

Study on cholesterol metabolism in lifestyle diseases ameliorated by food functional ingredients

袁, 興宇

<https://hdl.handle.net/2324/5068274>

出版情報 : Kyushu University, 2022, 博士 (農学), 課程博士
バージョン :
権利関係 :



Study on cholesterol metabolism in
lifestyle diseases ameliorated by food
functional ingredients

YUAN XINGYU

2022

Study on cholesterol metabolism in lifestyle diseases regulating by food functional ingredients

Contents

CHAPTER I	Introduction	1
CHAPTER II	α-Globulin-rich rice cultivar, low glutelin content-1 (LGC-1), decreases serum cholesterol concentration in exogenously hypercholesterolemic rats	18
CHAPTER III	The effects of dietary linoleic acid on atherosclerosis development and aortic inflammation in different dietary cholesterol levels	38
CHAPTER IV	The effect of egg yolk phosphatidylcholine on the biomarker expression of Alzheimer's disease through regulating oxysterol and cholesterol metabolism	57
CHAPTER V	Summary and Conclusions	83
ACKNOWLEDGEMENTS.....		86
LITERATURE CITED.....		87

CHAPTER I Introduction

1.The characteristics and evolution of lifestyle diseases

Lifestyle diseases can be defined as diseases linked with one's lifestyle and are also known as non-communicable diseases (NCDs) which are chronic and cannot be communicated from one person to another [1]. The following four points summarize the current characteristics of lifestyle diseases [1]. Firstly, the pathogenesis of lifestyle diseases is highly complex and diverse. Most are related to natural factors such as lack of physical activity, unhealthy dietary habits, alcohol, substance use disorders, and global population aging. Secondly, several risk factors lead to the development of lifestyle diseases. They can be mainly divided into three primary risk factors: modifiable risk factors, non-modifiable risk factors, and metabolic risk factors. Modifiable risk factors include smoke, physical inactivity, unhealthy dietary habits, and disturbed biological clock [2]. An epidemiological survey reports that occupational environment and workplace stress are also seen as potential risk factors for the aggravation of lifestyle diseases [3]. Non-modifiable risk factors include age, ethnic background, family history of genetic predisposition, and gender [3]. Metabolic risk factors contain increased levels of triglyceride (TG), cholesterol and glucose in the blood, obesity, and high blood pressure [3]. These factors are considered direct causative or pathological features for several lifestyle diseases. Thirdly, lifestyle diseases are almost long-duration and cause disability or functional impairment. Patients are forced to undergo nursing and medical treatment in their later stages of the disease [4]. This characteristic poses a tremendous burden on labor and medical resources. Fourthly, lifestyle diseases are of non-contagious origin and cannot disseminate in population or country. Therefore, lifestyle diseases do not cause the spread of organisms and global epidemics.

Pathological and epidemiological studies define several lifestyle diseases, including Alzheimer's disease, hypercholesterolemia, atherosclerosis, asthma, arthritis, chronic liver disease or cirrhosis, colitis, irritable bowel syndrome, type 2

diabetes, heart disease, hypertension, metabolic syndrome, chronic kidney failure, osteoporosis, stroke, depression, obesity, and vascular dementia [5]. At present, lifestyle diseases cause 40 million deaths each year, accounting for about 70% of the global total, and have evolved into one of the greatest threats to the health of human beings [6]. Lifestyle-related diseases are now the leading causes of mortality and morbidity in developed countries. The incidence of lifestyle diseases also has increased dramatically in developing countries due to economic development and changes in dietary habits [7]. However, the types and characteristics of lifestyle diseases are different between developed and developing countries. In developed countries, people consume too many red meats, dairy products, sugary foods and beverages, alcoholic beverages, and other industrialized food, all of which contain an excess of fat, sugar, saturated and trans fatty acids, refined carbohydrates, and caffeine. This dietary component intake significantly increases the risk of cardiovascular disease (CVD), type 2 diabetes, abnormal lipid metabolism, and alcoholic and non-alcoholic fatty liver disease (AFLD and NAFLD) [8]. At the same time, developed countries face many social problems, for example, population aging. It significantly increases the incidence of Alzheimer's disease, osteoporosis, and prostate disease [9]. In contrast, people in developing countries, whose diets still depend on low-sugar starchy foods with little meat or fat, are accompanied by unemployment, insecurity, poor social environment, and unfavorable working conditions. Disturbed by insufficient nutrient supplementation and a poor living environment, the incidence of chronic gastrointestinal inflammation and osteoporosis in the growth period rocketed in developing countries [10]. Based on the above research, lifestyle diseases in developed and developing countries are different and closely related to their dietary structure and living environment.

2. Common lifestyle diseases and related pathological characteristics in Japan

Japanese dietary intake habits and characteristics have powerful ethnic features and are composite. The elder population prefers traditional Japanese foods, including

grilled fish, rice, eggs, pickles, miso, and natto, which have high salt and cholesterol levels [11]. The high-salt diet (HSD) and high cholesterol dietary intake are risk factors for CVD, hypertension, and metabolic syndromes [12]. The young population prefers red meat, alcohol, and sugar-sweetened beverages, which contain high levels of sugar, fat and trans fatty acid [13]. A mass of clinic and pathological research proves that the western diet obviously aggravates hypercholesterolemia and then develops atherosclerosis in mice and humans [14-16]. The western diet also promotes the development of cognitive impairment and Alzheimer's disease [17]. Moreover, rice is the food that contributes most to total carbohydrate and energy intake (43% and 29%, respectively) in Japan, which brings different dietary glycemic index (GI) and glycemic load (GL: $GI \times \text{available carbohydrate content}$) compared with western people [18]. Recent results from observational studies have suggested that diets with a low GI, a low GL, or both have a beneficial effect on several metabolic risk factors for cardiovascular disease and type 2 diabetes, such as body mass index (BMI; in kg/m^2) [19], HDL cholesterol [20] and TG [21]. In addition, average life expectancy in Japan hit record highs for both women and men in 2020, with 87.74 years and 81.64 years, respectively [22]. The aging population brings several lifestyle diseases such as osteoporosis, joint inflammation, and Alzheimer's disease. These lifestyle diseases lead to disability and physical dysfunction that require medical care and treatment. According to the results and points mentioned above, the rapid growth in the incidence of lifestyle diseases in Japan are invariably linked with the changes in dietary habits and population aging.

The Ministry of Health, Labor, and Welfare published a comprehensive survey of living conditions and reported basic subjects such as health, medical care, welfare, and pension. These primary data are used to plan and manage health, labor, and welfare administration [23]. The latest comprehensive survey of living conditions in 2019 reports that the incidence of hypercholesterolemia ranks second among women and fourth among men. The hypercholesterolemia patients among male patients increased by 18%, and among female patients increased by 11%, respectively, in comparison with the 2016 survey [23]. Hypercholesterolemia is closely linked to blood

cholesterol levels. Cholesterol is an essential lipid that constitutes biological membranes [24] and is the precursor of steroid hormones and bile acids [25]. Since cholesterol is insoluble in water, it is transported in the blood within lipoproteins [26]. Lipoproteins are classified by their density: Chylomicron (CM), very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) [26]. Hypercholesterolemia is defined by an elevation of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) or non-high-density lipoprotein cholesterol (HDL-C) (defined as the subtraction of HDL-C from TC) in the blood [27]. Hypercholesterolemia might accompany a decrease in HDL-C, an increase in TG, or qualitative lipid abnormalities [28]. Hypercholesterolemia is classified as serum TC, LDL-C, TG, apolipoprotein B (APOB), lipoprotein A concentrations above the 90th percentile, HDL-C, or HDL-C or apolipoprotein A-I concentrations below the 10th percentile for the general population [29]. However, these classic percentile cut-off points should not be used too rigidly in defining hypercholesterolemia [29]. For example, evidence suggests that lipoprotein(a) ≥ 80 th percentile is abnormal and linked to elevated cardiovascular risk [30]. Hypercholesterolemia is typically due to a combination of environmental and genetic factors [31]. Ecological factors include weight, diet, and stress [31]. Familial hypercholesterolemia is due to the additive effects of multiple genes. In familial hypercholesterolemia, mutations may be present in the APOB gene (autosomal dominant), the autosomal recessive LDL receptor adapter protein 1 (LDLRAP1) gene, autosomal dominant familial hypercholesterolemia (HCHOLA3) variant of the proprotein convertase subtilisin/Kexin type 9 (PCSK9) gene, or the LDL receptor (LDLR) gene [32]. Although hypercholesterolemia is asymptomatic, the long-standing elevation of serum cholesterol can lead to atherosclerosis. Atherosclerosis is a pattern of the disease arteriosclerosis [33] in which the wall of the artery develops abnormalities, called lesions. These lesions may narrow the vascular due to the buildup of atheromatous plaque, resulting in coronary artery disease, stroke, peripheral artery disease, or kidney problems [34]. There are different stages in the progression of atherosclerosis. Atherosclerosis can be divided into five key steps (1)

endothelial dysfunction, (2) the formation of lipid layer or fatty streak within the intima, (3) the migration of leukocytes and smooth muscle cells into the vessel wall, (4) foam cell formation and vascular inflammation (5) the degradation of extracellular matrix [35]. According to these consecutive steps, an atherosclerotic plaque is formed. The formation of the plaque can also be divided into three major stages (1) fatty streak, which represents the initiation, (2) plaque progression, which represents adaption and development, and (3) plaque disruption, which represents the clinical complication of atherosclerosis [36]. Since the 20th century, cardiovascular disease (CVD) has grown to be the major culprit of death and disability across the globe, illustrated by 17.3 million deaths per year in 2008 [37]. Atherosclerosis vascular disease begins early in life and can lead to obstructive arterial disease [38]. Once atherosclerotic lesions become clinically significant, severe complications such as ischemic heart disease, aneurysm, chronic occlusion, and stroke may occur.

Research on future estimates of the elderly population with dementia or cognitive impairment in Japan, funded by the Ministry of Health, Labor, and Welfare, was implemented in 2014 [39]. The research created a three variables mathematical model and estimated the future Japanese cognitive impairment patients through the results of the dementia survey in Hisayama, Fukuoka. The number of dementia patients in Japan is predicted to grow, reaching 6.5-7 million in 2025, 8-9.5 million in 2040, and 85-11.5 million in 2060 [40]. Alzheimer's disease (AD), considered the final stage of dementia, was remarkably increased [41]. Furthermore, it was suggested that the number of dementia patients requiring care would continue to rise. It is necessary to promote further basic and clinical research to elucidate the pathophysiology of dementia [41]. AD is a neurodegenerative disease that usually starts slowly and progressively worsens [42]. The most common early symptom is difficulty in remembering recent events. As the disease worsens, symptoms may include problems with language, disorientation (including easily getting lost), and behavioral issues [43]. As an AD condition declines gradually, patients' bodily functions are lost, ultimately leading to death. Although the speed of progression varies, the typical life expectancy following diagnosis ranges from three to nine years

[44]. The direct pathological causes of AD remain undetermined. Current pathological and neurological studies have mainly proposed the two hypotheses to explain the pathogenesis of AD. Firstly, the Amyloid Hypothesis. The amyloid hypothesis (also known as the amyloid cascade hypothesis, the amyloid β ($A\beta$) hypothesis, etc.) has been the mainstream explanation for the pathogenesis of AD over the past 25 years [45]. In normal subjects, $A\beta$ is excised from APP by β - and γ -secretase and released outside the cell, where it is rapidly degraded or removed [46]. However, in aged subjects or under pathological conditions, the metabolic ability to degrade $A\beta$ are weakened, and $A\beta$ peptides may accumulate [46]. $A\beta_{40}$ and $A\beta_{42}$ (more hydrophobic than $A\beta_{40}$), containing 40 and 42 amino acid residues, respectively, are major components of the accumulated $A\beta$ [47]. An increase in the level of $A\beta_{42}$ or in the ratio of $A\beta_{42}$ induces $A\beta$ amyloid fibril formation, and the accumulated $A\beta$ amyloid fibrils develop into senile plaque, causing neurotoxicity and induction of tau pathology, leading to neuronal cell death and neurodegeneration [48]. Secondly, the Tau Hypothesis. Tau is one of the microtubule-associated proteins that regulate the stability of tubulin assemblies [49]. The human tau gene is localized in chromosome 17. Six tau isoforms are expressed in the adult human brain due to mRNA alternative splicing, with or without exons 2, 3, and 10. The adult human brain expresses 3R and 4R tau isoforms, located mainly in the axons of adult neurons under normal physiological conditions [50]. In the brain of AD, 3R and 4R tau are accumulated in a hyperphosphorylated state in the pathological inclusions [51]. Tau pathologies have also been observed in other neurodegenerative dementing disorders, including frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease (PID), argyrophilic grain disease (AGD), tangle-only dementia, and chronic traumatic encephalopathies (CTE) [52]. In the central nervous system, apoE is produced and secreted by astrocytes, and microglia binds to lipoprotein and is taken up into nerve cells via the apoE receptor during the developmental stage of the central nervous system and the repair period after neuronal damage [53]. The apoE4, as a sub-type allele, is a genetic risk factor for sporadic AD. As the number of $\epsilon 4$ genes increase, the age of onset of AD declines while the incidence of AD grows [54].

The incidence of AD increases by 3-4 and 8-12 times for one or two copies of the allele, respectively. It is considered that impaired apoE4 function affects the clearance pathway of A β [54] and modulates A β -induced effects on inflammatory receptor signaling [55], including amplification of detrimental pathways and suppression of beneficial pathways [56]. ApoE also plays an important role in tau-mediated neurodegeneration and neuroinflammation, with apoE4 causing more severe damage [57]. ApoE4 affects neurodegeneration independently of A β and A β amyloid in the context of tau pathology [58]. It is the principal cholesterol carrier protein in the brain. PDAPP mice hemizygous for apoE were found to have a 60% reduction in amyloid deposition, whereas no nonfibrillar or fibrillar A β was detected in any brain region of homozygous knockout mice [58]. The absence of apoE affected the extracellular A β accumulation, but not the synthesis. Several in vitro and in vivo studies have shown that apoE can bind A β [59-60], and this phenomenon's pathophysiological importance remains unclear. ApoE may also mediate the internalization of A β through its binding to the LDL receptor-related protein [61-62]. In addition to directly facilitating A β internalization and aggregation, apoE4 may alter brain cholesterol homeostasis by modifying lipoprotein-particle formation. In summary, there is a strong connection between the dysregulation of cholesterol homeostasis and the development of AD.

3. Cholesterol homeostasis regulation pathways

According to the above research, cholesterol homeostasis disorder can induce hypercholesterolemia and cause atherosclerosis. The destruction of cholesterol homeostasis and the catabolic derivative of cholesterol in the brain can aggravate cognitive impairment (CI) and AD. Cholesterol plays an essential physiological role in the pathological processes of the above-mentioned disease. Cholesterol is synthesized in all animal cells and performs multiple physiological functions in cells and organs. It is required to build and maintain membranes and modulates membrane fluidity in physiological temperatures. Cholesterol promotes membrane packing, which alters membrane fluidity [63] and maintains membrane integrity according to interaction with the fatty-acid chains of phospholipid [63]. Cholesterol regulates the

biological process of substrate presentation and the enzymes that use substrate presentation as an activation mechanism. Cholesterol serves as precursor molecules in many physiological metabolisms, including synthesis of vitamin D and steroid hormones synthesis [64]. The metabolism and homeostatic regulation of cholesterol are shown in Figure I-1. The size of the cholesterol pool in the human is determined among the processes of cholesterol *de novo* biosynthesis, cholesterol catabolism (bile acid synthesis and enterohepatic circulation), cholesterol esterification, cholesterol transportation, cholesterol dietary intake and intestinal absorption.

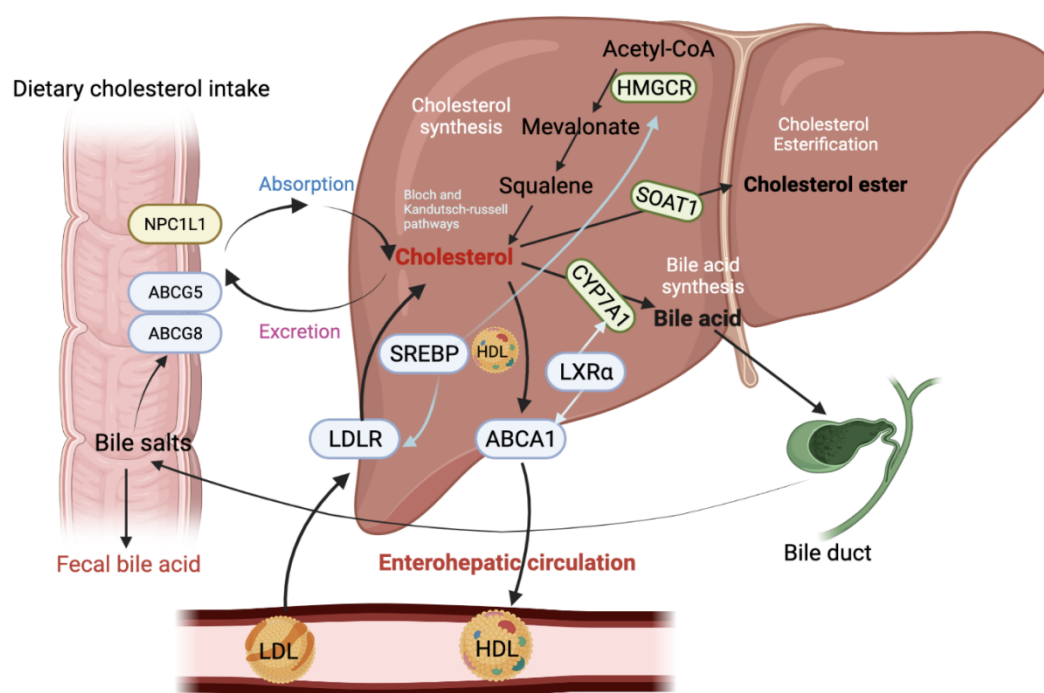


Figure I-1 Metabolic pathways of cholesterol in human

The processes of cholesterol *de novo* biosynthesis is shown in Figure I-2. Cholesterol *de novo* biosynthesis within the human starts with the mevalonate pathway where molecules of acetyl-CoA form 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) [65]. HMG-CoA is then reduced to mevalonate by enzyme HMG-CoA reductase (HMGCR). HMGCR is the rate-limiting step and the primary site of feedback inhibition and is the site of action for statins which is a classic

cholesterol-lowering drug [66]. Mevalonate is then converted to isopentenyl pyrophosphate (IPP) through two phosphorylation steps and one decarboxylation step that requires and finally condenses to form squalene by the action of squalene synthase in the endoplasmic reticulum [67]. Squalene is converted to lanosterol by squalene monooxygenase (SQLE) and Lanosterol synthase (LSS) [68]. Then the conversion of lanosterol to cholesterol in the following stages of the pathway probably occurs *via* two routes: in one route, the Kandutsch-Russell pathway, where the immediate precursor of cholesterol is 7-dehydrocholesterol; in the other route, the Bloch pathway, where the immediate precursor of cholesterol is desmosterol. These two pathways are independently regulated but share many enzymes. A deficiency of one of these enzymes will seriously disrupt cholesterol synthesis [69].

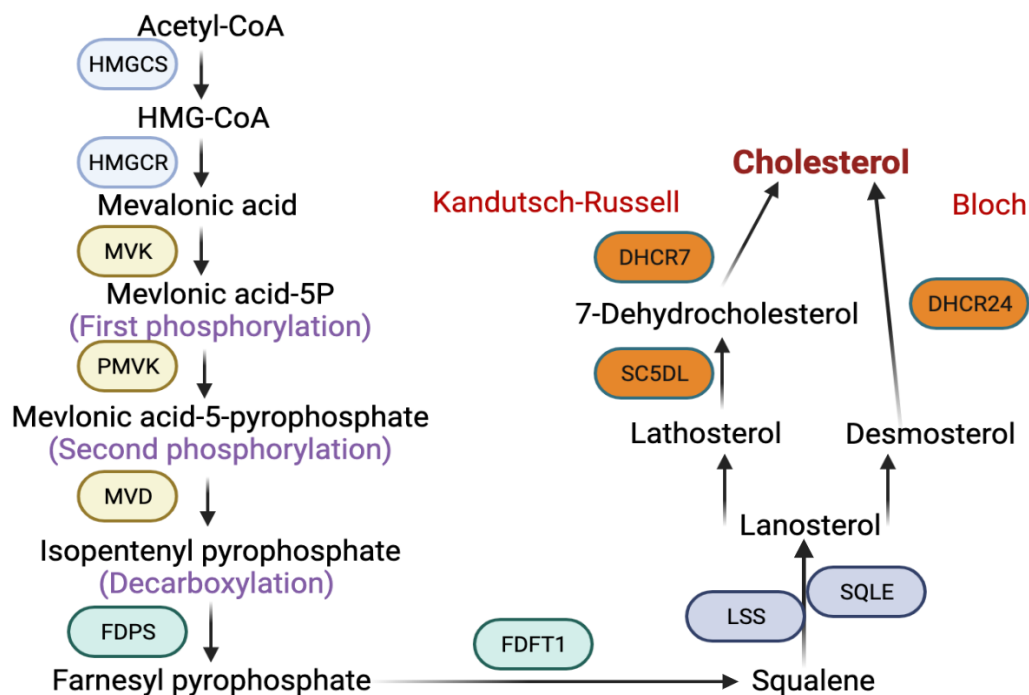


Figure I-2 Metabolic process and intermediates of *de novo* cholesterol synthesis

Bile acid (BA) synthesis is the dominant metabolic pathway of cholesterol catabolism, and biosynthetic reactions occur in the endoplasmic reticulum, mitochondria, cytoplasm, and peroxisomes. The metabolism process is shown in Figure I-3. The Biosynthesis of BAs takes place through four different pathways:

classic, alternative, Yamasaki, and 25-hydroxylation [70]. The exact sequence of the biosynthetic step has still not been defined as many intermediates share the same enzymes. Herein, we mainly discuss the classic bile acid synthesis pathway. The classic pathway of the bile acid synthesis cascade is responsible for producing 90% of the total amount of BAs, with less than 10% of the total bile acids coming from the alternative pathway during routine physiological conditions. In the classic pathway, cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized almost equally [71]. Cytochrome P450 family 7 subfamily A member 1 (CYP7A1) is a crucial enzyme in this catabolic pathway, catalyzing the hydroxylation of cholesterol to 7 α -hydroxycholesterol. Hydroxylation reaction occurs at different sites of 7 α -hydroxycholesterol. When the hydroxylation reaction takes place in the C₁₂ position with sterol, 7 α -hydroxycholesterol is converted to CA by cytochrome P450 family 8 subfamily B member 1 (CYP8B1) [71]. However, if there is no hydroxylation in this sites, 7 α -hydroxycholesterol is converted to CDCA [71]. CA and CDCA are known as primary bile acids. As the synthesis of bile acids fail to meet the demands of digestion and physiological metabolism, enterohepatic circulation is required. CA and CDCA are conjugated (reversibly attached) mainly to glycine or taurine molecules to form water-soluble primary conjugated bile acids, also known as "bile salts." These bile acids travel to the gall bladder during the inner digestive phase for storage and to the descending part of the duodenum *via* the common bile duct through the major duodenal papilla during digestion [72]. The efficiency of this enterohepatic circulation recycles and minimizes fecal and urinary bile acid loss by excretion.

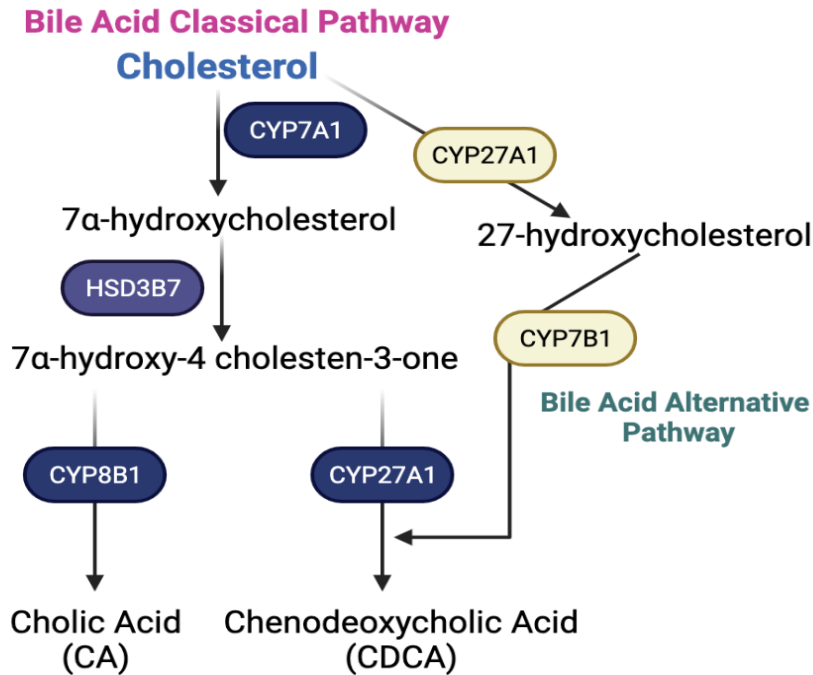


Figure I-3 Metabolic process and intermediates of bile acid synthesis

Cholesterol esterification is a universal mechanism to store and transport large quantities of cholesterol between organs and tissues and to avoid the toxicity of excess cellular cholesterol. Intended for transport and storage and thus to be inert, cholesteryl esters (CEs) reside in hydrophobic cores of circulating lipoproteins and intracellular lipid droplets [73]. In hepatic cells, excess cholesterol is esterified in the ER by the enzyme acyl CoA cholesterol acyltransferase 1 (ACAT1), also called sterol O-acyltransferase 1 (SOAT1), and the newly synthesized CEs are stored in lipid droplets after the threshold level in cellular cholesterol mass has been reached [73].

Cholesterol transportation is the most important pathway for the internal circulation to maintain cholesterol homeostasis and is divided into the endogenous pathway, the exogenous pathway, and the reverse transport pathway. The metabolism process is shown in Figure I-4. The exogenous pathway is considered the primary source of dietary cholesterol and transports food-derived cholesterol to the internal environment. Dietary lipids are cleaved into fatty acids and small-molecule glycerol by pancreatic lipase and absorbed by the enterocytes [74]. Then these lipids are assembled with ApoB48 and then become CM. As they circulate through the

lymphatic vessels, nascent chylomicrons bypass the liver circulation and are drained *via* the thoracic duct into the bloodstream [75]. In the bloodstream, CM particles interact with HDL particles, resulting in HDL donation of apolipoprotein C-II and ApoE to the nascent chylomicron. Mature CM activates lipoprotein lipase (LPL), an enzyme on endothelial cells lining the blood vessels *via* apolipoprotein C-II, and LPL catalyzes the hydrolysis of TG to release glycerol and fatty acids from the CM [76]. Glycerol and fatty acids can then be absorbed in peripheral tissues, especially adipose and muscle, for energy and storage. Initial metabolism and lipoprotein assembly of the endogenous pathway occurs in the liver. In hepatocytes, TG and CE assemble with ApoB100 to form nascent VLDL. The VLDL is released into the bloodstream through a process that depends on ApoB100. In the bloodstream, the VLDL binds to HDL particles, which donates lipoprotein C-II and lipoprotein E to the VLDL particles. At this time, the VLDL granules are considered mature, and the VLDL granules circulate, and encounter LPL expressed on endothelial cells, resulting in the hydrolysis of VLDL and the release of glycerol and fatty acids, which are taken up by peripheral tissues, mainly fat and muscle. The hydrolyzed VLDL particles are now referred to as ADLs. IDL residues can be circulated and absorbed by the liver through the interaction between apoE and remnant receptors, or they can be further hydrolyzed by hepatic lipase (HL). The further hydrolysis of IDLs releases glycerol and fatty acids and leaves IDL residues, also known as LDLs, which contain relatively high levels of cholesterol. The circulation of LDLs is uptake by the liver and surrounding cells through endocytosis *via* LDLR. Reverse cholesterol transport mediates the transport of excess cholesterol from peripheral tissues back to the liver *via* entering the lymphatic system, then the bloodstream [77]. Cholesterol from non-hepatic peripheral tissues is transferred to HDL *via* ATP-binding cassette transporter protein (ABCA1). ABCA1 also mediates hepatic HDL assemble and efflux [76]. ApoA-1 is considered as the major protein component of HDL. Cholesterol is converted to CE by the enzyme LCAT (lecithin-cholesterol acyltransferase) and CEs are transferred to other lipoproteins in exchange for triglycerides with the help of cholesteryl ester transfer protein (CETP). Then the excessive cellular cholesterol or CEs are transported into the liver then

converts to bile acid or CEs for further storage and utilization. Cholesterol reverse transport promotes the excretion of excess intracellular cholesterol to the liver then converts to bile acid or CEs for further storage and utilization. This process has significant clinical implications in hypercholesterolemia and atherosclerosis development according to the transport of cholesterol from foam cells to the liver for secretion into the bile [77].

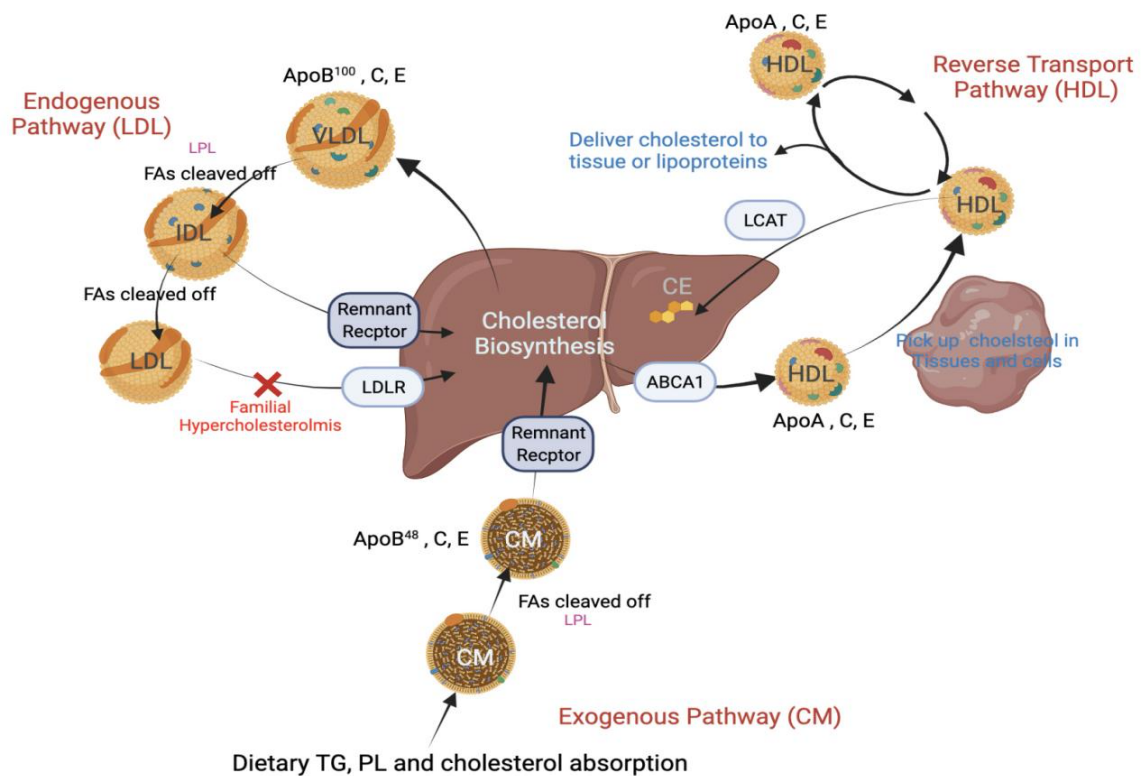


Figure I-4 Metabolic process of cholesterol transportation

Cholesterol dietary intake and intestinal absorption is the most important source of cholesterol other than autologous cholesterol synthesis. Dietary cholesterol is absorbed from bile salt micelles with fatty acids and lysophospholipids in the proximal parts of the enterocytes. Several key transportation proteins have been found to mediate cholesterol transport in the intestine. Niemann-Pick C1-Like 1 (NPC1L1) is a transporter protein mainly expressed in intestinal tract epithelial cells and is considered the main cholesterol absorption regulator [78]. NPC1L1 has the special structure of the *N*-terminal domain, namely the *N*-terminal domain, which can

specifically sense cholesterol and then induce the endocytosis of the NPC1L1–flotillin–cholesterol membrane microdomains to mediate endocytosis, which is an early step in cholesterol absorption [78]. Inhibiting NPC1L1 expression can effectively prevent cholesterol absorption in the intestine and has been proved to be the target of the cholesterol-lowering drug ezetimibe [78]. ATP-binding cassette sub-family G member 5 and 8 (ABCG5 and ABCG8) are expressed in a tissue-specific manner in the intestines and are considered as a half transporter to limit cholesterol intestinal absorption and promote the biliary excretion of sterol and cholesterol [79]. The Liver X receptor is the major positive regulator of ABCG5 and ABCG8 expression. Mutations in either the ABCG5 or ABCG8 cause sitosterolemia, a condition in which cholesterol and plant sterols accumulate in the circulation leading to premature cardiovascular disease. Over expression of ABCG5 and ABCG8 retards diet-induced atherosclerosis because of reduced circulating and hepatic cholesterol [79].

4. Cholesterol-lowering food functional ingredients and aims of this research

In summary, cholesterol homeostasis is maintained by a combination of several metabolic pathways mentioned above. Cholesterol homeostasis and its metabolites also are important causative factors for hypercholesterolemia, atherosclerosis, and AD. At present, types of cholesterol-regulating drugs include Statins, PCSK9 inhibitors, Fibric acid derivatives, Bile acid sequestrants, Selective cholesterol absorption inhibitors, Adenosine triphosphate-citrate lyase (ACL) inhibitors, and so on. These medicines regulate cholesterol metabolism *via* a different regulation pathway mentioned above paragraphs. However, some side effects may occur with prolonged use of the drug, such as muscle reactions, gastrointestinal reactions, skin rashes, increased blood sugar, and so on. Over recent years, many types of research reported that food functional ingredients are possible alternative nutritional therapies for regulating cholesterol metabolism through a similar mechanism to cholesterol-lowering medicine, especially for patients, whose blood cholesterol level is marginally high but not high enough to warrant the prescription of cholesterol-lowering

medication. The food source with cholesterol cholesterol-lowering bioactivity and related mechanisms are shown in Table I-1.

Table I-1. Summary of cholesterol-lowering food source and their functional ingredients and action mechanisms

Food Source	Functional ingredients	Mechanism	Cites
<i>Lupinus angustifolius</i>	Protein	Hepatic synthesis inhibition and Bile acid synthesis promotion	[80][81]
Flaxseed	Peptides (≤1kDa protein hydrolysates, IPPF, IIPAF, LLGTL, IIF.etc)	SREBP2-regulated mevalonate pathway inhibition, Intestinal absorption inhibition, Bile acid synthesis promotion, and re-absorption inhibition	[82][83]
Buckwheat	Protein	Bile acid re-absorption inhibition	[84]
Conjugated linoleic acids (CLA)	t10, c12-CLA, c9, t11-CLA	Inhibition on intestinal ACAT activity.	[85][86]
Dietary phytosterols	Stigmasterol, β-Sitosterol, Campesterol, Brassicasterol	Competitive absorption of intestinal cholesterol (NPC1L1 inhibition)	[87]
Soybean	Proteins, peptides, genistein, daidzein and glycitein	Hepatic synthesis inhibition; Endogenous transportation activation	[88][89]
Tea	Catechins: EGCG, ECG, EGC, and EC	SREBP2-regulated mevalonate pathway inhibition, Intestinal transports inhibition, Bile acid synthesis promotion, Hepatic cholesterol activation	[90][91]

Red yeast rice	Monacolin K and its relative derivatives	Hepatic synthesis inhibition (HMGCR)	[92]
Maize and Wheat Brans	Non-digestible polysaccharides, Short chain fatty acids	Hepatic synthesis inhibition, Bile acid synthesis promotion, and re-absorption inhibition	[93][94]
Egg yolk	Phospholipids (PL)	Alters the physicochemical properties of mixed micelles, Intestinal transports inhibition	[95]
Rice bran oil	Tocotrienols, γ -oryzanol	Hepatic synthesis inhibition	[96]
Oats	β -Glucan	Bile acid excretion promotion	[97]
Garlic	S-Allyl cysteine, etc Organosulphur compounds	Hepatic synthesis inhibition, enhance reverse cholesterol transport.	[98][99]
Marine foods	Polysaccharide, n-3 Polyunsaturated fatty acid (n-3 PUFA)	Accelerate the decomposition of cholesterol, Bile acid synthesis promotion and re-absorption inhibition, Hepatic synthesis inhibition, Endogenous transportation inhibition	[100][101] [102]

According to the research which showed on Table I-1, the food functional ingredients such as proteins, unsaturated fatty acids, and PL have certain cholesterol-lowering activities through different cholesterol regulation pathways. Food functional ingredients which regulate cholesterol metabolism play an important role in preventive medicine and nutrition interventions and contribute to reducing the medical burden and the aging population. At present, the development of these foods' functional ingredients sparks the interest from both the public and the scientific

community. Rice is the most important staple food and a major source of carbohydrates in Japan [17]. Safflower oil is the highest dietary intake oil containing n-6 unsaturated fatty acid (linoleic acid) [103]. Eggs are an important food source of protein and phospholipids in Japan [104]. Rice α -Globulin, linoleic acid (LA), and egg yolk phosphatidylcholine (PC) are high content in rice, safflower oil, and egg, respectively. The effect of rice α -globulin, LA, and egg yolk PC on the development of hypercholesterolemia, atherosclerosis, and AD through regulating cholesterol and its derivatives are researched in this subject and divided into three sub-projects. Some studies investigating the bioactivity of food functional ingredients were supplemented out of normal dietary intake through gavage or intravenous injection. In this subject, food functional ingredients with disease-prevent activities were evaluated as a part of the diet to simulate human dietary intake habits to explore whether functional ingredients exert their bioactivity under the interference of other nutrients and different diet composition. In Chapter II, the effect of a new Japanese α -globulin-rich rice cultivar, low glutelin content-1 (LGC-1), on exogenously hypercholesterolemia as the form of rice flour was investigated. In Chapter III, the effect of LA, which has two-side effects of lowering serum cholesterol and promoting vascular inflammation, on the development of atherosclerosis and aortic inflammation in different dietary cholesterol levels was evaluated. In Chapter IV, the effect of egg yolk PC on the biomarker expression of AD development through regulating oxysterol and cholesterol metabolism was assessed. We expect that the research can be applied to clinical nutrition intervention and novel functional food production in the future.

CHAPTER II α -Globulin-rich rice cultivar, low gluten content-1 (LGC-1), decreases serum cholesterol concentration in exogenously hypercholesterolemic rats

1. Introduction

Cholesterol is *de novo* synthesized in all animal cells and serves as an essential structural component of animal cell membranes and also as a precursor for the biosynthesis of steroid hormones and bile acid [1-2]. It has been reported that excessive cholesterol intake increases the risk of hypercholesterolemia, which leads to atherosclerosis [3]. Risk factors for atherosclerosis include hypercholesterolemia, hypertension, and oxidative stress, among others [4]. An epidemiological study has revealed that lowering serum cholesterol levels is a critical factor in the prevention of atherosclerosis [5]. There is a significant value in understanding ways to reduce cholesterol levels using diet or supplements to help prevent and treat atherosclerosis.

Several nutrients have been confirmed to have cholesterol-lowering activity, such as unsaturated fatty acids and proteins [6]. Plant protein intake shows higher cholesterol-lowering activity than unsaturated fatty acids and animal protein intake [7]. Globulin proteins are widely distributed among plants and have been shown to exhibit a lowering effect on serum cholesterol levels. For example, soy β -conglycinin, a soy globulin, has been shown to reduce serum cholesterol levels by promoting LDL clearance in a human study [8]. As is known to all, rice is a globally valuable staple food that serves as the primary carbohydrate source for millions of people. Furthermore, due to the increasing demand for gluten-free ingredients worldwide, rice has been attracting increasing attention in the form of rice flour in recent years. Rice endosperm contains large quantities of α -globulin. Our previous study demonstrated that rice α -globulin decreases serum cholesterol levels in rats fed with a hypercholesterolemic diet [9]. The peptide, GEQQQQPGM, derived from rice α -globulin, decreases total cholesterol levels to reduce the risk of atherosclerosis in hamsters fed with a hypercholesterolemic diet [10]. In this study, we evaluated the

hypocholesterolemic activity of rice α -globulin as a dietary ingredient and used an α -globulin-rich rice cultivar, LGC-1. LGC-1 is a new rice cultivar developed in Japan that contains high α -globulin levels in its endosperm (10.6 mg/g rice flour, calculated the data from reference 10), which is approximately 1.5 times higher than the α -globulin content of Koshihikari, one of the most widely distributed rice cultivars in Japan [11]. LGC-Jun is a non-globulin cultivar developed from LGC-1 [12]. In previous studies [9,10], the hypocholesterolemic effect of LGC-1 was evaluated with a hypercholesterolemic model with 0.2% dietary bile salt. However, supplementation of bile salt is not physiological. In this study, we designed the evaluation for the anti-hypercholesterolemic effect of LGC-1 with exogenously hypercholesterolemic (ExHC) rats. ExHC rats were established from Sprague-Dawley (SD) rats as a model of diet-induced hypercholesterolemia [13]. Their serum total cholesterol levels increase only when fed a cholesterol-containing diet without dietary bile salt as a result of disorders in carbohydrate metabolism [14], not of excessive cholesterol synthesis in the liver. Therefore, ExHC rats enables to design more physiological, bile-salt-free dietary condition. In this study, we compared the effects of LGC-Jun and LGC-1 on cholesterol metabolism in ExHC rats that were fed a 0.5% cholesterol-containing diet. In addition, SD rats were fed diets containing LGC-Jun or LGC-1, and their cholesterol and lymphatic cholesterol transport levels were measured using permanent cannulation of the thoracic lymph duct. Our results will facilitate the comprehensive development and utilization of LGC-1 in addition to providing a theoretical basis to support the development of cholesterol-lowering functional foods.

2. Materials & Methods

2.1 Materials

The LGC-1 (α -globulin-rich) and LGC-Jun (α -globulin-deficient) rice lines provided by the National Institute of Agrobiological Sciences (NIAS) were cultivated in the rice fields of the Kyushu University Farm. Harvested seeds from this cultivation were used in the following analyses. Trimethylsilylation (TMS) reagent solution, a

mixture of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1), was purchased from Supelco (MO, USA). Commercial enzyme assay kits (Cholesterol E test kit, Free Cholesterol E test kit, Triglyceride E test kit, Glucose CII test kit, and Total Bile Acid test kit) were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2 Animals and diets

ExHC rat colonies were maintained at our institute through brother-sister mating. Four-week-old male ExHC rats ($n = 5$ /group) were used for the diet-based experiments. We used 7-week-old male SD rats (Kud:SD) ($n = 5$ /group) for thoracic lymph duct cannulation. The SD rats weighed between 230 and 240 g and were purchased from Kyudo Co. Ltd. (Kumamoto, Japan). The rats were individually housed in metal cages and kept in a temperature-controlled room (22-25°C) with a 12 h light cycle. Before the experimental period, the rats had free access to a commercial non-purified diet (NMF, Oriental Yeast Co., Tokyo, Japan) and deionized water. Table II-1 lists the composition of the experimental diets. In the feeding experiment, ExHC rats were fed an experimental diet for 2 weeks. Animals were then sacrificed by withdrawal of aortic blood under pentobarbitone anesthesia (0.08 mg/100 g body weight), followed by immediate excision of liver and intestinal mucosa for use in subsequent experiments. To isolate serum, blood was collected, incubated at room temperature for 30 min, and centrifuged at $1,750 \times g$ for 10 min at 4°C. The handling and euthanasia of all animals was carried out in accordance with nationally prescribed guidelines and experimentation was carried out following ethical approval from the Animal Care and Use Committee of Kyushu University (authorization number: A22-160-2).

Table II-1. Composition of experimental diets

	LGC-Jun	LGC-1
	(g/kg diet)	
Sucrose	95	95
Casein	200	200
Rice flour of LGC-Jun	500	-
Rice flour of LGC-1	-	500
Cellulose	50	50
Mineral mixture (AIN76™)	35	35
Vitamin mixture (AIN76™)	10	10
Cholesterol	5	5
DL-Methionine	3	3
Choline bitartrate	2	2
Olive oil	100	100

2.3 Analysis of serum and liver parameters

Serum levels of total cholesterol, free cholesterol, TG, and glucose were measured using commercial enzyme assay kits (Cholesterol E test kit, Free Cholesterol E test kit, Triglyceride E test kit, Glucose CII test kit, and Total Bile Acid test kit). Serum cholesterol ester levels were calculated as the difference between total cholesterol and free cholesterol levels. For measurement of serum HDL-C cholesterol levels, HDL fractions were collected as described previously [15] and then measured using the Cholesterol E test kit. Non-HDL cholesterol levels were calculated as the difference between total and HDL cholesterol levels. Total lipids from the liver were extracted using the method described by Folch *et al.* [16]. Total liver and free cholesterol levels were measured using the method of Sperry [17]. One point five mL and 2 mL concentrated liver lipid extract were added into cholesterol tubes for total cholesterol and free cholesterol measurement, respectively. After drying up, 0.5 mL of

ethanol: acetone = 1:1 solution was added into tubes and shaken well. Only for total cholesterol measurement, 2 drops of 50% KOH solution (1 g KOH / 2 mL H₂O) were added and well mixed by glass rod. Alkaline hydrolysis was carried out at 45°C with stirring using glass rod every 10 min. After cooling and returning to room temperature, pH was confirmed by adding 0.1% phenolphthalein solution into the tube and observe whether the solution became red. The solution was neutralized with 10% acetic acid solution until colorless and transparent, further 1 drop of acid is added to make the solution acidic. 0.2 mL of 0.5% hydrofluoric acid solution was added into the tubes and stirred using a glass rod for 5 min later to react at room temperature for 30 min. Glass rod was raised while washing with ethanol: acetone = 1:1 solution. The ethanol: acetone = 1:1 solution was added up to two thirds of the tube. Add a silicone plug onto the tube and put it in a dark room overnight. The next day, only for free cholesterol measurement, samples were centrifuged at 15°C and 1,610 × *g* for 15 min, and the upper layer was removed using a pathtool pipette. Then, 1 mL acetone: diethyl ether = 1:2 solution was added into each tube and capped (The same operation was performed below for measuring total cholesterol). After centrifugation at 15°C and 1,610 × *g* for 15 min, the supernatant was removed with a Pasteur pipette. After adding a small amount of diethyl ether and vortexing, diethyl ether was added to about two-thirds of the tube. The mixture was capped and centrifuged at 1,610 × *g* for 5 min at 15°C, and the supernatant was removed with a Pasteur pipette. The glass rod was placed in a tube and placed in a dryer at 110°C for 30 min to remove diethyl ether. Then, while the tube was hot, 0.5 mL of acetic acid was added and stirred well with a glass rod to dissolve the precipitate. (The same operation was performed for the standard solution below.) After returning to room temperature, add 1 mL of a coloring reagent (acetic anhydride: concentrated sulfuric acid = 20:1 solution) to the tube every 30 seconds, and the reaction was accurately carried out at 25°C for 30 min. The absorbance was measured at 620 nm every 30 seconds. Liver CE levels were calculated as the difference between total and free cholesterol levels. Liver TG levels were measured using the TG E test kit.

2.4 Determination of serum phytosterol levels

Serum lipids were extracted using the method described by Folch *et al.* [16]. Phytosterol levels in the extracted serum lipids were measured using the method of Jones *et al.* [18]. Add 5 α -cholestane, which is the internal standard substance, to a round mouth tube to 10 μ g / tube (liver) and 5 μ g / tube (serum). Add 1 mL of liver lipid extract and 100 μ L of serum and mix gently. Only the liver sample was dried to nitrogen. 1 mL of 0.71 M KOH (in 95% EtOH) was added, filled with argon gas, and incubated in a hot water bath at 65°C for 1 hour with stirring every 10 min for saponification. 0.5 mL of miliQ and 2 mL of hexane were added, filled with argon gas, shaken for about 15 seconds, and then centrifuged at 18°C, 1,610 $\times$ g for 10 min. Only the hexane layer (upper layer) was sucked up with Pasteur so as not to suck the aqueous layer (lower layer) and dispensed into a gas chromatograph tube. Extraction was performed twice, and a total of 4 mL of hexane was dried up with nitrogen gas. For temporary storage, samples were dissolved in an organic solvent such as hexane or purified EtOH, sealed with argon gas, and stored in a freezer. After drying up, the steroid extract extracted by the above procedure, add 400 μ L of BSTFA + TMCS, 99:1 (heat TMS reagent), vortex, and heated at 70°C for 1 hour using a heat block with stir every 5 min. After drying up with nitrogen gas, sample was dropped to 30 μ L and 1 μ L was injected into the GC-FID. For liver samples, gas chromatograph (GC-2014s, Shimadzu Corporation, Kyoto, Japan) was used for sterol analysis. The column used was SPB-1 fused silica capillary column (60 m $\times$ 0.25 mm, 0.25 mm phase thickness, Supelco, USA). Temperature of the column was risen to 275°C and hold for 30 min (Homeothermic analysis). The temperature of the injector and detector (interface) were 275°C and 300°C, respectively. The entrance pressure was 335 kPa. The Column flow rate and total flow rate were 1.55 mL/min and 35.6 mL/min, respectively. The split ratio was 20 and the flow velocity was 32.5 cm/sec. For serum samples, gas chromatograph (GC-2014s, Shimadzu Corporation, Kyoto, Japan) was used for sterol analysis. The column used was InertCap 5MS/NP-VS2 (30 m $\times$ 0.25 mm i.d., 0.25 μ m f.t., GL Sciences). Initial temperature of the column was 180°C and was hold for 1 min; Temperature was risen to 250°C with heating rate of 20°C per minute, and then risen

to 290°C with heating rate of 5°C per minute and hold for 18 min. The temperature of both the injector and detector (interface) were 300°C. The entrance pressure was 171.4 kPa. The Column flow rate and total flow rate were 1.52 mL/min and 35.0 mL/min, respectively. The split ratio was 40 and the flow velocity was 40.0 cm/sec.

2.5 Measurement of oxysterols in the liver

Hepatic oxysterols were derivatized to trimethylsilyl ethers using a mixture of trimethylchlorosilane, hexamethyldisilazane, and dried pyridine (1:3:9, v/v/v), followed by quantification using GC-MS, as previously described [19]. To quantify each oxysterol, calibration curves were produced, which measured the peak area of each oxysterol versus that of the internal standard (19-hydroxycholesterol) [20]. Peak identification was confirmed using the relative retention time and mass spectral comparison with an authentic standard [16]. Lipid extraction 1 mL of liver lipid extract and 1.0 mL of 19-hydroxycholesterol (100 ng / mL) as an internal standard were added into a threaded mouth tube, dried under nitrogen, and 4 mL of 1M KOH ethanol solution was added. The tube was shaken overnight at room temperature. Add 4 mL of deionized water and 4 mL of hexane into the tube, shake for 5 min, and centrifuge at $1,610 \times g$ for 5 min. The upper layer was separated into a gas chromatograph tube (glass, inner diameter 1 cm x length 10 cm, tip 1.5 cm pointed). Four mL of hexane was added again, and the mixture was shaken and centrifuged to separate the upper layer. During the operation, the sample tube was filled with argon gas in order to prevent oxidation. The collected upper liquid is stored at -25°C. The saponified sample was dissolved in 1 mL of toluene and then adsorbed on a silica cartridge (Sep-pal silica cartridge, Waters, USA) equilibrated with 2 mL of hexane. Then, after washing with 1 mL of hexane and 10 mL of 0.5% isopropanol (in hexane), the oxysterol fraction was eluted with 5 mL of 30% isopropanol (in hexane). Samples are dried under nitrogen, concentrated on the bottom of a gas chromatograph tube, and 0.2 mL trimethylsilyl derivatized (TMS) reagent (dried pyridine: hexamethyldisilazane: trimethylchlorosilane (9:3:1, v/v/v) was added. The mixture was sealed and reacted at room temperature for 30 min for derivatization. During derivatization, light stirring was performed every 5 min. After removing the

solvent under nitrogen, about 1 mL of acetic anhydride was added and the mixture was centrifuged at $1,610 \times g$ for 5 min. The upper layer was collected and stored at -30°C . Gas chromatograph (GC-2014s Ver.3), and mass spectrometer with an electron impact ionization source (Shimadzu QP2020A, Shimadzu Corporation) were used for analysis for oxysterol. Carrier gas was high purity He (purity 99.9999% or higher, Asahi Oxygen, Fukuoka). The column used was SPB-1 fused silica capillary column (60 m $\times$ 0.25 mm, 0.25 mm phase thickness, Supelco, USA). Initial temperature of the column was 180°C and was hold for 1 min; Temperature was risen to 250°C with heating rate of 20°C per minute, then risen to 290°C with heating rate of 5°C per minute and hold for 32.5 min. The temperature of both the injector and detector (interface) were 300°C ; the split ratio was 10 and the flow velocity was 1.5 mL/min. After determining the concentrations of various cholesterol oxide standard substance solutions by GC, a calibration curve was prepared. The ions were subjected to GC-MS by scanning, the retention time and mass spectrum of each oxysterol were confirmed, and then the ions to be used in the SIM method were selected with reference to the literature values and past detection data. A calibration curve was prepared for each oxysterol using the ions selected in this way. Waveform processing, peak area and concentration calculation were all performed using GC-MS Solution Ver.1.20 (Shimadzu Corporation) software.

2.6 Measurement of cholesterol synthesis intermediates in the liver

The cholesterol synthesis intermediates in the liver were analyzed by previous method which described in the part of 2.4 with slight modification. After saponification with ethanolic KOH and derivatization using the TMS reagent solution (99% BSTFA, 1% TMCS), hepatic cholesterol synthesis intermediates here analyzed using a GC-FID system (GC-2014s; Shimadzu) equipped with an SPB-1 column using 5α -cholestane as an internal standard. The temperatures of the injection port and detector were set at 300°C . The column temperature was set at 275°C .

2.7 Measurement of fecal lipid contents

Feces were collected for 3 days at the end of the experiment, lyophilized, weighed, and stored at -30°C until further analysis. Fecal neutral steroids were

derivatized into trimethylsilyl ethers and quantified using GC-2014s, as described previously [18]. The data for neutral steroids was the sum of cholesterol and coprostanol. Fecal acidic steroids were determined using the Total Bile Acid test kit.

2.8 Permanent thoracic lymph duct cannulation and lymphatic cholesterol transport measurements

After meal-feeding training, SD rats were anesthetized using sodium prior to permanent cannulation of the thoracic lymph duct, as described previously [21]. After a 20 min collection period (blank lymph), the rats were given experimental diets for 30 min. Following that, the lymph was collected every hour for 7 h. Lymphatic cholesterol levels were measured using the Cholesterol E test kit.

2.9 Determination of mRNA levels in the liver

Total RNA was isolated from liver tissues using the phenol/chloroform method, as described previously [20]. Complementary DNA (cDNA) was synthesized from 1.0 µg total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Berlin, Germany). Gene expression levels for genes were analyzed using quantitative real-time reverse transcription polymerase chain reaction with a SYBR[®] Premix EX Taq II Kit (Takara, Shiga, Japan) and a Thermal Cycler Mx3000P QPCR System (Agilent, CA, USA). The mRNA levels were normalized using β -actin (*Actb*) gene as an internal standard. Primer sequences and annealing temperatures used for the analysis are shown in Table II-2.

Table II-2. Primers for real-time RT-PCR

Gene	Protein	Forward primer	Reverse primer
<i>Actb</i>	β -actin	TCAGGTCATCACTATCGGCA	TCATGGATGCCACAGGATTC
<i>Hmgcr</i>	Hydroxymethylglutaryl-CoA reductase	TGTGGGAACGGTGACACTTA	GCTGACGCAGGTTCTGGAA
<i>Cyp7a1</i>	Cholesterol 7 α -hydroxylase	CTGTCATACCACAAAGTCTTATGTCA	ATGCTTCTGTGTCCAAATGCC
<i>Ldlr</i>	Low-density lipoprotein receptor	CACTGTGGCAGTAGTGAGTG	GGCTACCGTGAATACAGGAG
<i>Soat1</i>	Sterol O-acyltransferase 1	GATGGGGTTATGTTGCTATGC	GGGCTCCTGTTTGATATTCCG
<i>Soat2</i>	Sterol O-acyltransferase 2	TGTGTCCCTGTCTTTGCCAA	TGTCTCCAAACCGCAGCATC

2.10 Statistical analysis

Data have been expressed as mean $\pm$ standard error of mean. Differences between the two groups were analyzed using Student's *t*-test and were considered statistically significant at $p < 0.05$ and trend at $0.05 < p < 0.1$.

3. Results

3.1 Effects of the LGC-1 diet on growth parameters

The two groups of rats had similar food intake and relative liver weights after a 2-week feeding period (Table II-3). However, the final body weight, body weight gain, and food efficiency of the LGC-1 group were significantly lower when compared with those of the LGC-Jun group (Table II-3) ($p < 0.05$).

Table II-3. Growth parameters[†]

	LGC-Jun	LGC-1	<i>p</i> value
Body weight (g)			
Initial	75.2 ± 1.4	72.2 ± 3.0	0.395
Final	176.5 ± 4.2	160.3 ± 4.6*	0.032
Gain	101.4 ± 2.9	88.1 ± 3.6*	0.021
Daily food intake (g/day)	14.1 ± 0.2	13.5 ± 0.3	0.169
Food efficiency (%)	51.3 ± 0.8	46.6 ± 1.4*	0.021
Relative liver weight (g/100 g b.w.) [‡]	5.11 ± 0.08	5.18 ± 0.08	0.540

[†]Values have been expressed as mean ± SEM (n= 5 /group). Asterisks show a significant difference at *p* < 0.05, as calculated using Student's *t*-test.

[‡]b.w.: body weight

3.2 Effects of the LGC-1 diet on serum parameters

The serum esterified cholesterol, and thus total cholesterol levels were significantly lower in the LGC-1 group than those in the LGC-Jun group (Table II-4) (*p* < 0.05). Serum free cholesterol levels were similar between the two groups. In the lipoprotein fractions, serum non-HDL-cholesterol levels were significantly lower in the LGC-1 group than that in the LGC-Jun group (*p* < 0.05). In case of the serum HDL-cholesterol levels, there were no significant differences between the groups. Serum levels of phytosterols (campesterol, and β-sitosterol), lathosterol, TG, and glucose were not significantly different between the two groups.

Table II-4. Serum parameters[†]

	LGC-Jun	LGC-1	<i>p</i> value
	(mg/dL)		
Total cholesterol	467 ± 24	384 ± 24*	0.042
Free cholesterol	82.0 ± 5.3	71.5 ± 2.2	0.105
Cholesterol ester	385 ± 20	313 ± 22*	0.043
HDL cholesterol	19.5 ± 1.3	19.2 ± 0.9	0.831
Non-HDL cholesterol	447 ± 25	365 ± 24*	0.043
Lathosterol	0.623 ± 0.080	0.505 ± 0.037	0.213
Campesterol	0.683 ± 0.015	0.755 ± 0.048	0.194
β-Sitosterol	1.50 ± 0.06	1.38 ± 0.20	0.582
Triglyceride	101 ± 11	106 ± 16	0.768
Glucose	159 ± 3	170 ± 6	0.118

[†]Values have been expressed as mean ± SEM (n= 5 /group). Asterisks show a significant difference at $p < 0.05$, as calculated using Student's *t*-test.

3.3 Effects of the LGC-1 diet on hepatic lipid parameters

Hepatic levels of 7-dehydrocholesterol were higher in the LGC-1 group than that in the LGC-Jun group with $p = 0.161$. Hepatic squalene levels were significantly higher in the LGC-1 group compared to those in the LGC-Jun group (Table II-5) ($p < 0.05$). Among oxysterol species, hepatic levels of 7α-hydroxycholesterol and β-Epoxycholesterol tended to decrease in the LGC-1 group, as compared to the LGC-Jun group, with $p = 0.098$ and 0.078 , respectively (Table II-5).

Table II-5. Hepatic lipid parameters[†]

	LGC-Jun	LGC-1	<i>p</i> value
Cholesterol (mg/g liver)	36.7 ± 4.6	34.2 ± 2.6	0.804
Triglyceride (mg/g liver)	26.8 ± 1.4	26.0 ± 2.6	0.429
Esterified Cholesterol (mg/g liver)	30.9 ± 4.9	26.1 ± 3.7	0.475
Cholesterol synthesis intermediates			
Squalene (µg/g liver)	81.8 ± 1.7	107 ± 7.0*	0.010
Cholestanol (µg/g liver)	84.7 ± 3.8	93.6 ± 5.4	0.214
8-Dehydrocholesterol (µg/g liver)	9.02 ± 0.32	8.93 ± 0.77	0.919
Desmosterol (µg/g liver)	28.2 ± 0.2	29.2 ± 1.6	0.554
7-Dehydrocholesterol (µg/g liver)	78.8 ± 2.7	85.9 ± 3.7	0.161
Lathosterol (µg/g liver)	28.4 ± 1.3	29.4 ± 2.9	0.778
Oxycholesterol			
7α-Hydroxycholesterol (µg/g liver)	25.3 ± 2.6	20.0 ± 1.9	0.098
7β-Hydroxycholesterol (µg/g liver)	1.07 ± 0.08	1.05 ± 0.08	0.864
β-Epoxycholesterol (µg/g liver)	6.07 ± 0.48	4.93 ± 0.41	0.079
α-Epoxycholesterol (µg/g liver)	1.98 ± 0.16	1.71 ± 0.27	0.384
4β-Hydroxycholesterol (µg/g liver)	1.08 ± 0.05	1.21 ± 0.09	0.188
6-Ketocholestanol (µg/g liver)	0.152 ± 0.019	0.167 ± 0.061	0.794
7-Ketocholesterol (µg/g liver)	0.343 ± 0.056	0.353 ± 0.090	0.915
25-Hydroxycholesterol (µg/g liver)	0.962 ± 0.107	1.015 ± 0.061	0.648
Phytosterol			
Campesterol (µg/g liver)	90.6 ± 39.6	82.0 ± 40.4	0.356
β-Sitosterol (µg/g liver)	65.2 ± 7.6	58.0 ± 9.8	0.088
Stigmasterol (µg/g liver)	8.28 ± 0.71	9.99 ± 2.09	0.727

[†]Values have been expressed as mean ± SEM (n= 5 /group). Asterisks show a significant difference at *p* < 0.05, as calculated using Student's *t*-test.

3.4 Effects of the LGC-1 diet on fecal lipid parameters

Fecal dry weights and fecal contents of neutral and acidic steroids were similar between the two groups (Table II-6). Fecal acidic steroid contents lower in the LGC-1 group than that in the LGC-Jun group (Table II-6).

Table II-6. Fecal parameters[†]

	LGC-Jun	LGC-1	<i>p</i> value
Dry weight (g/day)	2.97 ± 0.08	2.97 ± 0.14	0.966
Neutral steroids (mg/day)	29.6 ± 1.4	28.7 ± 1.5	0.679
Acidic steroids (mg/day)	49.2 ± 3.6	41.0 ± 4.8	0.204

[†]Values have been expressed as mean ± SEM (n= 5/group). The value of fecal neutral steroids represents the sum of fecal cholesterol and coprostanol.

3.5 Effects of the LGC-1 diet on lymphatic cholesterol transport

Cumulative lymphatic cholesterol amounts per hour and total amount of lymphatic cholesterol amounts of the LGC-1 group were not significantly difference from those of the LGC-Jun group (Figure II-1).

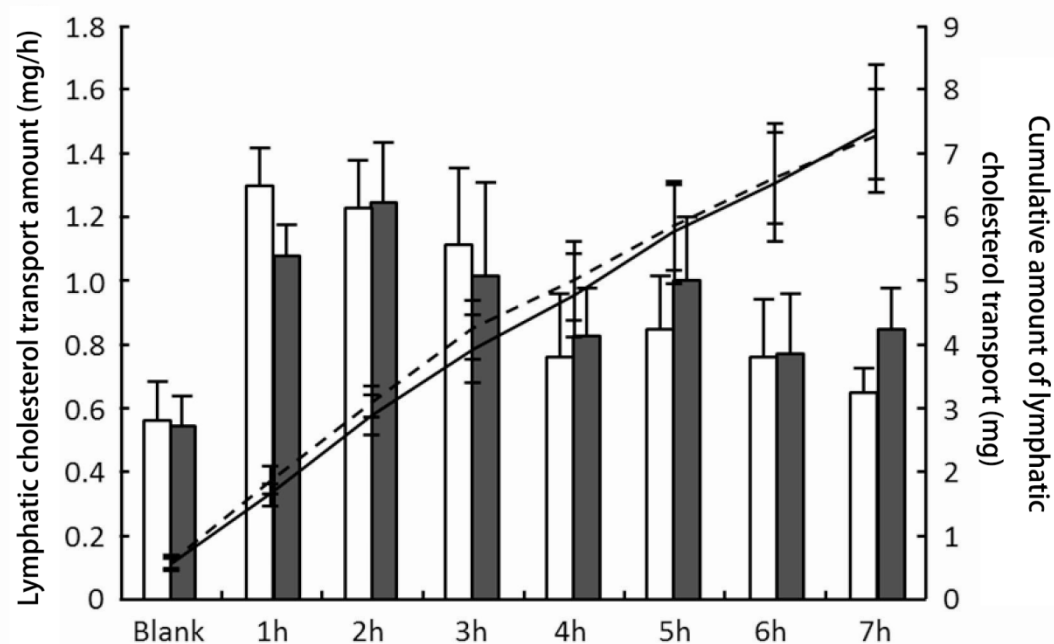


Figure II-1. Lymphatic transport of cholesterol in rats that were fed diets containing LGC-Jun and LGC-1.

(Line graphs: right axis) Values have been represented as cumulative amount of lymphatic cholesterol transport in LGC-Jun (solid line) and LGC-1 (dashed line) groups. (Bar graphs: left axis) Values have been represented as lymphatic cholesterol flow rate of LGC-Jun (white bars) and LGC-1 (black bars) groups at each hour. All values have been expressed as mean $\pm$ SEM (n=5/group).

3.6 Effects of the LGC-1 diet on expression levels of genes related to cholesterol and bile acid metabolism in the liver

Hepatic *Cyp7a1* levels were significantly lower in the LGC-1 group than that in the LGC-Jun group (Table II-7). The mRNA levels of the other genes did not differ between the two groups (Table II-7).

Table II-7. Expression levels of genes related to cholesterol and bile acid metabolism in the liver[†]

gene	LGC-Jun	LGC-1	<i>p</i> value
(Arbitrary unit)			
<i>Hmgcr</i>	1.00 ± 0.16	0.94 ± 0.08	0.744
<i>Cyp7a1</i>	1.00 ± 0.19	0.51 ± 0.06*	0.038
<i>Ldlr</i>	1.00 ± 0.07	1.03 ± 0.08	0.771
<i>Soat1</i>	1.00 ± 0.10	0.82 ± 0.10	0.230
<i>Soat2</i>	1.00 ± 0.07	0.95 ± 0.03	0.516

[†]Values have been expressed as mean ± SEM (n= 5 /group). Asterisks show a significant difference at *p* < 0.05, as calculated using Student's *t*-test.

4. Discussion

In this study, we examined the hypocholesterolemic effect of LGC-1, a rice cultivar containing high α -globulin in its endosperm. After the experimental period, LGC-1 feeding significantly decreased serum cholesterol levels in ExHC rats. The hypocholesterolemic effect of α -globulin, which has been evaluated under less physiological conditions in previous studies [9-10], was retained even when administered as a dietary ingredient.

LGC-1 have a certain weight-loss effect because the final body weight and body weight gain were significantly reduced in the LGC-1 group, as compared to the LGC-Jun group (Table II-3). Our previous study showed that rice α -globulin decreased serum cholesterol but had no effect on body weight gain in atherosclerotic mice [9]. The peptide, GEQQQQPGM, derived from rice α -globulin, decreased serum total cholesterol levels, but had no effect on body weight gain in hamsters [10]. There was no significant difference in body weight gain between ExHC and SD rats with the same dietary energy intake [22]. Important differences between this study and our previous studies are the techniques and dosages of α -globulin administrations. In a

previous study, α -globulin was orally administered at 100 mg/kg body weight/day and had no effect on body weight loss in mice [9]. In this study, we fed α -globulin as rice flour at about 614 mg/kg body weight/day in rats. Therefore, the dose-dependent increase in α -globulin intake might be the main reason for the 13% body weight loss in ExHC rats. A previous study demonstrated that a diet containing 2% peptides (mg/kg body weight/day) isolated from black soybeans (*Rhynchosia. volubilis* Lour.) inhibited weight gain by 11% and improved lipid profiles in rats that were fed a high-fat, high-cholesterol diet for 28 days [23]. The body weight loss and weight gain inhibition mediated by black soybean peptides occurred in a concentration-dependent manner [23]. The above results suggest that plant protein or peptide intake exerts a weight-loss effect in rats.

We investigated the mechanism of LGC-1 on cholesterol-lowering in ExHC rats. The size of the cholesterol pool in the body is determined as a result of balance among the processes of *de novo* cholesterol synthesis, catabolism (bile acid synthesis), cholesterol esterification, dietary intake, and intestinal absorption. The effect of LGC-1 on *de novo* cholesterol synthesis was examined. Cholesterol biosynthesis in mammals, following conversion of squalene to lanosterol, is undertaken by two pathways known as the Kandutsch-Russell and Bloch pathways [24]. Hepatic levels of the intermediates, such as desmosterol, in the Bloch pathway were similar between the two groups (Table II-5). Dietary LGC-1 only significantly increased hepatic levels of squalene in the rats, but not of the other cholesterol synthesis intermediates (Table II-5). At the same time, we find that LGC-1 diet did not decrease the expression of *hmgcr* and there is no significant difference on cholesterol content in the liver. A previous study reported that squalene levels in the liver are increased when the activity of squalene monooxygenase (SM), the second rate-limiting enzyme of cholesterol synthesis, is suppressed [25]. Using model cell systems, the study found that cholesterol caused the accumulation of the squalene, suggesting that SM may serve as a controlling enzyme beyond HMG-CoA reductase (HMGR) in *de novo* cholesterol synthesis [26]. Therefore, SM activities in the liver of ExHC rats being fed the LGC-1 diet may be inhibited. The LGC-1 diet only affected

the squalene level, resulting from partial inhibition of cholesterol synthesis. However, the LGC-1 diet did not affect the hepatic *Hmgcr* mRNA level. In general, HMGCR mRNA and protein levels are regulated by several factors like SREBP2 with a circadian rhythm [27]. Furthermore, the enzyme activity is regulated by phosphorylation and dephosphorylation [28]. It is considered that the amount of mRNA and protein does not always increase and decrease proportionally. Therefore, we measured the level of intermediates in *de novo* cholesterol synthesis as indicators of the rate of the synthetic system. As a result, an increase in the level of squalene was observed. This increase indicated the LGC-1 diet partially inhibited *de novo* cholesterol synthesis. Then the effect of LGC-1 on bile acid synthesis was examined. In bile acid synthesis, which follows cholesterol synthesis in the liver, *Cyp7a1* is the first step limiting enzyme [29]. Dietary LGC-1 significantly decreased *Cyp7a1* mRNA levels in the liver (Table II-7). 7 α -Hydroxycholesterol is the product of cholesterol 7 α -hydroxylase [30]. The LGC-1 diet decreased the hepatic levels of 7 α -hydroxycholesterol (Table II-5, $p = 0.098$) and also decreased the fecal acidic steroid contents (Table II-6). These results indicated that the LGC-1 diet inhibits bile acid synthesis in the liver. A study showed that an inhibition of bile acids synthesis caused enterohepatic circulation inhibition [31]. Enterohepatic circulation inhibition further regulates cholesterol reabsorption and reduces serum cholesterol level [31]. Taken together, the reducing effect of LGC-1 on serum cholesterol level is exerted as a result of partial inhibitions of bile acid synthesis and cholesterol *de novo* synthesis in the liver.

Then the effect of LGC-1 on cholesterol esterification was examined because a large amount of cholesterol is esterified and packaged into lipoprotein. Sterol O-acyltransferase (SOAT), also called acyl-coenzyme A: cholesterol acyltransferase, which is the first step catalytic enzyme for cholesterol esterification, converts cholesterol to cholesterol esters and can be activated by cholesterol [32-33]. SOAT1 and SOAT2, two isoforms of SOAT, have been identified in various mammals, including humans and rats, and regulate hepatic cholesterol esterification to protect against the unnecessary buildup of free cholesterol within the cell membranes [34]. In

this study, dietary LGC-1 did not change hepatic *Soat1* and *Soat2* mRNA levels (Table II-7) and there is no significant difference of hepatic. Thus, these results indicated that LGC-1 has no effect on hepatic cholesterol esterification.

Finally, the effect of LGC-1 on dietary cholesterol intake and intestinal absorption was investigated. The LGC-Jun and LGC-1 groups had the same dietary cholesterol intake. Then campesterol and β -sitosterol concentrations in the liver and serum [35], fecal neutral steroid excretion [9], and lymphatic cholesterol transport [21] were measured to evaluate dietary cholesterol absorption. Dietary LGC-1 did not change these indicators (Tables II-4 and II-6 and Figure II-1). These results suggest that LGC-1 has no effect on dietary cholesterol intake and intestinal absorption. The latest research show that cholesterol metabolism and intestinal microbes have a close connection. A variety of gut microbes have been shown to reduce the risk of atherosclerosis, non-alcoholic fatty liver disease and type 2 diabetes which cause by high fructose and high cholesterol diet in mammals [36-37]. Dietary amino acids or protein are not only absorbed and metabolized by enterocytes but also available to microbes in the gut in mammals [38]. Grape seed proanthocyanidin reduces serum cholesterol level *via* changing gut microflora and enhancing propionate production in weaned pigs [39]. A study showed that propionate is involved in cholesterol synthesis and affects intestinal cholesterol absorption [40]. LGC-1 contains a larger amount of α -globulin, which is considered to be a functional component of its serum cholesterol-lowering effect. LGC-1 intake may affect the intestinal propionate-producing microbes and then lower serum cholesterol. The functionality of proteins is generally determined by their amino acid sequence and spatial structure. The peptide, GEQQQQPGM, derived from rice α -globulin, has been shown to decrease serum total cholesterol levels and serum HDL cholesterol in hamsters that were fed a hypercholesterolemic diet [10], consistent with the findings of this study. In either hypothesis, LGC-1 can be used as a new functional food with a very broad research value.

5. Conclusion

In conclusion, a rice cultivar LGC-1, containing higher α -globulin content, significantly decreased serum cholesterol levels in ExHC rats. The mechanism for the serum cholesterol-lowering effect of LGC-1 is suggested to be through partial inhibition of bile acid and cholesterol synthesis in the liver. LGC-1 can be used as a new functional food ingredient. The findings of this study can effectively improve the economic value of LGC-1 and provide a theoretical basis and guidance for the development and utilization of Japanese rice cultivars.

CHAPTER III The effects of dietary linoleic acid on atherosclerosis development and aortic inflammation in different dietary cholesterol levels

1. Introduction

Atherosclerosis and related complications are among the disorders contributing to the highest rates of human mortality and have become a substantial threat to human health [1]. The signs and symptoms of Atherosclerosis are chronic degeneration of arteries and gradual changes in arterial walls [2]. Excessive cholesterol and fatty acids accumulate inside and outside the cells, making the arterial wall hard and thick. The arteries become thin and lose their elasticity in the final stage of atherosclerosis with oxidative stress and inflammation. The size of aortic lesions, accompanied by elevated levels of serum cholesterol and aortic inflammation, has been found to be a key variable in atherosclerosis [2]. Numerous clinical studies have shown that reducing serum cholesterol and aortic endothelial inflammation are salient factors in the prevention and treatment of this disease [3].

Epidemiological studies have indicated that compared with an intake of saturated fatty acids (SFAs), n-6 polyunsaturated fatty acid (n-6 PUFA) intake is associated with a lower risk of cardiovascular disease [4]. Among the latter, linoleic acid (C18:2, n-6) (LA) is considered a potent dietary fatty acid that reduces plasma total cholesterol levels in humans [5]. Research has also indicated that diets extremely high in LA (16% to 29% of dietary fat) contribute to a significant lowering of plasma low-density lipoprotein cholesterol levels in humans by between 16% and 22% compared with diets high in SFAs (19% to 30% of dietary fat) and low in LA (only 2.3% to 4.5% of dietary fat) [5]. In animal studies, the structured fat containing LA has been found to show a protective effect against atherosclerosis in C57BL/6J mice when fed high LA diets for 15 weeks [6]. In contrast, the findings of several studies have shown that an increased intake of LA leads to elevated levels of inflammation, which may be associated with endothelial dysfunction, given that LA is a precursor of arachidonic

acid (AA) and inflammatory eicosanoids [7]. The conversion of LA to AA in healthy humans is about 1% [8]. An excess LA intake could promote the conversion to produce a non-negligible amount of AA, and sequentially intermediates of the pro-inflammatory cascade [8]. The inflammatory impact of the linoleic acid intake and conversion has been observed in experimental studies using the rat as the model [9]. *In vivo*, the pro-inflammatory marker isoprostane is formed primarily via the non-enzymatic free radical-catalyzed oxidation of AA. Turpeinen *et al.* have shown that in healthy subjects, urinary 8-iso-prostaglandin F2 α (8-iso-PGF2 α), a reliable biomarker of inflammation, increases with consumption of an LA-rich diet [10]. Collectively, the findings of the aforementioned studies indicate that an increased intake of LA leads to increased inflammation and oxidative stress, which may be associated with endothelial dysfunction and promotes the development of atherosclerosis in animal models.

LA is in ambivalent status on the serum cholesterol-lowering and aortic pro-inflammation. The findings of a previous study by Sato *et al.* have revealed that LA-rich safflower oil reduces the development atherosclerosis beyond its oxidative and inflammatory stress-increasing effects in apolipoprotein E (apoE)-deficient mice [11]. The essence of this dietary LA activity is assumed to be associated with its effect on lowering serum cholesterol levels. Consequently, dietary LA may promote arteriosclerosis when serum cholesterol levels are comparable those of a SFA diet by controlling the cholesterol content of the LA diet. In the present study, we used apoE-deficient (ApoE^{-/-}) mice fed a high-cholesterol diet as an atherogenic animal model to investigate whether LA can induce the development of aortic atherosclerosis in response to the provision of a high-cholesterol LA diet.

2. Materials & Methods

2.1 Materials

Trimethylsilylation reagent solution, a mixture of *N*, *O*-bis (trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (99:1), was purchased from Merck-Supelco (Burlington, MA, USA). Commercial enzyme assay kits (Cholesterol E,

Free Cholesterol E, and Triglyceride E test kits) were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). 15-F2t-IsoPM [$^{18}\text{O}_2$], as a stable isotope labeled 15-F2t-IsoPM (dinor) which is used for analyzing the content of 15-F2t-IsoPM in urine by GC-MS, was kindly donated by Dr. J.D. Morrow (Department of Medicine and Pharmacology, Vanderbilt University, Nashville, USA).

2.2 Animals and diets

ApoE^{-/-} mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). A total of 30 male and female ApoE^{-/-} mice (18 males and 12 females, 7 weeks old) with initial weights of 20.5 and 19.1 g, respectively, were raised in the Animal Laboratory Building (AG12) of the Department of Bioresources and Bioenvironmental Science, Faculty of Agriculture, Kyushu University. The mice were individually housed in metal cages and maintained in a temperature-controlled room (22-25°C) under a 12-h light cycle (dark period 20:00-08:00). Prior to the experimental period, the mice were given free access to a commercial non-purified diet (NMF; Oriental Yeast Co., Tokyo, Japan) and deionized water for 1 week. Following this preliminary phase, the 30 male and female ApoE^{-/-} mice were randomly divided into three groups (each containing six males and four females). These mice were fed one of the following three AIN-76 diets containing different fat and cholesterol contents: saturated fatty acid-rich fat (50 g palm oil and 50 g lard/kg) and low cholesterol content (0.4 g/kg) (the SFA group); LA fat (100 g high-linoleic safflower-seed oil/kg) and low cholesterol content (0.4 g/kg) (the LALC group; and LA fat (100 g high-linoleic safflower-seed oil/kg) and high cholesterol content (1 g/kg) (the LAHC group). The overall and fatty acids compositions of the three experimental diets are listed in tables III-1 and III-2, respectively. In the feeding experiment, the male and female mice were fed an experimental diet for 9 weeks, after which, the animals were then sacrificed by withdrawal of aortic blood under pentobarbitone anesthesia (0.08 mg/100 g body weight), followed by immediate excision of the liver and the aorta for use in subsequent experiments. To isolate serum, blood was collected, incubated at room temperature for 30 min, and centrifuged at $1,750 \times g$ for 10 min at 4°C. The handling and euthanasia of all animals were carried out in accordance with nationally

prescribed guidelines and all experiments were conducted following ethical approval from the Animal Care and Use Committee of Kyushu University (authorization number: A02-005-01), as well as in accordance with law no. 105 and notification no. 6 of the Government of Japan.

Table III-1. Compositions of the experimental diets

Ingredients (g/kg diet)	SFA	LALC	LAHC
Sucrose	449.6	449.6	449.0
Casein	200	200	200
Corn starch	150	150	150
Palm oil	50	-	-
Lard	50	-	-
High linoleic acid safflower oil	-	100	100
Cellulose	50	50	50
Mineral mix (AIN-76)	35	35	35
Vitamin mix (AIN-76)	10	10	10
DL-Methionine	3	3	3
Choline bitartrate	2	2	2
Cholesterol	0.4	0.4	1.0
Total	1000	1000	1000

Table III-2. Fatty acid compositions of the experimental diets

	SFA	LALC	LAHC
		(%)	
12:0	0.1	-	-
14:0	1.2	-	-
16:0	33.0	7.2	7.2
16:1	2.6	0.2	0.2
18:0	9.7	2.6	2.6
18:1	40.9	16.1	16.1
18:2 (n-6)	11.4	71.6	71.6
18:3 (n-3)	-	0.2	0.2
20:5	-	-	-
22:6	-	-	-
Others	1.1	2.1	2.1
SFA	44.0	9.8	9.8
n-6 PUFA	11.6	71.6	71.6

2.3 Morphometric determination of atherosclerosis

To determine the size and cross-sectional area of aortic lesions, hearts containing aortic roots were processed for quantitative atherosclerosis assays as previously described by Tomoyori *et al.* [12]. ApoE^{-/-} mice were perfused with 50 mL of phosphate-buffered saline (pH 7.4) *via* a cannula inserted into the left ventricle and following dissection of the aorta and its main branches from the aortic valve to the iliac bifurcation, perfusion of the heart was immediately continued with 50 mL of 10% (v/v) neutral formalin buffer solution (pH 7.4). The heart was removed and fixed in 10% (v/v) neutral formalin buffered solution and stored at 4°C for approximately 1 week. The heart and the aorta were removed and fixed in 10% neutral formalin-buffered solution. The percentage of the aortic surface covered by lesions was determined using an

enface preparation as described previously. To determine the cross-sectional lesion area, hearts containing aortic roots were processed for the quantitative atherosclerosis assay using a modification of the method described by Paigen *et al.* Briefly, the heart was cut along a plane between the tip of two atria and the top half was embedded in paraffin. Serial sections (10 mm thick) were prepared from the ascending aorta to the aortic sinus until the aortic tissue was invisible. The sections were mounted on glass slides and stained with elastic Van Gieson and haematoxylin as described previously. Five sections of each heart were selected for intimal area determination. The first and most distal sections to the heart were taken where the aortic valve was just visible, from this section, moving to the base of the heart, the 15th, 30th, 45th and 60th sections were also photographed along with the first one, using a video camera mounted on an Olympus IX70 light microscope (Olympus Optical, Tokyo, Japan). The intimal area was measured by NIH image/68k 1.57 software (National Institute of Health, Bethesda, MD, USA) on a Macintosh computer. The mean intimal area was calculated for each animal and subsequently for each group.

2.4 Analysis of serum and liver parameters

The levels of total cholesterol in serum were measured using commercial enzyme assay kits (Cholesterol E test kit), and to determine high-density lipoprotein (HDL) cholesterol levels, HDL fractions were collected as described previously by Tomoyori *et al.* [12] and then measured using the Cholesterol E test kit. Total lipids from the liver were extracted using the method described by Folch *et al.* [13] and total liver and free cholesterol levels were measured using a method previously described by Tomoyori *et al.* [12]. Liver cholesterol ester levels were calculated as the difference between total and free cholesterol levels. Liver triacylglycerol levels were measured using a triglyceride E test kit, liver phospholipids were chemically determined as described previously by Tomoyori *et al.* [12]. One point five mL of liver lipid extract was sampled, and the organic solvent was dried up. A 70% perchloric acid solution was added and heated at 180°C until the solution became colorless. After adding 5 mL of deionized water, 1 mL of 2.5% (w/v) ammonium molybdate aqueous solution, and 1

mL of 10% (w/v) ascorbic acid aqueous solution in sequence, the mixture was heated in a boiling water bath for 5 min. After cooling, the absorbance was measured at 820 nm to quantify lipid phosphorus in the sample. In addition, potassium dihydrogen phosphate was used as a standard. The fatty acid composition of hepatic phospholipids was determined according to the methods described by Carvajal *et al.* [14].

2.5 Determination of aortic Monocyte chemoattractant protein-1 (*Mcp-1*) mRNA levels

Total RNA was isolated from the aorta using the phenol/chloroform method, as described previously by Yuan *et al.* [15]. Complementary DNA was synthesized from 1.0 µg total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Berlin, Germany). Gene expression levels were analyzed based on quantitative real-time reverse transcription polymerase chain reaction using a SYBR® Premix EX Taq II Kit (Takara, Shiga, Japan) and an Mx3000P QPCR Thermal Cycler System (Agilent, CA, USA). mRNA levels were normalized using the β -actin (*Actb*) gene as an internal standard. The sequences of primers used for analyses are showed follow: *Mcp-1* (Forward primer: CACCAGCAAGATGATCCCAATG and Reverse primer: AAGGCATCACAGTCCCAGTCACAC); *Actb* (Forward primer: GGATCCCAGATCATGTTTGAGACCTTCAA and Reverse primer: GAATTCGGAGAGCATAGCCCTCGTAGATGG).

2.6 Determination of urinary isoprostaglandins

Purification and measurement of urinary 8-iso-PGF₂ α and 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F₂ α (15-F₂t-IsoPM, dinor) were carried out using a Shimadzu QP2020A GC/MS system (Shimadzu, Kyoto, Japan) as previously described by Sato *et al.* [11]. GC was performed using a 30-m, 0.25-mm-diameter, 0.25-µm-film-thick, SPB-20 fused silica capillary column (Supelco). The column temperature was initially maintained at 100°C for 2 min. The column was then heated to 250°C in 7 min and then to 290°C at 2°C/min and maintained at this temperature. Isobutane was used as a reactant gas for negative chemical ionization and helium as a carrier gas at 1.1 mL/min. The ion source temperature was 290°C and the electron

energy was 70 ev. The ion monitor for endogenous 8-iso-PGF2a or 15-F2t-IsoP-M was set at m/z 569 (M-181) and m/z 547, respectively. 8-Iso-PGF2a-d4 (Cayman Chemicals) or 15-F2t-IsoP-M[$^{18}\text{O}_2$] (kindly donated by Dr J. D. Morrow, Department of Medicine and Pharmacology, Vanderbilt University, Nashville, TN 37 232, USA) was used as an internal standard and ions at m/z 573 or m/z 547 were monitored. The quantification of endogenous 8-isoPGF2a or 15-F2t-IsoP-M was accomplished by SIM analysis of the ratio of m/z 569 to m/z 573 or m/z 543 to m/z 547, where the lower limit of detection (signal: noise ratio of 4:1) of 8-isoPGF2a and 15-F2t-IsoP-M were 28 and 109 pg, respectively. A standard curve was constructed by adding varying amounts of unlabeled 8-iso-PGF2a to 1ng 8-isoPGF2a-d4 or 15-F2t-IsoP-M to 1ng of 15-F2t-IsoP-M[$^{18}\text{O}_2$]. The concentration of urine isoprostanes was expressed as a function of urinary creatinine (Wako Pure Chemicals, Osaka, Japan).

2.7 Statistical analysis

All data are presented as the means $\pm$ standard error of the mean (SEM) unless otherwise indicated. Male and female groups were compared using one-way ANOVA with Fisher's Protected Least Significant Difference (PLSD) test to identify individual differences. Analyses were performed using GraphPad 8.3 with significance set at $p < 0.05$, and $0.05 < p < 0.1$ being considered indicative of a trend. In subsequent analyses, we did not distinguish between the sexes, and analysis of the correlation between related indicators was performed using a correlation set at $p < 0.05$.

3. Results

3.1 Growth parameters and relative organ weights

As shown in Table III-3, there were no significant differences in dietary intake, feed efficiency, liver weight, or aorta weight between males and females among the three groups. In male mice, the final body weight was significantly higher in those in the LALC group than in the LAHC group, and body weight gain tended to decrease in the LAHC group ($p = 0.052$) compared with that in the LALC group. Contrastingly, there are no significant differences in body weight gain or final body weight among female mice in the three experimental groups.

Table III-3. Growth parameters assessed in the study

	male			female		
	SFA	LALC	LAHC	SFA	LALC	LAHC
Body weight (g)						
Initial	20.3 ± 1.9	21.5 ± 0.5	20.7 ± 1.1	18.8 ± 0.8	19.3 ± 1.2	19.0 ± 0.2
Final	32.4 ± 1.7 ^{ab}	34.6 ± 1.6 ^a	29.6 ± 1.2 ^b	27.3 ± 0.5	28.7 ± 1.7	25.8 ± 0.6
Gain	12.1 ± 1.7	13.2 ± 1.4	8.9 ± 1.1	8.6 ± 1.0	9.5 ± 1.0	6.8 ± 0.8
Daily food intake (g/day)	4.5 ± 0.1	4.5 ± 0.2	4.2 ± 0.1	5.0 ± 0.2	4.6 ± 0.1	4.5 ± 0.3
Feed efficiency (%)	42.5 ± 5.3	46.0 ± 4.0	33.7 ± 4.6	26.9 ± 2.5	32.5 ± 3.1	24.7 ± 4.5
Aorta (mg)	12 ± 1	17 ± 2	13 ± 2	19 ± 3	16 ± 5	14 ± 3
Liver weight (g)	1.65 ± 0.19	1.45 ± 0.11	1.49 ± 0.12	1.16 ± 0.14	1.25 ± 0.07	1.29 ± 0.15
Relative liver weight (g/100 g b.w*.)	5.07 ± 0.46	4.20 ± 0.32	4.98 ± 0.23	4.26 ± 0.52	4.39 ± 0.34	4.99 ± 0.50

Values are the means ± SEM (n = 4 or 6 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using Fisher's PSLD method. *b.w.: body weight.

3.2 Lesion size and cross sections in the aortic root

As shown in Figure III-1, in male mice, the lesion size and cross sections of aortic root lesions were significantly reduced in the LALC group compared with the SFA group and were significantly increased in the LAHC group compared with the LALC group. However, in female mice, there were no significant differences among females with respect to lesion size and cross section between SFA and LALC groups, although these parameters were significantly increased in the LAHC females compared with those in the LALC group.

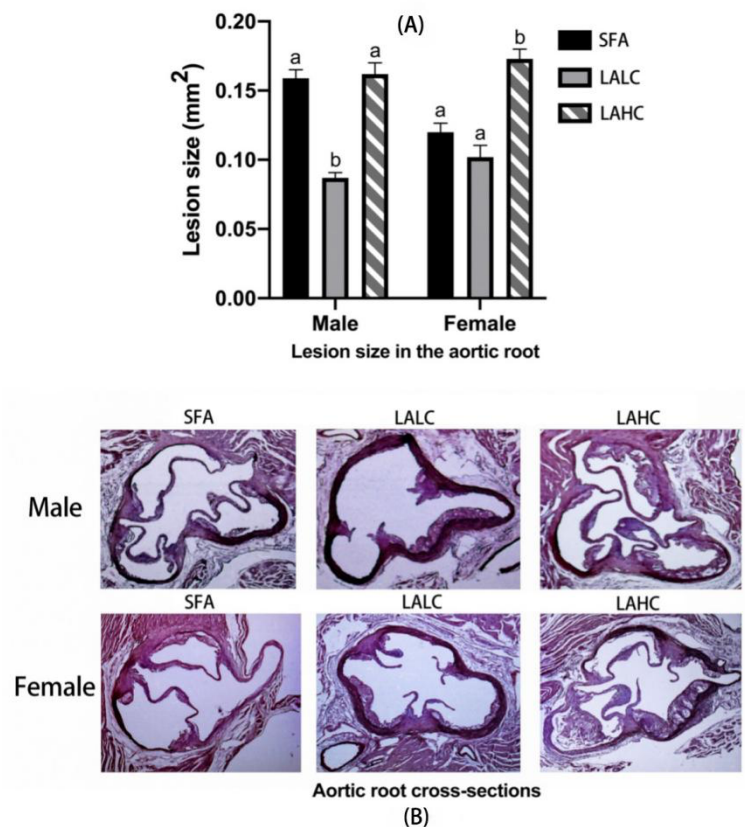


Figure III-1. Lesion sizes in the aortic root (A) and aortic root cross-sections (B) from male and female ApoE^{-/-} mice

Values are the means $\pm$ SEM ($n = 4$ or 6 /group). Different letters above bars indicate a significant difference at $p < 0.05$, as determined using Fisher's PSLD method.

3.3 Serum, hepatic, and urinary parameters

As shown in Table III-4, in male mice, serum and hepatic total cholesterol levels were significantly lower in the LALC group than in the SFA group and significantly higher in the LAHC group than in the LALC group. Hepatic cholesterol ester levels were significantly lower in the LAHC group than in the LALC group. Analogous results were obtained for the female groups with respect to serum and hepatic total cholesterol levels. Urinary 8-iso-PGF2 α levels in the female LALC group were found to be significantly higher than those in the LAHC group. There was no significant difference among the three male groups with respect to urinary 8-iso-PGF2 α levels. Conversely, in male mice, urinary dinor levels were significantly higher in the LALC

group than those in the LAHC group, whereas no significant difference in urinary dinor levels were detected among the three female groups.

Table III-4. Serum cholesterol, hepatic lipid parameters, and urinary isoprostane levels

	male			female		
	SFA	LALC	LAHC	SFA	LALC	LAHC
Serum	(mg/dL)					
Total cholesterol	799.0 ± 27.3 ^a	640.7 ± 41.1 ^b	790.4 ± 57.7 ^a	575.0 ± 38.3 ^{ab}	429.4 ± 50.1 ^a	667.5 ± 55.1 ^b
HDL cholesterol	47.5 ± 12.2	38.6 ± 9.0	46.1 ± 7.9	20.7 ± 6.5	32.7 ± 7.0	39.6 ± 13.8
Liver	(mg/g liver)					
Total cholesterol	10.12 ± 1.15 ^a	5.59 ± 0.43 ^b	9.60 ± 0.59 ^a	8.04 ± 0.33 ^a	5.14 ± 0.50 ^b	10.38 ± 1.10 ^c
Free cholesterol	2.58 ± 0.2	2.51 ± 0.09	2.74 ± 0.30	3.13 ± 0.25 ^a	1.95 ± 0.07 ^b	2.87 ± 0.19 ^a
Cholesterol ester	7.54 ± 1.05 ^a	3.08 ± 0.40 ^b	6.88 ± 0.43 ^a	4.91 ± 0.31 ^{ab}	3.19 ± 0.57 ^a	7.51 ± 1.16 ^b
Triglyceride	85.5 ± 16.8	86.9 ± 11.2	81.5 ± 17.4	76.8 ± 14.7	70.5 ± 8.3	73.1 ± 17.1
Phospholipid	27.2 ± 2.0	30.1 ± 0.6	30.4 ± 1.1	29.4 ± 0.5 ^a	32.5 ± 0.6 ^b	33.0 ± 1.3 ^b
Urinary	(ng/mg creatinine)					
8-iso-PGF2 α *	0.30 ± 0.03	0.39 ± 0.09	0.39 ± 0.09	0.71 ± 0.16 ^{ab}	0.84 ± 0.04 ^a	0.54 ± 0.06 ^b
dinor**	3.90 ± 0.69 ^a	12.86 ± 2.38 ^b	5.01 ± 1.77 ^a	14.62 ± 0.99	23.78 ± 4.50	23.57 ± 2.31

Values are the means ± SEM (n = 4 or 6 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using Fisher's PSLD method.

8-iso-PGF2 α *: 8-iso-prostaglandin F2 α ; dinor**: 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F2 α .

3.4 Aortic *Mcp-1* mRNA level

As shown in Figure III-2, in male mice, the levels of aortic *Mcp-1* mRNA were significantly higher in the LALC group than in the SFA group, whereas no significant differences between the male LALC and LAHC groups were observed. In female mice, consumption of the different experimental diets had no effects on *Mcp-1* mRNA levels.

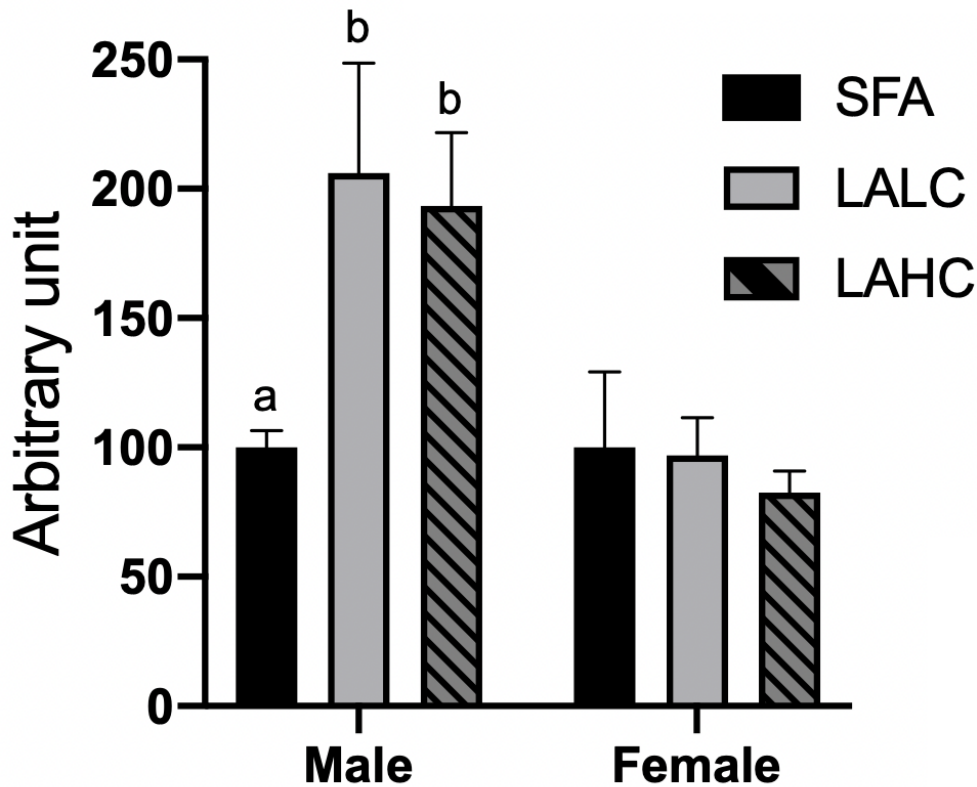


Figure III-2. Aortic *Mcp-1* mRNA levels

Values are the means $\pm$ SEM ($n = 4$ or 5 /group). Different letters above bars indicate a significant difference at $p < 0.05$, as determined using Fisher's PSLD method.

3.5 Fatty acid composition of liver phospholipids

As shown in Table III-5, both in male and female mice, the levels of arachidonic acid (C20:4 n-6, AA) were significantly higher in the LALC and LAHC groups than in the SFA group, and a similar pattern was observed regarding n-6 PUFA. We also found that the 20:4/18:2 ratio in the male LAHC group was significantly lower than that in the LALC group, whereas no significant difference in this ratio was detected

between the female LALC and LAHC group.

Table III-5. Fatty acid composition of hepatic phospholipids

	male			female		
	SFA	LALC	LAHC	SFA	LALC	LAHC
	(%)					
16:0	17.5 ± 0.8	16.5 ± 0.8	16.9 ± 0.6	16.8 ± 0.4 ^a	14.8 ± 1.0 ^{ab}	13.5 ± 0.8 ^b
16:1	1.7 ± 0.1 ^a	0.8 ± 0.0 ^b	0.9 ± 0.0 ^b	1.2 ± 0.1 ^a	0.5 ± 0.1 ^b	0.5 ± 0.0 ^b
18:0	14.2 ± 0.5 ^a	15.8 ± 0.3 ^b	15.2 ± 0.2 ^{ab}	16.8 ± 0.5	19.2 ± 1.0	18.9 ± 0.8
18:1(n-9)	14.0 ± 0.2 ^a	6.3 ± 0.2 ^b	6.7 ± 0.2 ^b	13.1 ± 0.7 ^a	5.5 ± 0.2 ^b	5.9 ± 0.2 ^b
18:1(n-7)	3.5 ± 0.2 ^a	1.7 ± 0.1 ^b	1.8 ± 0.1 ^b	2.9 ± 0.2 ^a	1.3 ± 0.1 ^b	1.4 ± 0.1 ^b
18:2(n-6)	10.0 ± 0.4^a	16.7 ± 0.8^b	19.8 ± 0.9^c	9.9 ± 0.4^a	15.4 ± 0.4^b	16.7 ± 0.4^c
20:3(n-6)	3.1 ± 0.2 ^a	2.2 ± 0.1 ^b	2.7 ± 0.2 ^{ab}	2.4 ± 0.1 ^a	1.5 ± 0.2 ^b	1.8 ± 0.1 ^b
20:4(n-6)	20.9 ± 0.5^a	26.0 ± 0.7^b	23.9 ± 0.4^c	21.7 ± 0.4^a	26.7 ± 0.7^b	26.2 ± 0.4^b
20:5(n-3)	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
22:4(n-6)	0.4 ± 0.0 ^a	0.8 ± 0.1 ^b	0.6 ± 0.0 ^b	0.2 ± 0.1 ^a	0.7 ± 0.1 ^b	0.7 ± 0.0 ^b
22:5(n-6)	2.6 ± 0.5 ^a	4.5 ± 0.6 ^b	2.8 ± 0.5 ^a	2.7 ± 0.2 ^a	3.8 ± 0.3 ^b	2.8 ± 0.4 ^{ab}
22:5(n-3)	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.0
22:6(n-3)	7.0 ± 0.5 ^a	6.2 ± 0.4 ^b	5.4 ± 0.3 ^{ab}	8.7 ± 0.4	8.4 ± 0.6	8.0 ± 0.4
SFA	31.8 ± 1.1	32.3 ± 0.8	32.2 ± 0.7	33.6 ± 0.8	34.0 ± 1.6	32.4 ± 1.4
PUFA(n-6)	37.0 ± 0.9 ^a	50.1 ± 0.6 ^b	49.8 ± 0.6 ^b	37.0 ± 0.1 ^a	48.1 ± 0.9 ^b	48.2 ± 0.8 ^b
20:4/18:2 ratio	2.09 ± 0.02^a	1.53 ± 0.05^b	1.21 ± 0.02^c	2.19 ± 0.03^a	1.73 ± 0.01^b	1.60 ± 0.03^b

Values are the means ± SEM (n = 4 or 6 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using Fisher's PS�D.

3.6 Correlation between lesion size in the aortic root, serum parameters, and inflammation biomarkers

As shown in Table III-6, The lesion size in the aortic root was positively correlated with serum cholesterol levels. There is no correlation between lesion size in aortic root and serum HDL cholesterol levels. Moreover, there is no correlation between lesion size in aortic root and inflammation biomarkers, such as aortic *Mcp-1* mRNA level and urinary isoprostane levels.

Table III-6. Correlation between lesion size in the aortic root and levels of serum cholesterol, aortic *Mcp-1* mRNA, and urinary isoprostane

	R ² value	P value
Serum cholesterol levels (n = 31)		
Total cholesterol	0.562	<0.001
HDL cholesterol	0.090	0.63
Aortic <i>Mcp-1</i> mRNA levels (n = 28)	-0.226	0.25
Urinary isoprostane levels (n = 31)		
8-iso-PGF2 α *	-0.348	0.06
dinor**	-0.187	0.31

8-iso-PGF2 α *: 8-iso-prostaglandin F2 α .

dinor**: 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F2 α .

4. Discussion

In this study, we established that compared with SFA, dietary LA intake was associated with a significant prevention of atherosclerosis development in male and female ApoE^{-/-} mice only in the condition of the lower content (0.04 % in the diet) of dietary cholesterol. However, this positive effect of LA on atherosclerosis development was found to be negated by consumption of a diet containing a high content of cholesterol. Imaizumi reviewed the relationship between dietary components and atherosclerosis in ApoE^{-/-} mice [16]. The analysis of 50 studies were examined that

the relationship between serum cholesterol levels and aortic lesion sizes in ApoE^{-/-} mice fed 50 different kinds of dietary components and a correlation coefficient (*r*) of 0.43 (*P* < 0.01) was obtained for the association between serum cholesterol levels and aortic lesion sizes [16]. Notably, compared with other effective dietary supplements, supplementation with LA was found to contribute to the lowest level of serum cholesterol, although gave rise to the largest aortic lesions. We consider that, as a precursor of AA and inflammatory eicosanoids, an increase of dietary LA content leads to an increase in aortic inflammation and further promotes the development of atherosclerosis. Saini and Keum have reported that the n-3 and n-6 PUFA ratio in a diet strongly affects eicosanoid production, and a reduced consumption of n-3 fatty acids contributes to an enhanced synthesis of inflammatory eicosanoids from AA [17]. In the present study, the fatty acid contents of the LALC and LAHC experimental diets comprised 76% n-6 PUFA, which would further promote an inflammatory response. Accordingly, serum cholesterol levels and aortic root inflammation are considered to be the most important pathogenic factors contributing to the development of atherosclerosis in ApoE^{-/-} mice.

In the present study, we observed that differences in the levels of serum cholesterol are associated with differences in the size of aortic lesions in both male and female ApoE^{-/-} mice. Specifically, we detected significant increases in serum cholesterol levels and aortic root lesion sizes in male and female mice fed the LAHC diet compared with those fed the LALC diet (Figure III-1, Table III-4). Such elevated serum cholesterol levels are considered a high-risk pathogenic factor for atherosclerosis [18]. Animal model studies have shown that a high dietary cholesterol intake increases total serum cholesterol levels in ApoE^{-/-} mice and ExHC rats [19-20]. Additionally, compositions of dietary fatty acids are also an important factor for serum cholesterol levels. Generally, dietary fatty acids can both increase and decrease when dietary oils are changed. In this study, the change in oils, from palm oil and lard to high-LA-safflower oil, increased LA in the diet, but at the same time it was accompanied by a decrease in SFA and oleic acid (OA). Therefore, it is quite difficult to conclude the cause of the change in serum cholesterol levels to the change in the

composition of a single fatty acid. Yu *et al.* established a multiple regression formula by meta-analysis of epidemiological studies on the effects of changes in dietary fatty acid composition on serum cholesterol levels [21]. It is not suitable to analyze the results of animal experiments with the equation established from human epidemiological studies. However, it is considered that the weighting of each fatty acids does not change significantly between species. Based on that, the effects of dietary fatty acid changes were estimated. In our research, the major changes in dietary concentrations of SFA {mainly palmitic acid (C16:0) and stearic acid (C18:0)} and MUFA {mainly OA (C18:1)} caused the changes of serum cholesterol levels between SFA and LALC groups. However, considering the coefficient of Δ PUFA in the formula of Yu is negative. LA levels were increased 7-fold in the LALC group compared to the SFA group. We conclude that the levels of LA increase also contributed to serum cholesterol decreased between SFA and LALC groups. At the same time, Keys *et al.* also reported that changes in serum cholesterol levels are affected by changes of dietary SUFA, PUFA and cholesterol levels [22]. The dietary SFA and PUFA levels are the same between LALC and LAHC groups. The serum cholesterol levels increased only due to the increase of dietary cholesterol between LALC and LAHC groups. We demonstrated that dietary cholesterol contributed directly to an increase in serum cholesterol levels and exacerbated atherosclerosis in mice. Moreover, aortic lesion size was found to be positively correlated with serum cholesterol levels (Table III-6). Cooper *et al.* found that conjugated LA had no effect on atherosclerosis but had adverse effects on lipoprotein and liver lipid metabolism in ApoE^{-/-} mice fed a 1.25% cholesterol diet [23]. We confirmed that dietary high cholesterol intake had the effect of negating the serum cholesterol level-lowering effect of LA, thereby exacerbating the development of atherosclerosis.

A large number of studies have reported the increase of inflammatory biomarkers such as C-reactive protein (CRP), Tumor necrosis factor (TNF), Inducible nitric oxide synthase (iNOS), etc. as the development of atherosclerosis [24]. However, in the present study, we selected aortic *Mcp-1* mRNA as a marker of aortic inflammatory response. The aortic *Mcp-1* levels of which are considered an index of the

inflammatory response of the vascular wall, is transcriptionally regulated by nuclear factor-kappa B (*NF- κ B*), a transcriptional regulator that responds to pro-inflammation [25]. Therefore, aortic *Mcp-1* mRNA was considered to represent the inflammatory status in the vascular wall more accurately than other serum markers. Arterial inflammation is associated with atherosclerosis, and this association has been found to be sex-dependent [26]. In male mice, we found that male mice fed the LALC diet were characterized by elevated levels of aortic *Mcp-1* mRNA, thereby indicating an aortic inflammatory response, whereas in contrast, no similar response was detected in male mice provisioned with the LAHC diet (Figure III-2). We confirmed that the higher levels of cholesterol in the LAHC diet appeared to have no significant effect on aortic inflammation in male ApoE^{-/-} mice. It has been reported that endogenous AA or inflammatory eicosanoids are transported from the liver to the aorta by lipoproteins and albumin, thereby promoting the development of atherosclerosis [27]. Our observations indicated that LAHC diet not promoting inflammatory responses in the aortic root may conceivably be attributed to a reduction in liver-derived AA, and consequently, we further analyzed the composition of hepatic fatty acids. The AA in hepatic phospholipids is derived from inflammatory eicosanoids and is naturally incorporated into structural phospholipids in the cell membrane [28]. In the present study, we analyzed the fatty acid composition of hepatic phospholipids as a means of evaluating AA synthesis as a desaturation index, and accordingly found that consumption of the LAHC diet had the effect of significantly reducing the levels of hepatic AA and urinary dinor (an AA metabolite) and contributed to a reduction in the hepatic 20:4/18:2 ratio. LA is acted upon by desaturation enzymes (Δ -6 fatty acid desaturase and Δ -5 fatty acid desaturase) and is subsequently converted to AA in the liver [29]. Several studies have reported that high cholesterol intake suppresses the action of these desaturases during the conversion of LA to AA [30]. We thus assume that the high cholesterol levels in LAHC diet inhibits the conversion of LA to AA in the livers of male mice. High level of cholesterol in this diet did not aggravate the aortic inflammatory response due to inhibition of AA synthesis in the liver, which further reduces AA transported to the aorta. In contrast to the male mice, we found

that the different experimental diets had no significant effect on aortic *Mcp-1* mRNA levels in female mice. Moreover, the arterial *Mcp-1* mRNA levels in male mice were found to be significantly higher than those in female mice. These phenomena indicated that arterial inflammation in female mice was reduced compared with that in male mice. We believe that this reduction in arterial inflammation in females might be attributed to the action of oestrogen, which has been established to be a regulator of inflammation and exerts anti-inflammatory effects [31]. There are a few reports about using female animal models to evaluate atherosclerosis. 17 β -estradiol treatment has been shown suppress *Mcp-1* protein and gene expression in ovariectomized (OVX) rabbits [32], and oestrogen treatment has been found to attenuate vascular re-modelling by promoting the synthesis of *Mcp-1* mRNA in endothelial cells [33]. Moreover, it has been demonstrated that oestrogen reduces *Mcp-1* mRNA levels in female OVX apoE^{-/-} mice, thereby preventing the development of atherosclerosis [34]. On the basis of the foregoing discussion, it is thus reasonable to assume that high dietary cholesterol of LAHC diets does not aggravate the aortic inflammatory response in either male or female ApoE^{-/-} mice, although there may be differences in the underlying mechanisms. Moreover, we established that the size of aortic lesions size shows no appreciable correlation with the levels of either aortic *Mcp-1* mRNA or urinary isoprostane. (Table III-6). We accordingly speculate that the increase in aortic lesion size and cross-section are attributable to elevated levels of serum cholesterol, but not aortic inflammation. Consequently, serum cholesterol levels might be considered a more pathogenic factor than aortic inflammation in promoting atherosclerosis development.

At present LA is still controversial in terms of cardiovascular prevention and pro-inflammation in humans. GH Johnson and K Fritsche have reported that no evidence was available from randomized, controlled intervention studies among healthy, noninfant human beings to show that addition of LA to the diet increases the concentration of inflammatory markers [35]. Unfortunately, no clinical study in which LA is the unique variable is available, which would allow to conclude on its pro-inflammatory effects. There is also no research reported that high dietary LA

intake increases the concentration of inflammatory markers among cardiovascular patients. On the other hands, anti-effects of cardiovascular disease by dietary LA were discovered with observation studies [36]. However, it has not been designed that dietary LA directly affects inflammatory status in human nutritional intervention studies. We expect that our study provides a research direction or theoretical basis for future clarification of the physiological role of LA in humans.

5 Conclusion

In conclusion, the findings of this study indicate that dietary intake of high cholesterol negates the serum cholesterol concentration-lowering effect of LA and has no significant effect on the inflammatory response on aortic root vessel walls in either male or female ApoE^{-/-} mice. We believe that the inhibitory effect of LA on arteriosclerosis is cancelled by higher dietary cholesterol due to a direct increase in the level of serum cholesterol.

CHAPTER IV The effect of dietary egg yolk phosphatidylcholine on the biomarker expression of Alzheimer's disease through regulating oxysterol and cholesterol metabolism

1. Introduction

Alzheimer's disease (AD) is a complex, heterogeneous, progressive disease and is the most common type of neurological dementia [1]. Pathological observations of AD development suggest neuronal atrophy and loss in AD are associated with toxic amyloid-beta ($A\beta$) oligomers and protein aggregates, intra-neuronal neurofibrillary tangles consisting of hyperphosphorylated microtubule-associated protein Tau, regionally specific reduction of cerebral glucose metabolism, synaptic dysfunction, and mitochondrial dysfunction in AD patients or different animal models of AD [2]. These complex and numerous pathological markers, and metabolic mechanisms cause the ultimate etiology of AD to remain obscure. The mainstream pathological hypotheses for AD mainly include as follows amyloid hypothesis, tau propagation hypothesis, mitochondrial cascade hypothesis, calcium homeostasis hypothesis, neurovascular hypothesis, inflammatory hypothesis, and metal ion hypothesis [3]. The development of AD goes through three stages: the pre-symptomatic stage, the prodromal stage of mild cognitive impairment (MCI), and the clinical form of AD [4]. AD accounts for 50–70% of cases of common neurodegenerative dementia. It is estimated that about 44 million people worldwide are living with dementia, and this number could triple by 2050 due to an aging population [5]. Healthcare spending to care for people with dementia is estimated at \$305 billion in 2020 [5]. The cost of AD in developed countries currently exceeds that of cancer or cardiovascular disease (CVD) [6].

The Brain contains about 20% of the total cholesterol in the human body [7]. As the blood-brain barrier (BBB) does not allow the passage of plasma lipoproteins, almost all cholesterol in the brain derives from *de novo* synthesis [7], and its

homeostasis requires a complex interplay between critical players involved in its cellular biosynthesis, its trafficking between astrocytes and neurons, and its disposal from the brain [8]. Cholesterol plays different structural roles in the brain: it is an important component of the myelin sheath surrounding axons produced by oligodendrocytes and an important constituent of neuronal membranes [9]. Cholesterol is a major component of synaptic vesicles which is formed, shaped, and released in the central nervous system (CNS) [10]. Moreover, a reduced amount of cholesterol on the postsynaptic surface can impair neurotransmission and induce a loss of dendrite spines and synapses [11]. In tissues outside CNS, cholesterol is acquired both from endogenous synthesis and from exogenous lipoproteins delivered from the circulation. However, plasma lipoproteins do not cross the intact blood-brain barrier (BBB), nearly all cholesterol in the brain is synthesized in astrocytes and then transported to neurons by ApoE [12]. The characteristics of cholesterol in the CNS explain why cholesterol homeostasis in the CNS is regulated independently of that in the peripheral circulation. Unlike cholesterol, oxysterols and its metabolites can cross the BBB from the peripheral circulation into the brain and vice-versa. The main oxysterols in the circulation are 24S-hydroxycholesterol (24S-OH) and 27-hydroxycholesterol (27-OH) [13].

Several clinical and animal research reported that some neural diseases including Alzheimer's, Huntington's, and Parkinson's diseases are thought to involve changes in brain oxysterols (mainly 24S-OH and 27-OH) and cholesterol metabolism disorder [13]. Tolppanen *et al.* performed a randomized study on mid-life subjects and showed that high total serum cholesterol levels could be a significant predictor of dementia, cognitive impairment, and Alzheimer's disease during aging [14]. Sparks *et al.* proved that statin (inhibitors of cholesterol synthesis) administration improved the decline of cognitive performance and memory capabilities in AD patients [15]. 27-OH promotes A β accumulation *via* altering A β metabolism in mild cognitive impairment patients and APP/PS1 mice [16]. 24S-OH is found to be inhibitors of β -secretase and decline A β secretion in rat primary cortical neurons [17]. At present, there is no effective drug to treat AD effectively. Maintaining cholesterol and

oxycholesterol metabolism in brain may be potential ways to prevent the development of AD pathology and related complications such as memory loss and disability.

Eggs are an important food source of protein and lipids for the Japanese. Eggs yolks contain some functional lipids, such as egg yolk phospholipids (PL) and fatty acids (FAs) [18]. The egg yolk PL has various bioactivities including anti-inflammatory, cholesterol lymphatic transportation inhibition, and neuroprotective and cardiovascular protection effect. The main component of the egg yolk phospholipid is phosphatidylcholine (PC), which accounts for about 85% of the egg yolk PL. PC is decomposed to produce choline which is critical in neurotransmission [19]. Several studies have reported that egg yolk PC ingestion improves brain function. Egg-derived PC (100 mg in 0.3 mL of water) increased the acetylcholine concentration and enhanced the memory of passive avoidance performance in rats, maintaining neurological function to a certain extent [20]. Intake of egg PC (5 g/100 g of diet) effectively improved maze cognitive ability and brain function in elder mice [21]. On the other hand, PC consists of a choline head group and glycerophosphoric acid, with a variety of FAs. The functional FAs such as DHA and EPA are also reported to increase brain function according to anti-inflammation and neurodevelopment promotion [22]. At present, few studies report that PC improves brain function through regulating cholesterol and oxycholesterol metabolism in the brain. There are also few studies assessing which PC with choline head groups and fatty acids composition of PC performed more effective physiological efficacy on improving brain function, respectively. In this research, we used 24-week elder and 6-week young SD rats to evaluate the effect of egg yolk PC on biomarker of AD through regulating oxycholesterol and cholesterol metabolism in different generations. We also use different oils and fatty acid methyl esters to simulate the fatty acid composition of egg yolk PC to confirm which of the PC with choline head groups and fatty acids composition of PC exerts more physiological efficacy on preventing AD development.

2. Materials & Methods

2.1 Materials

Egg yolk PC is provided by the Kewpie Corporation. Trimethylsilylation (TMS) reagent solution, a mixture of *N*, *O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1), was purchased from Supelco (MO, USA). Commercial enzyme assay kits (Cholesterol E test kit, HDL-Cholesterol E test kit, Triglyceride E test kit, Glucose CII test kit, Human/Rat β Amyloid (42) ELISA kit) were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2 Analysis and preparation of mixed oils

In this study, the fatty acid composition of egg yolk PC was simulated with different oils (corn oil, lard, and palm oil), fatty acid triglycerides pure products (Tripalmitin and Tristearin), and fatty acid methyl esters pure products (arachidonyl ethyl ester and docosahexaenoyl ethyl ester) then analysis by GC-FID.

One drop of the different oil, fatty acid triglycerides pure products, fatty acid methyl esters pure products, and egg yolk PC was methylated by sulfuric acid and methanol (1:155, v/v) at 80°C and shake every 30 min. The reaction was carried out for 2 hours to transmethyrate the fatty acids into the methyl ester. After cooling to room temperature in a water bath, added 2 mL of saturated sodium hydroxide solution and 2 mL of hexane, sealed with argon gas, mixed well with a shaker for 5 min, and centrifuge at $1,610 \times g$ for 5 min to separate the upper hexane layer.

After the recovered solvent was dried up with nitrogen gas, 1 μ L (1/100 μ L) of the fatty acid methyl ester (FAME) dissolved in 100 μ L of hexane was subjected to GC-FID (GC-2014, Shimadzu Corporation, Kyoto, Japan). The column was CP-Sil 88 for FAME (100 m $\times$ 0.25 mm, 0.25 μ m, Agilent). The temperature of the column was 140-240°C (4°C/min). The temperature of both the injector and detector (interface) was 250°C. The entrance pressure was 335 kPa. The total flow rate was 1.55 mL/min. The split ratio was 10 and the flow velocity was 40.0 cm/sec. Chromato-PRO (Runtime Instruments Co., Ltd.) was used for data analysis. The reagents used for fatty acid identification were Supelco 37 Component FAME Mix and Bacterial Acid Methyl Esters Mix, which were purchased from SUPELCO (Bellefonte, PA, USA).

According to the calculation, several oils and fatty acid pure products were added in proportion to the triangular flask with magnetons, filled with argon, and sealed with aluminum foil and sealing film. The flask was then placed in a water bath equipped with magnetic agitation and mixed at 45°C, away from light, for 6h. The mixed oil was analyzed by GC-FID one more time. The correlation of dietary fatty composition between the egg yolk PC group and the fatty acid adjustment group was shown in Figure IV-1.

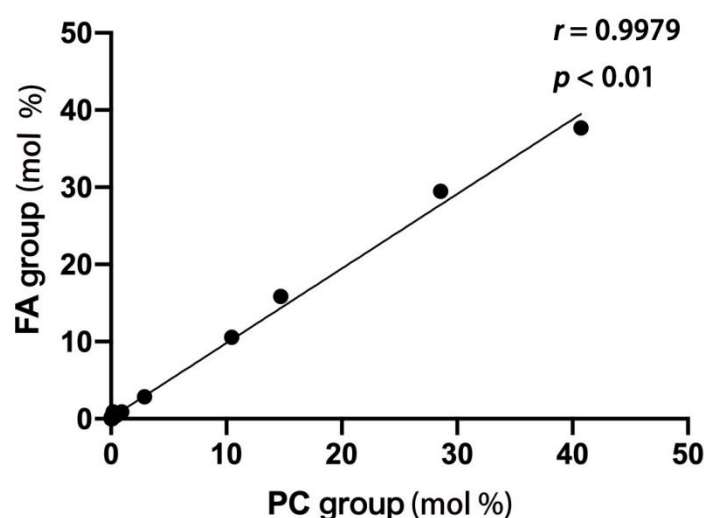


Figure IV-1. Correlation of dietary fatty acid composition between egg yolk PC group and the fatty acid adjustment group.

2.3 Animals and diets

Twenty four week-old male elder ($n = 22$) and 6-week-old male young ($n = 24$) SD rats were used for the diet-based experiments. The elder SD rats weighed between 650 and 665 g and the young SD rats weighed between 150 and 165 g both were purchased from Kyudo Co. Ltd. (Tosu, Saga, Japan) and were raised in the Animal Laboratory Building (AG12) of the Department of Bioresources and Bioenvironmental Science, Faculty of Agriculture, Kyushu University. The rats were individually housed in metal cages and kept in a temperature-controlled room (22-25°C) with a 12 h light cycle. Before the formal feeding experimental period, were pre-fed for 2 weeks with commercial solid feed (NMF Experimental animal solid feed: Oriental yeast). After that, the elder rats ($n = 22$) were randomly divided into three groups ($n = 7$ to 8 for each

group) without a difference in feeding period and body weight: control group, egg yolk PC group (PC group), and fatty acid adjustment group (FA group). Young rats ($n = 24$) performed the same grouping as the elder rats ($n = 8$ for each group). Table IV-1 lists the experimental diets composition and Table IV-2 lists the fatty acid composition of each diet. In the feeding experiment, elder and young rats were fed the experimental diet for 4 weeks. Animals were then sacrificed by the withdrawal of aortic blood under pentobarbitone anesthesia ($0.08 \text{ mg}/100\text{g}$ body weight), followed by immediate excision of the brain and other tissues for use in subsequent experiments. To isolate plasma, blood was collected, incubated at room temperature for 30 min, and centrifuged at $1,750 \times g$ for 10 min at 4°C . The handling and euthanasia of all animals were carried out under nationally prescribed guidelines and experimentation was carried out following ethical approval from the Animal Care and Use Committee of Kyushu University (authorization number: A20-255-0), as well as in accordance with law no. 105 and notification no. 6 of the Government of Japan.

Table IV-1 Compositions of the experimental diets

Ingredients (g/kg diet)	Control	PC	FA
Sucrose	449	449	449
Casein	200	200	200
Cornstarch	150	50	50
Cellulose	50	50	50
Lard	100	60	72.9
Corn Oil	-	-	8.3
Palm Oil	-	-	8.7
Tripalmitin	-	-	6.4
Tristearin	-	-	1.9
Arachidonyl ethyl ester	-	-	1.5
Docosahexaenoyl ethyl ester	-	-	0.2
Egg yolk PC	-	40	-
Mineral mix ¹	35	35	35
Vitamin mix ²	10	10	10
DL-Methionine	3	3	3
Choline bitartrate	2	2	2
Cholesterol	1	1	1
Total	1000	1000	1000

1, 2 are based on the AIN-76 formula.

Table IV-2 Fatty acid composition of experiment diets

	Control	PC	FA
	mol (%)		
Capric acid (C10:0)	0.06	0.04	0.04
Lauric acid (C12:0)	0.13	0.08	0.12
Myristic acid (14:0)	1.93	1.23	1.52
Myristoleic acid (14:1)	0.27	0.16	0.20
Pentadecanoic acid (15:0)	0.13	0.11	0.08
Palmitic acid (16:0)	27.84	33.04	31.77
Palmitoleic acid (16:1)	2.75	2.02	2.01
Heptadecanoic acid (17:0)	0.49	0.35	0.35
Heptadecenoic acid (17:1)	-	-	0.05
Stearic acid (C18:0)	14.21	12.72	12.76
Oleic acid (C18:1 n-9)	43.17	37.26	37.69
Linoleic acid (C18:2 n-6)	8.00	10.70	11.14
γ-Linolenic acid (C18:3 n-6)	-	0.04	-
α-Linolenic acid (C18:3 n-3)	0.17	0.14	0.10
Arachidonic acid (C20:0)	0.33	0.20	0.30
Eicosenoic acid (C20:1)	0.15	0.09	0.09
Eicosadienoic acid (C20:2 n-6)	0.23	0.27	0.27
Dihomo-γ-linolenic acid (C20:3 n-6)	0.05	0.16	0.15
Arachidonic acid (C20:4 n-6)	-	1.17	1.15
Docosenoic acid (C22:1 n-9)	0.09	0.05	0.05
Docosahexaenoic acid (C22:6 n-3)	-	0.19	0.18
Total	100	100	100
n-3 PUFA	0.17	0.33	0.28
n-6 PUFA	8.28	12.34	12.71

2.4 Analysis of plasma parameters

Plasma levels of total cholesterol, TG, and glucose were measured using commercial enzyme assay kits (Cholesterol E test kit, Triglyceride E test kit, and Glucose CII test kit). For the measurement of plasma (HDL) cholesterol levels, HDL fractions were collected as described previously and then measured using the Cholesterol E test kit. Non-HDL cholesterol levels were calculated as the difference between total and HDL cholesterol levels.

2.5 Measurement of cholesterol and cholesterol synthesis intermediates in the brain cortex

The cholesterol and cholesterol synthesis intermediates level of brain cortex in elder and young rats were analyzed by the method which is described in detail in Chapter II 2.6. After the saponification with ethanolic KOH and derivatization using the TMS reagent solution (99% BSTFA, 1% TMCS), cholesterol synthesis intermediates of the brain cortex were analyzed *via* GC-FID system (GC-2014s; Shimadzu) equipped with an SPB-1 column using 5 α -cholestane as an internal standard. The temperatures of the injection port and detector were set at 300°C. The column temperature was set at 275°C.

2.6 Measurement of oxysterol in the plasma and brain cortex

The oxysterol levels of plasma and cerebral cortex in elder and young rats were analyzed by the method which is described in detail in Chapter II 2.5. Plasma and cerebral cortex oxysterol were derivatized to trimethylsilyl ethers using a mixture of trimethylchlorosilane, hexamethyldisilazane, and dried pyridine (1:3:9, v/v/v), followed by the quantification using GC-MS, as previously described in. To quantify each oxidized cholesterol, calibration curves were produced, which measured the peak area of each oxysterol versus that of the internal standard (19-hydroxycholesterol). Peak identification was confirmed through the relative retention time and mass spectral comparison with an authentic standard.

2.7 Measurement of A β 42 concentration in the brain cortex of elder rats

The measurement of cortical A β 42 concentration was performed according to the method of the Human/Rat β Amyloid (42) ELISA Kit Wako High-Sensitive kit instruction. Zero point one g of the cortex was weighted and stored at -30°C. The weighed sample was placed in a 1.5 mL microtube, and 500 μ L of Tris buffer (5 mM Tris, 13.8 mM NaCl, 0.27 mM KCl: all from Nacalai Tesque) was added. Meanwhile, polytron crushing was performed twice for 10 seconds at maximum speed on ice until no tissue fragment was visible. Tissue fragments were then crushed in a centrifuge at 20,000 $\times g$ for 90 minutes at 4°C. The supernatant was collected as the TBS buffer-soluble fraction. Next, 70% formic acid solution was added to the precipitates, and polytron crush was performed again under the same conditions. The sonicator crush was performed (DUTY 30, OUTPUT 50, 10 times $\times$ 2 times). The supernatant was collected by centrifugation at 18°C, 20,000 $\times g$, for 2 hours. Tris-base buffer (1M Tris, 0.5M Na₂HPO₄, both from Nacalai Tesque) was added to the supernatant at a ratio of 1:20, and the fraction was considered as the TBS buffer-insoluble fraction. The soluble or insoluble fraction was added in 150 μ L portions to a solid-phase ELISA 96-well plate, and 50 μ L of dilution buffer was added. For the calibration curve, 150 μ L of 70% formic acid: Tris-base buffer = 1:20 solution was added for each 50 μ L of standard diluent to ensure that the buffer composition has no difference between the sample well and standard well. One hundred μ L of mixture solution was taken to make a calibration curve. After agitation, the wells were incubated overnight at 4°C and light shielded. After washing 5 times with 300 μ L of washing buffer, 100 μ L of HRP-conjugated antibody was added, mixed, and incubated at 4°C, then shielded from light for 2 hours. After washing again under the same conditions, 100 μ L of TMB solution was added and allowed to stand in the dark at room temperature for 30 minutes. After washing, 100 μ L of TMB solution was added and allowed to stand for 30 minutes in the dark at room temperature. Then 100 μ L of reaction stopper reagent was added and the wavelength at 450 nm was measured with a multiscan plate reader.

2.8 Measurement of plasma A β 42 concentration in elder rats

Human/Rat β Amyloid (42) ELISA Kit Wako high-sensitive was used for the assay. Samples were diluted 3-fold with standard diluent. One hundred μ L of each standard solution and diluted samples were added to a solid-phase ELISA 96-well plate, agitated, and allowed to stand overnight at 4°C and light-shielded. After washing 5 times with 300 μ L of washing buffer, 100 μ L of HRP-conjugated antibody was added, mixed, and incubated at 4°C, then shielded from light for 1 hour. After washing again under the same conditions, 100 μ L of TMB solution was added, stirred, and allowed to stand in the dark at room temperature for 30 minutes. After adding 100 μ L of reaction stopper reagent and stirring, the wavelength at 450 nm was measured with a multiscan plate reader.

2.10 Statistical analysis

All data are presented as the means $\pm$ standard error of the mean (SEM) unless otherwise indicated. Elder and young groups were compared using one-way ANOVA with the Tukey-Kramer test to identify individual differences. Analyses were performed using GraphPad 8.3 with significance set at $p < 0.05$, and $0.05 < p < 0.1$ being considered indicative of a trend.

3. Results

3.1 Growth parameters and relative organ weights

As the results are shown in Table IV-3-1 and 3-2, there were no significant differences in body weight, total food intake, feed efficiency, and brain weight (left and right) among the three groups of elder or young rats.

Table IV-3-1 Growth parameters and brain weight in elder rats

	Control	PC	FA
Body weight (g)			
Initial	658.23 ± 12.63	655.41 ± 11.44	660.30 ± 9.19
Final	718.39 ± 14.19	714.96 ± 10.69	721.87 ± 4.64
Gain	60.16 ± 5.79	59.54 ± 6.10	61.57 ± 6.04
Total food intake (g)	691.91 ± 7.52	694.87 ± 5.38	703.19 ± 0.91
Food efficiency (%)	8.72 ± 0.87	8.56 ± 0.86	8.76 ± 0.86
Left brain (g)	1.09 ± 0.01	1.06 ± 0.02	1.06 ± 0.02
Right brain (g)	1.09 ± 0.03	1.06 ± 0.01	1.06 ± 0.02

Values are the means ± SEM (n = 7 to 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

Table IV-3-2 Growth parameters and brain weight in young rats

	Control	PC	FA
Body weight (g)			
Initial	158.12 ± 12.6	158.06 ± 1.33	157.86 ± 1.41
Final	396.59 ± 4.38	387.76 ± 5.23	387.43 ± 4.94
Gain	238.40 ± 3.76	229.70 ± 4.81	229.56 ± 4.40
Total food intake (g)	571.46 ± 7.39	571.31 ± 4.96	565.88 ± 6.87
Food efficiency (%)	41.75 ± 0.69	40.21 ± 0.80	40.59 ± 0.80
Left brain (g)	1.03 ± 0.02	1.00 ± 0.03	1.01 ± 0.01
Right brain (g)	0.94 ± 0.02	0.92 ± 0.01	0.93 ± 0.02

Values are the means ± SEM (n = 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

3.2 Effects of the egg yolk PC diet on plasma parameters

As the results are shown in Table IV-4-1, the plasma total cholesterol levels of elder rats in the FA group significantly increased compared with the control and PC groups. The HDL-cholesterol levels of elder rats in FA group significantly increased compared with the control group. There was no significant difference between non-HDL-cholesterol, PL, TG, and glucose among the three groups in elder rats.

Table IV-4-1 Plasma parameters in elder rats

	Control	PC	FA
	(mg/dL)		
Total cholesterol	123.21 ± 10.22 ^a	133.81 ± 8.53 ^a	167.05 ± 12.40 ^b
HDL-cholesterol	57.66 ± 8.28 ^a	68.46 ± 9.68 ^{ab}	90.25 ± 5.44 ^b
Non-HDL-cholesterol	65.55 ± 12.28	57.18 ± 10.65	76.80 ± 10.68
Phospholipids	210.01 ± 16.48	213.82 ± 12.87	256.55 ± 10.63
Triglyceride	311.65 ± 31.84	332.52 ± 44.85	365.40 ± 38.26
Glucose	265.43 ± 18.87	270.51 ± 9.08	273.43 ± 27.53

Values are the means ± SEM (n = 7 to 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

As the results are shown in Table IV-4-2, the plasma HDL-cholesterol levels of young rats in the FA group significantly increased compared with control group. There was no significant difference between total cholesterol, non-HDL-cholesterol, PL, TG, and glucose among the three groups in young rats.

Table IV-4-2 Plasma parameters in young rats

	Control	PC	FA
	(mg/dL)		
Total cholesterol	115.77 ± 8.24	126.41 ± 10.82	140.82 ± 5.24
HDL-cholesterol	54.29 ± 3.34 ^a	63.11 ± 3.35 ^{ab}	68.95 ± 3.31 ^b
Non-HDL-cholesterol	61.48 ± 1.97	63.30 ± 12.94	72.77 ± 5.55
Phospholipids	207.06 ± 14.07	210.69 ± 12.20	214.83 ± 9.96
Triglyceride	244.50 ± 23.34	265.77 ± 18.03	277.99 ± 40.81
Glucose	265.99 ± 10.94	255.14 ± 12.62	268.47 ± 19.73

Values are the means ± SEM (n = 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

3.3 Effects of the egg yolk PC diet on cholesterol and cholesterol synthesis intermediates in the brain cortex

As the results are shown in Table IV-5-1 and 5-2, the cholesterol and cholesterol synthesis intermediates were no difference among the three groups both in the brain cortex of elder and young rats. Egg yolk PC dietary intake has no effect on cholesterol and cholesterol synthesis intermediates levels in the brain cortex.

Table IV-5-1 Brain cholesterol and its synthesis intermediates levels in elder rats

	Control	PC	FA
	(mg/g tissue)		
Squalene	0.047 ± 0.015	0.048 ± 0.014	0.034 ± 0.010
Cholesterol	31.659 ± 3.223	30.667 ± 2.937	31.722 ± 1.811
7-DHC	0.023 ± 0.003	0.048 ± 0.020	0.029 ± 0.050
Desmosterol	0.209 ± 0.023	0.198 ± 0.030	0.146 ± 0.031
Lathosterol	0.061 ± 0.005	0.061 ± 0.006	0.053 ± 0.005
Campesterol	0.069 ± 0.006	0.069 ± 0.005	0.060 ± 0.008
β-Stiosterol	0.038 ± 0.002	0.047 ± 0.004	0.073 ± 0.029

Values are the means ± SEM (n = 7 to 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

Table IV-5-2 Brain cholesterol and its synthesis intermediates levels in young rats

	Control	PC	FA
	(mg/g tissue)		
Squalene	0.051 ± 0.013	0.029 ± 0.04	0.034 ± 0.006
Cholesterol	36.257 ± 8.881	22.274 ± 3.812	25.722 ± 4.284
7-DHC	0.024 ± 0.006	0.025 ± 0.008	0.018 ± 0.004
Desmosterol	0.402 ± 0.113	0.233 ± 0.040	0.231 ± 0.050
Lathosterol	0.179 ± 0.048	0.095 ± 0.014	0.097 ± 0.017
Campesterol	0.025 ± 0.014	0.011 ± 0.002	0.013 ± 0.002
β-Stiosterol	0.022 ± 0.006	0.015 ± 0.003	0.018 ± 0.005

Values are the means ± SEM (n = 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

3.4 Effects of the egg yolk PC diet on oxysterol levels in the brain cortex

As the results shown in Table IV-6-1, β -Epoxy, α -Epoxy, 24S-OH, 4 β -OH, and 6-Keto levels of the brain cortex were significantly increased in the PC group compared with the control group in elder rats. 4 β -OH levels of the brain cortex were significantly increased in the PC and FA groups compared with the control group. The other oxysterol levels have no significant difference among three groups in elder rats. The 27-OH/24S-OH ratio of the PC and FA groups were significantly decreased compared with the control group in elder rats.

As the results are shown in Table IV-6-2, β -Epoxy was significantly increased in the PC group compared with the control and FA groups in young rats. α -Epoxy was significantly increased in FA group compared with the PC and control groups in young rats.

Table IV-6-1 Brian oxysterol levels in elder rats

	Control	PC	FA
	(µg/ tissue)		
β-Epoxycholesterol (β-Epoxy)	0.63 ± 0.12 ^a	1.17 ± 0.13 ^b	0.81 ± 0.06 ^{ab}
4β-Hydroxycholesterol (4β-OH)	1.81 ± 0.32 ^a	4.51 ± 0.47 ^b	3.51 ± 0.48 ^b
α-Epoxycholesterol (α-Epoxy)	0.88 ± 0.16 ^a	1.58 ± 0.19 ^b	1.19 ± 0.09 ^{ab}
β-Cholestanetriol (β-Triol)	0.97 ± 0.48	0.89 ± 0.12	0.92 ± 0.14
24S-Hydroxycholesterol (24S-OH)	12.42 ± 2.72 ^a	28.64 ± 3.24 ^b	20.80 ± 3.52 ^{ab}
6-Ketocholestanol (6-Keto)	0.12 ± 0.01 ^a	0.17 ± 0.01 ^b	0.13 ± 0.01 ^{ab}
25-Hydroxycholesterol (25-OH)	0.75 ± 0.18	1.33 ± 0.27	0.95 ± 0.23
7-Ketocholesterol (7-Keto)	0.42 ± 0.08	0.68 ± 0.07	0.68 ± 0.08
27-Hydroxycholesterol (27-OH)	0.72 ± 0.07	0.73 ± 0.09	0.76 ± 0.09
27-OH/24S-OH ratio	0.07 ± 0.01 ^a	0.03 ± 0.00 ^b	0.04 ± 0.01 ^b

Values are the means ± SEM (n = 7 to 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

Table IV-6-2 Brian oxysterol levels in young rats

	Control	PC	FA
	(µg/ g tissue)		
β-Epoxycholesterol (β-Epoxy)	1.46 ± 0.12 ^a	2.08 ± 0.21 ^b	1.41 ± 0.14 ^{ac}
4β-Hydroxycholesterol (4β-OH)	2.42 ± 0.27	3.10 ± 0.27	2.05 ± 0.42
α-Epoxycholesterol (α-Epoxy)	2.21 ± 0.24 ^a	3.06 ± 0.32 ^b	2.08 ± 0.23 ^a
β-Cholestanetriol (β-Triol)	3.35 ± 0.82	4.19 ± 1.13	4.46 ± 1.13
24S-Hydroxycholesterol (24S-OH)	38.71 ± 3.85	48.37 ± 3.37	33.49 ± 5.21
6-Ketocholestanol (6-Keto)	0.26 ± 0.04	0.33 ± 0.03	0.29 ± 0.05
25-Hydroxycholesterol (25-OH)	1.98 ± 0.19	2.41 ± 0.17	1.74 ± 0.23
7-Ketocholesterol (7-Keto)	1.08 ± 0.11	1.32 ± 0.06	1.14 ± 0.12
27-Hydroxycholesterol (27-OH)	1.25 ± 0.14	1.20 ± 0.19	1.20 ± 0.13
27-OH/24S-OH ratio	0.03 ± 0.01	0.03 ± 0.00	0.05 ± 0.02

Values are the means ± SEM (n = 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

3.5 Effects of the egg yolk PC diet on plasma oxysterol levels

As the results shown in Table IV-7-1 and 7-2, 27-OH levels in the PC group significantly increased compared with FA and control groups in elder rats. 4β-OH levels in the PC group significantly increased compared with control groups in elder rats. The other oxysterol levels have no significant difference among the three groups both in elder and young rats. The 27-OH/cholesterol ratio of control and PC groups significantly increased compared with the FA group in elder rats.

Table IV-7-1 Plasma oxysterol levels in elder rats

	Control	PC	FA
		($\mu\text{g/ mL}$)	
7 α -Hydroxycholesterol (7 α -OH)	0.222 $\pm$ 0.046	0.280 $\pm$ 0.019	0.170 $\pm$ 0.022
7 β -Hydroxycholesterol (7 β -OH)	0.091 $\pm$ 0.016	0.122 $\pm$ 0.016	0.105 $\pm$ 0.009
β -Epoxycholesterol (β -Epoxy)	0.503 $\pm$ 0.075	0.574 $\pm$ 0.112	0.553 $\pm$ 0.110
α -Epoxycholesterol (α -Epoxy)	0.383 $\pm$ 0.095	0.461 $\pm$ 0.135	0.354 $\pm$ 0.91
4 β -Hydroxycholesterol (4 β -OH)	0.379 $\pm$ 0.077 ^a	0.791 $\pm$ 0.055 ^b	0.756 $\pm$ 0.158 ^{ab}
β -Cholestanetriol (β -Triol)	0.044 $\pm$ 0.014	0.040 $\pm$ 0.007	0.047 $\pm$ 0.014
6-Ketocholestanol (6-Keto)	0.034 $\pm$ 0.005	0.032 $\pm$ 0.002	0.045 $\pm$ 0.011
7-Ketocholesterol (7-Keto)	0.134 $\pm$ 0.028	0.133 $\pm$ 0.012	0.130 $\pm$ 0.020
24S-Hydroxycholesterol (24S-OH)	0.024 $\pm$ 0.004	0.027 $\pm$ 0.002	0.034 $\pm$ 0.009
25-Hydroxycholesterol (25-OH)	0.029 $\pm$ 0.004	0.031 $\pm$ 0.004	0.031 $\pm$ 0.003
27-Hydroxycholesterol (27-OH)	0.023 $\pm$ 0.003 ^a	0.035 $\pm$ 0.003 ^b	0.032 $\pm$ 0.002 ^{ab}
27-OH/24S-OH ratio	1.071 $\pm$ 0.183	1.339 $\pm$ 0.080	1.208 $\pm$ 0.155
24S-OH/cholesterol ratio	0.020 $\pm$ 0.004	0.021 $\pm$ 0.002	0.015 $\pm$ 0.001
27-OH/cholesterol ratio	0.023 $\pm$ 0.002 ^a	0.023 $\pm$ 0.003 ^a	0.019 $\pm$ 0.002 ^b

Values are the means $\pm$ SEM (n = 7 to 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

Table IV-7-2 Plasma oxysterol levels in young rats

	Control	PC	FA
	(µg/ mL)		
7α-Hydroxycholesterol (7α-OH)	0.198 ± 0.030	0.209 ± 0.027	0.245 ± 0.026
7β-Hydroxycholesterol (7β-OH)	0.100 ± 0.011	0.106 ± 0.011	0.098 ± 0.004
β-Epoxycholesterol (β-Epoxy)	0.219 ± 0.025	0.246 ± 0.023	0.321 ± 0.066
α-Epoxycholesterol (α-Epoxy)	0.128 ± 0.016	0.135 ± 0.013	0.185 ± 0.047
4β-Hydroxycholesterol (4β-OH)	0.389 ± 0.075	0.464 ± 0.127	0.432 ± 0.093
β-Cholestanetriol (β-Triol)	0.019 ± 0.002	0.024 ± 0.003	0.020 ± 0.001
6-Ketocholestanol (6-Keto)	0.029 ± 0.003	0.028 ± 0.002	0.026 ± 0.001
7-Ketocholesterol (7-Keto)	0.088 ± 0.007	0.099 ± 0.005	0.104 ± 0.008
24S-Hydroxycholesterol (24S-OH)	0.022 ± 0.003	0.023 ± 0.001	0.021 ± 0.001
25-Hydroxycholesterol (25-OH)	0.044 ± 0.004	0.052 ± 0.005	0.047 ± 0.005
27-Hydroxycholesterol (27-OH)	0.026 ± 0.003	0.028 ± 0.003	0.027 ± 0.003
27-OH/24S-OH ratio	1.243 ± 0.100	1.191 ± 0.107	1.277 ± 0.088
24S-OH/cholesterol ratio	0.019 ± 0.002	0.020 ± 0.003	0.021 ± 0.006
27-OH/cholesterol ratio	0.019 ± 0.003	0.027 ± 0.003	0.019 ± 0.001

Values are the means ± SEM (n = 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

3.6 Effects of the egg yolk PC diet on Amyloid β-42 levels in plasma and brain cortex of elder rats

As the results shown in Table IV-8, the plasma Aβ-42 levels of PC groups were significantly increased compared with control and FA groups. The total Aβ-42 levels of the brain cortex have no difference among the three groups. The insoluble Aβ-42 levels of FA group ($p = 0.1$) tend to decrease compared with control groups and PC group ($p = 0.37$) showed similar result, but no statistical difference.

Table IV-8 Plasma and brain Amyloid β -42 levels in elder rats

	Control	PC	FA
	(pmol/L)		
Plasma total A β -42 levels	10.27 $\pm$ 0.60 ^a	13.05 $\pm$ 1.12 ^b	9.39 $\pm$ 0.25 ^a
	(pmol/mg tissue)		
Brain soluble A β -42 levels	27.45 $\pm$ 2.05	31.36 $\pm$ 3.71	34.53 $\pm$ 4.02
Brain insoluble A β -42 levels	35.62 $\pm$ 3.65	31.03 $\pm$ 2.55	28.10 $\pm$ 2.57
Brain total A β -42 levels	62.28 $\pm$ 2.40	60.66 $\pm$ 4.22	60.98 $\pm$ 5.40
Brain soluble/insoluble ratio	0.78 $\pm$ 0.15	0.99 $\pm$ 0.13	1.20 $\pm$ 0.15

Values are the means $\pm$ SEM (n = 7 to 8 /group).

Different superscript letters indicate a significant difference at $p < 0.05$, as determined using the Tukey-Kramer method.

4. Discussion

In this research, we confirmed the effect of egg yolk PC can alter oxysterol metabolism and elevate the 24S-OH levels in brain cortex of elder rats. Oxysterol, as cholesterol oxidation product, has been reported to have a close connection with AD. To maintain cholesterol homeostasis in the brain, and because the brain cannot degrade cholesterol, there must be several mechanisms to eliminate excess cholesterol, transporting it through the BBB into the systemic circulation to prevent accumulation [9]. One important mechanism that operates the conversion of cholesterol to oxysterol. Cholesterol is converted to 24S-OH and 27-OH by cytochrome P450 family 46 subfamily A member 1 (CYP46A1) and cytochrome P450 family 27 subfamily A member 1 (CYP27A1), respectively [13]. In addition to being the main by-product of brain cholesterol catabolism, 24S-OH is considered a potential AD biomarker in human and animal models [23]. Elevated 24S-OH levels improve study and memory ability in human and AD animal models [24]. Reduction in the levels of 24S-OH in the brain reflects neuronal dysfunction during AD development. A large amount of clinical observation studies reported that the 24S-OH levels significantly

decreased in biological samples (brain, blood, and CSF) subjects with different AD severity in both male and female patients [24-25]. 24S-OH levels also decreased in critical areas of post-mortem brains of AD patients (frontal and occipital cortex, basal ganglia, and pons), as well as in aged mice expressing the A β protein precursor APP751 with the London (V717I) and Swedish (K670M/N671L) mutations [26-27]. Research reported that the probable mechanism of 24S-OH in preventing AD development is considered as inhibiting A β production by modulating the expression of genes coding APP and β -secretase, an important enzyme in the amyloidogenic APP processing pathway in human neuroblastoma SH-SY5Y cells [28]. Thus, elevating 24S-OH levels in the brain are considered a potential way to improve and treat AD and other neurological disorders. In this research, the PC group has higher 24S-OH levels compared with control group in elder rats (Table IV-6-1). At the same time, the PC, FA, and control groups have the same plasma 24S-OH levels (Table IV-7-1). As 24S-OH only synthesized in the brain, we confirmed that dietary egg yolk PC intake promotes the 24S-OH synthesis and accumulation in the brain cortex of elder rats to prevent AD development. On the other hand, the control, PC, and FA groups have similar 27-OH levels in the brain cortex of elder rats and the cholesterol synthesis intermediates have no difference among the three groups in the brain cortex of elder rats. Research reported that 27-OH has been suggested as an inhibitor of brain cholesterol synthesis in mice [29]. A review lists an association between high levels of 27-OH and memory deficits, Alzheimer's disease (AD), and other neurodegenerative processes [30]. The results of this study are consistent with our results. Thus, we confirmed that dietary intake of egg yolk PC has no effect on 27-OH and cholesterol metabolism levels in the brain cortex of elder and young rats. We also found that the PC group has higher plasma 27-OH levels compared with FA and control groups. Research reported that 27-OH promotes A β accumulation *via* altering A β metabolism and increasing neurotoxicity and apoptosis [31]. Phospholipid-modified liposome particles can alter the permeability of the BBB and perform drug delivery to brain tissue [32]. In this research, we speculate that PC may alter the permeability of the BBB and enhance the circulation of 27-OH from the brain

to the blood, removing excess 27-OH from the cerebral cortex and thus reducing the neurotoxicity of 27-OH. The effect of egg yolk PC on BBB permeability and 27-OH cerebral blood circulation will be performed in the future.

We found that dietary intake of egg yolk PC altered oxysterol homeostasis and significantly elevated 24S-OH levels in the brain cortex of elder rats. The level of 24s-OH is closely related to the synthesis and secretion of A β 42 [23]. Considering the egg yolk PC and FAs composition of PC dietary intake have not obviously effect on oxysterol and cholesterol levels in young rats and brain A β -42 levels of young rats is too low to test. We investigated the A β 42 levels of plasma and brain cortex only in elder rats. Deposition of A β in the brain is a pathological biomarker of AD. There are two major isoforms of A β : the 42-residue A β 42 and the 40-residue A β 40 [33]. The amyloid plaques in AD patients consist of mostly A β 42 and some plaques contain only A β 42 [34]. The development of AD results in the excessive production of A β peptide, leading to its gradual aggregation into plaques [35]. APP is initially cleaved by β -secretase to yield C99, a 99-residue peptide fragment that is further processed by γ -secretase to afford either the 40- or 42-residue variant of A β peptide. Then A β peptide is released into the brain cortex and hippocampus and finally forms amyloid plaques [35]. In this research, the control, PC, and FA group have the same total A β 42 levels in the brain cortex. The egg yolk PC and FA composition of PC do not affect total A β 42 synthesis in the brain although the level of 24S-OH in the brain cortex increased. We also observed that the FA group ($p = 0.10$) tends to reduce insoluble A β -42 levels in the brain cortex (Table IV-8) and the PC group ($p = 0.37$) exhibits similar results but no statistical differences. A study reported that insoluble A β 42 is more likely to exacerbate AD development than soluble A β 42 [36]. PC and fatty acid composition of PC might have the physiological activity on reducing the proportion of insoluble A β 42 levels in total A β 42 levels of elder rats' brain to prevent A β 42 accumulation in the brain and prevent the development of AD. However, this speculation needs further verification and related mechanism is still unclear. The reason for the lack of statistical difference of A β 42 was considered to be caused by the only 4-weeks of dietary intake of PC and FA diets. The feed experiment period is

too short. We may extend the duration of dietary interventions in the future. We consider the increased levels of n-3 PUFA in PC and FA groups might be the reason for the insoluble A β 42 decrease in FA and PC groups. Several types of research reported that n-3 PUFA may play a role in the prevention of AD and other neuro diseases. Enriched endogenous n-3 PUFA alleviates cognitive and behavioral deficits in a mice model of Alzheimer's disease [37]. Diet n-3 PUFA enrichment prevents amyloid beta-induced depressive-like phenotype. Dietary n-3 PUFA can modify neural maturation and enhance brain functioning in healthy C57BL/6J mice [38]. Otherwise, we also observed that the FA groups have higher brain insoluble A β 42 levels compared with PC groups. We considered that the reason may be the n-6/n-3 ratio in PC group (37.09) lower than that in the FA group (45.39) which was showed in Table IV-2. Research reported the role of n-6 PUFA and oxidized eicosanoid derivatives of n-6 PUFA (mainly AA and prostaglandin) have recently been reviewed as contributing to β -amyloid deposition [39]. Enhanced consumption of n-6 PUFA leads to excessive production of the proinflammatory cytokines derived from AA through COX and LOX enzymatic activity which leads to brain damage [40]. Therefore, a dietary pattern consisting of lower n-6 PUFA and higher n-3 PUFA or a more balanced n-6: n-3 PUFA ratio may be therapeutic in the pathogenesis of AD.

In addition, we observed the PC group has higher plasma total A β 42 levels compared with the FA groups. The A β 42 is almost synthesized in the brain and consider as the main source of blood A β 42. We confirmed that the egg yolk PC could promote the excretion of A β 42 from the brain into the blood to prevent A β 42 accumulation and then ameliorate AD development. Previous studies suggested that the A β peptide might interact with PL membranes directly [41]. Liposomes formed from phospholipids can transport complexes across the BBB into the blood [32]. We speculate that egg yolk PC can form a complex with A β 42 to reduce A β 42 accumulation or alter the BBB permeability to remove excess A β 42 from the brain to the blood. However, whether PC can bind with A β 42 and related molecular structure, the mechanism of the PC-A β complexes crossing the BBB, and whether PC can change the BBB permeability remain unclear and will be investigated in further studies.

We expect to discover new metabolic pathways for PC to prevent A β 42 accumulation in the brain. We also observed that the total A β 42 levels had no significant difference in brain cortex. This result indicated that the synthesis of A β 42 in the brain did not increase. Besides the brain, A β peptide might also be generated in peripheral organs or tissues including skeletal muscle and bone [42]. A part of A β 42 is stored in cerebrospinal fluid (CSF) and other tissues [42]. The A β 42 from CSF and other tissues might also increase the plasma A β 42 levels in this research. We plan to analyze A β 42 levels in CSF and skeletal muscle in the future. Otherwise, due to limited experimental conditions and deficient levels of samples, we did not separate plasma total A β 42 into soluble and insoluble fractions. The part source of A β 42 in the plasma might be the insoluble A β 42 fraction from the brain.

Egg yolk PC has an appreciable impact on oxysterol metabolism and improves brain function in the brain cortex of elder rats. However, the egg yolk PC has no effect on the indicators of oxysterol and A β 42 metabolism in the cerebral cortex of young rats. We consider the possible reason lies in the decreased ApoE synthesis in elder rats. ApoE synthesis levels in tissues and organs are on the decline with age [43]. ApoE coordinates the mobilization and redistribution of cholesterol in the repair, growth, and maintenance of neuronal membranes during the development process or after injuries in the peripheral nervous system [44]. Clinical research reported that normal APOE4 carriers over 50 years are more likely to show significant cognitive impairment than APOE4-negative subjects [45]. Phospholipids of APOE lipoproteins activate microglia in an isoform-specific manner in preclinical models of AD [46]. In addition to promoting the synaptic linkage of aging neuronal cells, egg yolk PC dietary intake could further activate microglia which can have beneficial effects on assisting A β clearance and forming a barrier surrounding amyloid plaques [47]. The increase in APOE levels also further regulates the metabolism of oxidized cholesterol in the brain, thus further reducing the accumulation of A β in the brain and regulating the cholesterol homeostasis in the brain. We will further measure APOE4 and total APOE levels in the brain in the future. Compared with the elder rats, the cholesterol homeostasis and apolipoprotein levels in the brain of young rats are in a normal

metabolic state. Therefore, egg yolk PC intake did not affect brain oxysterol metabolism in young rats. We also observed that the PC and FA intake reduce the brain cholesterol levels in young rats but without statistical differences. We considered that the cholesterol metabolism and clearance are normal in young rats. PC and some functional FAs of PC have the certain cholesterol-lowering activity. Dietary intake of FAs and PC further promotes cholesterol metabolism and excretion, thereby reducing the cholesterol levels in the brain of young rats. The reduction of cholesterol levels may also be related to the high expression of APOE in young mice. Both above speculations need further verification.

5. Conclusion

In conclusion, dietary intake of egg yolk PC increased brain 24S-OH concentration without plasma cholesterol increasing in 24-week elder rats. Egg yolk PC and fatty acid composition of PC ($p = 0.10$) reduced the levels of insoluble A β 42 in the brain of elder rats, but without statistical difference. Egg yolk PC also increased the plasma A β 42 levels in elder rats, representing the excretion of A β 42 from the brain into the blood. Egg yolk PC dietary intake had no effect on oxysterol levels and bio-marker expression of AD in young rats. We confirmed that egg yolk PC consumption may elevate 24S-OH levels in the brain and promote A β 42 excretion from the brain into the blood to protect brain function in elder rats. Egg yolk PC can be used as a new dietary ingredient to improve AD and other neurological disorders. Our research provides a theoretical basis for the development of the physiological activity of egg yolk PC to ameliorate AD and other neurological diseases.

CHAPTER V Summary and Conclusions

In the doctor research project, we investigated the effects of food functional ingredients that regulate cholesterol metabolism to ameliorate lifestyle diseases and confirmed related mechanisms.

In the chapter II, the effect of α -globulin-rich rice cultivar, low glutelin content-1 (LGC-1), on serum cholesterol concentration in ExHC rats was investigated. Rice α -globulin has been reported to have serum cholesterol-lowering activity in rats. However, it is still unclear whether α -globulin exerts this effect when taken as one of the dietary components. In this study, we investigated the effect of two cultivars of rice, low glutelin content (LGC)-1 and LGC-Jun on reducing serum cholesterol in exogenously hypercholesterolemic (ExHC) rats. LGC-1 is enriched in α -globulin (10.6 mg/g rice flour, which is about 1.5 times higher α -globulin content than in Koshihikari which is a predominant rice cultivar in Japan), while LGC-Jun is a globulin-negative cultivar. ExHC rats, the model strain of diet-induced hypercholesterolemia, were fed 50% LGC-1 or LGC-Jun and 0.5% cholesterol-containing diets for two weeks, followed by measurement of cholesterol metabolism parameters in serum and tissues. Serum cholesterol and non-HDL cholesterol levels were significantly lower in the LGC-1 group, as compared to the LGC-Jun group. Cholesterol intestinal absorption markers, hepatic and serum levels of campesterol and β -sitosterol, and lymphatic cholesterol transport were not different between the two groups. Levels of 7 α -hydroxycholesterol, an intermediate of bile acid synthesis, showed a downward trend in the livers of rats that were fed LGC-1 ($p = 0.098$). There was a significant decrease in the hepatic mRNA expression of *Cyp7a1* (a synthetic enzyme for 7 α -hydroxycholesterol) in the LGC-1 group, as compared to the LGC-Jun group. Dietary LGC-1 significantly decreased serum cholesterol levels in ExHC rats. The possible mechanism for the cholesterol-lowering activity of LGC-1 is partial inhibition of bile acid and cholesterol synthesis in the liver.

In the chapter III, the effects of dietary linoleic acid (LA) on atherosclerosis development and aortic inflammation in different dietary cholesterol levels were investigated. LA has a two-sided effect with regards to serum cholesterol lowering and pro-inflammation, although whether this fatty acid reduces serum cholesterol and the development of atherosclerosis under high-cholesterol conditions has yet to be ascertained. In this study, we examine the effects of dietary LA on reducing serum cholesterol and atherosclerosis development under high-cholesterol conditions. Male and female apolipoprotein E-deficient (ApoE^{-/-}) mice were fed AIN-76-based diets containing 10% saturated fatty acids and 0.04 % cholesterol (SFA group), 10% LA and 0.04% low cholesterol (LALC group), or 10% LA and 0.1% high cholesterol (LAHC group) for 9 weeks. The results revealed significant reduction in serum cholesterol levels and aortic lesions with increasing levels of pro-inflammatory biomarkers (urinary isoprostane and aortic *Mcp-1* mRNA) in male and female LALC group compared with those in the SFA group ($p < 0.05$). Furthermore, whereas there were significant increases in the serum cholesterol levels and aortic lesions ($p < 0.05$), there was no difference in aortic *Mcp-1* mRNA levels in male and female LAHC group compared with those in the LALC group. A high dietary intake of cholesterol eliminated the serum cholesterol-lowering activity of LA but had no significant effect on aortic inflammation in either male or female ApoE^{-/-} mice. The inhibitory effect of LA on arteriosclerosis is canceled by a high-cholesterol diet due to a direct increase in serum cholesterol levels. Accordingly, serum cholesterol levels might represent a more prominent pathogenic factor than aortic inflammation in promoting the development of atherosclerosis.

In chapter IV, the effect of egg yolk phosphatidylcholine on the bio-marker expression of Alzheimer's disease through regulating oxysterol and cholesterol metabolism was investigated. Studies have confirmed the relationship between cognitive impairment (CI) and cholesterol metabolism in the brain. Egg yolk contains about 26% phosphatidylcholine (PC). In this study, we investigated the effect of dietary egg yolk PC on improving brain function focusing on the alteration of brain oxysterol and cholesterol. Twenty four week-old elder (n = 22) and 6 week-old (n

= 24) young SD rats were randomly divided into 3 groups: Control; Egg yolk PC (PC) and Fatty acid adjustment (FA). The control group was fed the diet based on the AIN76 composition. PC group was fed a PC diet based on the diet of the control group which part of the dietary lipid was replaced by egg yolk PC. FA group was adjusted to have the same fatty acid composition as the PC diet. The feeding experiment period was 4 weeks. The results showed that dietary intake of egg yolk PC increased brain 24S-OH concentration without plasma cholesterol increasing in elder rats. Egg yolk PC and fatty acid composition of PC ($p = 0.10$) reduced the insoluble A β 42 levels in the brain of elder rats, but without statistical difference. Egg yolk PC also increased the plasma A β 42 levels in elder rats, representing the excretion of A β 42 from the brain into the blood. Egg yolk PC had no effect on oxysterol levels and biomarker expression of AD in young rats. We confirmed that egg yolk PC consumption show the protective effect on AD development through elevating 24S-OH levels in the brain and promoting A β 42 excretion from brain to blood in elder rats.

In conclusion, α -globulin riched rice cultivar, LGC-1, safflower oil riched LA, and egg yolk PC dietary intake ameliorate hypercholesterolemia, atherosclerosis, and Alzheimer's disease through regulating cholesterol and oxysterol metabolism. We expect our results to provide a theoretical basis for the development of functional foods and their application to clinical studies as adjunctive therapies and synergistic nutritional interventions to improve lifestyle diseases.

ACKNOWLEDGEMENTS

I wish to express my deep appreciations to Dr. Masao Sato, Professor of Laboratory of Nutrition Chemistry, for letting me join the laboratory and become his students. I also hope to express my deep gratitude for his kind guidance, critical directions, progressive advice, and warm encouragement thought the doctor research period. I have learned enormous things on academic, life attitude and behavior from him and make a solid foundation for my future research career.

I would also like to express my heartfelt thanks to Dr. Bungo Shirouchi, Associate Professor of University of Nagasaki, for his guidance, advice, and encouragement in my doctoral course, as well as his critical reading and comments of my doctoral thesis.

I also deeply grateful to Dr. Yasutake Tanaka, Assistant Professor of Laboratory of Nutrition Chemistry, for his detailed guidance and revision for my experiments and papers, as well as his kind and warm support during my doctoral research.

I am also deeply indebted to Dr. Shigeki Furuya, Professor of Laboratory of Division of Systems Bioengineering in Kyushu University, for his valuable instructions and suggestions for my doctoral thesis.

I am grateful to my mother, Wei Tian, and my father, Li Yuan, for giving the opportunity to study in Japan, providing financial support, and enlighten and encourage me in times of difficulty.

I wants to deeply acknowledge Japan Science and Technology Agency (JST) Support for Pioneering Research Initiated by the Next Generation (SPRING) (Grant Number: JPMJSP2136) for the financial support and research expenses, so that I had no financial burden and could concentrate on my study.

In addition, I acknowledge Zhe Jiang, Yuki Otsubo, Ai Takeyama, Tingyu Wang, Wenzhao He and all the other members of Laboratory of Nutrition Chemistry for their numerous help during the past three years and half.

LITERATURE CITED

Chapter I

1. Tabish S A. Lifestyle diseases: consequences, characteristics, causes and control. *Journal of Cardiology & Current Research*, 2017, 9(3): 00326.
2. Börnhorst C, Russo P, Veidebaum T, et al. The role of lifestyle and non-modifiable risk factors in the development of metabolic disturbances from childhood to adolescence. *International Journal of Obesity*, 2020, 44(11): 2236-2245.
3. Egger G, Dixon J. Beyond obesity and lifestyle: a review of 21st century chronic disease determinants. *BioMed research international*, 2014, 12-24
4. Fried L P, Guralnik J M. Disability in older adults: evidence regarding significance, etiology, and risk. *Journal of the American Geriatrics Society*, 1997, 45(1): 92-100.
5. Senapati S, Bharti N, Bhattacharya A. Modern lifestyle diseases: chronic diseases, awareness and prevention. *International Journal of Current Research and Review*, 2015, 3(3): 215-23.
6. Singh A, Banerjee P, Anas M, et al. Traditional nutritional and health practices targeting lifestyle behavioral changes in humans. *Journal of Lifestyle Medicine*, 2020, 10(2): 67.
7. Senapati S, Bharti N, Bhattacharya A. Modern lifestyle diseases: chronic diseases, awareness and prevention. *International Journal of Current Research and Review*, 2015, 3(3): 215-23.
8. Carrera-Bastos P, Fontes-Villalba M, O'Keefe J H, et al. The western diet and lifestyle and diseases of civilization. *Research Reports in Clinical Cardiology*, 2011, 2(1): 15-35.
9. Lloyd-Sherlock P, McKee M, Ebrahim S, et al. Population ageing and health. *The Lancet*, 2012, 379(9823): 1295-1296.
10. Khan Y, Bhutta Z A. Nutritional deficiencies in the developing world: current status and opportunities for intervention. *Pediatric Clinics*, 2010, 57(6): 1409-1441.
11. Tsugane S, Sawada N. The JPHC study: design and some findings on the typical Japanese diet. *Japanese journal of clinical oncology*, 2014, 44(9): 777-782.

12. Hoffmann I S, Cubeddu L X. Salt and the metabolic syndrome. *Nutrition, Metabolism and Cardiovascular Diseases*, 2009, 19(2): 123-128.
13. Arisawa K, Uemura H, Yamaguchi M, et al. Associations of dietary patterns with metabolic syndrome and insulin resistance: a cross-sectional study in a Japanese population. *The Journal of Medical Investigation*, 2014, 61(3.4): 333-344.
14. Zhao Y, Yang Y, Xing R, et al. Hyperlipidemia induces typical atherosclerosis development in Ldlr and Apoe deficient rats. *Atherosclerosis*, 2018, 271: 26-35.
15. Ghosh S S, Righi S, Krieg R, et al. High fat high cholesterol diet (western diet) aggravates atherosclerosis, hyperglycemia and renal failure in nephrectomized LDL receptor knockout mice: role of intestine derived lipopolysaccharide. *PLoS One*, 2015, 10(11): e0141109.
16. Han S N, Leka L S, Lichtenstein A H, et al. Effect of a therapeutic lifestyle change diet on immune functions of moderately hypercholesterolemic humans. *Journal of Lipid Research*, 2003, 44(12): 2304-2310.
17. Kanoski S E, Davidson T L. Western diet consumption and cognitive impairment: links to hippocampal dysfunction and obesity. *Physiology & Behavior*, 2011, 103(1): 59-68.
18. Murakami K, Sasaki S, Takahashi Y, et al. Dietary glycemic index and load in relation to metabolic risk factors in Japanese female farmers with traditional dietary habits. *The American Journal of Clinical Nutrition*, 2006, 83(5): 1161-1169.
19. Ma Y, Olendzki B, Chiriboga D, et al. Association between dietary carbohydrates and body weight. *The American Journal of Epidemiology* 2005; 161: 359–67.
20. Slyper A, Jurva J, Pleuss J, et al. Influence of glycemic load on HDL cholesterol in youth. *The American Journal of Clinical Nutrition*, 2005, 81(2): 376-379.
21. Wolever T M S, Nguyen P M, Chiasson J L, et al. Relationship between habitual diet and blood glucose and lipids in non-insulin dependent diabetes (NIDDM). *Nutrition Research*, 1995, 15(6): 843-857.
22. Aburto J M, Villavicencio F, Basellini U, et al. Dynamics of life expectancy and life span equality. *Proceedings of the National Academy of Sciences*, 2020, 117(10): 5250-5259.

23. Comprehensive Survey of Living Conditions. Ministry of Health, Labour and Welfare. [Accessed on August 13, 2022]. Available from URL: <https://www.mhlw.go.jp/english/database/db-hss/index.html>
24. Luo J, Yang H, Song B L. Mechanisms and regulation of cholesterol homeostasis. *Nature reviews Molecular cell biology*, 2020, 21(4): 225-245.
25. Russell D W. Cholesterol biosynthesis and metabolism. *Cardiovascular Drugs and Therapy*, 1992, 6(2): 103-110.
26. Attie A D. Lipoprotein/cholesterol metabolism. *Enzyme Mechanisms*, 2004: 116.
27. Illingworth D R. Management of hypercholesterolemia. *Medical Clinics of North America*, 2000, 84(1): 23-42.
28. FREDRICKSON D S. An international classification of hyperlipidemias and hyperlipoproteinemias. *Annals of Internal Medicine*, 1971, 75(3): 471-472.
29. Nordestgaard B G, Langlois M R, Langsted A, et al. Quantifying atherogenic lipoproteins for lipid-lowering strategies: consensus-based recommendations from EAS and EFLM. *Atherosclerosis*, 2020, 294: 46-61.
30. Cromwell W C, Otvos J D. Low-density lipoprotein particle number and risk for cardiovascular disease. *Current atherosclerosis reports*, 2004, 6(5): 381-387.
31. Lewis G F, Xiao C, Hegele R A. Hypertriglyceridemia in the genomic era: a new paradigm. *Endocrine Reviews*, 2015, 36(1): 131-147.
32. Varghese M J. Familial hypercholesterolemia: A review. *Annals of Pediatric Cardiology*, 2014, 7(2): 107.
33. Fishbein M C, Fishbein G A. Arteriosclerosis: facts and fancy. *Cardiovascular Pathology*, 2015, 24(6): 335-342.
34. Crowther M A. Pathogenesis of atherosclerosis. *ASH Education Program Book*, 2005, 6(1): 436-441.
35. Douglas G, Channon K M. The pathogenesis of atherosclerosis. *Medicine*, 2014, 42(9): 480-484.
36. Insull Jr W. The pathology of atherosclerosis: plaque development and plaque responses to medical treatment. *The American Journal of Medicine*, 2009, 122(1): S3-S14.

37. Gaziano J M, Prabhakaran D, Gaziano J M. Fundamentals of cardiovascular disease. Braunwald's heart disease: A textbook of cardiovascular medicine, 2008: 1-20.
38. Fuster V, Badimon L, Badimon J J, et al. The pathogenesis of coronary artery disease and the acute coronary syndromes. New England Journal of Medicine, 1992, 326(4): 242-250.
39. Ministry of Health Labour and Welfare of Japan. Japanese dementia strategy (New Orange Plan) Ministry of Health Labour and Welfare of Japan; 2015. [Accessed on August 13, 2022]. Available from: <http://www.mhlw.go.jp/stf/seisakunitsuite/bunya/nop1/>.
40. Nakahori N, Sekine M, Yamada M, et al. Future projections of the prevalence of dementia in Japan: results from the Toyama Dementia Survey. BMC geriatrics, 2021, 21(1): 1-10.
41. Rice D P, Fillit H M, Max W, et al. Prevalence, costs, and treatment of Alzheimer's disease and related dementia: a managed care perspective. American Journal of Managed Care, 2001, 7(8): 809-820.
42. Zvěřová M. Clinical aspects of Alzheimer's disease. Clinical Biochemistry, 2019, 72: 3-6.
43. Beckett M W, Stoeck K, Meguro K, et al. Advances in Alzheimer's Disease. Advances in Alzheimer's Disease, 2016: 360.
44. Buchman A S, Bennett D A. Loss of motor function in preclinical Alzheimer's disease. Expert review of Neurotherapeutics, 2011, 11(5): 665-676.
45. Selkoe D J, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO Molecular Medicine, 2016, 8(6): 595-608.
46. Pinnix I, Ghiso J A, Pappolla M A, et al. Major carboxyl terminal fragments generated by γ -secretase processing of the Alzheimer amyloid precursor are 50 and 51 amino acids long. The American Journal of Geriatric Psychiatry, 2013, 21(5): 474-483.
47. Qiu T, Liu Q, Chen Y X, et al. A β 42 and A β 40: similarities and differences. Journal of Peptide Science, 2015, 21(7): 522-529.

48. Kumar S, Walter J. Phosphorylation of amyloid beta (A β) peptides—A trigger for formation of toxic aggregates in Alzheimer's disease. *Aging (Albany NY)*, 2011, 3(8): 803.
49. Trinczek B, Biernat J, Baumann K, et al. Domains of tau protein, differential phosphorylation, and dynamic instability of microtubules. *Molecular Biology of the Cell*, 1995, 6(12): 1887-1902.
50. Goedert M, Jakes R. Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. *The EMBO journal*, 1990, 9(13): 4225-4230.
51. Andorfer C, Kress Y, Espinoza M, et al. Hyperphosphorylation and aggregation of tau in mice expressing normal human tau isoforms. *Journal of Neurochemistry*, 2003, 86(3): 582-590.
52. Spillantini M G, Goedert M. Tau protein pathology in neurodegenerative diseases. *Trends in Neurosciences*, 1998, 21(10): 428-433.
53. Holtzman D M, Pitas R E, Kilbridge J, et al. Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line. *Proceedings of the National Academy of Sciences*, 1995, 92(21): 9480-9484.
54. Heffernan A L, Chidgey C, Peng P, et al. The neurobiology and age-related prevalence of the ϵ 4 allele of apolipoprotein E in Alzheimer's disease cohorts. *Journal of Molecular Neuroscience*, 2016, 60(3): 316-324.
55. Dorey E, Bamji-Mirza M, Najem D, et al. Apolipoprotein E isoforms differentially regulate Alzheimer's disease and amyloid- β -induced inflammatory response in vivo and in vitro. *Journal of Alzheimer's Disease*, 2017, 57(4): 1265-1279.
56. Tai L M, Ghura S, Koster K P, et al. APOE-modulated A β -induced neuroinflammation in Alzheimer's disease: current landscape, novel data, and future perspective. *Journal of Neurochemistry*, 2015, 133(4): 465-488.
57. Litvinchuk A, Huynh T P V, Shi Y, et al. Apolipoprotein E4 reduction with antisense oligonucleotides decreases neurodegeneration in a tauopathy model. *Annals of Neurology*, 2021, 89(5): 952-966.

58. Bales K R, Verina T, Cummins D J, et al. Apolipoprotein E is essential for amyloid deposition in the APPV717F transgenic mouse model of Alzheimer's disease. *Proceedings of the National Academy of Sciences*, 1999, 96(26): 15233-15238.
59. Cedazo - Mínguez A. Apolipoprotein E and Alzheimer's disease: molecular mechanisms and therapeutic opportunities. *Journal of Cellular and Molecular Medicine*, 2007, 11(6): 1227-1238.
60. DeMattos R B, Cirrito J R, Parsadanian M, et al. ApoE and clusterin cooperatively suppress A β levels and deposition: evidence that ApoE regulates extracellular A β metabolism in vivo. *Neuron*, 2004, 41(2): 193-202.
61. Kanekiyo T, Bu G. The low-density lipoprotein receptor-related protein 1 and amyloid- β clearance in Alzheimer's disease. *Frontiers in Aging Neuroscience*, 2014, 6: 93.
62. Kang D E, Pietrzik C U, Baum L, et al. Modulation of amyloid β -protein clearance and Alzheimer's disease susceptibility by the LDL receptor-related protein pathway. *The Journal of Clinical Investigation*, 2000, 106(9): 1159-1166.
63. Israelachvili J N, Mitchell D J. A model for the packing of lipids in bilayer membranes. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1975, 389(1): 13-19.
64. Demer L L, Hsu J J, Tintut Y. Steroid hormone vitamin D: implications for cardiovascular disease. *Circulation Research*, 2018, 122(11): 1576-1585.
65. DeBose-Boyd R A. Feedback regulation of cholesterol synthesis: sterol-accelerated ubiquitination and degradation of HMG CoA reductase. *Cell Research*, 2008, 18(6): 609-621.
66. Mohassel P, Mammen A L. Statin-associated autoimmune myopathy and anti-HMGCR autoantibodies. *Muscle & Nerve*, 2013, 48(4): 477-483.
67. Goldstein J L, Brown M S. Regulation of the mevalonate pathway. *Nature*, 1990, 343(6257): 425-430.
68. Bloch K. The biological synthesis of cholesterol. *Science*, 1965, 150(3692): 19-28.
69. Brown A J, Ikonen E, Olkkonen V M. Cholesterol precursors: more than mere markers of biosynthesis. *Current Opinion in Lipidology*, 2014, 25(2): 133-139.

70. Di Ciaula A, Garruti G, Baccetto R L, et al. Bile acid physiology. *Annals of Hepatology*, 2018, 16(1): 4-14.
71. Molinaro A, Wahlström A, Marschall H U. Role of bile acids in metabolic control. *Trends in Endocrinology & Metabolism*, 2018, 29(1): 31-41.
72. Housset C, Chrétien Y, Debray D, et al. Functions of the Gallbladder. *Comprehensive Physiology*, 2016, 6(3): 1549-77.
73. Shrestha S, Wu B J, Guiney L, et al. Cholesteryl ester transfer protein and its inhibitors. *Journal of Lipid Research*, 2018, 59(5): 772-783.
74. Tso P, Fujimoto K. The absorption and transport of lipids by the small intestine. *Brain Research Bulletin*, 1991, 27(3-4): 477-482.
75. Randolph G J, Miller N E. Lymphatic transport of high-density lipoproteins and chylomicrons. *The Journal of Clinical Investigation*, 2014, 124(3): 929-935.
76. Ramasamy I. Recent advances in physiological lipoprotein metabolism. *Clinical Chemistry and Laboratory Medicine* , 2014, 52(12): 1695-1727.
77. Eisenberg S, Levy R I. Lipoprotein metabolism. *Advances in Lipid Research*, 1975, 13: 1-89.
78. Jia L, Betters J L, Yu L. Niemann-pick C1-like 1 (NPC1L1) protein in intestinal and hepatic cholesterol transport. *Annual Review of Physiology*, 2011, 73: 239-259.
79. Hazard S E, Patel S B. Sterolins ABCG5 and ABCG8: regulators of whole body dietary sterols. *Pflügers Archiv-European Journal of Physiology*, 2007, 453(5): 745-752.
80. Parolini C, Rigamonti E, Marchesi M, et al. Cholesterol-lowering effect of dietary *Lupinus angustifolius* proteins in adult rats through regulation of genes involved in cholesterol homeostasis. *Food Chemistry*, 2012, 132(3): 1475-1479.
81. Santos-Sánchez G, Cruz-Chamorro I, Álvarez-Ríos A I, et al. *Lupinus angustifolius* protein hydrolysates reduce abdominal adiposity and ameliorate metabolic associated fatty liver disease (MAFLD) in Western diet fed-ApoE^{-/-} mice. *Antioxidants*, 2021, 10(8): 1222.
82. Bao X, Yuan X, Li X, et al. Flaxseed-derived peptide, IPPF, inhibits intestinal cholesterol absorption in Caco-2 cells and hepatic cholesterol synthesis in HepG2

- cells. *Journal of Food Biochemistry*, 2022, 46(1): e14031.
83. Yuan X, Bao X, Liu X, et al. Flaxseed-derived peptides ameliorate hepatic cholesterol metabolism in Sprague–Dawley rats fed a high-cholesterol and high-fat diet. *Journal of the Science of Food and Agriculture*, 2022, 102 (12), 5348-5357.
 84. Kayashita J, Shimaoka I, Nakajoh M, et al. Consumption of buckwheat protein lowers plasma cholesterol and raises fecal neutral sterols in cholesterol-fed rats because of its low digestibility. *The Journal of Nutrition*, 1997, 127(7): 1395-1400.
 85. Yu-Poth S, Yin D, Zhao G, et al. Conjugated linoleic acid upregulates LDL receptor gene expression in HepG2 cells. *The Journal of nutrition*, 2004, 134(1): 68-71.
 86. Lam C K, Chen J, Cao Y, et al. Conjugated and non-conjugated octadecaenoic acids affect differently intestinal acyl coenzyme A: Cholesterol acyltransferase activity. *Atherosclerosis*, 2008, 198(1): 85-93.
 87. Jesch E D, Seo J M, Carr T P, et al. Sitosterol reduces messenger RNA and protein expression levels of Niemann-Pick C1-like 1 in FHs 74 Int cells. *Nutrition Research*, 2009, 29(12): 859-866.
 88. Cho S J, Juillerat M A, Lee C H. Cholesterol lowering mechanism of soybean protein hydrolysate. *Journal of Agricultural and Food Chemistry*, 2007, 55(26): 10599-10604.
 89. Sung J H, Choi S J, Lee S W, et al. Isoflavones found in Korean soybean paste as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Bioscience, Biotechnology, and Biochemistry*, 2004, 68(5): 1051-1058.
 90. Bursill C A, Abbey M, Roach P D. A green tea extract lowers plasma cholesterol by inhibiting cholesterol synthesis and upregulating the LDL receptor in the cholesterol-fed rabbit. *Atherosclerosis*, 2007, 193(1): 86-93.
 91. Lee M S, Park J Y, Freake H, et al. Green tea catechin enhances cholesterol 7 α -hydroxylase gene expression in HepG2 cells. *British Journal of Nutrition*, 2008, 99(6): 1182-1185.
 92. Man R Y K, Lynn E G, Cheung F, et al. Cholestin inhibits cholesterol synthesis and secretion in hepatic cells (HepG2). *Molecular and Cellular Biochemistry*, 2002, 233(1): 153-158.

93. Wong J M W, De Souza R, Kendall C W C, et al. Colonic health: fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology*, 2006, 40(3): 235-243.
94. Erkkilä A T, Lichtenstein A H. Fiber and cardiovascular disease risk: how strong is the evidence? *Journal of Cardiovascular Nursing*, 2006, 21(1): 3-8.
95. Cohn J S, Kamili A, Wat E, et al. Dietary phospholipids and intestinal cholesterol absorption. *Nutrients*, 2010, 2(2): 116-127.
96. Rukmini C, Raghuram T C. Nutritional and biochemical aspects of the hypolipidemic action of rice bran oil: a review. *Journal of the American College of Nutrition*, 1991, 10(6): 593-601.
97. Ellegård L, Andersson H. Oat bran rapidly increases bile acid excretion and bile acid synthesis: an ileostomy study. *European journal of clinical nutrition*, 2007, 61(8): 938-945.
98. Liu L, Yeh Y Y. S-alk (en) yl cysteines of garlic inhibit cholesterol synthesis by deactivating HMG-CoA reductase in cultured rat hepatocytes. *The Journal of nutrition*, 2002, 132(6): 1129-1134.
99. Kwon M J, Song Y S, Choi M S, et al. Cholesteryl ester transfer protein activity and atherogenic parameters in rabbits supplemented with cholesterol and garlic powder. *Life sciences*, 2003, 72(26): 2953-2964.
100. Oscarsson J, Hurt-Camejo E. Omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid and their mechanisms of action on apolipoprotein B-containing lipoproteins in humans: a review. *Lipids in health and disease*, 2017, 16(1): 1-13.
101. Mann N J, O'Connell S L, Baldwin K M, et al. Effects of seal oil and tuna-fish oil on platelet parameters and plasma lipid levels in healthy subjects. *Lipids*, 2010, 45(8): 669-681.
102. Qi H, Huang L, Liu X, et al. Antihyperlipidemic activity of high sulfate content derivative of polysaccharide extracted from *Ulva pertusa* (Chlorophyta). *Carbohydrate Polymers*, 2012, 87(2): 1637-1640.
103. Khalid N, Khan R S, Hussain M I, et al. A comprehensive characterisation of safflower oil for its potential applications as a bioactive food ingredient-A review. *Trends in Food Science & Technology*, 2017, 66: 176-186.

104. Sugano M, Matsuoka R. Nutritional viewpoints on eggs and cholesterol. *Foods*, 2021, 10(3): 494.

Chapter II

1. Gylling H. Cholesterol metabolism and its implications for therapeutic interventions in patients with hypercholesterolaemia. *International Journal of Clinical Practice*, 2004, 58(9): 859-866.
2. Peet D J, Turley S D, Ma W, et al. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR α . *Cell*, 1998, 93(5): 693-704.
3. Wang H H, Garruti G, Liu M, et al. Cholesterol and lipoprotein metabolism and atherosclerosis: recent advances in reverse cholesterol transport. *Annals of Hepatology*, 2017, 16: S27-S42.
4. van Rooy M J, Pretorius E. Obesity, hypertension and hypercholesterolemia as risk factors for atherosclerosis leading to ischemic events. *Current Medicinal Chemistry*, 2014, 21(19): 2121-2129.
5. Lecanu L, Yao Z X, McCourty A, et al. Control of hypercholesterolemia and atherosclerosis using the cholesterol recognition/interaction amino acid sequence of the translocator protein TSPO. *Steroids*, 2013, 78(2): 137-146.
6. Lupton J R, Brooks J A, Butte N F, et al. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids. National Academy Press: Washington, DC, USA, 2002, 5: 589-768.
7. Campbell T C. A plant-based diet and animal protein: questioning dietary fat and considering animal protein as the main cause of heart disease. *Journal of Geriatric Cardiology: JGC*, 2017, 14(5): 331.
8. Cabanos C, Kato N, Amari Y, et al. Development of a novel transgenic rice with hypocholesterolemic activity via high-level accumulation of the α' subunit of soybean β -conglycinin. *Transgenic Research*, 2014, 23(4): 609-620.
9. Tong L T, Fujimoto Y, Shimizu N, et al. Rice α -globulin decreases serum cholesterol concentrations in rats fed a hypercholesterolemic diet and ameliorates atherosclerotic

- lesions in apolipoprotein E-deficient mice. *Food Chemistry*, 2012, 132(1): 194-200.
10. Tong L T, Ju Z, Qiu J, et al. Peptide GEQQQQPGM derived from rice α -globulin reduces the risk of atherosclerosis in hamsters by improving vascular endothelial cells injury. *RSC Advances*, 2017, 7(78): 49194-49203.
11. Hashizume F, Hino S, Okajima T, et al. Suppressive expression of the glutelin A1-collagen peptide fusion gene in hybrid rice lines with the mutant line, Low Glutelin Content-1 (LGC-1). *Plant Biotechnology*, 2008, 25(5): 465-471.
12. Nishimura M, Kusaba M, Miyahara K, et al. New rice varieties with low levels of easy-to-digest protein, 'LGC-Katsu' and 'LGC-Jun'. *Breeding Science*, 2005, 55(1): 103-105.
13. Sato M, Yoshida S, Nagao K, et al. Superiority of dietary safflower oil over olive oil in lowering serum cholesterol and increasing hepatic mRNAs for the LDL receptor and cholesterol 7 α -hydroxylase in exogenously hypercholesterolemic (ExHC) rats. *Bioscience, Biotechnology, and Biochemistry*, 2000, 64(6): 1111-1117.
14. Tanaka Y, Ono M, Miyago M, et al. Low utilization of glucose in the liver causes diet-induced hypercholesterolemia in exogenously hypercholesterolemic rats. *PLoS One*, 2020, 15(3): e0229669.
15. Niibo M, Shirouchi B, Umegatani M, et al. Probiotic *Lactobacillus gasseri* SBT2055 improves insulin secretion in a diabetic rat model. *Journal of Dairy Science*, 2019, 102(2): 997-1006.
16. Folch J, Lees M, Stanley G H S. A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry*, 1957, 226(1): 497-509.
17. Sperry W M, Webb M. A revision of the Schoenheimer-Sperry method for cholesterol determination. *Journal of Biological Chemistry*, 1950, 187: 97-106.
18. Jones P J, Raeini-Sarjaz M, Ntanios F Y, et al. Modulation of plasma lipid levels and cholesterol kinetics by phytosterol versus phytostanol esters. *Journal of Lipid Research*, 2000, 41(5): 697-705.
19. Shirouchi B, Furukawa Y, Nakamura Y, et al. Inhibition of Niemann-Pick C1-like 1 by ezetimibe reduces dietary 5 β , 6 β -epoxycholesterol absorption in rats.

- Cardiovascular Drugs and Therapy, 2019, 33(1): 35-44.
20. Sato M, Ueda T, Nagata K, et al. Dietary kakrol (*Momordica dioica* Roxb) flesh inhibits triacylglycerol absorption and lowers the risk for development of fatty liver in rats. *Experimental Biology and Medicine*, 2011, 236(10): 1139-1146.
21. Shirouchi B, Nakamura Y, Furukawa Y, et al. Ezetimibe inhibits lymphatic transport of esterified cholesterol but not free cholesterol in thoracic lymph duct-cannulated rats. *Cardiovascular Drugs and Therapy*, 2012, 26(5): 427-431.
22. Tanaka Y, Nagao K, Nakagiri H, et al. Unavailability of liver triacylglycerol increases serum cholesterol concentration induced by dietary cholesterol in exogenously hypercholesterolemic (ExHC) rats. *Lipids in Health and Disease*, 2014, 13(1): 19.
23. Rho S J, Park S, Ahn C W, et al. Dietetic and hypocholesterolaemic action of black soy peptide in dietary obese rats. *Journal of the Science of Food and Agriculture*, 2007, 87(5): 908-913.
24. Mitsche M A, McDonald J G, Hobbs H H, et al. Flux analysis of cholesterol biosynthesis in vivo reveals multiple tissue and cell-type specific pathways. *elife*, 2015, 4: e07999.
25. Yoshioka H, Coates H W, Chua N K, et al. A key mammalian cholesterol synthesis enzyme, squalene monooxygenase, is allosterically stabilized by its substrate. *Proceedings of the National Academy of Sciences*, 2020, 117(13): 7150-7158.
26. Gill S, Stevenson J, Kristiana I, et al. Cholesterol-dependent degradation of squalene monooxygenase, a control point in cholesterol synthesis beyond HMG-CoA reductase. *Cell Metabolism*, 2011, 13(3): 260-273.
27. Fink M, Španinger K, Prosenc U, et al. High-fat Medium and Circadian Transcription Factors (Cryptochrome and Clock) Contribute to the Regulation of Cholesterogenic Cyp51 and Hmgcr Genes in Mouse Embryonic Fibroblasts. *Acta Chimica Slovenica*, 2008, 55(1).
28. Ma S, Sun W, Gao L, et al. Therapeutic targets of hypercholesterolemia: HMGCR and LDLR. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, 2019, 12: 1543.

29. Pandak W M, Schwarz C, Hylemon P B, et al. Effects of CYP7A1 overexpression on cholesterol and bile acid homeostasis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 2001, 281(4): G878-G889.
30. Schwarz M, Lund E G, Setchell K D R, et al. Disruption of Cholesterol 7 α -Hydroxylase Gene in Mice II. Bile acid deficiency is overcome by induction of Oxysterol 7 α -Hydroxylase. *Journal of Biological Chemistry*, 1996, 271(30): 18024-18031.
31. Arab J P, Karpen S J, Dawson P A, et al. Bile acids and nonalcoholic fatty liver disease: molecular insights and therapeutic perspectives. *Hepatology*, 2017, 65(1): 350-362.
32. Antalis C J, Arnold T, Rasool T, et al. High ACAT1 expression in estrogen receptor negative basal-like breast cancer cells is associated with LDL-induced proliferation. *Breast Cancer Research and Treatment*, 2010, 122(3): 661-670.
33. Dunn S E, LeBlanc G A. Hypocholesterolemic properties of plant indoles: Inhibition of acyl-CoA: cholesterol acyltransferase activity and reduction of serum LDL/VLDL cholesterol levels by glucobrassicin derivatives. *Biochemical Pharmacology*, 1994, 47(2): 359-364.
34. Rogers M A, Liu J, Song B L, et al. Acyl-CoA: cholesterol acyltransferases (ACATs/SOATs): enzymes with multiple sterols as substrates and as activators. *The Journal of steroid biochemistry and molecular biology*, 2015, 151: 102-107.
35. AbuMweis S S, Vanstone C A, Lichtenstein A H, et al. Plant sterol consumption frequency affects plasma lipid levels and cholesterol kinetics in humans. *European Journal of Clinical Nutrition*, 2009, 63(6): 747-755.
36. Chang E B, Martinez-Guryn K. Small intestinal microbiota: the neglected stepchild needed for fat digestion and absorption. *Gut Microbes*, 2019, 10(2): 235-240.
37. Heeney D D, Zhai Z, Bendiks Z, et al. *Lactobacillus plantarum* bacteriocin is associated with intestinal and systemic improvements in diet-induced obese mice and maintains epithelial barrier integrity in vitro. *Gut Microbes*, 2019, 10(3): 382-397.
38. Ma N, Ma X. Dietary amino acids and the gut-microbiome-immune axis: physiological metabolism and therapeutic prospects. *Comprehensive Reviews in*

Food Science and Food Safety, 2019, 18(1): 221-242.

39. Wu Y, Ma N, Song P, et al. Grape seed proanthocyanidin affects lipid metabolism via changing gut microflora and enhancing propionate production in weaned pigs. The Journal of Nutrition, 2019, 149(9): 1523-1532.

40. Davis R A. Participation of propionate in cholesterol biosynthesis by rat liver. Steroids, 1978, 31(4): 593-600.

Chapter III

1. Neeland I J, Ross R, Després J P, et al. Visceral and ectopic fat, atherosclerosis, and cardiometabolic disease: a position statement. The Lancet Diabetes & Endocrinology, 2019, 7(9): 715-725.

2. Markin A M, Sobenin I A, Grechko A V, et al. Cellular mechanisms of human atherogenesis: focus on chronification of inflammation and mitochondrial mutations. Advances in Medical Biochemistry, Genomics, Physiology, and Pathology, 2021: 405-424.

3. Jain S, Buttar H S, Chintameneni M, et al. Prevention of cardiovascular diseases with anti-inflammatory and anti-oxidant nutraceuticals and herbal products: an overview of pre-clinical and clinical studies. Recent Patents on Inflammation & Allergy Drug Discovery, 2018, 12(2): 145-157.

4. Wang D D. Dietary n-6 polyunsaturated fatty acids and cardiovascular disease: Epidemiologic evidence. Prostaglandins, Leukotrienes and Essential Fatty Acids, 2018, 135: 5-9.

5. Bowen K J, Kris-Etherton P M, West S G, et al. Diets enriched with conventional or high-oleic acid canola oils lower atherogenic lipids and lipoproteins compared to a diet with a western fatty acid profile in adults with central adiposity. The Journal of Nutrition, 2019, 149(3): 471-478.

6. George J, Mulkins M, Casey S, et al. The effects of N-6 polyunsaturated fatty acid supplementation on the lipid composition and atherogenesis in mouse models of atherosclerosis. Atherosclerosis, 2000, 150(2): 285-293.

7. Le H D, Meisel J A, de Meijer V E, et al. The essentiality of arachidonic acid and docosahexaenoic acid. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 2009, 81(2-3): 165-170.
8. Choque B, Catheline D, Rioux V, et al. Linoleic acid: between doubts and certainties. *Biochimie*, 2014, 96: 14-21.
9. Marchix J, Choque B, Kouba M, et al. Excessive dietary linoleic acid induces proinflammatory markers in rats. *The Journal of Nutritional Biochemistry*, 2015, 26(12): 1434-1441.
10. Turpeinen A M, Basu S, Mutanen M. A high linoleic acid diet increases oxidative stress in vivo and affects nitric oxide metabolism in humans. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 1998, 59(3): 229-233.
11. Sato M, Shibata K, Nomura R, et al. Linoleic acid-rich fats reduce atherosclerosis development beyond its oxidative and inflammatory stress-increasing effect in apolipoprotein E-deficient mice in comparison with saturated fatty acid-rich fats. *British Journal of Nutrition*, 2005, 94(6): 896-901.
12. Tomoyori H, Kawata Y, Higuchi T, et al. Phytosterol oxidation products are absorbed in the intestinal lymphatics in rats but do not accelerate atherosclerosis in apolipoprotein E-deficient mice. *The Journal of Nutrition*, 2004, 134(7): 1690-1696.
13. Folch J, Lees M, Sloane Stanley G H. A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, 1957, 226(1): 497-509.
14. Carvajal O, Nakayama M, Kishi T, et al. Effect of medium - chain fatty acid positional distribution in dietary triacylglycerol on lymphatic lipid transport and chylomicron composition in rats. *Lipids*, 2000, 35(12): 1345-1352.
15. Yuan X, Minobe Y, Tanaka Y, et al. α - globulin - rich rice cultivar, low glutelin content - 1 (LGC - 1), decreases serum cholesterol concentration in exogenously hypercholesterolemic rats. *Journal of the Science of Food and Agriculture*, 2021, 101(15): 6417-6423.
16. Imaizumi K. Diet and atherosclerosis in apolipoprotein E-deficient mice. *Bioscience, Biotechnology, and Biochemistry*, 2011, 75 (6): 1023-1035.

17. Saini R K, Keum Y S. Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance—A review. *Life sciences*, 2018, 203: 255-267.
18. Douglas G, Channon K M. The pathogenesis of atherosclerosis. *Medicine*, 2014, 42(9): 480-484.
19. Duivenvoorden I, Voshol P J, Rensen P C N, et al. Dietary sphingolipids lower plasma cholesterol and triacylglycerol and prevent liver steatosis in APOE* 3 Leiden mice. *The American Journal of Clinical Nutrition*, 2006, 84(2): 312-321.
20. Tanaka Y, Ono M, Miyago M, et al. Low utilization of glucose in the liver causes diet-induced hypercholesterolemia in exogenously hypercholesterolemic rats. *PLoS One*, 2020, 15(3): e0229669.
21. Yu S, Derr J, Etherton T D, et al. Plasma cholesterol-predictive equations demonstrate that stearic acid is neutral and monounsaturated fatty acids are hypocholesterolemic. *The American Journal of Clinical Nutrition*, 1995, 61(5): 1129-1139.
22. KEYS A, PARLIN R W. Serum cholesterol response to changes in dietary lipids. *The American Journal of Clinical Nutrition*, 1966, 19(3): 175-181.
23. Cooper M H, Miller J R, Mitchell P L, et al. Conjugated linoleic acid isomers have no effect on atherosclerosis and adverse effects on lipoprotein and liver lipid metabolism in apoE^{-/-} mice fed a high-cholesterol diet. *Atherosclerosis*, 2008, 200(2): 294-302.
24. Tuttolomondo A, Di Raimondo D, Pecoraro R, et al. Atherosclerosis as an inflammatory disease. *Current Pharmaceutical Design*, 2012, 18(28): 4266-4288.
25. Panee J. Monocyte Chemoattractant Protein 1 (MCP-1) in obesity and diabetes. *Cytokine*, 2012, 60(1): 1-12.
26. Fiechter M, Haider A, Bengs S, et al. Sex-dependent association between inflammation, neural stress responses, and impaired myocardial function. *European Journal of Nuclear Medicine and Molecular Imaging*, 2020, 47(8): 2010-2015.
27. Stein Y, Stein O. Lipoprotein lipase and atherosclerosis. *Atherosclerosis*, 2003, 170(1): 1-9.
28. Hanna V S, Hafez E A A. Synopsis of arachidonic acid metabolism: A review.

Journal of Advanced Research, 2018, 11: 23-32.

29. Hastings N, Agaba M, Tocher D R, et al. A vertebrate fatty acid desaturase with $\Delta 5$ and $\Delta 6$ activities. Proceedings of the National Academy of Sciences, 2001, 98(25): 14304-14309.

30. Das U N. Arachidonic acid in health and disease with focus on hypertension and diabetes mellitus: A review. Journal of Advanced Research, 2018, 11: 43-55.

31. Nilsson B O. Modulation of the inflammatory response by estrogens with focus on the endothelium and its interactions with leukocytes. Inflammation Research, 2007, 56(7): 269-273.

32. Pervin S, Singh R, Rosenfeld M E, et al. Estradiol suppresses MCP-1 expression in vivo: implications for atherosclerosis. Arteriosclerosis, Thrombosis, and Vascular Biology, 1998, 18(10): 1575-1582.

33. Nakagami F, Nakagami H, Osako M K, et al. Estrogen attenuates vascular remodeling in Lp (a) transgenic mice. Atherosclerosis, 2010, 211(1): 41-47.

34. Sato M, Sato H, Ogawa A, et al. Antiatherogenic effect of isoflavones in ovariectomized apolipoprotein E-deficient mice. Journal of Agricultural and Food Chemistry, 2007, 55(22): 8967-8971.

35. Johnson G H, Fritsche K. Effect of dietary linoleic acid on markers of inflammation in healthy persons: a systematic review of randomized controlled trials. Journal of the Academy of Nutrition and Dietetics, 2012, 112(7): 1029-1041. e15.

36. Ramkumar J, Sharma N. Low dose aspirin and omega-3 fatty acids in the pro-resolving pathway of cardiovascular disorders. Cardiology and Angiology: An International Journal, 2017, 6: 1-12.

Chapter IV

1. DeTure M A, Dickson D W. The neuropathological diagnosis of Alzheimer's disease. Molecular Neurodegeneration, 2019, 14(1): 1-18.

Danysz W, Parsons C G. Alzheimer's disease, β - amyloid, glutamate, NMDA receptors and memantine - searching for the connections. British Journal of Pharmacology, 2012, 167(2): 324-352.

3. Kametani F, Hasegawa M. Reconsideration of amyloid hypothesis and tau hypothesis in Alzheimer's disease. *Frontiers in Neuroscience*, 2018, 12: 25.
4. Petersen R C. Early diagnosis of Alzheimer's disease: is MCI too late?. *Current Alzheimer Research*, 2009, 6(4): 324-330.
5. Prince, Martin, et al. World Alzheimer report 2016: improving healthcare for people living with dementia: coverage, quality and costs now and in the future. 2016.
6. Castro D M, Dillon C, Machnicki G, et al. The economic cost of Alzheimer's disease: Family or public-health burden? *Dementia & Neuropsychologia*, 2010, 4: 262-267.
7. Rhea E M, Banks W A. Interactions of lipids, lipoproteins, and apolipoproteins with the blood-brain barrier. *Pharmaceutical Research*, 2021, 38(9): 1469-1475.
8. Zhang, Juan, and Qiang Liu. Cholesterol metabolism and homeostasis in the brain. *Protein & Cell* 6.4 (2015): 254-264.
9. Pfrieger F W, Ungerer N. Cholesterol metabolism in neurons and astrocytes. *Progress in Lipid Research*, 2011, 50(4): 357-371.
10. Goritz C, Mauch D H, Pfrieger F W. Multiple mechanisms mediate cholesterol-induced synaptogenesis in a CNS neuron. *Molecular and Cellular Neuroscience*, 2005, 29(2): 190-201.
11. Orr, Adam L., et al. Neuronal apolipoprotein E4 expression results in proteome-wide alterations and compromises bioenergetic capacity by disrupting mitochondrial function. *Journal of Alzheimer's Disease* 68.3 (2019): 991-1011.
12. Hauser P S, Narayanaswami V, Ryan R O. Apolipoprotein E: from lipid transport to neurobiology. *Progress in Lipid Research*, 2011, 50(1): 62-74.
13. Griffiths W J, Wang Y. Oxysterol research: a brief review. *Biochemical Society Transactions*, 2019, 47(2): 517-526.
14. Tolppanen A M, Solomon A, Soininen H, et al. Midlife vascular risk factors and Alzheimer's disease: evidence from epidemiological studies. *Journal of Alzheimer's Disease*, 2012, 32(3): 531-540.
15. Sparks D L, Sabbagh M, Connor D, et al. Statin therapy in Alzheimer's disease. *Acta Neurologica Scandinavica*, 2006, 114: 78-86.
16. Heverin M, Bogdanovic N, Lütjohann D, et al. Changes in the levels of cerebral

and extracerebral sterols in the brain of patients with Alzheimer's disease. *Journal of Lipid Research*, 2004, 45(1): 186-193.

17. Gamba P, Testa G, Sottero B, et al. The link between altered cholesterol metabolism and Alzheimer's disease. *Annals of the New York Academy of Sciences*, 2012, 1259(1): 54-64.

18. Xiao N, Zhao Y, Yao Y, et al. Biological activities of egg yolk lipids: A review. *Journal of Agricultural and Food Chemistry*, 2020, 68(7): 1948-1957.

19. Parducz A, Kiss Z, Joo F. Changes of the phosphatidylcholine content and the number of synaptic vesicles in relation to the neurohumoral transmission in sympathetic ganglia. *Experientia*, 1976, 32(12): 1520-1521.

20. Chung S Y, Moriyama T, Uezu E, et al. Administration of phosphatidylcholine increases brain acetylcholine concentration and improves memory in mice with dementia. *The Journal of Nutrition*, 1995, 125(6): 1484-1489.

21. Lim S Y, Suzuki H. Intakes of dietary docosahexaenoic acid ethyl ester and egg phosphatidylcholine improve maze-learning ability in young and old mice. *The Journal of Nutrition*, 2000, 130(6): 1629-1632.

22. Song C, Shieh C H, Wu Y S, et al. The role of omega-3 polyunsaturated fatty acids eicosapentaenoic and docosahexaenoic acids in the treatment of major depression and Alzheimer's disease: Acting separately or synergistically. *Progress in Lipid Research*, 2016, 62: 41-54.

23. Kulig W, Cwiklik L, Jurkiewicz P, et al. Cholesterol oxidation products and their biological importance. *Chemistry and Physics of Lipids*, 2016, 199: 144-160.

24. Liu Q, An Y, Yu H, et al. Relationship between oxysterols and mild cognitive impairment in the elderly: a case-control study. *Lipids in Health and Disease*, 2016, 15(1): 1-6.

25. Popp J, Meichsner S, Kölsch H, et al. Cerebral and extracerebral cholesterol metabolism and CSF markers of Alzheimer's disease. *Biochemical Pharmacology*, 2013, 86(1): 37-42.

26. Hascalovici J R, Vaya J, Khatib S, et al. Brain sterol dysregulation in sporadic AD and MCI: relationship to heme oxygenase-1. *Journal of Neurochemistry*, 2009, 110(4):

1241-1253.

27. Heverin M, Bogdanovic N, Lütjohann D, et al. Changes in the levels of cerebral and extracerebral sterols in the brain of patients with Alzheimer's disease. *Journal of Lipid Research*, 2004, 45(1): 186-193.

28. Prasanthi J R P, Huls A, Thomasson S, et al. Differential effects of 24-hydroxycholesterol and 27-hydroxycholesterol on β -amyloid precursor protein levels and processing in human neuroblastoma SH-SY5Y cells. *Molecular Neurodegeneration*, 2009, 4(1): 1-8.

29. Heverin M, Maioli S, Pham T, et al. 27-hydroxycholesterol mediates negative effects of dietary cholesterol on cognition in mice. *Behavioural Brain Research*, 2015, 278: 356-359.

30. Wu M, Zhai Y, Liang X, et al. Connecting the Dots Between Hypercholesterolemia and Alzheimer's Disease: A Potential Mechanism Based on 27-Hydroxycholesterol. *Frontiers in Neuroscience*, 2022, 16.

31. Zhang X, Xi Y, Yu H, et al. 27-hydroxycholesterol promotes $A\beta$ accumulation via altering $A\beta$ metabolism in mild cognitive impairment patients and APP/PS1 mice. *Brain Pathology*, 2019, 29(4): 558-573.

32. Ross C, Taylor M, Fullwood N, et al. Liposome delivery systems for the treatment of Alzheimer's disease. *International Journal of Nanomedicine*, 2018, 13: 8507.

33. Gu L, Guo Z. Alzheimer's $A\beta_{42}$ and $A\beta_{40}$ form mixed oligomers with direct molecular interactions. *Biochemical and Biophysical Research Communications*, 2021, 534: 292-296.

34. Iwatsubo, Takeshi, et al. Amyloid β protein ($A\beta$) deposition: $A\beta_{42}$ (43) precedes $A\beta_{40}$ in Down syndrome. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, 1995, 37(3): 294-299.

35. Gouras G K, Almeida C G, Takahashi R H. Intraneuronal $A\beta$ accumulation and origin of plaques in Alzheimer's disease. *Neurobiology of Aging*, 2005, 26(9): 1235-1244.

36. Russo C, Saido T C, DeBusk L M, et al. Heterogeneity of water-soluble amyloid β -peptide in Alzheimer's disease and Down's syndrome brains. *FEBS Letters*, 1997,

409(3): 411-416.

37. Wu K, Gao X, Shi B, et al. Enriched endogenous n-3 polyunsaturated fatty acids alleviate cognitive and behavioral deficits in a mice model of Alzheimer's disease. *Neuroscience*, 2016, 333: 345-355.

38. Janssen C I F, Zerbi V, Mutsaers M P C, et al. Impact of dietary n-3 polyunsaturated fatty acids on cognition, motor skills and hippocampal neurogenesis in developing C57BL/6J mice. *The Journal of Nutritional Biochemistry*, 2015, 26(1): 24-35.

39. Whelan J. (n-6) and (n-3) Polyunsaturated fatty acids and the aging brain: food for thought. *The Journal of Nutrition*, 2008, 138(12): 2521-2522.

40. Farooqui A A, Horrocks L A, Farooqui T. Modulation of inflammation in brain: a matter of fat. *Journal of Neurochemistry*, 2007, 101(3): 577-599.

41. D'Errico G, Vitiello G, Ortona O, et al. Interaction between Alzheimer's A β (25–35) peptide and phospholipid bilayers: The role of cholesterol. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 2008, 1778(12): 2710-2716.

42. Ullah R, Park T J, Huang X, et al. Abnormal amyloid beta metabolism in systemic abnormalities and Alzheimer' s pathology: Insights and therapeutic approaches from periphery. *Ageing Research Reviews*, 2021, 71: 101451.

43. Lopez A M, Chuang J C, Posey K S, et al. Suppression of brain cholesterol synthesis in male Mecp2-deficient mice is age dependent and not accompanied by a concurrent change in the rate of fatty acid synthesis. *Brain Research*, 2017, 1654: 77-84.

44. Poirier J, Hess M, May P C, et al. Cloning of hippocampal poly (A) RNA sequences that increase after entorhinal cortex lesion in adult rat. *Molecular Brain Research*, 1991, 9(3): 191-195.

45. De Leon M J, Convit A, Wolf O T, et al. Prediction of cognitive decline in normal elderly subjects with 2-[18F] fluoro-2-deoxy-D-glucose/positron-emission tomography (FDG/PET). *Proceedings of the National Academy of Sciences*, 2001, 98(19): 10966-10971.

46. Fitz N F, Nam K N, Wolfe C M, et al. Phospholipids of APOE lipoproteins activate

microglia in an isoform-specific manner in preclinical models of Alzheimer's disease. *Nature Communications*, 2021, 12(1): 1-18.

47. Hansen D V, Hanson J E, Sheng M. Microglia in Alzheimer's disease. *Journal of Cell Biology*, 2018, 217(2): 459-472.