 1 (HF1), PAZ fraction 2 (HF2), PAZ fraction 3 (HF3), and PAZ fraction 4 (HF4) for 4 weeks. Values are mean ± SE. * $P < 0.05$ as compared with HW group.

Ultracentrifugation was performed on pooled plasma (4-5 hamsters/group) and lipoprotein fractionation indicated that a higher proportion of cholesterol was carried in the HDL fraction in the HP group (Figure 3.8), as compared with HW group. Particle sizes for LDL (Figure 3.9) were calculated and there was no statistical difference between the groups that received PAZ or its subfractions, when compared with HW. However, when HDL particle size was measured, data showed that the particle sizes of HF3 and HF4 groups were significantly increased as compared with control group (Figure 3.10). Data augment the previous finding that HDL cholesterol concentration has been increased in these groups, but also the particle size was enlarged.
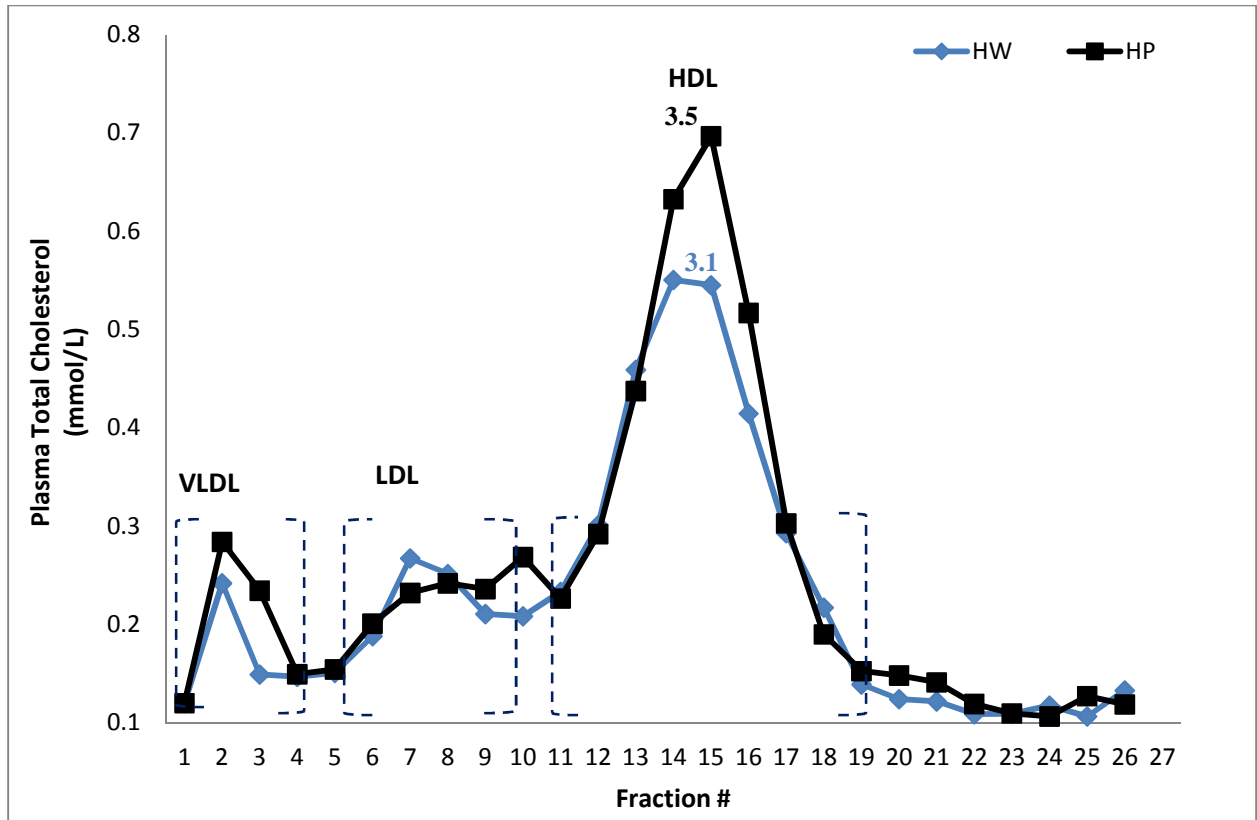
**Figure 3.8** Effect of PAZ on Lipoprotein Particle Distribution

**Note:** The graph depicts an increase in the high density particles in HP group (3.5 mmol/L) vs. HW (3.1 mmol/L) group. Total cholesterol concentrations were measured in all fractions (0.5 mL/fraction) using standard enzymatic reagents. Graph represents the mean of pooled plasma for HW and HP groups.
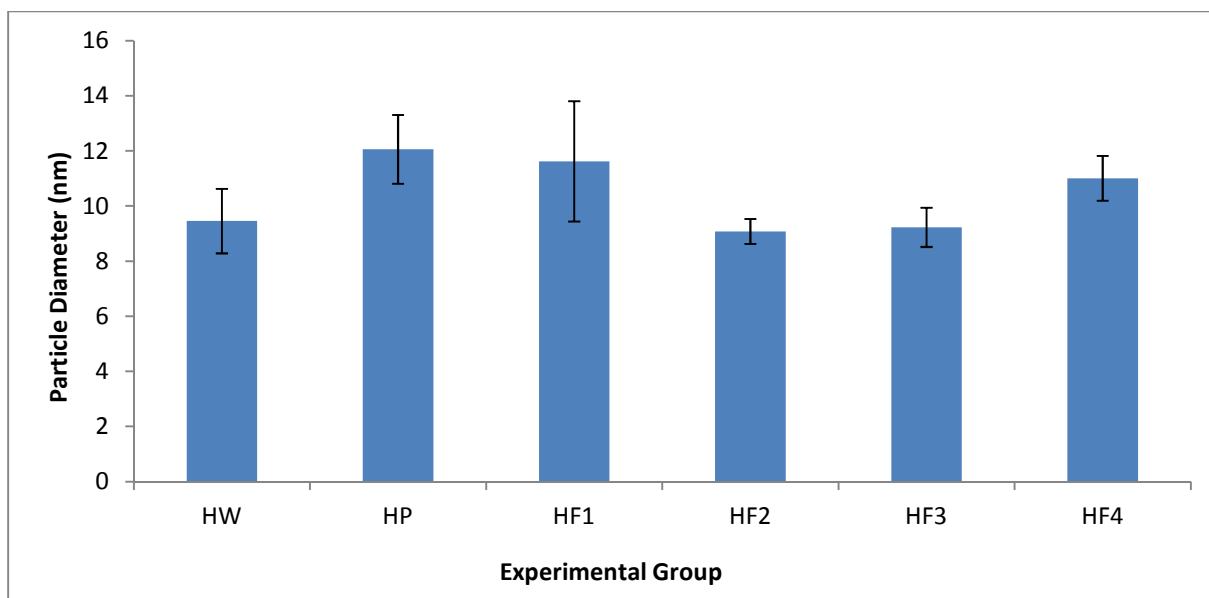
**Figure 3.9** Effect of PAZ and its fractions on LDL Particle Size

**Notes:** Values are mean ± SE. No sig. difference as compared with HW group. High fat diet along with water (HW), complete PAZ (HP), PAZ fraction 1 (HF1), PAZ fraction 2 (HF2), PAZ fraction 3 (HF3), and PAZ fraction 4 (HF4)
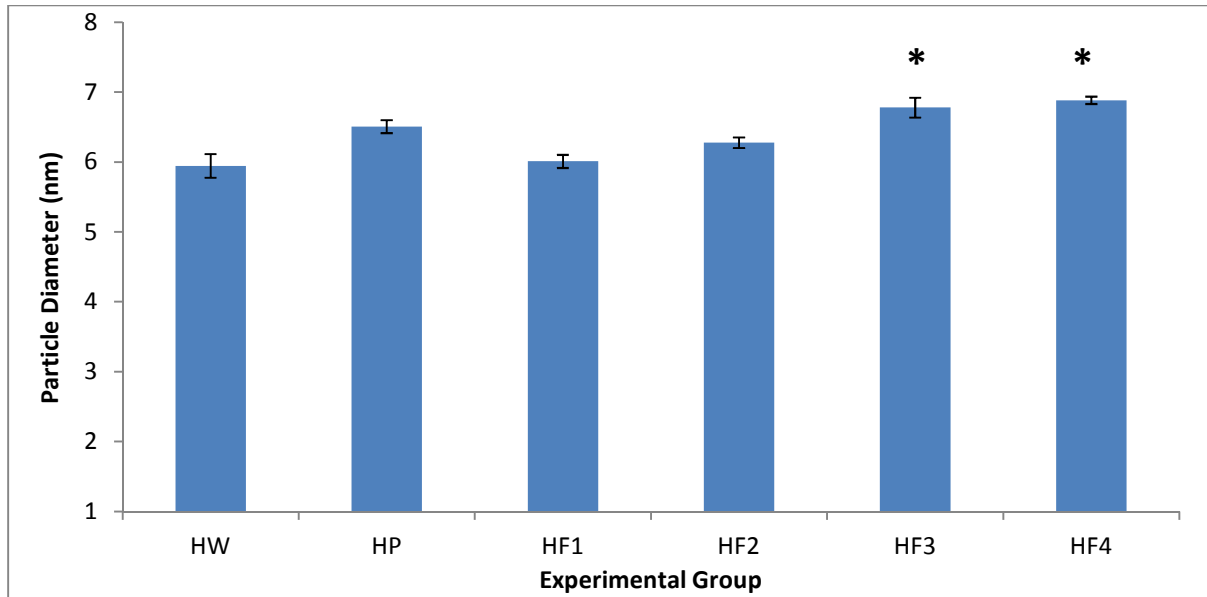
**Figure 3.10** Effect of PAZ and its fractions on HDL Particle Size

**Notes:** Values are mean ± SE. * $P < 0.05$ as compared with HW group. High fat diet along with water (HW), complete PAZ (HP), PAZ fraction 1 (HF1), PAZ fraction 2 (HF2), PAZ fraction 3 (HF3), and PAZ fraction 4 (HF4) for 4 weeks

### 3.1.C Effect of PAZ and its Subfractions on Gene Expression

In an attempt to elucidate the mechanism by which PAZ and its subfractions altered the lipoprotein profile by increasing HDL, the activities of ABCA1, Apo A1, SRB1, and CETP were assessed. When compared with the control group (HW), hamsters fed the PAZ (HP group) and fraction 4 (HF4 group) had the highest fold increase (approximately 5-fold) in Apo A1 expression, while the HP3 group showed a minor increase (2-fold, Figure 3.11a). ABCA1 sterol transporter expression showed a moderate increase in the HP3 and HP4 groups (1.7-fold and 1.8-fold, respectively, Figure 3.11b), as compared with HW group.

In addition, SRB1 activity levels were also modestly higher in the HP3 and HP4 groups (approximately 2-fold, Figure 3.11c) when compared with controls. The differences in the other treatment groups (HP1 and HP2) were not significant. The data suggest that the increase in HDL cholesterol concentrations in the HP, HP3, and HP4 groups was in part attributable to the increase in production of nascent HDL cholesterol particles and/or clearance via SRB1 receptors. Hepatic CETP expression was characterized by a 2-fold decrease only in the HP4 group as compared with those receiving water alone (Figure 3.11d). Inhibition of CETP is consistent with the decrease in plasma non-HDL lipoprotein along with the increase in HDL cholesterol.

Correlations between plasma lipid concentrations and hepatic gene expression levels were sought to identify potential relationships between molecular processes and circulating lipid concentrations (Table 3.2). TC, non-HDL cholesterol, and TC/HDL ratio were negatively correlated with hepatic expression of genes of ABCA1

and SRB1. There was a significant positive correlation between plasma HDL cholesterol concentrations and mRNA levels of Apo A1 (P < 0.01). In addition, positive correlations between HDL cholesterol concentrations and mRNA levels of SRB1 were observed.
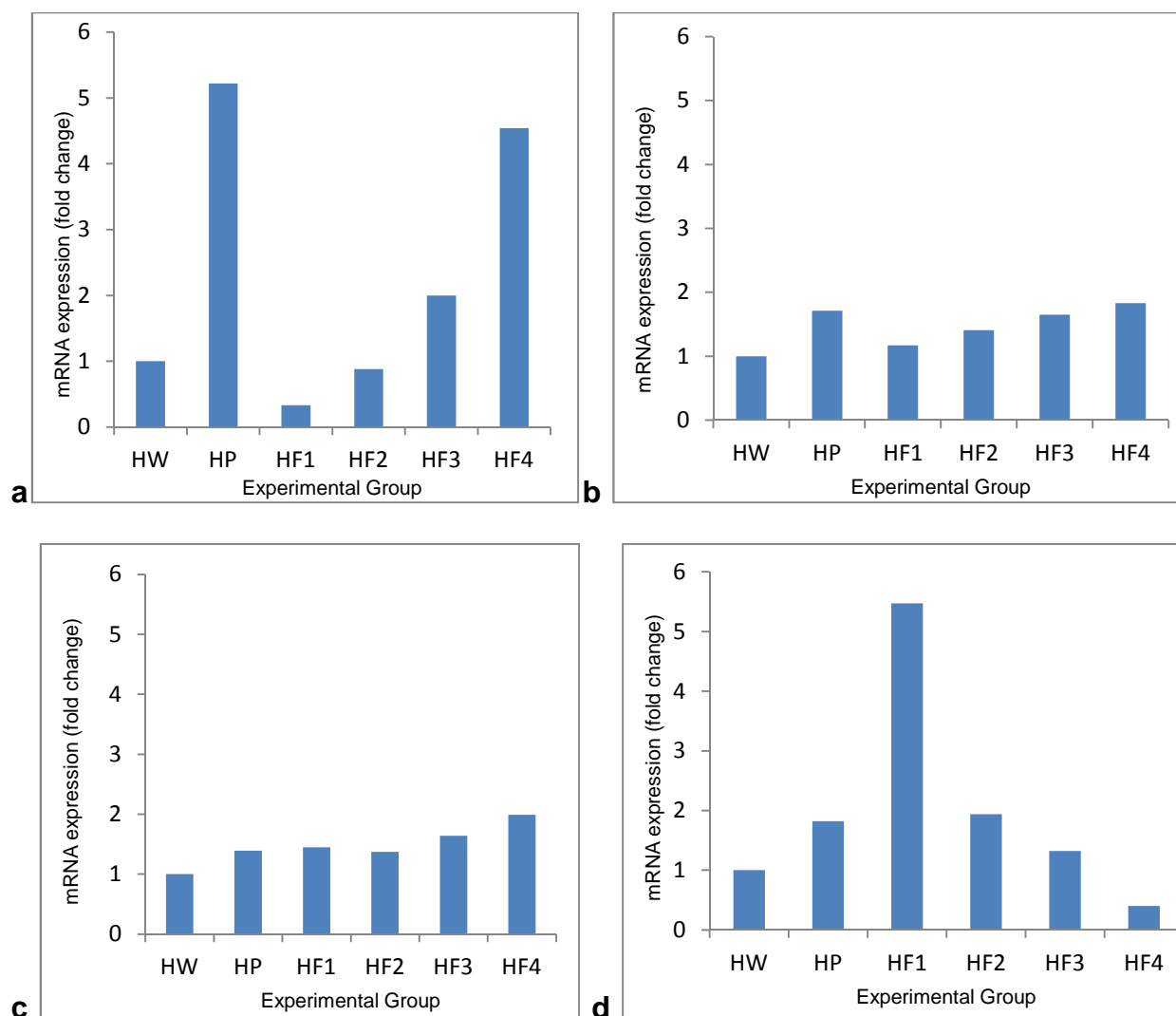
**Figure 3.11** Effect of PAZ and its fractions on Gene Expression

**Notes:** Relative levels of expression of genes that encode key proteins involved in the regulation of cholesterol and HDL metabolism in animals fed a high fat diet plus complete PAZ (HP) or one of the fractions, as compared with the control group on high fat diet and water (HW). Values are expressed as mean, n= 5 animals per group (n=10 for HP group); Apo A1, Apolipoprotein A1 (3.11a); ABCA1, ATP-binding cassette transporter A1 (3.11b); SRB1, Scavenger receptor class B member 1

(3.11c); CETP, Cholesteryl ester transfer protein (3.11d). Each mRNA was normalized with GAPDH and is expressed as a fold change.

**Table 3.2** Correlation between Plasma Cholesterol Concentrations and Expression of Hepatic Genes

| Genes | Total cholesterol | HDL cholesterol | TC/HDL ratio | non-HDL cholesterol |
|-------|-------------------|-----------------|--------------|---------------------|
| APO A1 | 0.153 | 0.399* | -0.342* | -0.243 |
| ABC A1 | -0.068 | 0.252 | -0.33* | -0.33* |
| SRB 1 | -0.190 | 0.252 | -0.420* | -0.459* |
| CETP | 0.040 | -0.055 | 0.056 | 0.099 |

**Note:** Values are Pearson correlation, N = 10. *P < 0.01

## 3.2 AIM 2: To investigate the change in plasma metabolomic profiles upon administration of PAZ and the biologically active fraction

### 3.2.A Multivariate Data Analysis

To further investigate the preventative effect of the algal infusion PAZ on the hypercholesterolemic hamsters, a metabolomic approach was applied to plasma samples from control (HW) and treatment groups HP and HF4. One-dimensional NMR spectra were acquired using the CPMG spin-echo sequence with presaturation, in order to attenuate broad signals acquired from proteins and lipoproteins. The resulting spectra have the signals from small metabolites only (Figure 3.12). During the pre-processing of the spectra, the acquired NMR files were edited, while auto-phasing and auto-baseline correction was applied (Figure 3.13). The spectra were further digitized to a table of common integrals and exported as a non-negative value text file for multivariate data analysis.
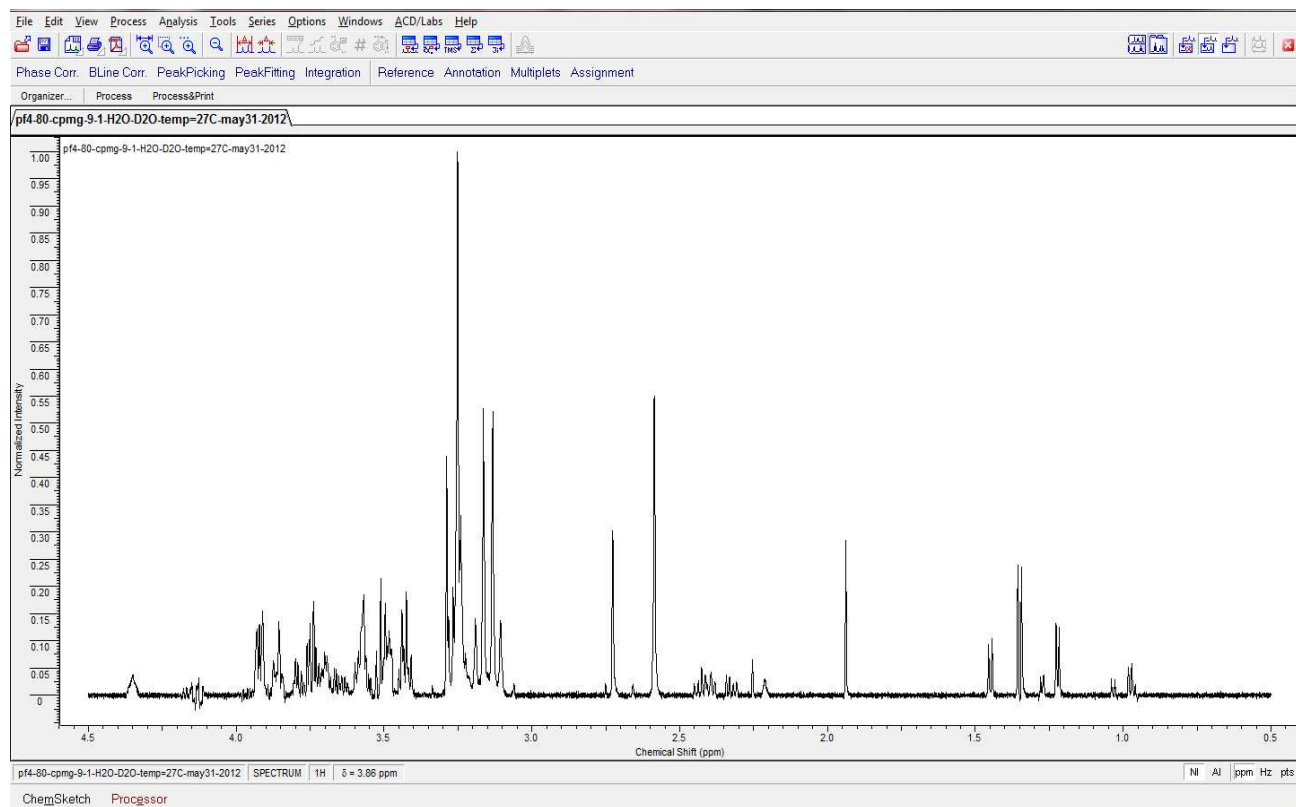
**Figure 3.12** NMR Spectrum obtained on 600 MHz Agilent Instrument, using CPMG Spin-Echo Sequence with Presaturation

78
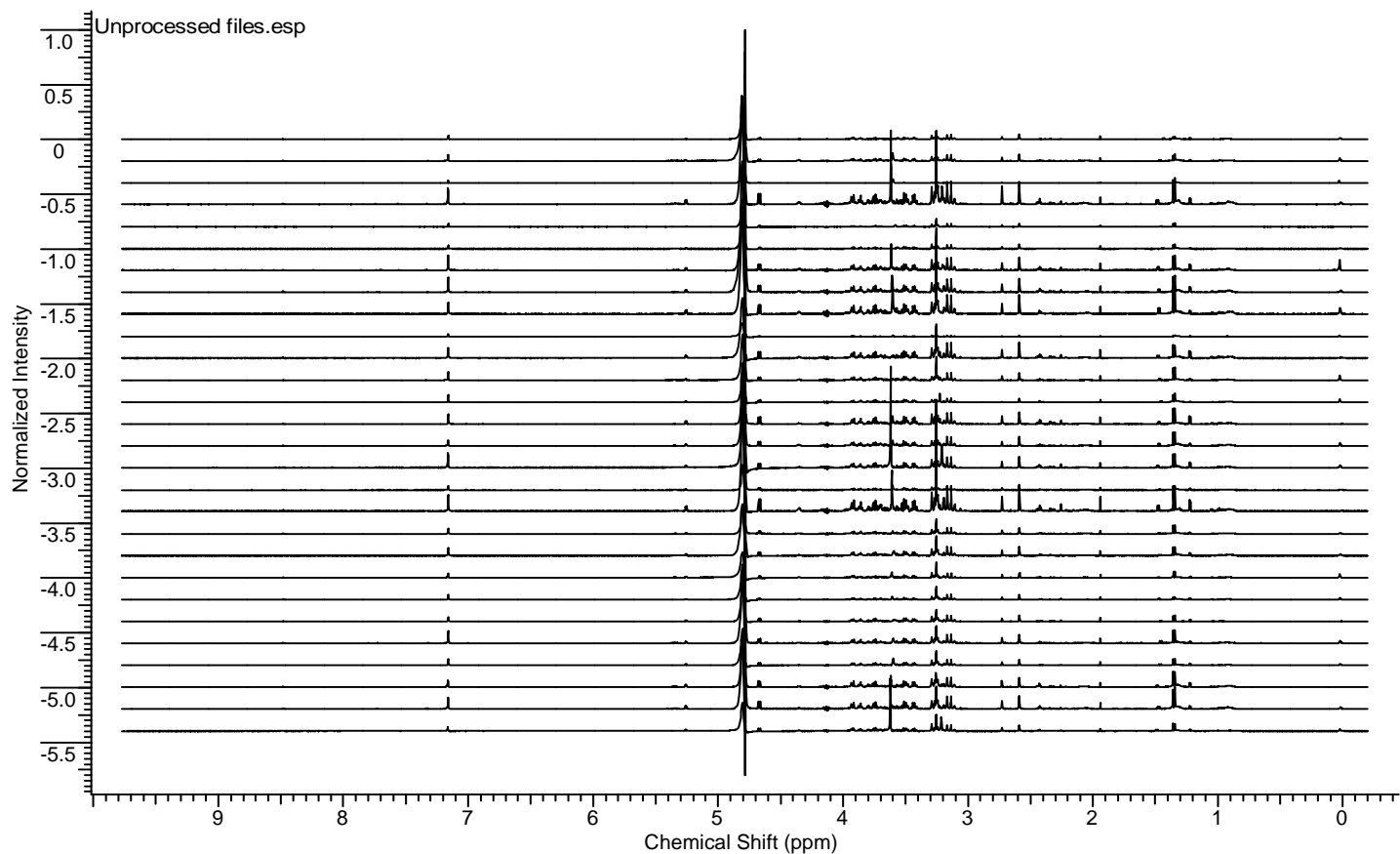


**Figure 3.13** ^{1}H NMR Spectra of HW, HP, and HF4 Groups

**Notes:** Spectra were stacked together as a group and transformed by Fourier transformation to a frequency domain. Spectra were processed by editing, auto-phasing, and auto-baseline correction. Further, intelligent binning was used to divide the edited spectra in 1000 bins.

To confirm that the metabolic profile of the hamsters that received high fat diet was changed, PCA was performed on the NMR data. Three-dimensional PCA plot (Figure 3.14) shows a clear separation between CW and HW groups, which represents the effect of the high fat diet. PLS-DA of the same two groups, reinforcing the effect of the diet is shown in Figure 3.15. We also analyzed CP and HP groups, to look at the effect of diet and addition of PAZ, and a clear separation of the two groups was observed (Figure 3.16).

Further, the focus was on the groups that received the high fat diet along with the complete PAZ infusion or fraction 4 and compare them with the control group that received water and high fat. Figure 3.17 represents the 3-dimensional PCA plot, providing overall information on metabolic changes of the hamsters due to PAZ or fraction 4 administrations, as compared with controls. To better analyze each treatment group as compared with the control animals, PCA and PLS-DA was conducted selecting two groups at the time.

The effect of complete PAZ infusion on the metabolic profile resulted in a clear separation of the HP and HW groups, as shown in Figure 3.18. The effect of administration of fraction 4 to the hamsters resulted in clustering of the HW and HF4 groups, showing an apparent separation based on their metabolic profiles (Figure 3.19a). The corresponding loading plot (Fig. 3.19b) provided significant information on the contribution of each variable to the pattern in the score plots, which can aid in metabolite identification in Chenomx software**.**

**Figure 3.14** Characterizations of the Plasma Metabolomic Changes Induced by High Fat Diet. 3-Dimensional CMPG_PCA score plot revealed that CW and HW groups were clearly separated. P < 0.05

**Notes:** CW, regular purified diet and water; HW, high fat diet and water. N = 8 hamsters/group.

**Abbreviations:** CPMG, Carr-Purcell-Meiboom-Gill; PCA, principal components analysis

Principal Component 1
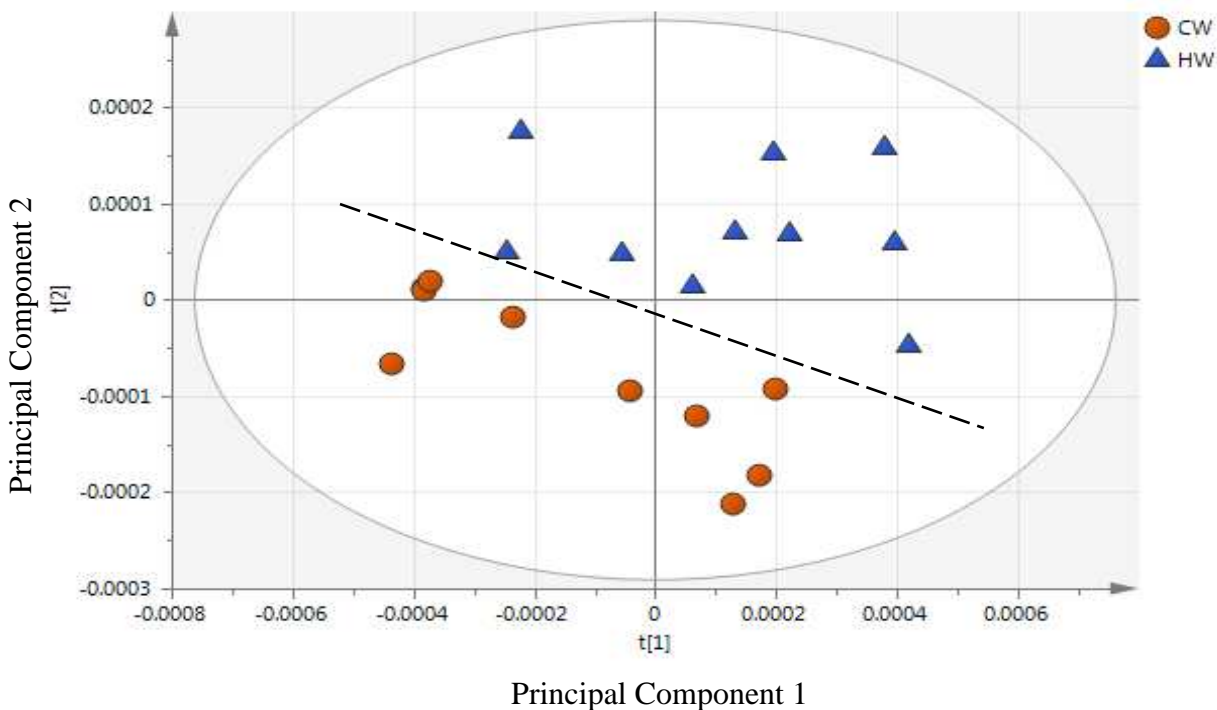
**Figure 3.15** Characterizations of the Plasma Metabolomic Changes Induced by High

Fat Diet. 2-Dimensional CMPG_PLS-DA score plot revealed that CW and HW groups

were clearly separated due to the diet received. P < 0.05

**Notes:** CW, regular purified diet and water; HW, high fat diet and water. N = 8

hamsters/group.

**Abbreviations:** CPMG, Carr-Purcell-Meiboom-Gill; PLS-DA, partial least squares –

discriminant analysis

Principal Component 1
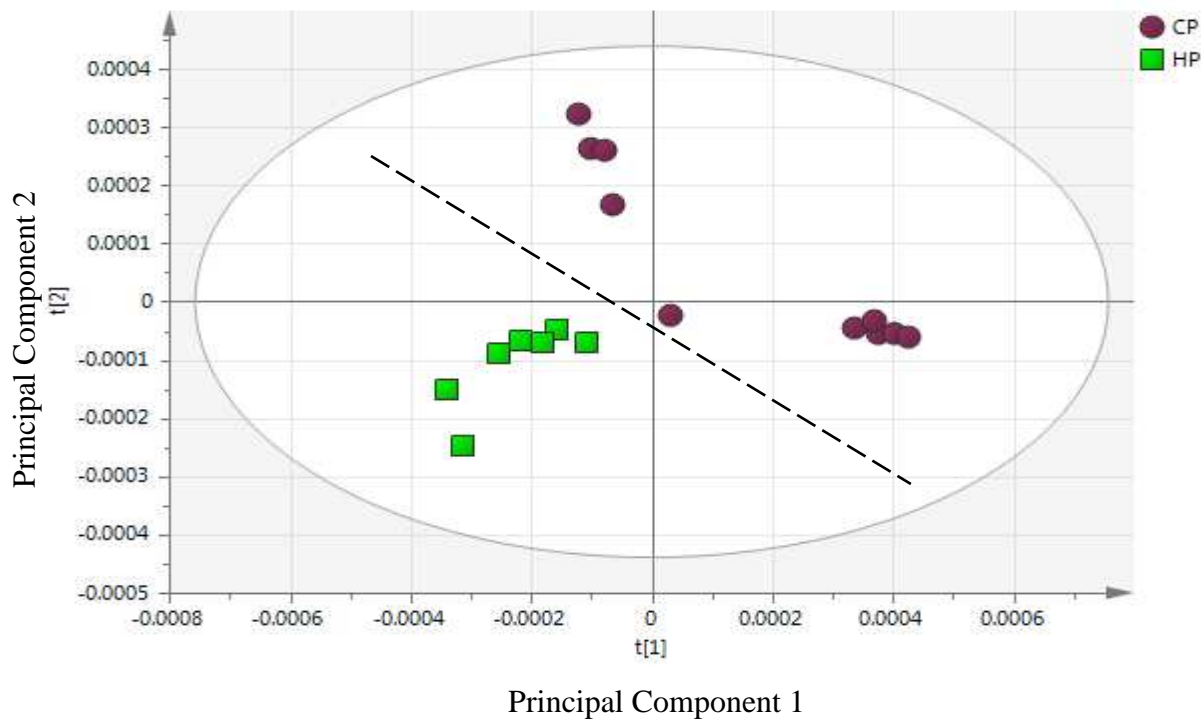
**Figure 3.16** Characterizations of the Plasma Metabolomic Changes Induced by Diet and PAZ Consumption. 2-Dimensional CMPG_PLS-DA score plot showing group discrimination (CP vs. HP) based on PAZ and the diet received. $P < 0.05$

**Notes:** CP, regular purified diet and PAZ; HP, high fat diet and PAZ. N = 8 hamsters/group.

**Abbreviations:** CPMG, Carr-Purcell-Meiboom-Gill; PLS-DA, partial least squares – discriminant analysis
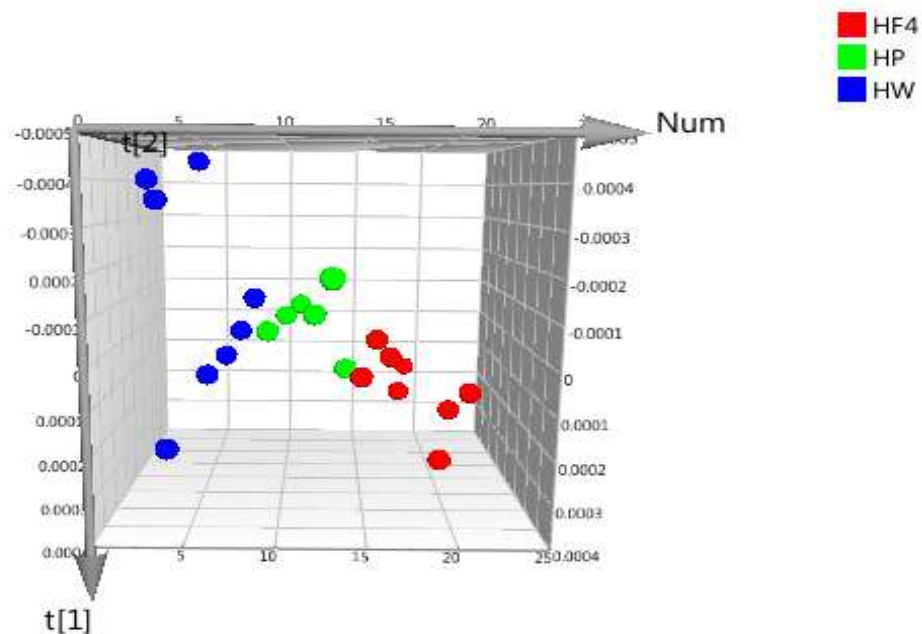
**Figure 3.17** Characterizations of the Plasma Metabolomic Changes Induced by High

Fat Diet and Drinking Fluid.  3-Dimensional CMPG_PCA score plot revealed that HW,

HP and HF4 groups were clearly separated. P < 0.05

**Notes:** HW, high fat and water; HP, high fat diet and PAZ; HF4, high fat and fraction

4. N = 8 hamsters/group.

**Abbreviations:** CPMG, Carr-Purcell-Meiboom-Gill; PCA, principal components

analysis
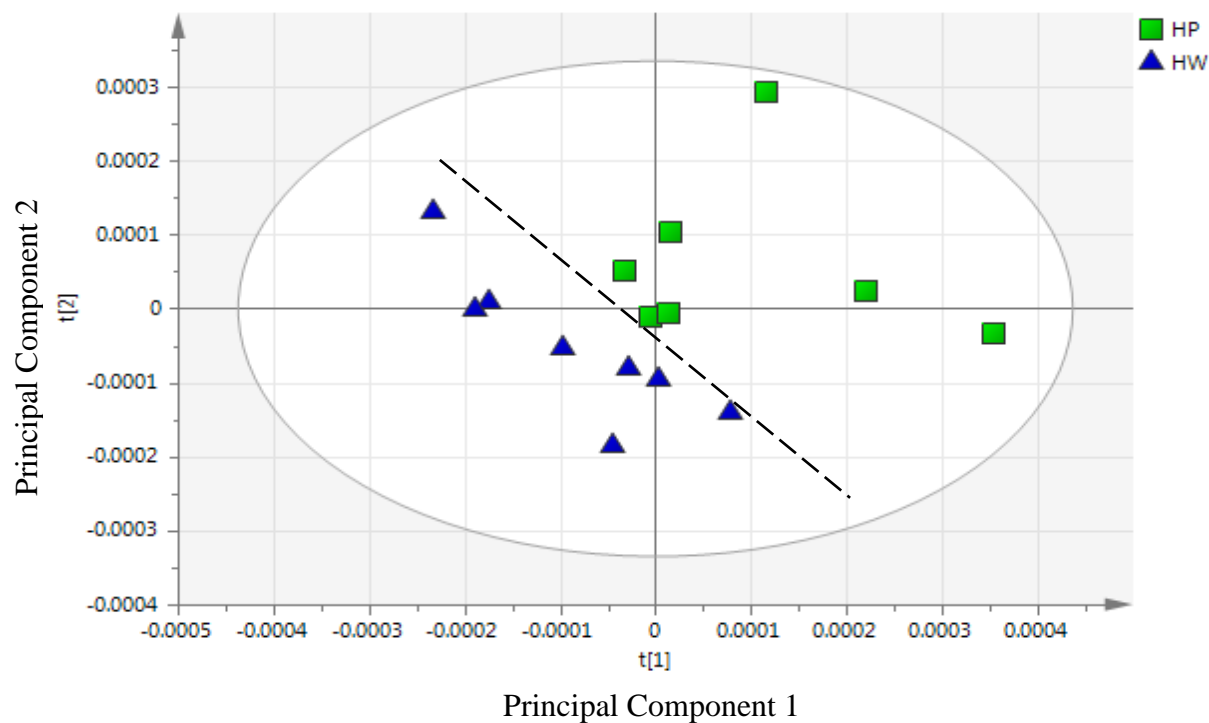
**Figure 3.18** Characterizations of the Plasma Metabolomic Changes Induced by PAZ.

2-Dimensional CMPG_PLS-DA score plot showing group discrimination (HW vs. HP)

based on PAZ administration. P < 0.05

**Notes:** HW, high fat diet and water; HP, high fat diet and PAZ. N = 8 hamsters/group.

**Abbreviations:** CPMG, Carr-Purcell-Meiboom-Gill; PLS-DA, partial least squares-
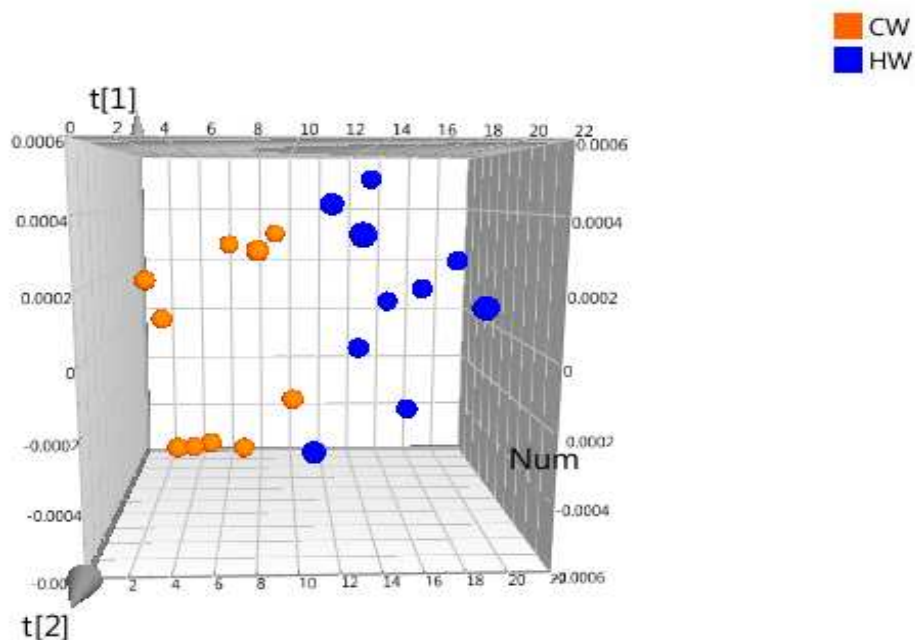
discriminant analysis
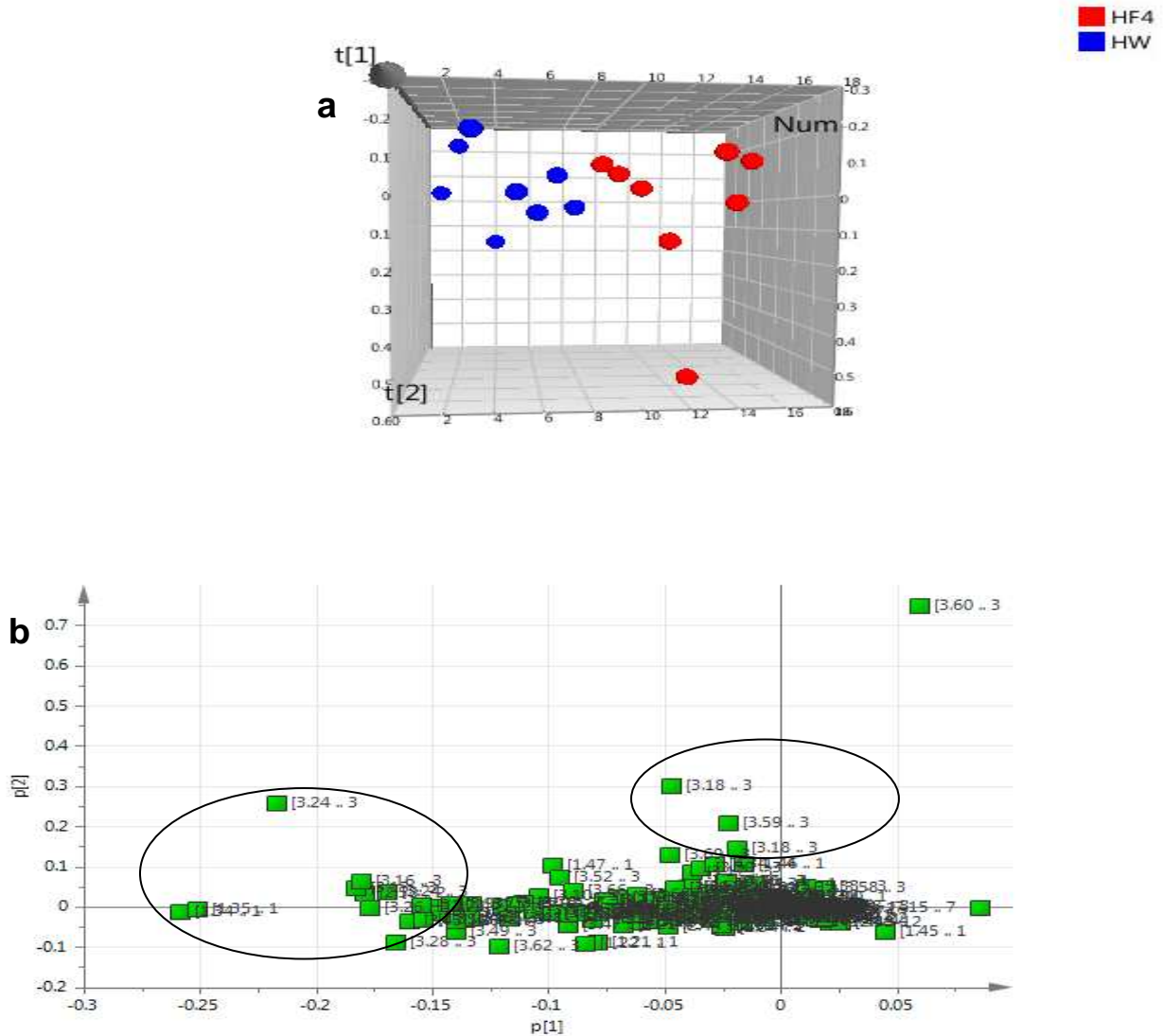
**Figure 3.19** Characterizations of the Plasma Metabolomic Changes Induced by Fraction 4. **3.19a**. 3-Dimensional CMPG_PCA score plot revealed that HW and HF4 groups were clearly separated. P < 0.05; **3.19b** CMPG_PCA corresponding loading plot indicating the regions of the spectra that are responsible for the group separation

**Notes:** HW, high fat diet and water; HF4, high fat diet and fraction 4. N = 8 hamsters/group.

**Abbreviations:** CPMG, Carr-Purcell-Meiboom-Gill; PCA, principal components analysis

Further, the discriminant analysis indicated that the treatment with PAZ and fraction 4 induced specific metabolites patterns that enabled class assignment of the hamsters (Figure 3.20). The PLS-DA score plot revealed that the group that received PAZ was clearly separated by the principal component 1 from the group that received fraction 4.

In addition, 2-dimensional PLS plot allowed for the evaluation of the PAZ and fraction 4 on changes in the plasma metabolome, inducing a significant coefficient of determination $R^2 = 0.6$, when the data were correlated with the plasma HDL concentrations (Fig. 3.21). The PLS regression between the plasma HDL concentration and the NMR variable revealed that 60 % of the variations in the plasma profile can be predicted by different treatment conditions (PAZ vs. fraction 4).

Furthermore, PLS analysis showed that HDL cholesterol concentrations were positively correlated with the plasma metabolic profile, indicating that the composition shift toward the higher density lipoproteins is reflected in the change in concentrations of some of the small metabolites present in plasma.

Principal Component 1
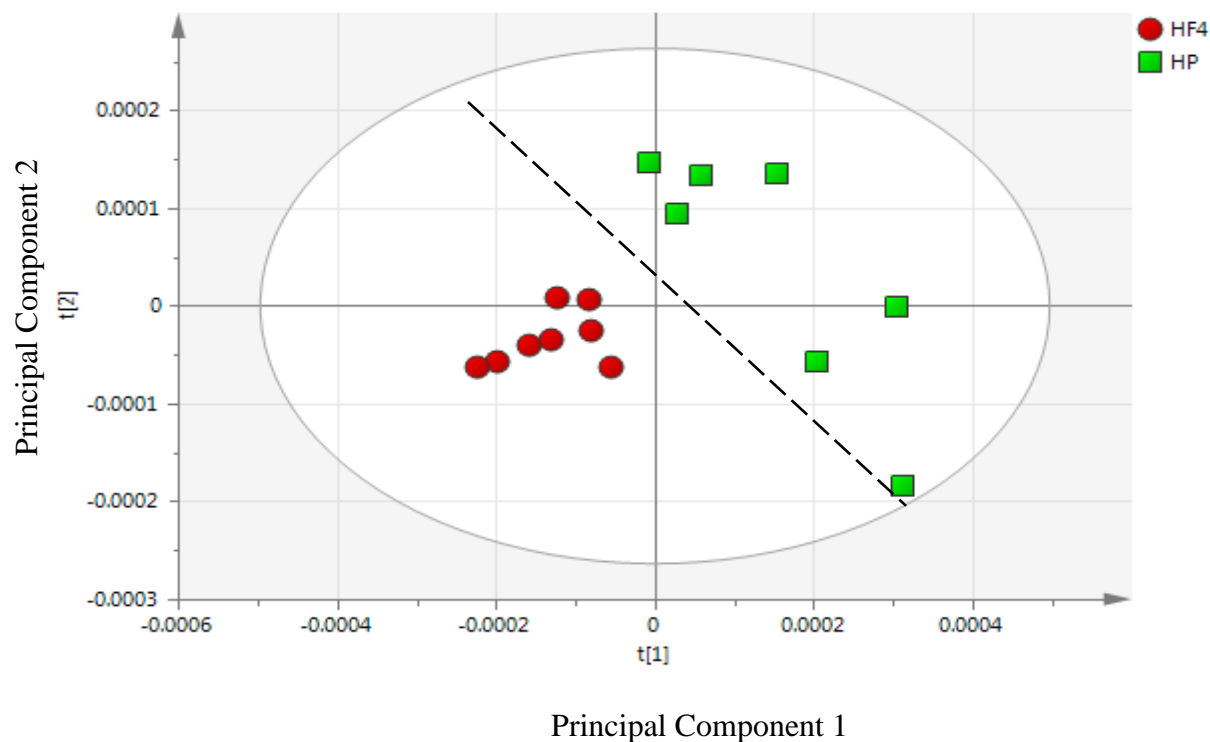
**Figure 3.20** Characterizations of the Plasma Metabolomic Changes Induced by PAZ and F4. 2-Dimensional CMPG_PLS-DA score plot showing group discrimination (HF4 vs. HP) based on PAZ and F4 administration. P < 0.05

**Notes:** HF4, high fat diet and fraction 4; HP, high fat diet and PAZ. N = 8 hamsters/group.

**Abbreviations:** CPMG, Carr-Purcell-Meiboom-Gill; PLS-DA, partial least squares – discriminant analysis
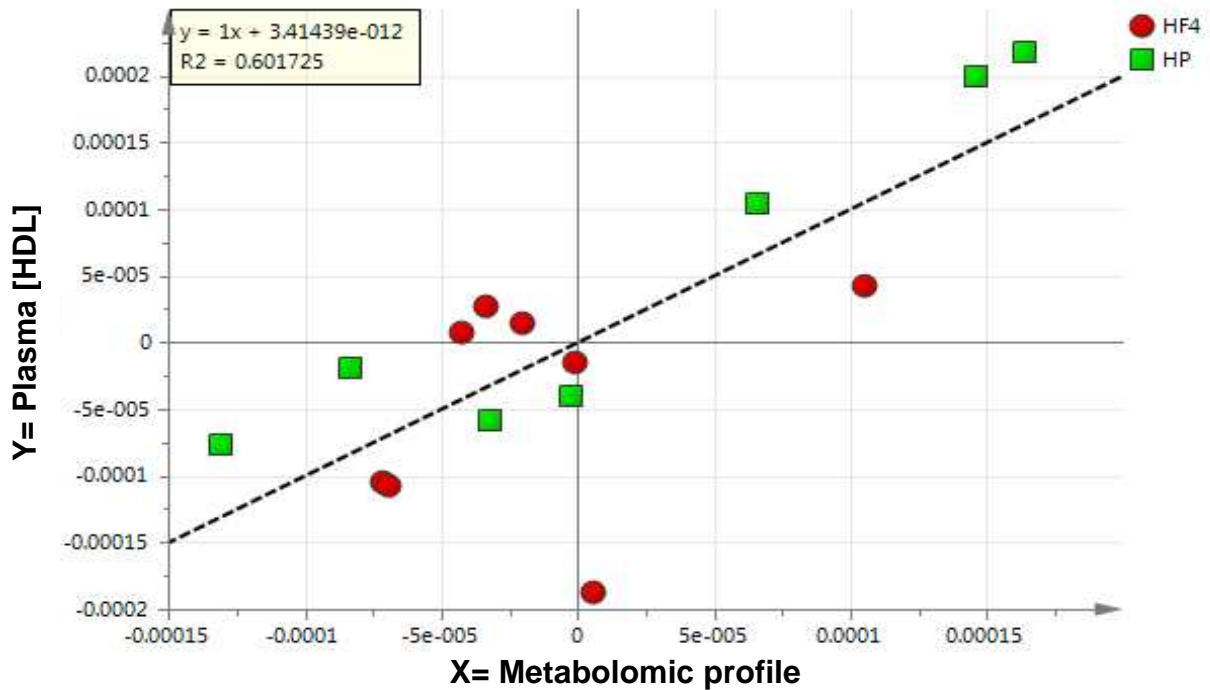
**Figure 3.21** Correlation of the PAZ fraction- induced plasma metabolomic and HDL cholesterol.

**Notes:** Changes among the metabolomic score values (X) of individual hamsters fed a high fat diet and different drinking fluids (F4 or PAZ fraction) are plotted along with the corresponding individual plasma HDL scores (Y), as determined by the PLS analysis. The coefficient of determination ($R^2$ = 0.6) among X and Y that was calculated after linearization of the relationship is indicated.

### 3.2.B Metabolite Identification and Quantification (Chenomx)

A total of fifty plasma metabolites of the control and treatment groups were identified and quantified using Chenomx NMR Suite software, including amino acids (leucine/isoleucine, valine), organic acids (3-hydroxybutyrate, lactate, acetate, acetoacetate, citrate, pyruvate, creatine, creatinine), carbohydrates (glucose, galactitol, glucitol), and phospholipids-associated molecules. The metabolites that had a significantly lower concentration in the group that received the algal infusion PAZ and in the group that received fraction 4 are summarized in Table 3.4 and 3.5, respectively.

Results indicated that the concentrations of betaine, phosphocholine, and glycerol-phosphocholine were significantly lowered in HP group as compared with HW. Treatment with the algal infusion PAZ on hypercholesterolemic hamsters also resulted in decreased levels of several amino acids, such as arginine, leucine, isoleucine, threonine, taurine.

When the experimental animals received the fraction 4 of PAZ (HF4 group), the concentration of additional metabolites resulted to be significantly lower, as compared with the control group. Besides the metabolites that were found to be significantly lower in HP group, we determined that sixteen additional metabolites were decreased in the group that received fraction 4 (HF4) as a preventative agent. Additionally to the phospholipids-associated metabolites betaine, phosphocholine, and glycerol-phosphocholine, which recently have been linked to atherosclerosis, we also found that also choline, carnitine, and trimethylamine N-oxide (TMAO) have been significantly reduced in HF4 group.

**Table 3.3** Metabolites with Significantly Lower Concentration in Hypercholesterolemic Hamsters that Received PAZ for 4 Weeks as a Preventative Agent as Compared with HW

| Metabolites | Peak Regions (ppm) |
|---|---|
| Acetoacetate | 2.3, 3.4 |
| Arginine | 1.6, 1.7, 1.9, 3.2, 3.8 |
| Betaine | 3.3, 3.9 |
| Glucitol | 3.6, 3.7, 3.8 |
| Glutamine | 2.1, 2.4, 3.8, 6.9, 7.6 |
| Glycerol | 3.6, 3.8 |
| Isoleucine | 0.9, 1.0, 1.2, 1.5, 2.0, 3.7 |
| Leucine | 0.9, 1.7, 3.7 |
| Glycero-phosphocholine | 3.2, 3.6, 3.7, 3.9, 4.3 |
| Taurine | 3.2, 3.4 |
| Threonine | 1.3, 3.6, 4.3 |

**Table 3.4** Metabolites with Significantly Lower Concentration in Hypercholesterolemic Hamsters that Received F4 as a Preventative Agent as Compared with HW

| Metabolites | Peak Regions (ppm) |
| --- | --- |
| 2-Aminobutyrate | 1.0, 1.9, 3.7 |
| 3-Hydroxybutyrate | 1.2, 2.3, 2.4, 4.1 |
| Acetate | 1.9 |
| Acetoacetate | 2.3, 3.4 |
| Alloisoleucine | 0.9, 1.0, 1.3, 1.4, 2.1, 3.7 |
| Arabinitol | 3.6, 3.7, 3.8, 3.9 |
| Arginine | 1.6, 1.7, 1.9, 3.2, 3.8 |
| Betaine | 3.3, 3.9 |
| Carnitine | 2.4, 3.2, 3.4, 4.6 |
| Choline | 3.2, 3.5, 4.1 |
| Galactitol | 3.7, 4.0 |
| Glucitol | 3.6, 3.7, 3.8 |
| Glucose | 3.2, 3.4, 3.5, 3.7, 3.8, 3.9, 4.7, 5.2 |
| Glutamine | 2.1, 2.4, 3.8, 6.9, 7.6 |
| Glycerol | 3.6, 3.8 |
| Isoleucine | 0.9, 1.0, 1.2, 1.5, 2.0, 3.7 |
| Lactate | 1.3, 4.1 |
| Leucine | 0.9, 1.7, 3.7 |
| Malonate | 3.1 |
| O-Phosphocholine | 3.2, 3.6, 4.1 |
| Pyruvate | 2.4 |
| Glycero-phosphocholine | 3.2, 3.6, 3.7, 3.9, 4.3 |
| Succinate | 2.4 |
| Taurine | 3.2, 3.4 |
| Threonine | 1.3, 3.6, 4.3 |
| Trimethylamine N-oxide | 3.2 |
| Valine | 1.0, 2.3, 3.6 |

**3.3 AIM 3: To determine the therapeutic effect of the biologically active fraction on hypercholesterolemia**

**3.3.A Metabolic Effects of High Fat Diet and Treatment with Fraction 4**

Animals were fed a high fat diet and water for the first four weeks of the study, time that represented the end point for T0. After four weeks, groups T3, T7, T10, T14, and T21 received the biologically active fraction of PAZ as their drinking fluid for 3, 7, 10, 14, and 21 days, respectively, while continuing the high fat diet. Animals were sacrificed at different time points, based on the number of days they were designed to receive treatment.

The net body weight gain of the animals for the first 4 weeks of the study, as well as the food efficiency ratio (g gained/ g feed) were statistically significant in group T7 ($P < 0.05$, Table 3.5). The ratio of fluid/water intake and the ratio of liver weight/body weight were not statistically different when compared with T0 group (Table 3.5). While on treatment with PAZ fraction, all animals survived the duration of the study and no abnormal characteristics related to the physiology of the animals were noted.

**Table 3.5** Anthropometrics of Male Hamsters Fed High Fat Diet and Water for 4 Weeks, Followed by High Fat and F4 Treatment for 0, 3, 7, 10, 14, and 21 Days

|  | T0 | T3 | T7 | T10 | T14 | T21 |
|---|---|---|---|---|---|---|
| Body weight gain, g/4 weeks | 37.2±2.1 | 40.8±3.7 | 50.6±6.2* | 44.2±3 | 34.4±3.5 | 39.8±2.9 |
| Food intake, g/d | 7.1±0.1 | 7.4±0.2 | 7.5±0.2 | 7.4±0.2 | 6.9±0.2 | 7.4±0.1 |
| Food efficiency ratio, g gain/g feed | 0.16±0.01 | 0.2±0.02 | 0.22±0.01* | 0.2±0.01 | 0.17±0.01 | 0.19±0.01 |
| Fluid intake, PAZ/water | 1.0±0.0 | 0.96±.04 | 0.95±.05 | 1.1±.04 | 1.0±.06 | 1.1±.04 |
| Liver/body weight | 4.6 ±0.1 | 4.9±0.1 | 4.7±0.1 | 4.8±0.1 | 4.7±0.1 | 4.5±0.2 |

**Notes:** Values are mean ± SE, N = 10/group (T0), N = 8/group (T3, T7, T10, T14, T21). *$P < 0.05$ when compared with T0. Statistical program ANOVA with Tukey's procedure was used, SPSS software

### 3.3.B Plasma Lipid Profile upon Therapy with Fraction 4

Plasma lipid profile of the hypercholesterolemic hamsters was enzymatically measured to determine the therapeutic effect of fraction 4 when administrated as the drinking fluid. Plasma TC (Figure 3.22) and TG (Figure 3.23) were not significantly reduced in the treatment groups while receiving the fraction 4, as compared with the control group. However, the TC/HDL ratio was significantly lower in all treatment groups when compared with the control T0 group ($P < 0.001$, Figure 3.24). Moreover, the concentration of HDL cholesterol was significantly increased in T3 group ($P < 0.05$), as well as in group T21 ($P < 0.001$), when compared with the control group (Figure 3.25).

Consumption of the biologically active fraction of PAZ, F4 also significantly reduced the non-HDL cholesterol in all groups, as compared with the control animals ($P < 0.001$ in T3, T7, and T10; $P < 0.05$ in T14 and T2, Figure 3.26). These results corroborates with our previous findings that ingestion of PAZ and its biologically active fraction can improve the plasma lipoprotein profile by significantly increasing the HDL cholesterol concentrations, while decreasing the  non-HDL cholesterol and TC/HDL ratio in hypercholesterolemic hamsters.

**Figure 3.22** Effect of Fraction 4 on Total Cholesterol Concentration

**Notes:** Plasma total cholesterol concentrations in male hamsters fed high fat diet for 4 weeks, followed by high fat and F4 for 0, 3, 7, 10, 14, and 21 days. Values are mean ± SE. No sig. difference as compared with T0 group.
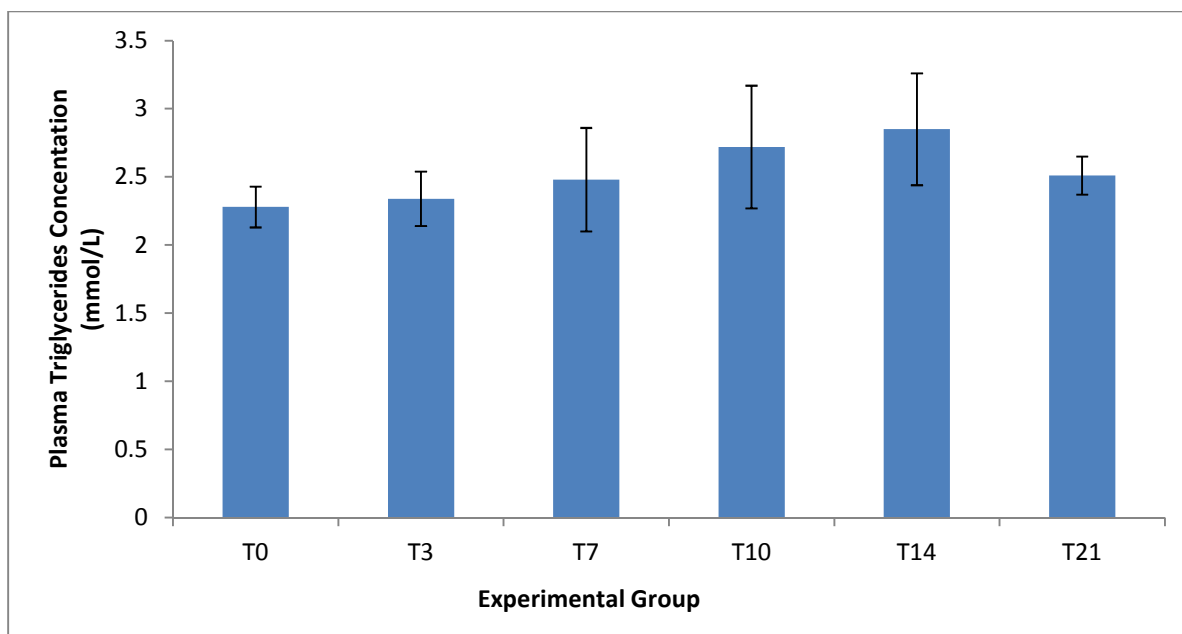
**Figure 3.23** Effect of Fraction 4 on Triglycerides Concentration

**Notes:** Plasma triglyceride concentrations in male hamsters fed high fat diet for 4 weeks, followed by high fat and F4 for 0, 3, 7, 10, 14, and 21 days. Values are mean ± SE. No sig. difference as compared with T0 group.
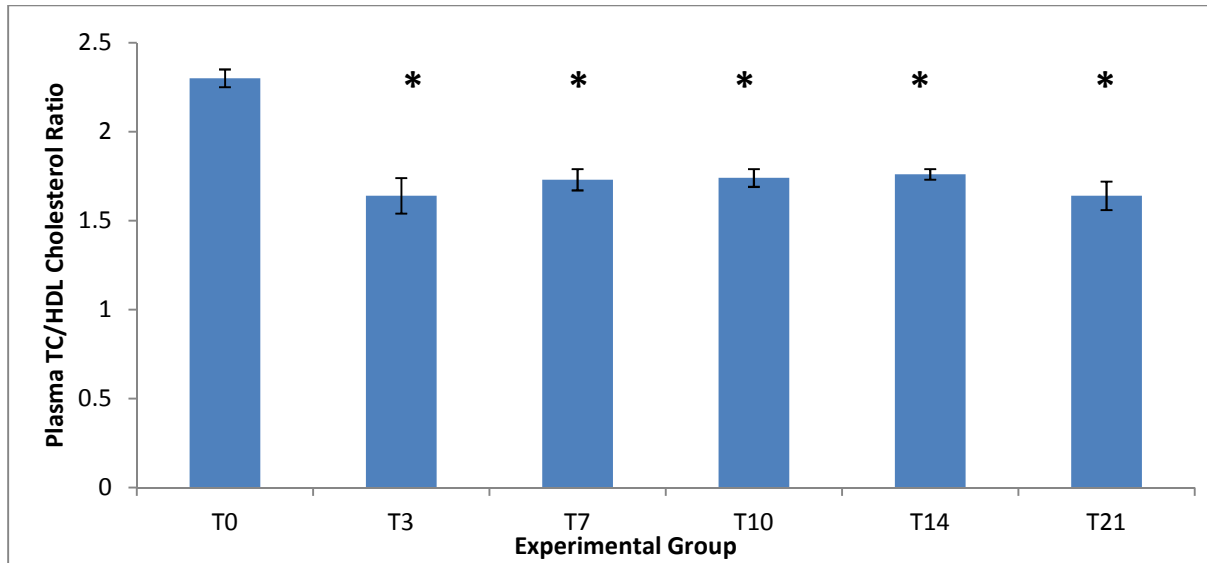
**Figure 3.24** Effect of Fraction 4 on TC/HDL Cholesterol Concentration

**Notes:** Plasma TC/HDL cholesterol concentrations in male hamsters fed high fat diet for 4 weeks, followed by high fat and algal treatment for 0, 3, 7, 10, 14, and 21 days. Values are mean ± SE. *P < 0.001 as compared with T0 group.
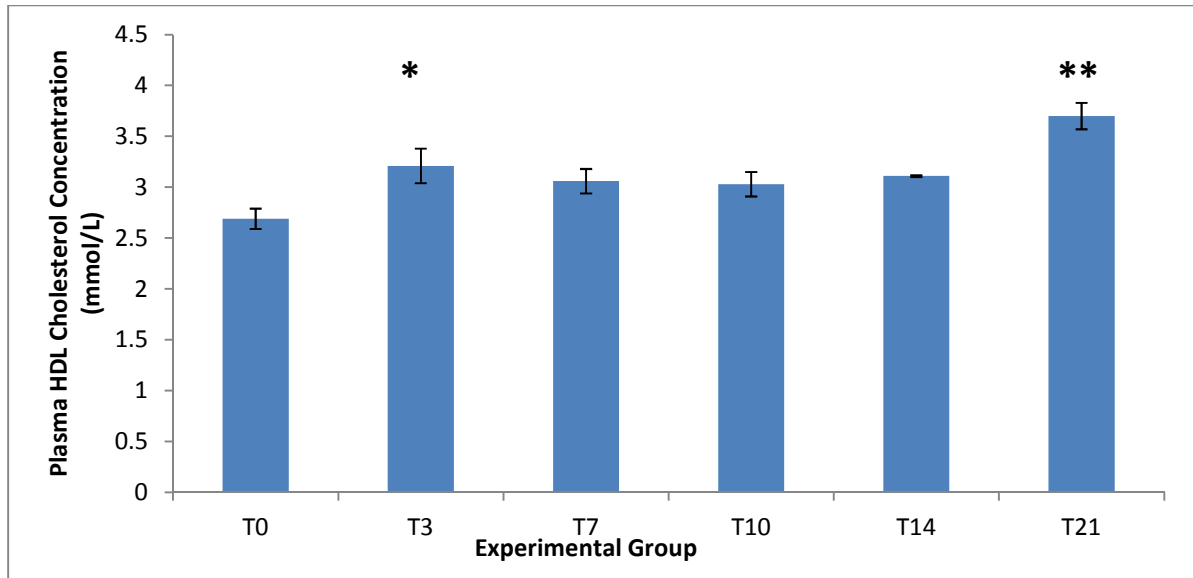
**Figure 3.25** Effect of Fraction 4 on HDL Cholesterol Concentration

**Notes:** Plasma HDL cholesterol concentrations in male hamsters fed high fat diet for 4 weeks, followed by high fat and algal treatment for 0, 3, 7, 10, 14, and 21 days. Values are mean ± SE. *$P < 0.05$, **$P < 0.001$ as compared with T0 group.

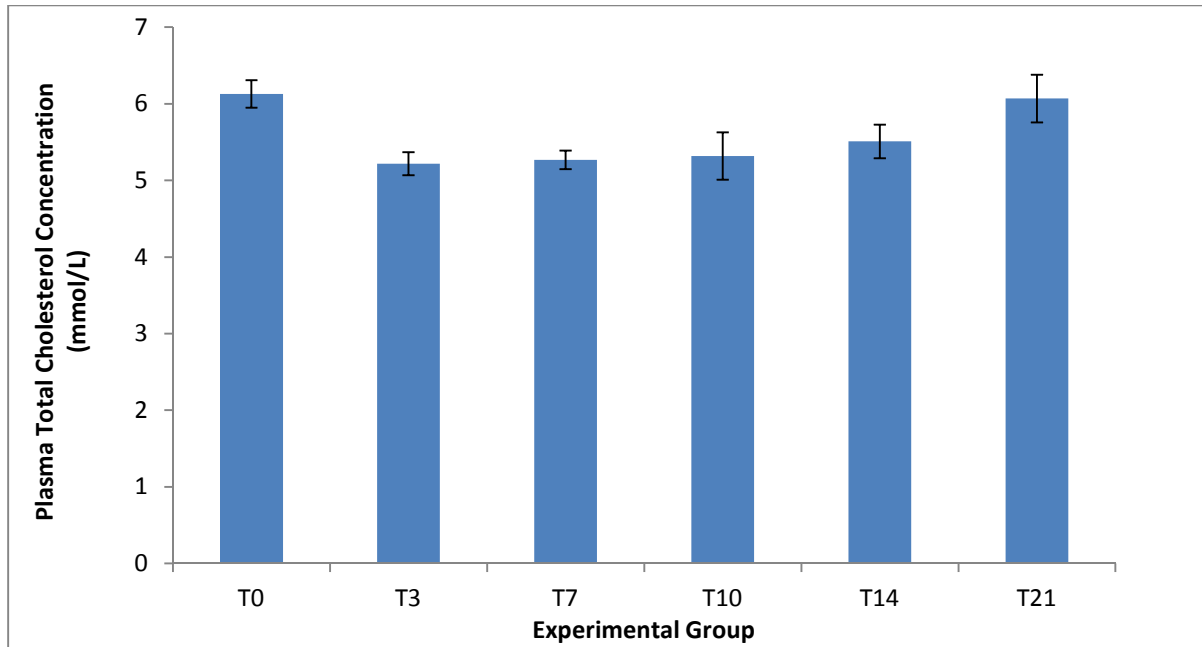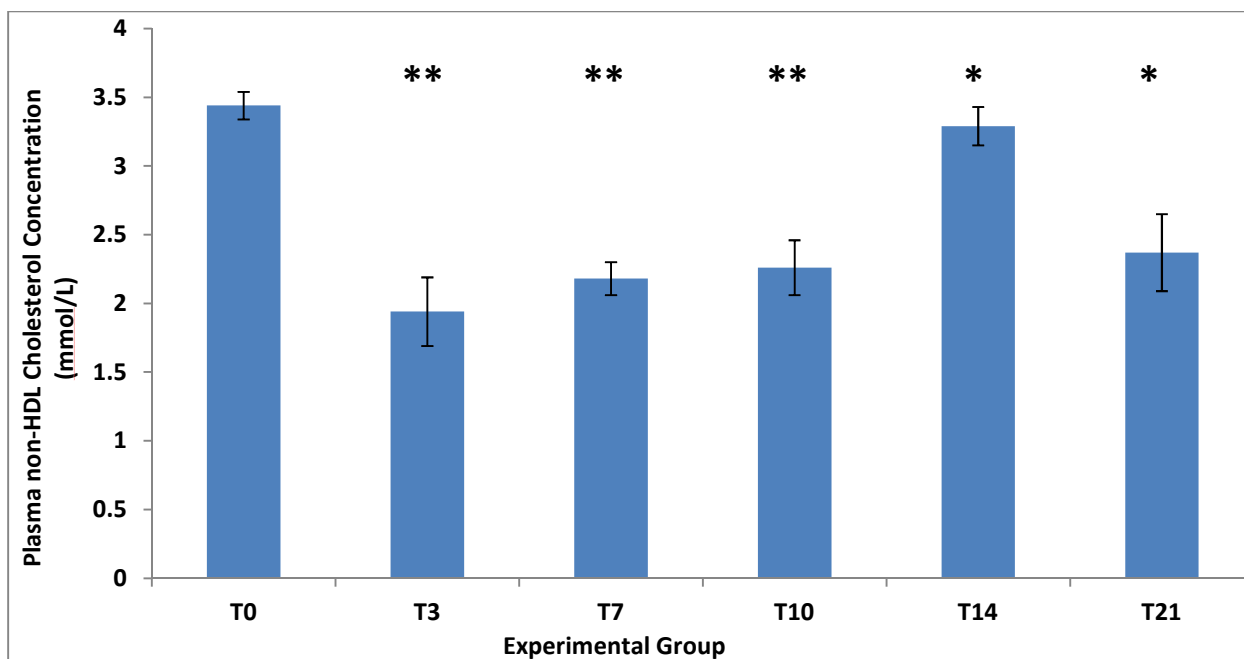**Figure 3.26** Effect of Fraction 4 on non-HDL Cholesterol Concentration

**Notes:** Plasma non-HDL cholesterol concentrations in male hamsters fed high fat diet for 4 weeks, followed by high fat and algal treatment for 0, 3, 7, 10, 14, and 21 days. Values are mean ± SE. *P < 0.01, **P < 0.001 as compared with T0 group.

**3.3.C Therapeutic Effect of the Biologically Active Fraction (F4) on Apo A1 Gene Expression**

In order to determine the therapeutic potential of the biologically active fraction of PAZ at the molecular level and to validate the significant increase in HDL cholesterol concentration, the activity of Apo A1 gene expression was evaluated. Also, the earliest time point at which the genetic expression is altered was determined. Apo A1 gene expression analysis showed that hamsters fed the biologically active fraction for 10 days (T10) had a moderate (3-fold) increase, while hamsters fed the PAZ fraction for 21 days exhibit an approximate 6-fold increase (Figure 3.27).

These results correlate with the increase in the plasma concentration of HDL cholesterol, as Apo A1 is responsible for the production of nascent HDL cholesterol particles. Thus the active fraction of PAZ increased HDL concentration at least in part by increased production of nascent HDL particles both when given as a treatment or preventative agent [100].
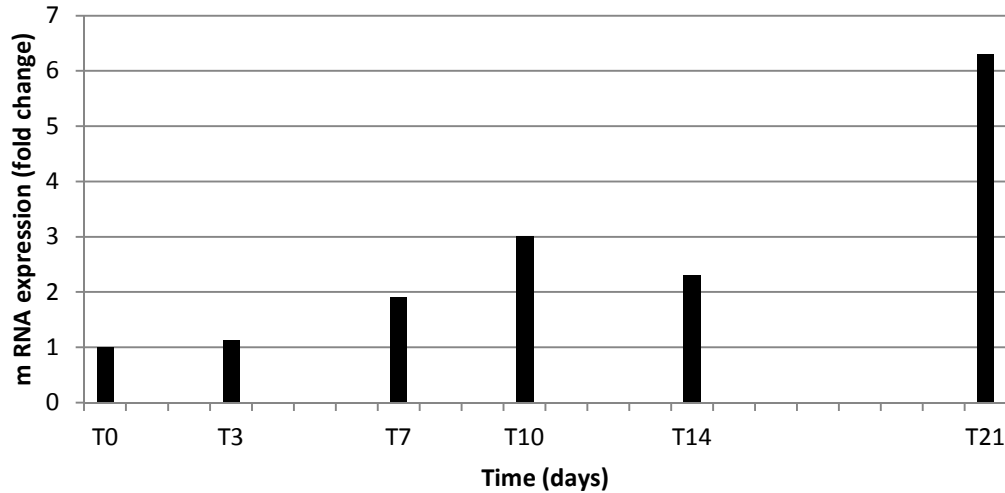
**Figure 3.27** Relative Levels of Hepatic mRNA Expression of Apo A1 Gene in Hypercholesterolemic Hamsters.

**Notes:** Animals were fed a high fat diet for 4 weeks, followed by high fat diet plus F4 for 0, 3, 7, 10, 14, and 21 days, respectively. Values are expressed as mean of threshold cycle (ct) values; n=8 animals per group (n=5 for T0 group). Each mRNA was normalized with GAPDH and is expressed as a fold change.

**Abbreviations:** ApoA1, Apolipoprotein A1

## 3.4 Aim 4: To investigate the change in plasma metabolomic profiles upon therapy with the biologically active fraction F4

### 3.4.A Multivariate Data Analysis

To investigate the therapeutic effect of the biologically active fraction on the hypercholesterolemic hamsters, a metabolomic approach was applied to plasma samples from controls (T0) and 21 days treatment group (T21) by $^1$H NMR spectroscopy. PCA was performed on the NMR data to get the overall information on metabolomic profile of hamsters, due to fraction 4 administration. The clustering of the T0 and T21 groups showed a clear separation of the hamsters in PCA plots (Figure 3.28a). Due to the fact that one of the samples in group T21 was outside of the ellipse of Hotelling's 95% confidence interval, it was considered a strong outlier and removed from the PCA analysis. The corresponding loading plot (Figure 3.28b) along with the variable importance on projection (VIP) plot (Figure 3.28c) provided significant information on the contribution of each variable to the pattern in the score plots, which can aid in metabolite identification in Chenomx software**.**

The discriminant analysis (PLS-DA) indicated that the treatment with PAZ fraction for 21 days induced specific metabolites patterns that enabled class assignment of the hamsters (PLS-DA, Figure 3.29). The PLS-DA score plot revealed that the control and treatment group were clearly separated by the principal component 1. In addition, the 2- dimensional PLS plot (Figure 3.30 a, b) allowed for the evaluation of the F4 treatment on changes in the plasma metabolome. For instance, the treatment with the active fraction for 21 days induced a significant coefficient of determination $R^2 = 0.7$, when the data were correlated with the plasma

lipid profile (Figure 3.30a). The PLS regression between the NMR variables and the plasma lipid profile revealed that 70% of the variations in the lipid profile can be predicted by different treatment conditions (water vs. F4).

Furthermore, as determined by the PLS analysis, HDL cholesterol concentrations were positively correlated with the plasma metabolomic profile ($R^2 = 0.62$), indicating that there is a positive correlation between the higher density lipoproteins concentrations and some of the small metabolites present in plasma (Figure 3.30b). Moreover, the orthogonal projections to latent structures (OPLS) aides in the process of identifying statistically significant and potentially biochemical significant metabolites based on contribution to the model and their reliability. The OPLS_S Plot (Figure 3.31) provided important information on the contribution of each variable to the pattern in the score plots, specifically was used to identify possible metabolites that correlate the metabolomic profile to HDL cholesterol concentration.
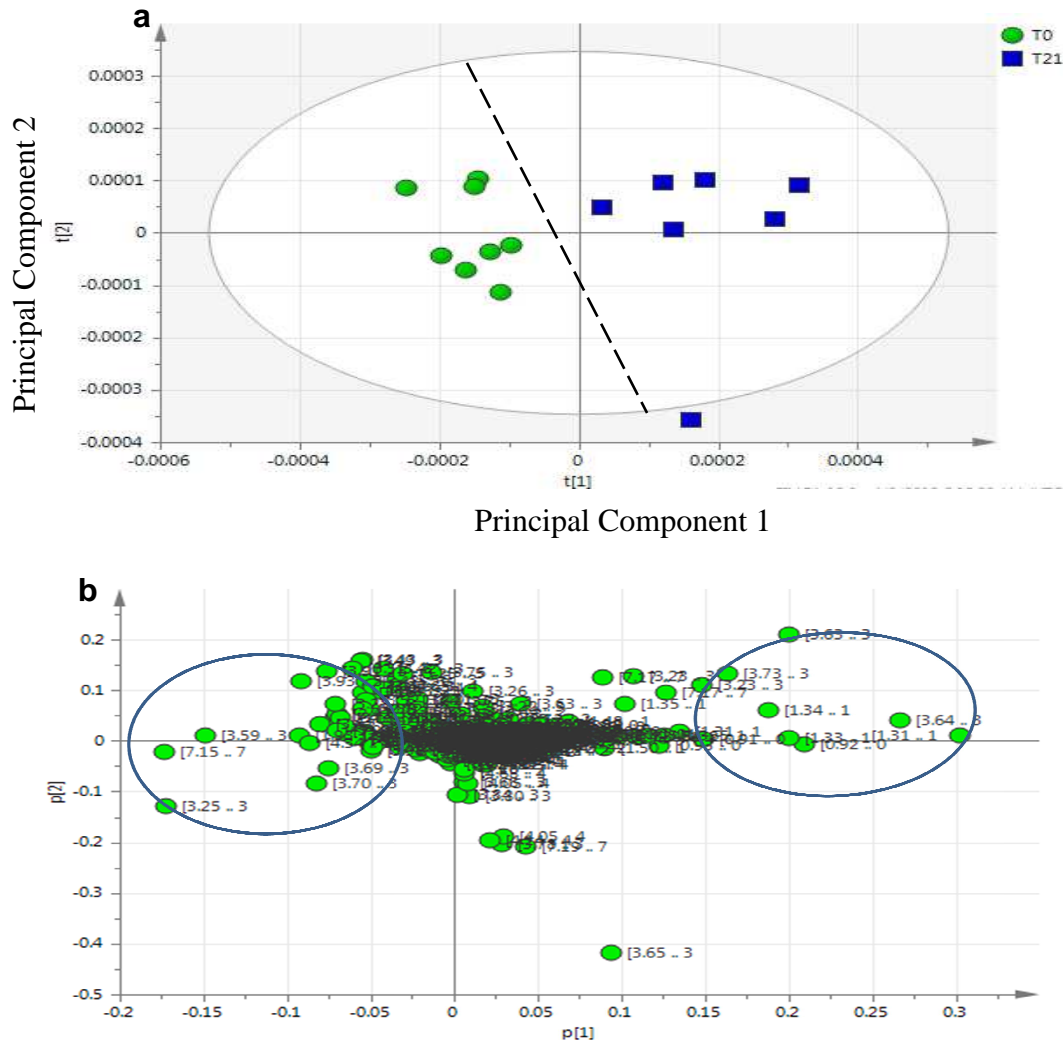
**Figure 3.28** Characterizations of the Plasma Metabolomic Changes Induced by F4

**3.28a**. CMPG_PCA score plot revealed that T0 and T21 groups were clearly separated

along [t1] and [t2]. P < 0.05; **3.28b**. CMPG_PCA corresponding loading plot indicating

the regions of the spectra that are responsible for the group separation

**Notes:** T0, high fat diet and water; T21, high fat diet and F4 for 21 days.

N = 8/hamsters per group.

**Abbreviations:** CPMG, Carr-Purcell-Meiboom-Gill; PCA, principal components analysis;

PLS-DA, partial least squares-discriminant analysis
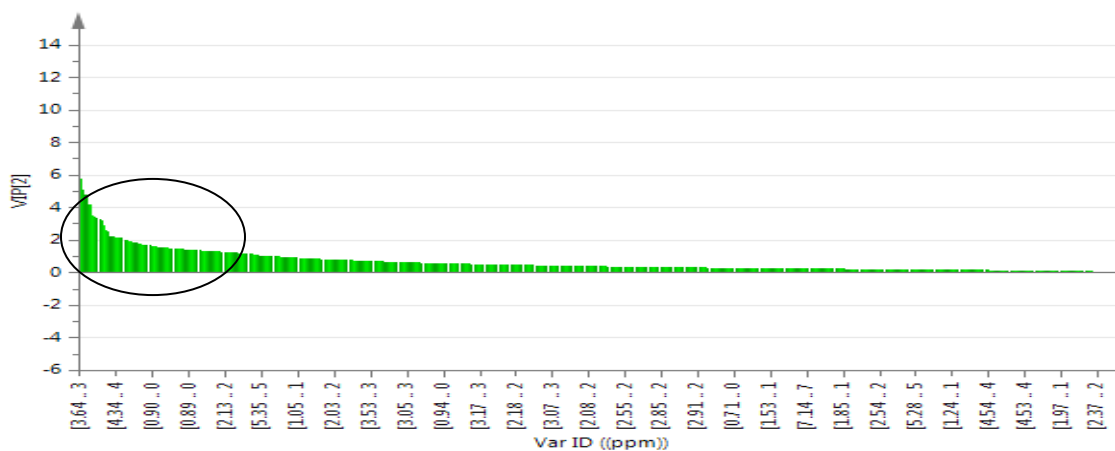
**Figure 3.28c.** Characterizations of Plasma Metabolomic Changes Induced by F4.

**Notes:** Variable importance on projection (VIP) plot shows the contribution of each variable to the pattern, with the regions of spectra in the far most left of the plot carrying most of the weight.

Principal Component 1
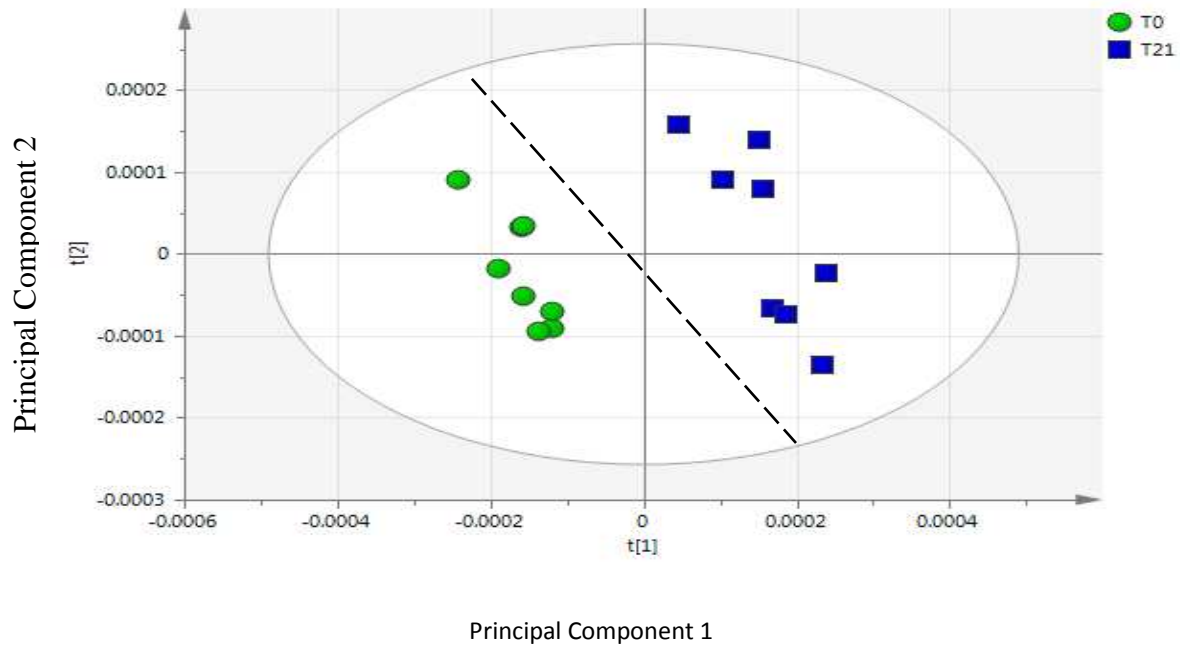
**Figure 3.29** Characterizations of the Plasma Metabolomic Changes Induced by F4. Two-dimensional PLS-DA score plot showing group discrimination based on treatment received. $P < 0.05$

**Notes:** T0, high fat diet and water; T21, high fat diet and F4 for 21 days. N = 8 hamsters per group.

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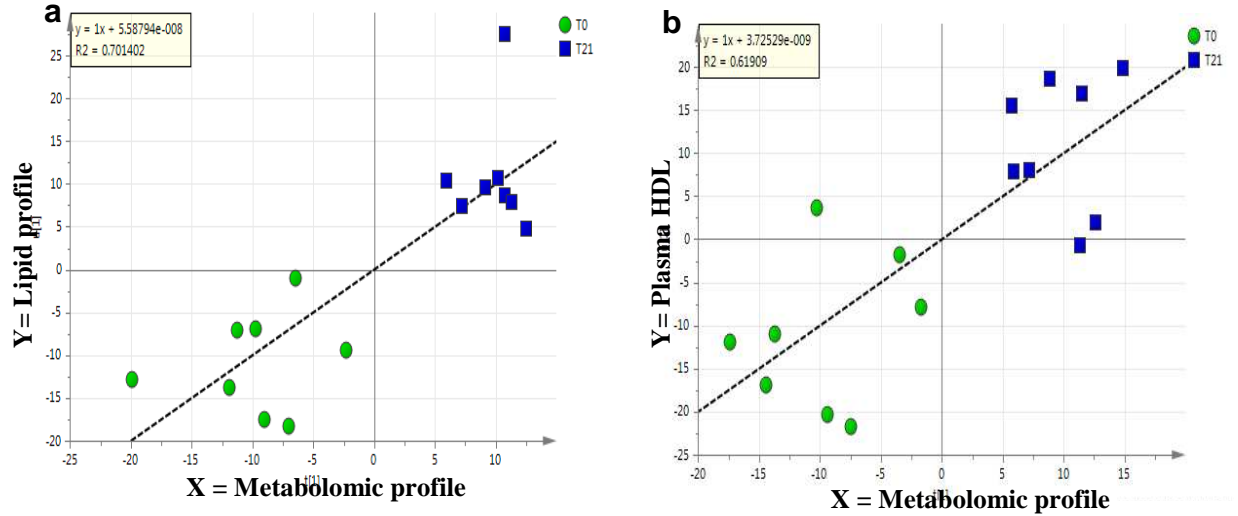


**Figure 3.30** Correlation of the Fraction 4-Induced Plasma Metabolomic and Lipid Profile.

**Notes:** Changes among the metabolomic score values (X) of individual hamsters fed a high fat diet and different drinking fluids (water or F4) are plotted along with the corresponding individual plasma lipid profile scores (Y), as determined by the PLS analysis. The coefficient of determination ($R^2$) among X and Y that was calculated after linearization of the relationship is indicated; **3.30a** Relationship between the plasma metabolomic and complete lipid profile. ($R^2 = 0.7$); **3.30b** Relationship between the plasma metabolomic profile and HDL cholesterol. ($R^2 = 0.62$); N = 8 hamsters per group.

**Abbreviations:** T0, high fat diet and water; T21, high fat diet and fraction 4 for 21 days. PLS, partial least squares
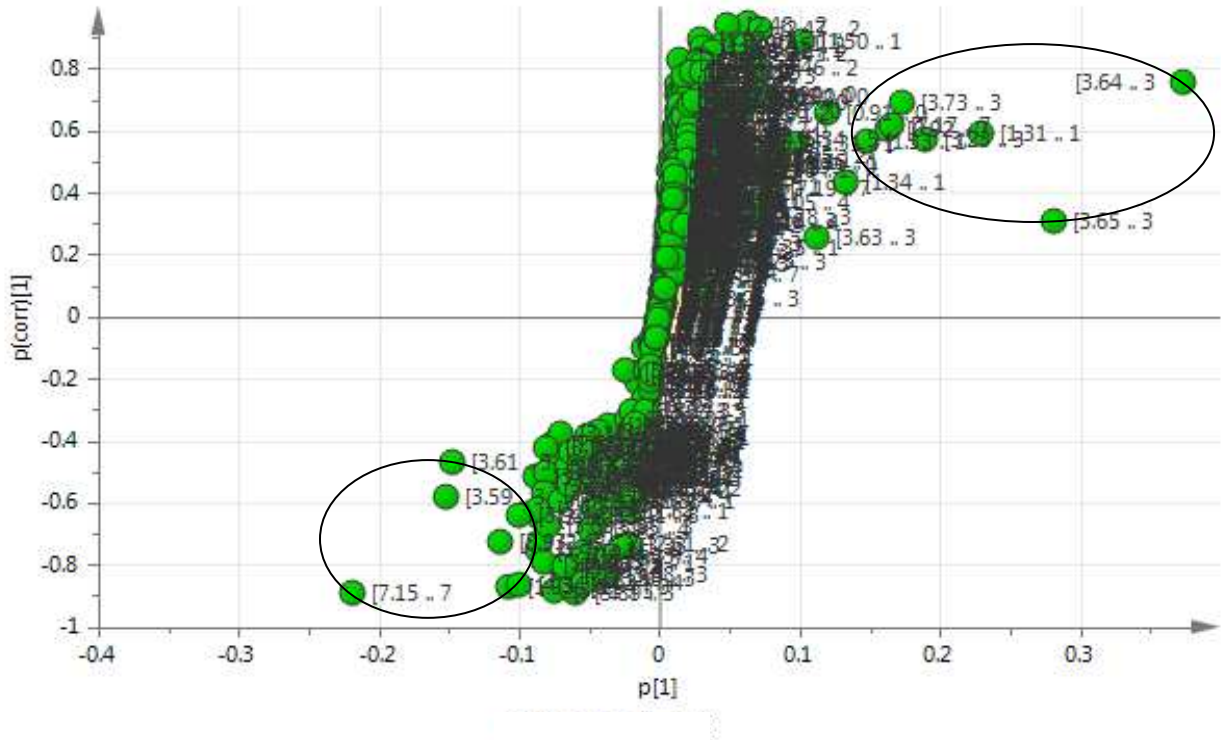
**Figure 3.31** Characterizations of the Plasma Metabolomic Changes Induced by F4.

**Notes:** CPMG_OPLS-S plot used to identify possible metabolites that correlate the metabolomic profile to HDL cholesterol metabolism

**Abbreviations:** OPLS, orthogonal partial least square

### 3.4.B Metabolite Identification and Quantification (Chenomx)

A total of fifty plasma metabolites of the control and treatment groups were identified and quantified using Chenomx NMR Suite software, including amino acids (leucine/isoleucine, valine), organic acids (3-hydroxybutyrate, lactate, acetate, acetoacetate, citrate, pyruvate, creatine, creatinine), carbohydrates (glucose, galactitol, glucitol), phospholipids-associated molecules. The metabolites that had a significantly lower concentration in the group that received the biologically active fraction for 21 days are summarized in Table 3.6.

Results indicated that the concentrations of choline, phosphocholine, glycerol-phosphocholine, betaine, and carnitine, were significantly lowered in T21 group as compared with T0. Treatment with the biologically active fraction of PAZ on hypercholesterolemic hamsters also resulted in decreased levels of several amino acids (arginine, leucine, isoleucine, threonine, taurine), as well as some other important molecules (3-hydroxy-butyrate, acetate, glycerol) involved in fatty acids metabolism.

**Table 3.6** List of Plasma Metabolites Quantified to be at Lower Concentrations After 21 Days of Treatment

| Metabolites | Peak regions (ppm) | Metabolites | Peak regions (ppm) |
|---|---|---|---|
| 2-Aminobutyrate | 1.0, 1.9, 3.7 | Glucitol | 3.6,3.7,3.8 |
| 3-Hydroxybutyrate | 1.2, 2.3,2.4,4.1 | Glycerol | 3.6,3.8 |
| Acetate | 1.9 | Isoleucine | 0.9,1.0,1.2,1.5,2.0,3.7 |
| Arabinitol | 3.6,3.7,3.8,3.9 | Lactate | 1.3,4.1 |
| Arginine | 1.6,1.7,1.9,3.2,3.8 | Leucine | 0.9,1.7,3.7 |
| Betaine | 3.3,3.9 | Phosphocholine | 3.2,3.6,4.1 |
| Carnitine | 2.4,3.2,3.4,4.6 | Glycero-phosphocholine | 3.2,3.6,3.7,3.9,4.3 |
| Choline | 3.2,3.5,4.1 | Taurine | 3.2,3.4 |
| Galactitol | 3.7,4.0 | Threonine | 1.3,3.6,4.3 |

**Notes:** Plasma metabolites found to be significantly lower in T21 group as compared with T0 group. The metabolites were quantified using the Chenomx 7.6 NMR Suite database and significance was obtained using 2 tailed Student t-tests, $P < 0.05$.

T0, high fat diet and water; T21, high fat diet and F4 for 21 days, N = 4 hamsters/group for quantification of metabolites.

# CHAPTER 4

## DISCUSSION

The current study was designed to examine the preventative and therapeutic effect of the algal infusion product Proalgazyme and its subfractions in a diet-induced hypercholesterolemic hamster model. More specifically, the objective was to investigate the possible mechanism of action whereby certain fractions of PAZ can influence the cholesterol metabolism pathway. In addition, the scope was also to determine the plasma metabolomic profile and compare the changes produced with the plasma lipid profile, and possibly identify new relationship between smaller metabolites and hypercholesterolemia.

A hamster model was used in this study given that it is the most appropriate model to study lipoprotein metabolism. Due to interspecies differences in the lipoprotein metabolism, it is important to study the metabolic changes in a non-primate model that develops the most similarities to the human disease. The hamster model used in the current study has been previously found to be suitable for exploring hypercholesterolemia associated with dietary changes [97] and the high fat diet used was proven to induce a hypercholesterolemic state in the hamster model [100]. As previously mentioned, plasma lipoprotein metabolism in hamsters is comparable to humans due to similar component and metabolism of both lipoproteins and bile acids [101, 102].

Different types of algal cellular biomass and algal extracts have been studied for their ability to lower circulating cholesterol concentrations in hamsters consuming

hypercholesterolemic diets [103] and in humans [104], and showed a reduction in plasma total cholesterol, non-HDL cholesterol , and/or triglycerides concentrations.

Previous findings [68] when PAZ was administrated to humans for 10 weeks to evaluate its effect on cardiovascular risk factors associated with the metabolic syndrome, showed a decrease in plasma total cholesterol, non-HDL cholesterol, and triglycerides concentrations. Moreover, the levels of HDL cholesterol were significantly increased, and PAZ was well tolerated with no notable adverse effects [68]. However, the large difference in the lipid profile of treatment versus the control group needed more validation. The mechanism of action required further investigation as well. Therefore, one of the objectives of the current study was to determine the hypolipidemic effects of PAZ and its subfractions. Moreover, the research investigated the mechanism by which plasma HDL cholesterol was augmented and if the increase can be detected in the hepatic mRNA expression of the key genes involved in HDL/RCT metabolism. Further, the plasma metabolomic profile was analyzed and correlated with the lipid profile of the hypercholesterolemic hamsters.

The results of the current study corroborate previous findings on plasma lipoprotein concentrations upon administration of dietary PAZ [68]. Administration of PAZ and its subfractions for 4 weeks as a preventative agent for hypercholesterolemia resulted in a significant improvement in plasma lipid profile and an alteration in the genes involved in HDL/ RCT metabolism. Results showed a highly significant reduction in non-HDL cholesterol and TC/HDL ratio in animals that received fraction 3 (HF3 group) and 4(HF4 group), and a moderate decrease in

groups HP (non HDL and TC/HDL), HF1 and HF2 (TC/HDL). In addition, the HDL cholesterol concentration was significantly increased in HP, HF3, and HF4 groups. To confirm the increase in HDL cholesterol plasma concentrations in the treatment groups, plasma ultracentrifugation was performed on pooled plasma of all hamsters. An increase in the high density portion of cholesterol was observed after TC assay was performed on all fractions, confirming the raise in HDL cholesterol plasma concentrations.

An important objective of the study was to explain the improvement in plasma lipid profile upon administration of complete PAZ and its subfractions to hamsters fed a high fat diet. Evaluation was performed on the genes involved in HDL metabolism (Apo A1, ABCA1, SRB1, and CETP) in liver tissues collected from hamster. Apo A1 is involved in the production of nascent HDL particles, while ABAC1 transports lipids from peripheral tissues to nascent HDL to form larger HDL particles. The mature HDL particle is removed via SRB1 receptor on the liver into bile, clearance from plasma occurs, and Apo A1 molecule is recycled. CETP transfers lipids from HDL to non-HDL particles, and partial inhibition of this enzyme is beneficial for lowering cholesterol concentrations in plasma. Using a real time RT-PCR technique, we have examined the ability of ProAlgaZyme to transcriptionally regulate these genes involved in HDL/RCT metabolism in hepatic tissue.

Administration of PAZ and its subfraction 4 did alter the ABCA1, APO A1, SRB1 and CETP hepatic mRNA levels, regulating the transcription of genes that encode specific proteins that control cholesterol levels. Transcription of Apo A1, ABCA1, SRB1 genes was up-regulated, whereas transcription of the gene encoding CETP

was down-regulated after 4 weeks dietary intervention with PAZ and/or its subfraction 4. Therefore, high non-HDL cholesterol and TC concentrations, and low HDL cholesterol can be treated by up-regulating ABCA1, APO A1, and SRB1, and down-regulating CETP expression.

In summary, the real-time RT-PCR analysis of gene regulation using mRNA from hamster liver samples corroborates the changes in lipid plasma profile noted in the first part of the study. Therefore, we concluded that the biologically active fraction of PAZ is fraction 4, and it was used to complete further aims in the project. The composition of fraction 4 is, however uncertain at this point. One-dimensional $^1$H NMR spectroscopy of the sample resulted in a spectrum with major peaks found around 1.2- 2.0 ppm, as well as around 7.3 ppm. Current literature describes most common classes of secondary metabolites in red and green algae as phytosterols, phenylpropanoids and various phenolic compounds [105, 106]. Since the $^1$H NMR spectra also showed prominent peaks in the aromatic regains as well as in the region associated with alcohol groups, presence of phenols or phytosterols is possible.

Further, the metabolomic plasma profile of hypercholesterolemic hamsters treated with PAZ and fraction 4 was analyzed, using $^1$H NMR spectroscopy and multivariate data analysis. Previous studies identified metabolic perturbations associated with abnormal lipoprotein profile, kidney disease associated with type I diabetics, insulin resistance, and atherosclerosis using NMR spectroscopy, this being a method widely used in the last decade [83, 88].

Plasma includes both high molecular weight proteins and lipoproteins, as well as low molecular weight metabolites. Hence, the standard plasma 1-dimentional $^1$H NMR spectrum is dominated by broad resonance peaks of the high molecular weight components. The CPMG echo pulse technique was used to suppress the resonance from the macromolecules and emphasize the low molecular weight metabolites, thus revealing subtle biochemical information of the plasma samples. A clear separation of the groups was obtained with the PCA and PLS-DA score plots, showing changes in plasma profiles due to the PAZ intervention treatment. To identify the metabolites responsible for this strong separation of the groups, PCA loading plot was analyzed to identify the unique regions in the spectra that generate this separation. In addition, Chenomx NMR Suite software along with the loading, VIP and S plot assisted in identification and qualification of the low molecular weight metabolites present.

It is now well established that a high fat diet is highly correlated with an atherogenic outcome. Independent from the effect of a high fat diet on lipid profile, a number of recent metabolomic studies have identified abnormalities in branched chain amino acids [107], choline, betaine, and TMAO metabolism [108-110] as being highly increased in subjects with stable atherosclerosis, heart failure, and other cardiovascular diseases. After screening more than 2000 metabolites from a large cohort study (n= 1,876), Wang *et al* [110] found that a unique cluster of three phospholipid-associated molecules, more specifically choline, betaine, and the final metabolite, TMAO are linked to CVD risk. It has been shown by Wang *et al* [110] that increased levels of these metabolites promoted up-regulation of several macrophage scavenger receptors that correlated with atherosclerosis, making these

phospholipid metabolites independent predictors for the risk of a clinical vascular event. Even though the key culprits in atherosclerosis remain cholesterol and triglycerides, the new findings had shifted the attention of scientists towards phosphatidylcholine biosynthesis pathway for additional information related to risk for CVD. Plasma levels of choline and betaine are dependent on the proatherosclerotic phospholipid-rich diet, and are considered a key risk factor, rather than a direct marker of CVD [111]. After quantification of these metabolites in our plasma samples, we determined that the group that received the high fat diet and the PAZ and/or fractions 4 had significantly lower levels of betaine, choline, phosphocholine, glycerol-phosphocholine, as compared with the animals that received the high fat diet and water. In addition, the group that received fraction 4 had a significantly lower concentration of TMAO, demonstrating that the biologically active fraction provides enhanced benefits to the hypercholesterolemic animals. TMAO was demonstrated to promote accelerated atherosclerosis and has been proposed that TMAO induces up-regulation of macrophage scavenger receptors and can contribute to augment the "forward cholesterol transport" [112].

Another recently studied metabolite that contains a trimethylamine structure similar to that of choline is L- carnitine. Its fundamental role is to transport fatty acids into the mitochondrial compartment [112], and it has been associated with potential health risk related to CVD [113]. It has been shown that TMAO, and its precursors choline and carnitine suppress *in vivo* reverse cholesterol transport, and elevated levels of plasma carnitine in humans are significantly associated with risk for coronary artery disease, peripheral artery disease, and overall CVD [114]. Our

metabolomics analysis showed a significantly lower plasma concentration of carnitine in the group that received fraction 4 as compared with the group that received a high fat diet and water. This finding is of extreme importance, as it appends to the increase in plasma HDL cholesterol, along with the decrease in non-HDL and TC/HDL ratio, all considered contributory factors towards prevention of heart disease.

Furthermore, Aim 3 was designed to examine the therapeutic effect of fraction 4 in a diet-induced hypercholesterolemic hamster model. More specifically, the objective of this study was to determine the efficacy of fraction 4 on plasma lipid and metabolomic profile of animals that have been previously induced to a hypercholesterolemic state. Also, the aim was to determine the earliest time point at which the plasma profile was modified after treatment with fraction 4. Also, this is the first study examining the plasma metabolomic profile of hypercholesterolemic hamsters treated with fraction 4; therefore it is very important to determine the changes in the concentration of small molecular weight metabolites due to fraction 4 therapeutics and how they correlate with the results from the preventative study.

The results of this therapeutic investigation reveal an improvement in the plasma lipoprotein profile upon administration of fraction 4, showing a significant reduction in non-HDL cholesterol and total cholesterol/HDL ratio in all treatment groups, as well as a significant increase in plasma HDL cholesterol concentration. In addition, there was a moderate (T7, T10, and T14 groups) and a highly significant increase (T21 group) in the hepatic mRNA levels of Apo A1 gene, which is involved in the production of nascent HDL particles. Further, we analyzed the metabolic plasma

profile of hypercholesterolemic hamsters treated with the biologically active fraction of PAZ, using $^1$H NMR spectroscopy and multivariate data analysis. A clear separation of the groups was obtained with the PCA and PLS-DA score plots, showing changes in blood plasma profiles due to therapy with fraction 4. To identify the metabolites responsible for this strong separation of the groups, PCA loading plot was analyzed to identify the unique regions in the spectra that generate this separation. Chenomx NMR Suite software along with the PCA loading, VIP and S plots assisted in identification and qualification of important low molecular weight metabolites. After quantification of these metabolites in our plasma samples, it was noticed that the group that received the high fat diet and fraction 4 for 21 days had significantly lower levels of betaine, choline, phosphocholine, and glycerol-phosphocholine, as compared with the animals that received the high fat diet and water. Furthermore, our metabolomics approach showed a significant lower plasma concentration of carnitine in the group that received BAF for 21 days as compared with the group that received a high fat diet and water.

The concentration of these metabolites was also altered when PAZ and fraction 4 were administrated as a preventative agent in hypercholesterolemic hamsters, therefore we can suggest that the phosphocholine-containing molecules and their pathways are being altered by administration of PAZ and/or BAF. Figure 4.1 presents an overview of these pathways and the relationship between these molecules and lipid metabolism.

Several researchers investigated the phosphocholine-containing molecules and TMAO production and revealed that these molecules are gut-flora-dependent and

several pathogens are responsible for the production of TMAO [110, 114, 115]. Previous studies have also shown that the intestinal microbial community can influence the efficacy of utilizing energy from the diet, and ultimately increase the susceptibility to obesity [116]. More metabolomics studies have demonstrated the active role of gut microbiota with the development of complex metabolic aberrations, such as insulin resistance and non-alcoholic fatty liver disease [117].

Using a targeted metabolomics approach, Wang *at el* [110] and Koeth *at el* [114] identified a novel pathway connecting dietary lipid intake, intestinal microflora and atherosclerosis (Figure 4.2). This pathway represents a distinctive additional contribution to the pathogenesis of atherosclerosis, demonstrating that gut mirobiota engages in the metabolism of phospholipids to yield a molecule (trimethylamine), which is further metabolized to TMAO in the host and ultimately contribute to formation of atherosclerosis. Thus, the current findings are of extreme importance, demonstrating that PAZ and its BAF can alter not only the concentration of important phosphocholine-containing molecules, but may also change the gut microbiota leading to altered production of TMAO.
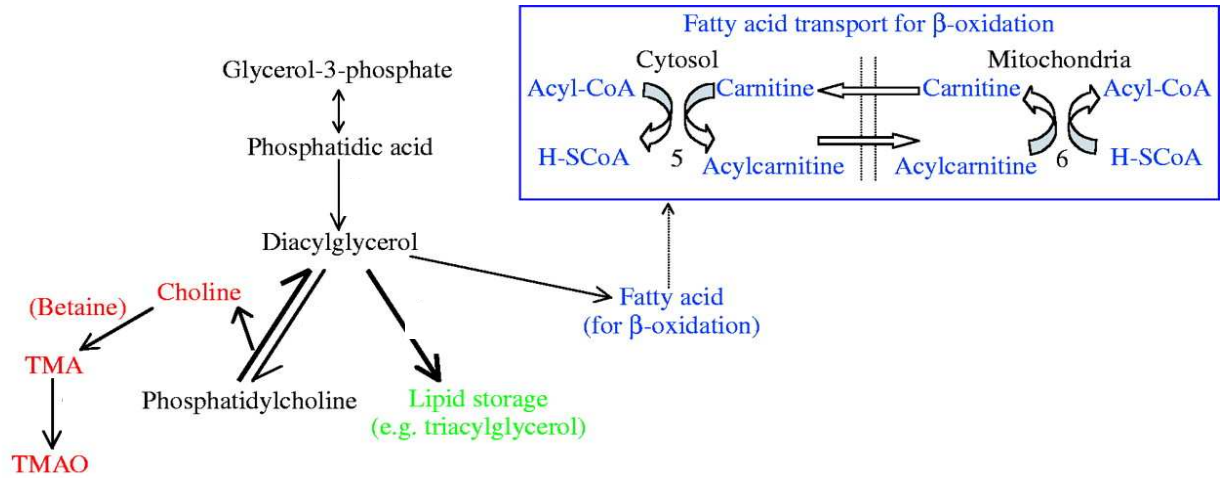
**Figure 4.1** Diagram of the Glycerolphosphate Pathway and Phosphatidylcholine Synthesis [118]
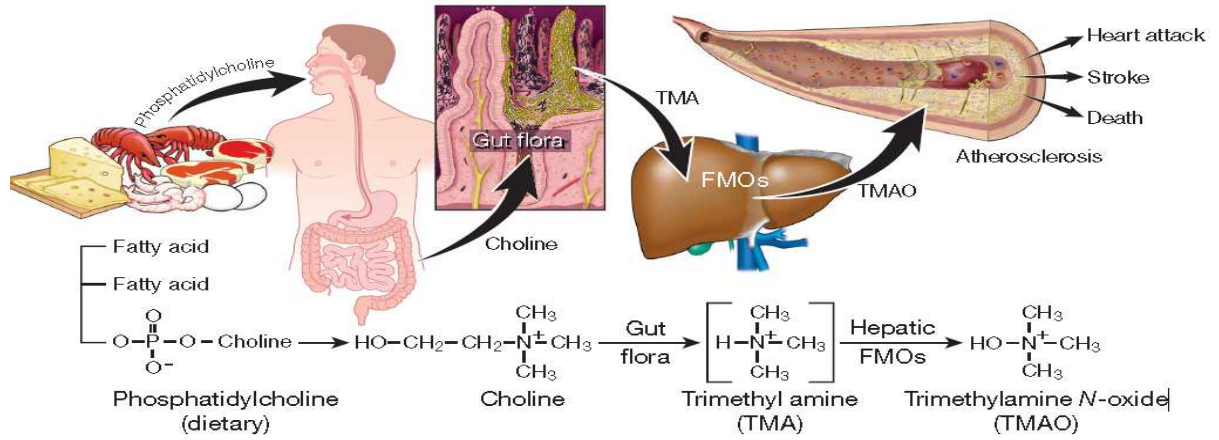
**Figure 4.2** Gut-flora Dependent Metabolism of Phosphocholine and Atherosclerosis.

## CONCLUSION

The present study has shown that dietary administration of PAZ and/or its biologically active fraction can improve the plasma lipoprotein profile, hepatic mRNA expression of genes involved in HDL/ RCT mechanism, as well as the concentration of low molecular weight metabolites in plasma. These modifications were achieved when fraction 4 of PAZ was administrated both as a preventative and as a therapeutic agent, suggesting the potential benefit of this agent in hypercholesterolemia.

The data suggest that administration of PAZ and the biologically active fraction results in a favorable lipoprotein profile in hamsters, primarily due to the effects on multiple targets in the reverse cholesterol transport pathway. In addition to improving the well known risk factors associated with CVD, the potentially valuable effect of the algal infusion on relatively new predictors of atherosclerosis, such as branched chain amino acids and the phosphocholine- containing molecules was identified.

It was also shown that the biologically active fraction can alter the pathway linking the phospholipids, intestinal microflora and atherosclerosis. The pro-atherogenic gut-flora-generated metabolite TMAO was significantly reduced when fraction 4 was administrated along with high fat diet as a preventative agent to hypercholesterolemic hamsters.

This study therefore further supports the dietary use of PAZ and/or its biologically active fraction for the prevention and management of dyslipidemia-related diseases such as cardiovascular disease and metabolic syndrome. Also, this research illustrates how the metabolomics approach can drive biomarker discovery and

generate new hypothesis for new treatments, opening exciting avenues for future research.

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**ABSTRACT**

**THE EFFECT OF PROALGAZYME AND ITS SUBFRACTIONS ON LIPID METABOLISM IN DIET INDUCED HYPERCHOLESTEROLEMIC HAMSTRES: CORREALTION WITH PLASMA METABOLOMIC PROFILE**

by

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**Major:** Nutrition and Food Science

**Degree:** Doctor of Philosophy

**Background:** Plasma HDL cholesterol levels are inversely related to cardiovascular disease, which is the leading cause of death worldwide. This study investigated the preventative effect of an algae infusion, ProAlgaZyme (PAZ) and its subfractions (F1, F2, F3, F4) on plasma HDL in a hamster model. Further, the study aimed to identify the biologically active fraction of PAZ and to determine the therapeutic efficacy of the fraction in diet induced hypercholesterolemic hamsters over time. Also, the current study investigated the changes in plasma metabolomic profile produced due to the interventions, and correlated the results with the lipoprotein profile of the hamsters.

**Methods:** Eighty male golden Syrian hamsters (8 weeks old) were randomized into controls (CW and CP) or high fat diet (HW, HP, HF1, HF2, HF3, and HF4). During the preventative intervention, an infusion of either 5% (HF1, HF2, HF3) or 20% (HP, HF4) concentration (v/v) was administered via the drinking water for 4 weeks, while the hamsters were being fed a high-fat diet (30% of calories from fat). Plasma lipids were

assayed and liver samples subjected to reverse transcription polymerase chain reaction (RT-PCR) to determine the relative transcription levels of genes involved in HDL/reverse cholesterol transport metabolism, ie, ApoA1, ABCA1, CETP, and SRB1. Lipid profile was correlated with plasma metabolomic profile using 1D $^1$H NMR spectroscopy and the biologically active fraction has been identified.

Further, the study aimed to determine the therapeutic effect of the active fraction of PAZ. For this, 40 male Golden Syrian hamsters were fed a high fat diet for 4 weeks prior to randomization into 5 groups, based on the number of days they received the treatment. Thus animals in T3, T7, T10, T14, and T21 groups received the active fraction for 3, 7, 10, 14, and 21 days, respectively, as their drinking fluid. HW group from previous study was considered as control (T0, high fat diet and the active fraction for 0 days) for this study. Plasma lipid profile was assayed enzymatically, while RT-PCR provided the alternative transcription levels of Apolipoprotein (Apo) A1 gene. Plasma metabolomic profile was determined using $^1$H nuclear magnetic resonance (NMR) spectroscopy and results correlated with the lipid profile of the hamsters.

**Results:** Non-HDL cholesterol was significantly reduced in the HP ($P < 0.05$), HF3 and HF4 ($P < 0.001$) groups as compared with the HW group, while HDL cholesterol showed a significant increase in the HP, HF3, and HF4 groups ($P < 0.001$). Moreover, the total cholesterol/HDL ratio was significantly improved in the HP, HF1, and HF2 ($P < 0.05$), and HF3 and HF4 ($P < 0.001$) groups. Real-time quantitative polymerase chain reaction showed a significant increase in hepatic ApoA1 (HP, HF4) and ABCA1 (HF3, HF4) expression, consistent with an increase in HDL production, biogenesis, and maturation. A two-fold increase in SRB1 expression indicates that HF4

further augments the reverse cholesterol transport mechanism. Reduction of CETP expression (HF4) is consistent with a decrease in the transfer of cholesteryl ester to LDL, further increasing the amount of cholesterol held as HDL particles. NMR metabolomics approach showed a significant decrease in the concentration of several small molecular weight molecules, including branched chain amino acids and phosphocholine-containing molecules, in groups HP and HF4, when compared with HW group. Since, F4 exhibited the most influence on plasma lipid and metabolomic profile, it was further tested for its therapeutic effect.

Plasma HDL was significantly increased in T3 ($P < 0.05$) and T21 ($P < 0.001$), while non-HDL cholesterol was significantly reduced in T3, T7, T10 ($P < 0.001$) and T14, T21 ($P < 0.01$). Moreover, the total cholesterol/HDL was significantly lower in all groups ($P < 0.001$) as compared with T0. Quantitative RT-PCR showed an increase in Apo A1 expression in T10 (3x) and T21 (6x) groups. NMR data followed by multivariate analysis showed a clear separation between T0 and T21 groups, indicating a difference in their metabolomic profiles. Plasma concentrations of choline, phosphocholine, glycerol-phosphocholine, betaine and carnitine metabolites were significantly lowered in T21 group. These metabolites are associated with a reduced risk for atherosclerosis and cardiovascular disease.

**Conclusion:** ProAlgaZyme and its subfractions significantly improved the plasma cholesterol profile by lowering non-HDL and increasing HDL, possibly via the reverse cholesterol transport mechanism. Also, the concentration of several pro-atherogenic small molecular weight metabolites has been decreased, indicating that PAZ and F4 can be used as a preventative agent for hypercholesterolemia and

atherosclerosis. Moreover, treatment with F4 also significantly improved plasma lipid profile by increasing HDL and lowering non-HDL cholesterol, and reducing key risk factor metabolites for atherosclerosis and cardiovascular disease.

## AUTOBIOGRAPHICAL STATEMENT

Andreea Geamanu is a PhD Candidate in the Nutrition and Food Science (NFS) Department, Wayne State University (WSU), Detroit, MI. She is expected to graduate in October 2013 with a doctor of philosophy degree in NFS and a minor in Evaluation and Research Statistics. Ms. Geamanu graduated from Wayne State University with an MS (2008) and Honors BS (2007) in NFS. Prior to her studies in the US, she was a medical student at Carol Davila School of Medicine, in Bucharest, Romania. During her graduate studies at WSU, Ms. Geamanu conducted research and training under the supervision of Dr. Smiti Gupta. She was involved in several projects investigating the synergistic effect of different nutraceuticals in the prevention and treatment of lung and pancreatic cancer.

For her doctoral dissertation, Ms. Geamanu evaluated the effect of an algal infusion on lipid metabolism. In addition, she utilized the metabolomics approach to observe the alterations in plasma NMR spectra from a diet-induced hypercholesterolemic animal model. Metabolomics approach was also used to evaluate biomarkers for early diagnosis of lung and pancreatic cancer. The change in metabolomic pattern or the biomarker profile enables targeting of specific metabolic pathways affected by the disease condition, leading to mechanistic insight. Ms. Geamanu's future research interests include diet, lipid metabolism, obesity, and cancer prevention and risk factors.