The Effects of Three Diets on Rabbit Lipoprotein Metabolism

by

Chai Sue Lee

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Committee in charge:

Professor Paola S. Timiras, Chair
Professor Hana Dan-Cohen
Professor Janet C. King

Fall 1997
The thesis of Chai Sue Lee is approved:

[Signature]
Chair

[Signature]
Date

[Signature]
Date

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INTRODUCTION

Atherosclerosis

Atherosclerosis, the most common form of arteriosclerosis (hardening of the arteries), is an insidious, slowly progressive vascular disease beginning in childhood\(^1\) characterized by the presence of fatty streaks followed by fibrous plaques and finally atherosclerotic plaques within the endotheliums, the innermost layer of arterial walls. It is not clinically evident until the arterial lesions precipitate organ injury, which usually occurs around middle age or later.

The basic lesion - the atherosclerotic plaque - consists of a raised focal plaque, having a central core of primarily cholesterol and cholesterol esters, and a superficial fibrous cap, which is composed primarily of modified smooth muscle cells, macrophage foam cells, and relatively dense connective tissue. Atherosclerotic plaques are sparsely distributed at first, but as the disease advances they become more and more numerous, sometimes covering the entire circumference of severely affected artery. Moreover, as they increase in size, the space-occupying feature of the atherosclerotic plaques eventually narrows the affected artery. If the atherosclerotic plaque fractures, ruptures, or ulcerates, or if the endothelial damage is severe, acute thrombosis, or blood clots, may be induced which may occlude the artery. The resultant ischemia of the tissues normally perfused by the occluded artery causes the death of the deprived tissues, known as an infarction.
Atherosclerotic lesions can cause clinical diseases by the following mechanisms:

- Slow, insidious narrowing of the vascular lumina, resulting in ischemia of tissues perfused by the involved vessels.
- Fractured fibrous cap providing a site for thrombosis and then embolism.
- Sudden occlusion of the lumen by superimposed thrombosis or hemorrhage into a plaque, producing ischemia and, if severe and prolonged, infarction of the tissues in the perfusion zone.
- Weakening the wall of a vessel, causing an aneurysm or rupture.

In large arteries, such as the aorta, the important complications of these plaques are large, mural thrombi that may dislodge and yield emboli into the distal circulation, most commonly noted in the kidneys, and aneurysms or ruptures due to destructive impingement of the plaques on the media weakening the affected arterial wall. In small arteries, however, the narrowing of the lumen by plaques, especially if accompanied by thrombosis or hemorrhage, can compromise blood flow to organs normally supplied by the arteries.

Although any artery in the body may be affected theoretically, symptomatic atherosclerosis is most often localized to the proximal portions of the coronary arteries, the larger branches of the carotid arteries, the circle of Willis, the large vessels of the lower extremities, and the renal and mesenteric arteries, supplying the heart, brain, lower extremities, kidneys, and small intestine, respectively.

The clinical manifestations of atherosclerosis are as varied as the vessels affected. The most significant consequence of
Atherosclerosis is ischemic heart disease and myocardial infarction, the most common cause of death for both women and men in the United States. Other significant complications include stroke from cerebral ischemia and infarction, ischemic bowel disease, peripheral vascular occlusive disease with findings varying from claudication to ischemic necrosis with gangrene, and renal arterial ischemia with secondary hypertension. Weakening of the vessel wall may lead to aneurysm formation.

Atherosclerosis overwhelmingly accounts for more deaths and serious morbidities in the Western world than any other disorder. Worldwide in distribution, the highest incidence occurs in Finland, Great Britain, other northern European countries, the United States, and Canada. The rates are remarkably lower in Asia, Africa, and South and Central America. For example, death rates from ischemic heart disease in Japan are one-sixth of those in the United States. However, Japanese who immigrate to the United States and adopt the lifestyles and dietary customs of their new home acquire the predisposition to atherosclerotic diseases of the American population. In Japan, mortality by strokes is higher than in the United States. It is probably safe to say that atherosclerosis affects every single individual in the world to some degree, although not all are symptomatic.

Deaths from cardiovascular disease in the United States rose from 14% of all deaths in 1937 to 54% in 1968, almost all cases being related to atherosclerosis. By 1975, for the first time, the rate showed a statistically significant decline, which has been maintained since. This downward trend is believed to be mediated largely by a
reduction in atherosclerosis influenced by changes in diet and lifestyle, better control of hypertension, and improved therapy for myocardial infarction and other complications of ischemic heart disease. Nevertheless, despite a reduction in mortality from myocardial infarction and other forms of ischemic heart disease, nearly 50% of all deaths in the United States continue to be attributed to atherosclerosis-related diseases.

Most people who die or are disabled because of atherosclerosis and subsequent coronary heart disease have one or more identifiable characteristics that predispose them to the development of these diseases. These characteristics, or risk factors, are classified as either primary or secondary, depending on the degree to which they affect the risk of developing coronary heart disease.

A number of prospective studies, most notably the famed Framingham (Massachusetts) Study and others (e.g., the Multiple Risk Factor Intervention Trial [MRFIT])\textsuperscript{2,3} have identified four primary, treatable risk factors that predispose to atherosclerosis and resultant ischemic heart disease. These are:

- Dyslipidemia, including elevated serum levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride, and decreased serum levels of high-density lipoprotein (HDL) cholesterol;
- Diabetes mellitus;
- Elevated blood pressure; and
- Cigarette smoking.

Secondary risk factors, some of which are modifiable, are:

- Obesity;
• Lack of physical activity;
• Psychosocial and behavioral factors (e.g., "type A" personality);
• Male gender;
• Increasing age;
• Estrogen depletion in women;
• Oral contraceptives;
• Hyperuricemia;
• Hyperhomocysteinemia;
• High carbohydrate intake;
• Heavy use of alcohol; and
• A family history of premature coronary heart disease.

According to the risk factor hypothesis, a person who has a risk factor for atherosclerosis is more likely to develop clinical manifestations of the disease and is likely to do so earlier than a person with no risk factors. Furthermore, if the risk factors present are eliminated or their impact is reduced, then the person’s risk of developing coronary heart disease and its related complications will also be reduced.

Each of the primary risk factors noted earlier contributes individually to the possible development of clinically significant atherosclerosis, but multiple factors exert a synergistic (more than additive) effect. All the risk factors mentioned above account for about 50% of cases.

Some patients with atherosclerosis-related diseases have no obvious risk factors. Furthermore, some individuals seem to be almost completely protected from developing this disease. As far as can be ascertained, they often ingest the same diet and receive the
same hemodynamic stimuli as do most of the persons who develop atherosclerotic lesions.

Why is it that some animals can withstand a severely hypercholesterolemic diet and show little or no elevation of serum cholesterol or little lesion development, when in the same experiment the majority of the same species of animals develops severe atherosclerosis? Even more puzzling, why is it that some animals have elevated cholesterol and LDL levels throughout the course of a study but develop no lesions, whereas other animals that are housed and fed in the same way and obtained at the same time from the same source develop progressive atherosclerosis? At present, we do not have the complete answers to these questions, although these phenomena have been demonstrated many times in people and in many animal models. Recent investigations indicate that the widely differing responses in individuals may be genetically determined. Nevertheless, the causes and pathogenesis of atherosclerosis remain subjects of lively speculation and controversy. For these reasons and because of the overall importance of atherosclerosis, our objective was to develop an animal model to be used to study atherosclerosis in our lab in the future.

Lipids and Lipoproteins

To understand the relationship between lipid abnormalities and the pathogenesis of atherosclerosis, it is desirable first to understand the normal process of cholesterol metabolism and transport. This section reviews in detail the lipoproteins and their metabolism and transport, the correlation between lipid elevations
and the development of coronary heart disease, and the possible mechanisms involved in this relationship.

All lipids, including cholesterol, are transported in plasma-complexed with proteins in a water-soluble structure called a lipoprotein. In its mature form, a lipoprotein is a spherical particle consisting of a hydrophobic core of triglycerides and cholesteryl esters surrounded by a polar surface layer of phospholipids, proteins, and cholesterols. The protein components of lipoproteins are known as apolipoproteins (apo), which serve a dual function. Not only do they bind and emulsify lipids, but they also contribute to the regulation of lipoprotein metabolism.

Plasma lipoproteins have been classified into five broad categories on the basis of their function and physical properties: the chylomicron, the very-low-density lipoprotein (VLDL), the intermediate-density lipoprotein (IDL), the low-density lipoprotein (LDL), and the high-density lipoprotein (HDL). They differ not only in density but also in size, ultracentrifugal properties, and electrophoretic characteristics (see later). The smaller the lipoprotein, the greater its density because the density of its outer coat is greater than that of its inner core. There is also heterogeneity within each family (see later).

Chylomicrons are the largest of the lipoproteins, ranging from 80 to 1000 nm in diameter, and have the highest sedimentation coefficient on ultracentrifugation (more than 400 Svedberg flotation units). These lipoproteins have the lowest density at an average of 0.95 g/mL. Chylomicrons do not migrate on paper or agarose gel electrophoresis, and normally they are absent from the blood after a
12-hour fast. Mature chylomicrons contain apo B\textsubscript{48}, apo A-I, A-II, A-IV, the apo Cs and apo E. Chylomicrons function to transport exogenous (in this case, dietary) triglycerides and cholesterol from the intestines to the tissues.

VLDLs, the next-largest lipoproteins, range from 30 to 80 nm in diameter. On paper and agarose gel electrophoresis, VLDL particles migrate with pre-\(\beta\) mobility (that is, just ahead of the \(\beta\)-globulin fraction). The primary apoprotein constituents of VLDL are apoB\textsubscript{100}, the apo Cs, and apo E, but they may also contain small amounts of apo A-I and apo A-II.

IDLs are 25 to 30 nm in diameter and have \(\beta\) mobility. They contain apo B\textsubscript{100} and apo E. IDLs (also called VLDL remnants) are produced by the hydrolysis of VLDL triglycerides.

Like IDLs, the LDLs are a product of VLDL hydrolysis. Approximately 20 to 25 nm in diameter, LDLs demonstrate \(\beta\) mobility, and they contain only apo B\textsubscript{100}. From chylomicrons to VLDLs followed by IDLs and finally LDLs, there is a progressive decrease in triglyceride content and a progressive increase in cholesteryl ester content. In LDLs, the core is predominantly cholesteryl ester; in chylomicrons and VLDLs, it is predominantly triglyceride. VLDL, IDL, and LDL are a group of related particles that function to transport endogenous (that is, internally supplied) triglycerides and cholesterol from the liver to the tissues.

HDL particles are the smallest (5 to 12 nm in diameter) of the major lipoproteins. There are two primary fractions of HDL, the lipid-rich, known as the HDL\textsubscript{2} (1.061 to 1.210 g/mL, 9 to 12 nm), and the lipid-poor, known as the HDL\textsubscript{3} (1.125 to 1.210 g/mL, 5 to 9 nm).
These two fractions can be further subdivided into HDL$_{2a}$, HDL$_{2b}$, HDL$_{3a}$, and HDL$_{3b}$; the last two are reduced in size and lipid content. The HDL particle contains nearly equal amounts of lipid and apoprotein in contrast to the LDL particle, which contains about 75% lipid and 25% apoprotein by weight. The lipid content of HDL is primarily cholesteryl ester and phosphatidylcholine (lecithin). HDL contains apo A-I, apo A-II, apo C, and apo E. On electrophoresis, HDLs migrate with the $\alpha$-globulins; the terms $\alpha$-lipoproteins and $\beta$-lipoproteins are sometimes used to refer to HDLs and LDLs, respectively. HDLs function to transport endogenous cholesterol from the tissues to the liver.

A lipoprotein called Lp[a] has a density between LDL and HDL. Lp[a] contains two apoproteins, apo B$_{100}$ and a glycoprotein specific to Lp[a] called apo [a], that are bound together by a disulfide bond. Elevated Lp[a] plasma levels are associated with increased risk of stroke and coronary heart disease. In addition, Lp[a] has been found in atherosclerotic plaques.

The various lipoproteins have different physiological functions as we discuss below. Dietary cholesterol and triglyceride are distributed to tissues in the form of chylomicrons. Newly formed chylomicrons incorporate apo A-I, apo A-IV, and apo B$_{48}$ from the intestinal wall, and they acquire apo Cs and apo E while circulating in the lymph or blood. Although apo B$_{48}$ is not recognized by any known cellular receptor, this apoprotein appears to be necessary for the incorporation of lipids into chylomicrons and for the secretion of chylomicrons by the cells of the intestinal wall. The chylomicrons are released from the intestine into the lymph and proceed through
the thoracic duct into the systemic circulation. Once they reach the capillaries, particularly those in adipose tissue and skeletal muscle, chylomicron core triglycerides are rapidly hydrolyzed to free fatty acids, which are taken up by the tissues, by the action of lipoprotein lipase, an enzyme attached to the capillary endothelium. Lipoprotein lipase requires apo C-II for activation. This apoprotein is thought to be transferred to the surface of the chylomicron from circulating HDL particles. At this point, the chylomicron’s surface components of apoproteins, phospholipids, and cholesterol are thought to be transferred to HDL particles. With this transfer of lipid and protein, HDL₃ particles are converted into the larger, more lipid-rich HDL₂ particles.

The chylomicrons remnants that remain after lipolysis are rich in cholesterol and are removed from the circulation by the liver. The removal mechanism has not been elucidated but is mediated in some way by apo E. It may be that apo E and lipoprotein lipase are added to partially digested chylomicron particles as those particles reach the liver. The exact receptor for the chylomicron remnant is not known, but a candidate is the LDL receptor-related protein (LRP)⁴. Hui et al⁵ classified these chylomicron remnants as β-VLDL particles because they exhibit some of the properties of VLDL particles and comigrate with the β-globulin fraction on paper and agarose gel electrophoresis. Chylomicrons therefore function to deliver dietary triglycerides to muscle and adipose tissue and dietary cholesterol to the liver.

Removal of chylomicron remnants is the means for transferring dietary cholesterol from the intestine to the liver. A rapid uptake of
these particles probably increases the intrahepatic supply of cholesterol and down-regulates the number of LDL receptors. Particles containing apo E₄ or apo E₃ are removed by the liver more rapidly than those containing only apo E₂. Thus, delayed clearance of chylomicron remnants containing only apo E₂ may have the effect of up-regulating the number of hepatic LDL receptors, leading to lower concentrations of LDL and total cholesterol in the blood. Particles containing only apo E₄ or apo E₃ would exert the opposite effect by causing a rapid increase of cholesterol in the liver cells.

Role of VLDLs: Approximately 7% of the body's cholesterol circulates in the plasma, predominantly in the form of LDL. As might be expected, the level of plasma cholesterol is influenced by its synthesis and catabolism. Figure 1 illustrates that liver plays a crucial role in both these processes. The first step in this complex sequence is the secretion of VLDL by the liver into the bloodstream. VLDL synthesis is stimulated by excessive consumption of calories, alcohol, or carbohydrates or by a high concentration of unesterified fatty acids reaching the liver. VLDL particles are rich in triglycerides, although they do contain lesser amounts of cholesteryl esters. Like chylomicrons, as VLDLs circulate through the capillaries of peripheral and adipose tissue, they are hydrolyzed by lipoprotein lipase, a process that extracts most of the triglyceride to peripheral sites, where the triglyceride may be used for energy or stored. During hydrolysis, the VLDL surface components of apoprotein, phospholipid, and cholesterol are transferred to HDL particles. The smaller, more dense VLDL remnants that remain are called IDLs. It is reduced in triglyceride content and enriched in cholesteryl esters,
but it retains two of the three apoproteins (B-100 and E) present in the parent VLDL particle. Some of the larger VLDL particles may be removed directly from the circulation by the liver, without proceeding through the IDL pathway.

After release from the capillary endothelium, the IDL particles have one of two fates. In the rat and many other animal species, removal by the liver is the major fate of VLDLs and IDLs. Approximately 50 to 70% of newly formed IDL is rapidly removed from circulation by the liver through a receptor-mediated transport. The receptor responsible for the binding of IDL to liver cell membrane recognizes both apo B-100 and apo E. It is called the LDL receptor, however, because it is also involved in the hepatic clearance of LDL, as described later. In the liver cell, IDL is recycled to generate VLDL. The IDL particles not taken up by the liver are subjected to further metabolic processing that removes most of the remaining triglycerides and apo E, yielding the cholesterol-rich LDL, a process thought to be mediated by hepatic lipase. It should be emphasized that IDL is the immediate and major source of plasma LDL. The proportion of IDLs removed by the liver versus the proportion converted to LDLs is a major determinant of the quantity of LDLs circulating in the blood. In those animal species in which most of the IDL particles are removed by the liver, the circulating LDL concentration is relatively low. These species tend to be relatively resistant to atherosclerosis. In humans, the higher concentration of circulating LDL predisposes to atherosclerosis.

Role of LDLs: The end product of VLDL catabolism is LDL, the preeminent atherogenic lipoprotein in humans. There appear to be
two mechanisms for removal of LDL from plasma, one mediated by an LDL receptor-dependent process and the other by an LDL receptor-independent pathway, described later. Approximately 70% of the plasma LDL appears to be cleared by the liver via the cell surface LDL receptors. Three separate processes are affected by the uptake of LDL cholesterols: (1) cholesterol suppresses endogenous cholesterol synthesis within the hepatic cells by inhibiting the activity of the enzyme 3-hydroxy-3-methylglutaryl (3HMG) CoA reductase, which is the rate-limiting enzyme in the cholesterol synthetic pathway; (2) the cholesterol activates the enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT), favoring esterification and storage of excess intracellular cholesterol; and (3) cholesterol suppresses the synthesis LDL receptors, thus protecting the cells from excessive accumulation of cholesterol. A decrease in the number or the activity of hepatic LDL receptors may result in a higher circulating concentration of LDL.

The transport of LDL not involving LDL receptors appears to occur at least in part into the cells of the mononuclear phagocyte system. Oxidation of the fatty acids or chemical modification of the free-lysine amino groups of apo B alters the LDL particle to the extent that it is no longer recognized by the LDL receptor. The modified LDL particle may be removed via another mechanism known as the scavenger receptor pathway. Monocytes and macrophages have receptors for chemically altered (e.g., acetylated or oxidized) LDLs. Normally, the amount of LDL transported along this "scavenger receptor" pathway is less than that mediated by the receptor-dependent mechanisms. In hypercholesterolemia, however,
there is a marked increase in the LDL receptor-independent traffic of LDL cholesterol into the cells of the mononuclear phagocyte system and possibly the vascular walls. This is responsible for the appearance of xanthomas and is thought to contribute to the pathogenesis of atherosclerosis. In support of this mechanism, deposits of oxidized LDLs have been found in atherosclerotic plaques by several investigators, and physical and chemical changes consistent with advanced oxidative damage in the arterial wall have been documented.

Role of HDLs: HDL has essentially the opposite function of LDL: it removes cholesterol from the tissues. HDL is secreted in an incomplete form (called nascent HDL) and is assembled in the plasma from components largely obtained through the degradation of other lipoproteins. The components of HDL are derived from multiple sources, including the liver, intestine, and the surfaces of chylomicrons and VLDLs. HDL contains phospholipid and some apolipoproteins. The HDL synthesized in the liver contains apo A-I, apo A-II, and apo E. The HDL synthesized in the intestine contains apo A-I and apo A-IV.

HDL is derived from the surface components of chylomicrons and VLDLs during their lipolysis. The apolipoproteins of HDL may be transferred to chylomicrons and VLDLs and then perform functions specific to the chylomicrons and VLDLs. For example, apo E transferred from HDLs to chylomicrons mediates the uptake of chylomicron remnants in the liver. Apo C-II transferred from HDLs to chylomicrons and VLDLs helps to activate lipoprotein lipase. It
has been suggested that the apo Cs prevent the premature uptake by the liver of chylomicron and VLDL remnants.

HDLs provide a reverse transport pathway for cholesterol from peripheral tissues to the liver, although this has not been proved as an antiatherogenic mechanism. However, epidemiological studies have demonstrated a negative association between atherosclerosis and HDL-cholesterol. Reverse transport would be mediated by an HDL receptor that recognizes apo A-I, apo A-II, and apo A-IV. Graham and Oram described a 100,000 dalton protein that has such binding properties and is induced by loading cells with cholesterol. The protein contains several repeat units of amphipathic helices, thus resembling the apoprotein structure. It has been suggested that binding of apo A-I or A-IV to this protein on the cell surface promotes the translocation of cholesterol to the cell surface, where it may be transferred to HDL₃. Binding by the HDL receptor does not appear to involve the internalization of a lipoprotein particle, in contrast to the LDL receptor.

Cholesterol removed from the cells and incorporated into HDL is converted to cholesteryl ester through the action of lecithin-cholesterol acyltransferase (LCAT), an enzyme that is activated by the HDL component apo A-I. HDL therefore functions as a cholesterol scavenger. Once the HDL particle contains sufficient cholesteryl ester, it is referred to as a mature particle. Mature HDLs are spherical when viewed with the electron microscope, whereas nascent HDLs are discoid. Once the mature HDL particle is formed, a lipid exchange may occur between the HDL and the VLDL, IDL, or LDL. This process is mediated by the cholesteryl ester transfer
protein (CETP). Triglycerides from the chylomicron remnants and VLDLs are transferred to LDL and HDL particles, while cholesteryl esters from the HDL particle are transferred to VLDL, IDL, or LDL. These cholesteryl ester-enriched particles can then be delivered to LDL receptors in the liver and removed from circulation. The liver is the only organ capable of disposing of significant quantities of cholesterol as bile acids. Thus, the process produces a larger class of LDL and HDL particles that are attacked by hepatic lipase, an enzyme that catalyzes the hydrolysis of triglycerides and phospholipids. The end result is small, dense, protein-enriched LDL and HDL particles. The concentration of HDL₂ and the total concentration of HDL are reduced. This sequence of events could produce small, dense LDL particles and the dyslipidemic features of syndrome X (described later). The metabolic condition of small, dense LDL and reduced HDL levels is thought to predispose a person to atherosclerosis.

HDL can also transport cholesteryl ester from tissues to the liver through direct interaction with the apo E receptor. Thus, the fraction of HDL that contains apo E could transport cholesteryl ester directly to the liver without the CETP-mediated transfer to LDL, which presumably occurs when CETP is completely absent, as in rats. Evidence for an alternative mechanism in humans comes from a recent description of several Japanese families who have a deficiency of CETP and extremely high HDL₂ levels, but premature coronary heart disease does not develop in these families.

Concentrations of the subfraction HDL₂ are higher in females than in males and are increased by exercise and by the presence of estrogen; androgens decrease HDL levels. Concentrations of HDL₂ are
also positively correlated with the activity of endothelial lipoprotein lipase and inversely correlated with the activity of hepatic lipase; HDL₂ may be the primary substrate for the latter enzyme. Ingestion of ethanol increases the circulating levels of both HDL subfractions, but it appears to exert the greater effect on HDL₃. Cigarette smoking decreases the concentration of HDL, especially HDL₂. Several drugs (including β-blockers) are known to decrease HDL concentrations. HDL levels have an inverse relationship to serum triglyceride concentrations.

**Hypercholesterolemia and Atherosclerosis**

Hypercholesterolemia and other abnormalities in lipid metabolism contribute a major risk factor in atherosclerosis. Four types of lipoprotein abnormality are frequently found in the population (and, indeed, one or more are present in 50 to 80% of myocardial infarction survivors): (1) increased LDL cholesterol levels, (2) decreased HDL cholesterol levels, (3) increased chylomicron remnants and IDL, and (4) increased levels of an abnormal lipoprotein Lp[a].

The evidence linking hypercholesterolemia and atherosclerosis takes many forms:

- Atherosclerosis plaques are rich in cholesterol and cholesterol esters, which are derived largely from lipoproteins in blood.
- Lesions of atherosclerosis can be induced in many experimental animals, including subhuman primates, by feeding them diets that raise the plasma cholesterol level.
• Genetic disorders causing severe hypercholesterolemia (e.g., congenital absence of either LDL receptors or HDL cholesterol) lead to premature atherosclerosis, often fatal in childhood, despite the absence of any other risk factor. Acquired diseases that cause hypercholesterolemia, such as the nephrotic syndrome and hypothyroidism, also increase the risk of ischemic heart disease.

• With few exceptions, populations having relatively high levels of blood cholesterol have higher mortality from ischemic heart disease, and the Framingham and other studies indicate increasing risk with increasing serum cholesterol concentrations.

• Finally, prospective studies, such as the Lipids Research Clinics Coronary Primary Prevention Trial, have shown that treatment with diet and cholesterol-lowering drugs reduces cardiovascular mortality in selected patients with hypercholesterolemia. A randomized, controlled trial of a similar treatment in patients with familial hypercholesterolemia yielded regression of coronary arterial lesions in both men and women.

Any single level of plasma cholesterol does not identify those individuals at risk. The higher the level, the higher the risk, although the risk rises more steeply once a level of approximately 200 mg/dl is exceeded (5.2 mmol/liter). The strongest association is with elevated plasma levels of LDL, the lipoprotein moiety richest in cholesterol; however, hypertriglyceridemia with increased concentrations of VLDL also appears to increase risk. In contrast, serum levels of HDL are inversely related to risk: the higher the level, the lower the risk. Thus, HDL is often called the "good cholesterol." In addition, there is evidence that other classes of
lipoproteins, such as IDL and Lp[a], are involved in the development of atherosclerosis\textsuperscript{16,17}.

**Pathogenesis of Atherosclerosis**

The physiological functions of endothelial cells are highly regulated but can change as a result of certain stimulation. The biological activity of endothelium-derived nitric oxide in atherosclerotic arteries has been shown to be impaired\textsuperscript{18}. The mechanisms leading to the defective activity of nitric oxide are not yet understood. The lack of biologically active nitric oxide in the endothelium has been associated with increased adhesion of leukocytes, which is now considered as a major initiating event in the atherosclerotic process. The accumulation of leukocytes in the vascular wall may, in turn, be responsible for a number of pathophysiological events, like generation of oxygen radicals, oxidation of LDL and subsequent foam cell formation, and release of cytokines and growth factors, resulting in smooth muscle cell migration and proliferation and subsequent intimal thickening\textsuperscript{18}. Moreover, further evidence suggests that the biological activity of nitric oxide is also decreased in early hypercholesterolemia, which may suggest that this biochemical defect is a cause, and not a consequence, of atherosclerotic disease\textsuperscript{19}. In addition, the lack of biologically active nitric oxide has been associated with increased vasospasm and impaired vasodilatation\textsuperscript{18}.

Increases in plasma levels of LDL or some component of hyperlipidemic serum may directly increase the rate of lipid penetration into the artery wall. Moreover, oxidized LDL can further
accelerate atherogenesis by several mechanisms: (1) It is chemotactic for circulating monocytes; (2) it increases monocyte adhesion; (3) it inhibits the motility of macrophages already in the lesions, thus favoring the recruitment and retention of macrophages in the lesions; (4) the enhanced rate of uptake of oxidized LDLs by resident macrophages leads to the formation of foam cells; (5) it is cytotoxic to endothelial cells and smooth muscle cells; (6) it stimulates the release of cytokines and growth factors which lead to further endothelial damage and smooth muscle cell proliferation; and (7) it may become antigenic and stimulate inflammatory autoimmune reactions. Hemodynamic forces, angiotensin II, carbon monoxide, catecholamines, homocystine, uremia, endotoxin, immune complexe, and virus also help produce sustained endothelial damage.

These and many other findings are interpreted as indicating that the vascular balance in the disease-free state reflects a multiplicity of functions of endothelial cells, smooth muscle cells, and inflammatory cells.

**LDL Heterogeneity and Risk of Coronary Artery Disease**

The association between plasma LDL level and coronary artery disease (CAD) is now believed to be causal. LDLs, however, comprise a heterogeneous spectrum of lipoprotein particles differing in size, density, and chemical composition. For most people, the LDL subclass pattern can be categorized as either pattern A or pattern B: pattern A is characterized by a predominance of larger, more buoyant, and lipid enriched LDL particles (diameter >255 Å), including LDL-I (d = 1.025 to 1.032 g/mL) and LDL-II (d = 1.032 to 20
1.038 g/mL) and pattern B is characterized by a major
electrophoretic peak of smaller, denser, lipid-depleted LDL particles
(diameter ≤255 Å), including predominantly LDL-III (d = 1.038 to
1.050 g/mL) and lesser amounts of LDL-IV (d = 1.050 to 1.063
g/mL)24,25.

Pattern B is associated with up to a threefold increase in the
susceptibility to developing CAD26-29. Furthermore, it has been
shown to be associated with increased levels of total cholesterol, LDL
cholesterol, plasma triglyceride, VLDL mass, IDL mass, and apo B and
with decreased levels of HDL cholesterol, HDL2 mass, and apo A-I,
each of which has been associated with increased risk of
CAD15,16,26,30-39.

Angiography and myocardial infarction case-control studies
have consistently found an association between small, dense LDL
particles and CAD26-29, 40-42. Interestingly, in all of these studies,
differences in LDL size or density distributions was statistically more
significant than the differences in LDL levels. Two prospective
studies have also shown an association between small, dense LDL and
the incidence of CAD. As a matter of fact, the presence of small LDL
particles precedes CAD42,43. The results from multivariate analyses
have been less consistent. Austin et al26 and Coresh et al29 reported
that LDL size was associated with CAD independent of other risk
factors, but not when triglyceride was included in the multivariate
models. Crouse et al27 and Campos et al28 reported similar findings
using different methods for measuring LDL-density fractions and
reported that LDL-III concentration was significantly associated with
CAD status after adjusting for triglyceride and other CAD risk factors.
Gardner et al\textsuperscript{43} reported that the case-control difference in LDL size was independent of other physiological CAD risk factors. Angiographic progression of CAD has also been shown to be associated with variations in the levels of IDL\textsuperscript{44,45}.

In animal models, particularly nonhuman primates, large, cholesteryl-ester-rich, apo E-containing IDL-like particles are most closely correlated with the extent of coronary atherosclerosis\textsuperscript{46}. Large, cholesteryl-ester-rich LDL have also been documented in spontaneously hypercholesterolemic pigs with a predisposition to developing CAD\textsuperscript{47}. Increased levels of cholesterol-rich β-VLDL have also been shown to be clearly atherogenic in other animal models of diet-induced atherosclerosis, such as rabbits and nonhuman primates as well as in human dysbetalipoproteinemia. It remains to be determined whether each of these atherogenic lipoprotein species promote different pathologic events in the development of atherosclerotic cardiovascular disease or that they share common features resulting in additive or overlapping effects on this process.

A predominance of small, dense LDL is commonly found in individuals with familial disorders of lipoprotein metabolism that are associated with an increased risk of premature CAD. These include familial combined hyperlipidemia\textsuperscript{48}, hyperapobetalipoproteinemia\textsuperscript{49}, and hypoalphalipoproteinemia\textsuperscript{50}. Thus, it is possible that the CAD risk in these syndromes is mediated in part by the metabolic changes associated with LDL subclass pattern B.
Small, Dense, Low-density Lipoprotein Phenotype

Pattern B is found in 30-35% of adult men, but is much lower in men less than 20 years of age. In premenopausal women, 5-10% have LDL subclass pattern B, with a slightly higher prevalence (15-25%) in postmenopausal women\textsuperscript{23,39,51}. These patterns have been found to be relatively stable, as evidenced by retention of LDL subclass patterns after 4 years in 75% of 268 subjects participating in a coronary disease intervention trial\textsuperscript{52}. Family studies indicate that pattern B is a genetically influenced trait\textsuperscript{24,53}. Multiple genes may contribute to the determination of LDL particle size, and the responsible genetic mechanisms may differ among affected families\textsuperscript{54}. Further investigations have suggested that linkage of the gene for LDL particle size with a locus on chromosome 19 near the LDL receptor gene\textsuperscript{55} as well as with other loci on chromosomes 11, 16, and 6\textsuperscript{56}. Estimates of heritability of LDL particle size as assessed by relative concordance in monozygotic versus dizygotic twins has indicated that genetic factors account for approximately half of the variation in LDL particle in both men\textsuperscript{57} and women\textsuperscript{58}, indicating the importance of nongenetic and environmental influences. Penetrance of the pattern B trait is strongly influenced by age and sex, with maximal expression after the age of 20 in men and after menopause in women\textsuperscript{24,53}. In addition to age and gender, effects on LDL particle size and density distribution have been shown with abdominal adiposity\textsuperscript{59}, presence of diabetes mellitus\textsuperscript{51,60,61}, oral contraceptive use\textsuperscript{62}, and hypertriglyceridemia occurring with acquired immunodeficiency syndrome\textsuperscript{63}. 

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There are strong associations between LDL subclass pattern B and a cluster of other CAD risk factors, consisting of glucose intolerance, hypertriglyceridemia, low HDL, and hypertension\textsuperscript{51,64-65}. These relationships persisted when differences in sex, age, and obesity were taken into consideration. This constellation of findings has been termed syndrome X and the underlying basis is postulated to be due to insulin resistance and hyperinsulinemia; however, the mechanism remains speculative at this time. A high prevalence of the small, dense LDL phenotype has been found in patients with non-insulin-dependent diabetes mellitus, and it is likely that this reflects the insulin resistance found in the majority of patients with this disease\textsuperscript{61,66}. In addition, a relative increase in LDL-III, in conjunction with increased levels of IDL, has been reported in patients with well-controlled insulin-dependent diabetes mellitus\textsuperscript{62}.

The association of small LDL particles with CAD thus appears to be consistent, prospective, graded, strong, and biologically plausible. These observations have led to the concept that LDL subclass pattern B can be viewed as a relatively discrete and stable marker for an atherogenic lipoprotein profile\textsuperscript{39,63}, and that the expression of this phenotype is influenced by multiple genetic and environmental factors. It is unclear yet whether small LDL particle size is an independent risk factor for CAD. Several studies have provided evidence which suggests that small LDL particle size and elevated triglyceride levels are linked through a causal pathway\textsuperscript{60,64-71}. Therefore, these may be 2 aspects of a common metabolic disorder; in which case, statistical independence is meaningless. In addition, interventions that lower triglyceride levels, such as weight reduction,
exercise, dietary change, and treatment with nicotinic acid, have also been shown to increase LDL size\textsuperscript{72-75}. Elevated triglyceride levels are also metabolically linked to other CAD risk factors, such as low HDL and insulin resistance\textsuperscript{64} as well as small, dense LDL\textsuperscript{76}. It remains to be determined the extent to which these metabolically related parameters are directly atherogenic vs high-risk markers and whether small LDL particles are directly atherogenic and causally related to CAD. However, such issues must be addressed using methods other than those employed in observational studies.

The biological plausibility of the epidemiologic association between LDL size and CAD has been supported through several mechanistic evidences from human, animal, and in vitro studies which suggest that smaller, more dense LDL particles are relatively more atherogenic than larger, more buoyant particles, including the greater susceptibility of smaller LDL particles to oxidative degradation in vitro\textsuperscript{77-79}, a lower fractional catabolic rate\textsuperscript{80}, a diminished affinity for LDL receptors, a longer period of residence in the plasma compartment\textsuperscript{81-84}, the modification of apo B\textsuperscript{85}, and an increased rate of binding to arterial proteoglycans\textsuperscript{86}.

A number of different intravascular enzymes are involved in the lipoprotein cascade leading to LDL formation. These lipoprotein-processing proteins include lipoprotein lipase, hepatic lipase, lecithin-cholesterol acyltransferase, and cholesterol ester transfer protein.

A reduced activity of lipoprotein lipase (LPL) has been found in individuals expressing the pattern B trait\textsuperscript{87}, and patients with heterozygous LPL deficiency have a lipoprotein phenotype that appears identical to that described in pattern B individuals\textsuperscript{88}. In
these individuals, reduced exogenous triglyceride clearance is independent of the fasting triglyceride level, suggesting that one or more factors responsible for retardation of triglyceride-rich lipoprotein metabolism may have an etiologic or contributory role in a high proportion of individuals with the small, dense LDL phenotype.

Hepatic lipase (HL) appears to be synthesized by the liver cells and bound to the luminal surface of the endothelial cells of the liver where the remodeling of small VLDL and IDL to LDL may take place. The strongest evidence supporting the role of HL in LDL formation comes from studies performed on human and animal models with HL deficiency. In these studies, low HL activity was associated with larger and more buoyant LDL particles, often extending into the IDL density range, whereas increased HL activity is associated with a shift to smaller and denser LDL particles, and a reduced LDL-free cholesterol content. This finding is observed in both normolipidemic men and those with CAD. Furthermore, both CAD and normolipidemic individuals with pattern B have higher HL activity than pattern A individuals, suggesting that HL may be involved in the expression of the LDL subclass patterns.

Cholesterol ester transfer protein (CETP) activity influences the distribution of the LDL subclasses. In patients with homozygous familial CETP deficiency, accumulation of a smaller, triglyceride-rich LDL subpopulation is found with a density distribution that overlaps with the predominant, larger LDL species. A similar phenomenon occurs in individuals with an alcohol-induced reduction in CETP activity. Following alcohol withdrawal, the particle distribution
becomes more homogeneous and the levels of CETP normalize\textsuperscript{97}. Recent evidence has indicated that a primary acceptor for CETP-mediated HDL cholesteryl ester transfer in normolipidemic individuals is a large, buoyant, triglyceride-enriched LDL subclass\textsuperscript{98}. Thus, it is possible that retention of such triglyceride-rich LDL and subsequent lipolytic processing contributes to the heterogeneous LDL subclass profile found in patients with CETP deficiency.

Although these metabolic factors appear to be important overall determinants of the LDL particle distribution, available evidence also implies that major aspects of LDL heterogeneity result from the production of differing forms of LDL from different VLDL and IDL precursors. At least two distinct IDL subspecies have been identified in normal humans that appear to be precursors of large (LDL-I) and intermediate (LDL-II) LDL subclasses, respectively\textsuperscript{99,100}. Parallel processing of particles in these two pathways by triglyceride enrichment and lipolysis may then give rise to smaller, denser species (LDL-III and LDL-IV). Two major LDL production pathways have been demonstrated in the pig, and evidence has been provided for a genetic determinant of the selective overproduction of the larger, more buoyant species of LDL, in a spontaneously hypercholesterolemic strain of pigs\textsuperscript{101}.

Lipoprotein transport is also controlled by lipoprotein receptors that recognize lipoproteins and mediate their cellular uptake and catabolism (LDL receptor, chylomicron remnant receptor, and the scavenger receptor). It is not known to what extent differences in receptor-mediated clearance of the LDL subclasses contribute to variations in the plasma LDL particle distribution. Differences in the
receptor-binding affinity of the various LDL subfractions have been reported, but the results have not been consistent\textsuperscript{102-106}. Swinkels et al\textsuperscript{102} found no differences in the receptor binding of LDL subfractions to LDL receptors on fibroblasts and HepG2 cells, but two other studies found that both buoyant (d=1.024 to 1.037 g/mL) and dense (d=1.036 to 1.047 g/mL) LDL subfractions had relatively reduced LDL receptor binding affinity compared with intermediate-density LDL subfractions (d=1.027 to 1.041 g/mL)\textsuperscript{104,106}. The weaker binding affinities of large, buoyant and small, dense LDL compared with LDL of medium size and density may have complex metabolic consequences that affect the atherogenicity of these particles. Decreased binding may be associated with increased plasma residence time and therefore possibly greater opportunity for potentially atherogenic intravascular and cellular modifications. Still other studies have found greater binding affinity for buoyant LDL subfractions (d=1.024 to 1.033 g/mL) compared with both medium and dense LDL subfractions (d=1.028 to 1.045 g/mL)\textsuperscript{103-105}. Campos et al\textsuperscript{107} found that the LDL receptor binding affinity was significantly greater in subjects with a high LDL levels compared with those with a normal LDL concentration. In addition, in individuals with normal LDL levels (\(<160 \text{ mg/dL})\), both large LDL-I and small LDL-III particles showed reduced binding affinity\textsuperscript{107}. In contrast, LDL-I and LDL-III did not show a reduced LDL receptor binding affinity in those individuals who had elevated LDL levels (\(\geq160 \text{ mg/dL}\)). Furthermore, the reduction in receptor-binding affinity for LDL-III relative to LDL-II was similar in pattern A and B subjects, while the binding affinity for LDL-I was significantly greater for pattern B.
Differences in nonreceptor-mediated LDL clearance among the LDL subpopulations may also contribute to variations in the LDL particle distribution.

Rabbits As Animal Models of Atherosclerosis

Many animals have been used in the past to study atherosclerosis, including nonhuman primates, pigs, rabbits, and mice. We chose the rabbit for our experimental animal model of atherosclerosis.

In normal rabbits, most of the VLDL and IDL are taken up by the liver and only a small fraction is converted to LDL. Consequently, they do not develop atherosclerosis. In 1908, Ignatowski\textsuperscript{108} first observed that a diet of milk, meat, and eggs produced atherosclerotic lesions in rabbits. Five years later, Anitschkow and Chalatow\textsuperscript{109} demonstrated that cholesterol was the atherogenic component of the diet. They induced atherosclerotic lesions in cholesterol-fed rabbits thereby establishing the rabbit as an animal model of atherosclerosis.

Traditionally, rabbits were fed a high level of cholesterol (2% to 4% by weight) to achieve rapid lesion formation. However, this resulted in a lipoprotein profile in rabbits which bears little resemblance to those seen in humans with atherosclerotic disease\textsuperscript{110-114}. In addition to exceedingly high total plasma cholesterol levels (> 1000 mg/dl), almost all of the cholesterol in the plasma of cholesterol-fed rabbits was found in β-VLDL particles, whereas in humans it is found primarily in the LDL particles. Furthermore, the atherosclerotic lesions which developed in these cholesterol-fed
rabbits were morphologically different from those seen in humans\textsuperscript{110-114}.

Several studies have reported that more human-like atherosclerotic lesions could be produced by reducing the level of dietary cholesterol (0.2\% to 2\% by weight) in New Zealand White\textsuperscript{115-117}, Chinchilla\textsuperscript{118}, and Dutch Belted rabbits\textsuperscript{119,120}. These lesions were shown to contain the large numbers of smooth muscle cells, extracellular matrix deposition, and cholesterol crystals that are typically seen in human lesions. Moreover, a cholesterol-free, casein-enriched diet induced an endogenous hypercholesterolemia that also resulted in atherosclerotic lesion formation\textsuperscript{121}. Furthermore, Daley et al showed that rabbits fed either a low level cholesterol diet or a casein diet formed the full spectrum of atherosclerotic lesions seen in humans, ranging from the classic early fatty streak to the more advanced atheromatous lesion\textsuperscript{122}. However, the cholesterol-fed rabbits had, in general, more advanced lesions than casein-fed rabbits with matched total plasma cholesterol levels. In addition, the entire spectrum of atherosclerotic lesions seen in humans, ranging from the classic early fatty streak to fibrous plaques to the more advanced atheromatous lesions, could be seen in rabbits as early as 6 months by the simple feeding regimen of a low-level cholesterol diet (0.125\% to 0.5\% by weight)\textsuperscript{122}.

Daley et al\textsuperscript{123} has shown that despite the similarity in lesion morphologies in cholesterol-fed and casein-fed rabbits with equal total plasma cholesterol levels, the excess plasma cholesterol in cholesterol-fed rabbits is equally distributed in VLDL, IDL, and LDL fractions while in casein-fed rabbits is present mainly in the LDL
fraction similar to that seen in humans. For this reason, the casein-fed rabbit has been used extensively in biochemical studies of lipoprotein metabolism. Furthermore, these results suggest that the rate of lesion formation (or lesion extent) is influenced by the major class of lipoprotein that carries cholesterol.

Other studies, however, have reported a primarily β-VLDL hypercholesterolemia in the cholesterol-fed rabbit\textsuperscript{124}. The discrepancy between these results and those of Daley et al\textsuperscript{123} may be related the level of total plasma cholesterol. Brattsand\textsuperscript{125} has shown that VLDL is the major carrier of cholesterol in cholesterol-fed rabbits only when the total plasma cholesterol levels are greater than 800 mg/dl. Moreover, with increasing total plasma cholesterol levels (up to 1400 mg/dl), VLDL carries an increasing percentage of cholesterol. In addition, Schwenke and Carew\textsuperscript{126} reported that with total plasma cholesterol levels of 250 to 600 mg/dl, LDL in cholesterol-fed rabbits carries 28% of the total plasma cholesterol.

Other studies have provided additional evidence that the rabbit is a suitable model for studying the regulation of lipoprotein metabolism, as related to human health and disease. As in human, rabbit VLDL, IDL, and LDL contain almost entirely apo B-100 and little if any B-48\textsuperscript{127}. The rabbit liver secretes little if any apo B-48\textsuperscript{128}. Furthermore, like the human, the rabbit has an active mechanism for transferring core lipids among lipoproteins\textsuperscript{129}. Finally, the rabbit converts an appreciable fraction of apo B-100 of VLDL to LDL and the extent of conversion is subject to regulation\textsuperscript{130}. In all these respects the rabbit seems to be a more suitable model for the study of human lipoprotein metabolism than the rat\textsuperscript{130}. 

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Only a few groups have characterized the lipoprotein profile of rabbits. The most extensive characterization involved separating lipoproteins into three fractions: VLDL, IDL, and LDL. However, it is now known that there is heterogeneity with each lipoprotein family. For example, LDLs are not a single chemical entity but are more accurately a family of particles that can be classified further as subforms of LDLs which differ in density, size, and chemical composition\textsuperscript{131-136}. As discussed previously, LDL heterogeneity is important because LDL size was found to be a strong and independent predictor of coronary artery disease status. LDL subclass pattern B is associated with up to a threefold increase in the susceptibility to developing coronary artery disease. Heterogeneity of human VLDL and IDL have also been demonstrated. Some forms of VLDL may be at least as atherogenic under some circumstances as the LDLs. In light of the differing physiological and pathological properties of individual lipoprotein subclasses, we felt that an analysis of levels and distributions of these subclasses can add considerably to the information provided by standard measurements of plasma cholesterol or LDL. Therefore, we investigated the effects of atherogenic diets on lipoproteins by characterizing the lipoprotein profile of cholesterol-fed and casein-fed rabbits using gradient gel electrophoresis, a technique to examine lipoprotein heterogeneity as a function of size and shape of lipoprotein particles. It is our goal to create an animal model to study the effects of LDL heterogeneity on lipoprotein metabolism and atherosclerosis.
METHODS

Animals and Diets

Male New Zealand White rabbits, six months of age, were used in the study. They were maintained on the chow diet (Dyets, Bethlehem, Pennsylvania) prior to switching to the atherogenic diets. Food and water were provided ad libitum.

Cholesterol Diet

Two male New Zealand White rabbits were fed a low-level cholesterol diet for 20 days. The cholesterol diet was composed of the chow diet mixed with 0.5% cholesterol. The chow diet is a base whose composition is never uniform.

Casein Diet

Four male New Zealand White rabbits from a different strain than the cholesterol-fed rabbits were gradually transferred over a period of 5 days from the chow diet to a low-fat, cholesterol-free, semipurified diet containing casein (Table 1). They were fed the casein diet for 8 weeks.

Casein+Fructose Diet

Four male New Zealand White rabbits were fed the casein+fructose diet. Three of the four rabbits were the same ones who participated in the casein diet experiment described above. A new fourth rabbit was added to this part of the study because the original rabbit was not in his optimal health condition after the casein diet experiment.

After eight weeks of casein diet feeding, the rabbits were switched over to the chow diet until plasma cholesterol levels fell
back to baseline. Subsequently, they were gradually transferred over a period of 5 days to the same casein diet except for the addition of fructose to the diet as the source of carbohydrate instead of cornstarch (Table 2). The rabbits were maintained on the casein+fructose diet for 8 weeks.

**Plasma Samples**

After the rabbits were fasted for 12 to 14 hours, between 6 to 10 ml of blood was collected from the ear vein into tubes containing disodium ethylene diaminetetraacetate (EDTA) at a final concentration of 1.5 mg/ml of blood. The tubes were immediately placed on ice, and the plasma was separated by low-speed centrifugation at 1500 rpm for 30 min at 4°C.

**Density Gradient Ultracentrifugation**

Major lipoprotein classes were isolated from the plasma by sequential preparative ultracentrifugation under standard conditions. Briefly, the plasma was adjusted to $d = 1.019 \text{ g/ml}$ with sodium bromide solution and centrifuged at 40,000 rpm for 20 hr at 10°C in a Beckman 40.3 rotor. Lipoprotein fraction of $d < 1.019 \text{ g/ml}$ was withdrawn from the top 2 ml and the subnatant fluid was adjusted to $d = 1.063 \text{ g/ml}$ with sodium bromide solution. After centrifugation of this mixture at 10°C for 24 hr at 40,000 rpm in a Beckman 40.3 rotor, the lipoprotein fraction of $d = 1.019 - 1.063 \text{ g/ml}$ was withdrawn from the top 1 ml and the next 1 ml was decanted. The subnatant was adjusted to $d = 1.210 \text{ g/ml}$ with sodium bromide solution and centrifuged at 40,000 rpm for 24 hr at
10°C in a Beckman 40.3 rotor. Lipoprotein fraction of $d = 1.063 - 1.210 \text{ g/ml}$ was withdrawn from the top 1 ml. Sodium bromide solutions used to adjust densities were prepared by adding appropriate amounts of solid NaBr to a base $d = 1.0063 \text{ g/ml}$ solution, consisting of 11.422 g/liter NaCl and 0.01% EDTA. The final pH was adjusted to 7.4 with 10 M NaOH, and densities were checked with a DMA 46 mettler/Paar density meter.

The isolated lipoprotein fraction of $d < 1.019 \text{ g/ml}$ was dialyzed to $d = 1.2100 \text{ g/ml}$ in NaBr solution at 4°C and subjected to nonequilibrium density gradient ultracentrifugation, as described in detail elsewhere\textsuperscript{138}. Briefly, a 4.5 ml sample was placed in a 12 ml Beckman SW41 rotor centrifuge tube (Beckman Instruments, Mountain View, California) and was sequentially overlaid with 3.0, 3.0, and 1.5 ml, respectively, of solutions of densities 1.020, 1.010, and 1.000 g/ml. The tubes were centrifuged for 6 hours in a Beckman SW41 swinging bucket rotor at 40,000 rpm and 22°C. Following centrifugation, the contents of the tube were withdrawn by pipetting two consecutive 0.5 ml fractions followed by three 1.0 ml fractions then four 0.5 ml fractions, designated 1 through 8 in order from the top of the tube. The first and second 0.5 ml fractions were designated 1a and 1b, respectively.

The isolated LDL fraction of $d = 1.019 - 1.063 \text{ g/ml}$ was dialyzed to $d = 1.0400 \text{ g/ml}$ in NaBr solution at 4°C and subjected to equilibrium density gradient ultracentrifugation, as described in detail elsewhere\textsuperscript{139}. Briefly, 2 ml of dialyzed LDL was layered carefully above 2.5 ml of NaBr solution of $d = 1.0540 \text{ g/ml}$ in a 7 ml Beckman SW45 rotor centrifuge tube (Beckman Instruments,
Mountain View, California), and 2.5 ml of NaBr solution of d = 1.0275 g/ml was layered above the LDL. The tubes were then centrifuged at 40,000 rpm for 40 hr in a Beckman SW 45 swinging bucket rotor at 17°C. Following centrifugation, the contents of the tube were withdrawn by sequentially pipetting the top 0.5 ml fraction, one 1.0 ml fraction, six 0.5 ml fractions, two 1.0 ml fractions, and the bottom 0.5 ml fraction, designated 0 through 10 in order from the top of the tube.

**Polyacrylamide Gradient Gel Electrophoresis**

Electrophoresis of plasma samples or lipoprotein fractions was performed on 2% to 16% polyacrylamide gradient gels (Pharmacia PAA 2/16, Piscataway, New Jersey) for 24 hours at 125 V in Tris (0.09 M)-boric acid (0.08 M)-Na$_2$ EDTA (0.003 M) buffer (pH 8.3) as described elsewhere$^{186}$. Gels were stained for proteins in a solution containing 0.1% Coomassie brilliant blue R-250, 50% ethanol, and 9% acetic acid (vol/vol), or for lipid with oil red O in 60% ethanol at 55°C$^{140}$. Gels were scanned at 555 nm for Coomassie blue staining or 530 nm for oil red O staining using a Transidyne RFT densitometer. Migration distances for each absorbance peak were determined and the molecular diameter corresponding to each peak was calculated from a calibration curve generated from the migration distances of standards of known diameter. These included carboxylated latex beads (Dow Chemical), thyroglobulin dimer, thyroglobulin, and apoferritin (Pharmacia) with molecular diameters of 380 Å, 236 Å, 170 Å, and 122 Å, respectively. The standard deviation for measurement of particle diameter (eight determinations on four
separated gel runs of an individual LDI sample) ranged from 2.0 Å to 2.8 Å (coefficient of variation, 0.8% to 1.0%)\textsuperscript{140}. 

**Chemical Composition Determination**

Plasma cholesterol and triglyceride concentrations were measured using enzymatic methods on a System 3500 Gilford Computer Directed Analyzer (Gilford Instruments, Oberlin, OH). Assay reagents used for the cholesterol assays were from Boehringer-Mannheim (Palo Alto, CA) and assay reagents for triglyceride were from Worthington Biochemical Corporation (Freehold, NJ).

**RESULTS**

The plasma cholesterol levels in the cholesterol diet, casein diet, and casein+fructose diet over time are shown in Figure 1. The plasma triglyceride levels in the cholesterol diet, casein diet, and casein+fructose diet over time are shown in Figure 2.

**Chow Diet**

On the chow diet, all rabbits showed a major peak with a particle diameter ranging from 271 to 277 Å. Two of the rabbits also contained a second peak with a particle diameter of 262 Å for one of the rabbit and 259 Å for the other rabbit. When the rabbits were put on chow for the second time, the major peak decreased in size slightly with a particle diameter from 253 to 267 Å which corresponded to the second major peak from the first time chow was
given. In addition, a second peak with particle diameter of 351 to 355 Å appeared in all the rabbits, which only appeared in one the rabbits the first time chow was given. Densitometric scan of representative gels for two chow-fed rabbits are shown in Figure 3.

**Cholesterol Diet**

Lipoprotein species in the plasma samples were characterized by 2% to 16% GGE. Densitometric scans of representative gels from one of the rabbits for day 3, 6, and 20 on the cholesterol diet are shown in Figures 4a and 4b. The findings for the other rabbit was similar. After 3 day on the cholesterol diet, the gradient gel electrophoretograms of whole plasma for one of the rabbits showed one major peak with particle diameters in the size range of large LDL to small IDL (279 Å). The other rabbit showed two overlapping peaks of 276 and 285 Å. Multiple smaller peaks of larger diameter in the size range of large IDL to VLDL were also present in both rabbits. The amount of the predominant species continued to diminish in mass and eventually increased slightly in size to 292 and 294 Å by day 20, while at the same time, the magnitude of the peaks of the larger-diameter lipoprotein species increased dramatically in mass, with the particle diameter measuring 368 and 380 being the predominant species for the two rabbits by day 20.

**Casein Diet**

**Whole Plasma**

Lipoprotein species in the plasma samples were characterized by 2% to 16% GGE. Densitometric scans of representative gels from
one of the rabbits for week 1, 3, 4, and 8 on the casein diet are shown in Figure 5a and 5b. The findings for the three other rabbits were similar. After 1 week on the casein diet, the gradient gel electrophoretograms of whole plasma showed one discrete peak with particle diameters in the size range of large LDL to small IDL (277 to 279 Å) in variable proportions in all but one of the rabbits which had an additional shoulder at 271 Å. The amount of this species increased dramatically over the following 7 weeks with a slight increase in its particle diameter (281 to 288 Å). By the end of the eighth week on the casein diet, multiple peaks of larger diameter were also present, with particle diameters ranging between 293 to 422 Å.

Lipoprotein Fractions

Eighth week plasma samples were fractionated by sequential ultracentrifugation at d = 1.019 g/ml, d = 1.063 g/ml, and d = 1.210 g/ml. Examples of such separations are shown in Figure 6. The VLDL and IDL fraction (d < 1.019) contained multiple peaks of diameter > 310 Å in variable proportions. The LDL fraction (1.019 < d < 1.063 g/ml) showed a predominant peak with particle diameter ranging between 276 to 289 Å with a shoulder at 298 to 304 Å.

VLDL and IDL Subfractions

Nonequilibrium density gradient ultracentrifugation of the d < 1.019 g/ml lipoproteins isolated from eighth week plasma was performed as described in Methods, and the fractions were analyzed by 2% to 16% GGE. For the most part, fraction 1a through 5 each contained a major peak of progressively smaller particle diameter, ranging from 375 to 310 Å. In addition, they showed a variable
number of peaks of greater particle diameters. Fraction 6-8 contained very little lipoproteins.

**LDL Subfractions**

Equilibrium density gradient ultracentrifugation of the 1.019 < d < 1.063 g/ml lipoproteins isolated from eighth week plasma was performed as described in Methods, and the fractions were analyzed by 2% to 16% GGE. Most of the 1.019 < d < 1.063 g/ml lipoproteins were in the top 4 fractions (fraction 0-3). The size range of the major subspecies in fractions 0-3 (269 to 291 Å) corresponded to that of the predominant, larger subspecies seen in ultracentrifugal fractionation. GGE showed very little amount of lipoprotein in fractions 4-8. Two of the rabbits showed a large peak in fraction 9 or 10 with a particle diameter of 273 to 287 Å.

**Casein+Fructose Diet**

**Whole Plasma**

Lipoprotein species in the plasma samples were characterized by 2% to 16% GGE. Densitometric scans of representative gels from one of the rabbits for week 1, 4, and 8 on the casein+fructose diet are shown in Figure 7a and 7b. The findings for the three other rabbits were similar. After 1 week on the diet, the gradient gel electrophoretograms of whole plasma showed either one or two discrete, small peaks in the size range between 275 to 268 Å. By week 4, a single, major peak with particle diameters in the size range of large LDL to small IDL (275 to 283 Å) appeared in all. Over the following four weeks, this predominant species continued to increase in mass, with multiple peaks of larger diameter appearing in some.
In all cases, the predominant species corresponded to peaks of similar size on electrophoretograms of whole plasma from week one. **Lipoprotein Fractions**

Eighth week plasma samples were fractionated by sequential ultracentrifugation at \(d = 1.019 \text{ g/ml}, \ d = 1.063 \text{ g/ml}, \) and \(d = 1.210 \text{ g/ml.}\) Examples of such separations are shown in Figure 8. The VLDL and IDL fraction \((d < 1.019)\) contained multiple peaks of diameter > 270 Å in variable proportions. The LDL fraction \((1.019 < d < 1.063 \text{ g/ml})\) showed a major peak with particle diameter ranging between 283 to 291 Å. In addition, the electrophoretograms of the LDL fraction revealed a smaller peak of particle diameter 270 Å in variable proportions in all but one of the rabbits, regardless of whether a discrete peak was present on GGE of whole plasma. **VLDL and IDL Subfractions**

Nonequilibrium density gradient ultracentrifugation of the \(d < 1.019 \text{ g/ml}\) lipoproteins isolated from eighth week plasma was performed as described in Methods, and the fractions were analyzed by 2% to 16% GGE. Fraction 1a and 1b showed multiple peaks of variable proportions with particle diameters > 340 Å. For the most part, fraction 2 through 7 each contained a relatively homogeneous lipoprotein band of progressively smaller particle diameter, ranging from 355 to 297 Å. Fraction 8 contained very little lipoproteins. **LDL Subfractions**

Equilibrium density gradient ultracentrifugation of the \(1.019 < d < 1.063 \text{ g/ml}\) lipoproteins isolated from eighth week plasma was performed as described in Methods, and the fractions were analyzed by 2% to 16% GGE. Most of the \(1.019 < d < 1.063 \text{ g/ml}\) lipoproteins...
were in the top 3 fractions (fraction 0-2). The size range of the major subspecies in fractions 0-2 (277 to 300 Å) corresponded to that of the predominant, larger subspecies seen in ultracentrifugal fractionation. Fractions 3 contained a peak of particle diameter 273 to 275 Å which corresponds to the smaller peak in the 1.019 < d < 1.063 g/ml ultracentrifugal fraction. Fraction 4 contained only small amounts of lipoprotein, of similar size to that in fraction 3. GGE showed no lipoprotein in fractions 4-10.

DISCUSSION

Chow Diet

Chow is the standard, cholesterol-free food given to lab rabbits. Rabbits do not develop atherosclerosis on this diet. What the GGE scans demonstrate is that there is heterogeneity of the LDL particles in rabbits, like humans. More specifically, there appears to be two forms of LDL, which are produced in variable amounts in different rabbits. These two LDLs are similar to human LDL-I and either LDL-II or III. In most rabbits, however, only one LDL form is present in plasma at any one time. A question to be addressed in a future study is why is there variation in distribution? Studies on humans seem to suggest that the individual differences may be due to genetics.

A small VLDL appeared in all the rabbits with the smaller LDL form. Because they appear together, this small VLDL may in fact be the precursor for the smaller LDL.
Cholesterol Diet

Plasma cholesterol level increased dramatically with cholesterol-feeding over time. Previous studies on rabbits have shown that with continued cholesterol-feeding, rabbit plasma cholesterol level will continue to rise to levels exceeding 1000 mg/dl. Hypercholesterolemia in the cholesterol-fed rabbit is the result of an accumulation of cholesterol of exogenous origin. These rabbits respond with a decrease in cholesterol synthesis\(^{141}\), an enhanced cholesterol-enriched VLDL (\(\beta\)-VLDL) production\(^{142}\), and most importantly, down-regulation of LDL receptors\(^{143}\). Down-regulation of LDL receptors causes a delayed uptake of VLDL remnants via this receptor and subsequently a marked rise of plasma VLDL levels\(^{142}\).

After 3 days on the cholesterol diet, the rabbits showed either one or two forms of large LDL. Multiple VLDL forms were also present. Over time, the amount of the LDLs continued to decrease while its diameter increased slightly to that of a small IDL. At the same time, the plasma level of the multiple VLDL forms increased dramatically. We hypothesized that these changes in the lipoprotein profile with cholesterol-feeding may be accounted for by the liver production of lipoproteins that are so cholesterol-enriched (\(\beta\)-VLDLs) that there are too little triglycerides left in the lipoprotein for the lipoprotein lipase and hepatic lipase to work on to make smaller particles. That is why at with cholesterol-feeding over time, we see a shift to the larger lipoprotein particles.
Casein Diet

Casein is a protein that is found in dairy products. The plasma cholesterol level increased over time with cholesterol-free, casein-enriched diet. However, the rate of increase is much less than that of cholesterol-feeding. The mechanism by which hypercholesterolemia is induced in the casein-fed rabbit involves a reduction in fecal bile acid excretion.\textsuperscript{144}

After one week on the casein diet, we see one large LDL form. The amount of this LDL increased dramatically over the following 7 weeks with a slight increase in its particle diameter to that of a small IDL. By the end of the eighth week on the casein diet, multiple VLDL forms were also present. These changes in the lipoprotein profile with casein-feeding may be accounted for by the liver responding to the casein diet with an increased production of VLDL, an increase in the direct synthesis of LDL, and delayed uptake of VLDL remnants (due to downregulation of hepatic LDL receptors).\textsuperscript{144} These VLDL remnants are subsequently converted to LDL. Most of the LDLS which accumulate in the plasma with casein-feeding over time is from the conversion of VLDL remnants to LDL with a small contribution from the direct LDL synthesis from the liver.

Previous studies using the traditional procedures for detecting and measuring lipoproteins have found that plasma cholesterol in casein-fed rabbits is present mainly in the LDL fraction, similar to that seen in humans. For this reason, the casein-fed rabbit has been used extensively in biochemical studies of lipoprotein metabolism. Our study shows that the LDLS produced in casein-fed rabbits are mainly large LDLS.
When the lipoprotein profiles of rabbits from the cholesterol and casein diets with similar plasma cholesterol levels were compared, the lipoprotein profiles from the two diets were similar. In other words, since the plasma cholesterol level increased at a much slower rate in casein-fed rabbits, the changes in the lipoprotein profile occurred at a slower rate when compared to the cholesterol-fed rabbits.

Casein+Fructose Diet

Both the cholesterol-fed and casein-fed rabbits produced large LDLs. The next step we took was to attempt to induce rabbits to make smaller LDLs in order to create an animal model to study LDL subclass pattern B in the future. We hypothesized that the lipoproteins produced by the rabbits on the atherogenic diets were so cholesterol-enriched that there were too little triglyceride left for the lipoprotein lipase and hepatic lipase to work on to make smaller particles. We decided to see what would happen if a triglyceride-raising agent was added to the casein diet. In previous research, fructose have been found to increase triglyceride levels in rabbits (personal communication with David Kritchevsky).

Plasma cholesterol level increased over time with casein+fructose-feeding. The cholesterol level is comparable to that of the casein diet. The addition of fructose to the casein diet only raised plasma triglyceride level slightly from 26 to 52 mg/dl.

After one week on the diet, either one or two large LDL forms was present. By week 4, a single large LDL became the predominant species. Over the following four weeks, the level of the large LDL
continued to increase, with multiple VLDL forms present. Adding fructose to the casein diet did not change the lipoprotein profile in rabbits.

The multiple forms of VLDL particles in both casein-fed and casein+fructose-fed rabbits can be divided into 3 groups. The first group consists of the large VLDL particles with diameter > 400 Å. The second group consists of the medium VLDL particles with diameters between 400 to 330 Å. The third group consists of the small VLDL particles with diameters between 330 to 300 Å.

CONCLUSION

These results may have implications for the use of rabbits as an animal model of atherosclerosis. Our characterization of the lipoprotein profile in the rabbits demonstrated for the first time that there is heterogeneity within each lipoprotein family like in humans. Furthermore, the proportion of each lipoprotein subclass within a lipoprotein family is subject to change with diet. With atherogenic diets, the rabbits tend to produce more of the larger subspecies within each lipoprotein family. This may be of importance in the face of the differing physiological and pathological properties of individual lipoprotein subclasses as seen in humans. In addition, there are also individual variations like in humans. This study supports the use of rabbits in the study of atherosclerosis, in particular as a model for pattern A.
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>sucrose</td>
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<tr>
<td>cornstarch</td>
<td>20.20%</td>
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<table>
<thead>
<tr>
<th>Protein</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>corn</td>
<td>1.00%</td>
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<tr>
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</tr>
<tr>
<td>Choline bitartrate</td>
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</tr>
<tr>
<td>Vitamins</td>
<td>1.00%</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.30%</td>
</tr>
<tr>
<td>Cellulose</td>
<td>15.00%</td>
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Table 2. Casein+Fructose Diet Composition

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<tbody>
<tr>
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</tr>
<tr>
<td>Cellulose</td>
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</table>
Figure 1. Plasma cholesterol levels of casein, fructose (casein+fructose), and cholesterol diets over time.
Figure 2. Plasma triglyceride levels of casein, fructose (casein+fructose), and cholesterol diets over time.
Figure 3. Densitometric scans of 2-16% gradient gels of plasma from chow-fed rabbits. Human LDL subspecies (LDL-I through LDL-IV) diameter range are included for comparison.
Figure 4a. Densitometric scans of 2-16% gradient gels of plasma from cholesterol-fed rabbits. Human LDL subspecies (LDL-I through LDL-IV) diameter range are included for comparison.
Figure 4b. Densitometric scans of 2-16% gradient gels of plasma from cholesterol-fed rabbits. Human LDL subspecies (LDL-I through LDL-IV) diameter range are included for comparison.
Figure 5a. Densitometric scans of 2-16% gradient gels of plasma from casein-fed rabbits. Human LDL subspecies (LDL-I through LDL-IV) diameter range are included for comparison.
Figure 5b. Densitometric scans of 2-16% gradient gels of plasma from casein-fed rabbits. Human LDL subspecies (LDL-I through LDL-IV) diameter range are included for comparison.
**VLDL and IDL Fraction**

![Graph of VLDL and IDL Fraction]

**Lipoproteins (Diameter Å)**

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<tr>
<th>Fraction</th>
<th>Diameter (Å)</th>
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<tbody>
<tr>
<td>VLDL</td>
<td>&gt; 300</td>
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<tr>
<td>IDL</td>
<td>300-280</td>
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<tr>
<td>LDL</td>
<td>280-220</td>
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<table>
<thead>
<tr>
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<th>Diam</th>
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<tbody>
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<tr>
<td>2</td>
<td>423.0</td>
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<tr>
<td>3</td>
<td>410.0</td>
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<tr>
<td>4</td>
<td>361.8</td>
</tr>
<tr>
<td>5</td>
<td>320.3</td>
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**LDL Fraction**

![Graph of LDL Fraction]

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<th>Diam</th>
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<td>3</td>
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Figure 6. Densitometric scans of 2% to 16% gradient gels of VLDL/IDL fraction obtained by nonequilibrium density gradient ultracentrifugation of \(d < 1.019\) g/ml lipoproteins and IDL fraction obtained by equilibrium density gradient ultracentrifugation of \(1.019 < d < 1.063\) g/ml lipoproteins from casein-fed rabbits.
Figure 7a. Densitometric scans of 2-16% gradient gels of plasma from casein+fructose-fed rabbits. Human LDL subspecies (LDL-I through LDL-IV) diameter range are included for comparison.
Figure 7b. Densitometric scans of 2-16% gradient gels of plasma from casein+fructose-fed rabbits. Human LDL subspecies (LDL-I through LDL-IV) diameter range are included for comparison.
Figure 8. Densitometric scans of 2% to 16% gradient gels of VLDL/IDL fraction obtained by nonequilibrium density gradient ultracentrifugation of $d < 1.019$ g/ml lipoproteins and IDL fraction obtained by equilibrium density gradient ultracentrifugation of $1.019 < d < 1.063$ g/ml lipoproteins from casein-fructose-fed rabbits.
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