ABSTRACT

Title of Document: COMPARATIVE STUDY OF LIPOPROTEIN METABOLISM IN MAREK'S DISEASE SUSCEPTIBLE AND RESISTANT LINES
Ping Yuan, Master of Science, 2010

Directed By: Assistant professor Dr. Jiuzhou Song,
Department of Animal and Avian Sciences

Marek's disease virus (MDV) infection causes atherosclerosis, and prior vaccination prevented the development of this disease. Two main strategies to resist Marek's disease (MD) have been demonstrated: vaccination and genetic resistance. However, little is known about the role of genetic resistance in the progression of MDV induced atherosclerosis. Atherosclerosis is primarily associated with lipoprotein metabolism. The purpose of this study was to investigate whether lipoprotein metabolisms are different in distinct MD susceptible and resistant chicken lines. Here, we studied different backgrounds of lipoprotein metabolism in the two lines and the changes of lipoprotein levels in response to MDV infection. The results showed that during chicken growth, the increase in total cholesterol was mostly due to the increasing (LDL+VLDL) in MD susceptible line, whereas it was mainly due to the elevating HDL in MD resistant line. These results suggested that different lipoprotein metabolisms exist in MD susceptible and resistant lines.

Comparative Study of Lipoprotein Metabolism in Marek's Disease Susceptible and Resistant Lines

By

Ping Yuan

Thesis submitted to the Faculty of the Graduate School
of the University of Maryland, College Park,
in partial fulfillment of the requirements for
Master of Science
2010

Advisory Committee:

Dr. Jiuzhou Song, Chair
Dr. Zhengguo Xiao
Dr. Scott M Updike

Dedication

To my parents, thank you for all your support

Acknowledgments

Thank you to my advisor Dr. Song, who has helped me a great deal in developing my scientific thinking and gave me solid support for my project.

To the other members of my committee, Dr. Xiao and Dr. Updike, who have given me valuable advice for my Master's work.

Thank you to the Song lab, for the help and friendship you have given me for the past two years.

Table of Contents

Chapter 1: Literature review

Introduction

Atherosclerosis is a progressive disease characterized by lipid accumulation in the large arteries, which causes about 50% of all deaths in westernized societies[1]. It has been demonstrated that atherosclerosis is a complex process which involves the interaction of several genetic and environmental factors, including diabetes, hypercholesterolemia and cigarette smoking[2]. Since atherosclerotic lesions are thought to be initiated by injury to the vascular endothelium, considerable research has been focused on studying the mechanisms responsible for this initial injury[2]. Herpesviruses have been proposed as potential initiators of arterial injury. This statement was based on several studies in the late 1970's, which revealed that Marek's disease virus (MDV), an avian herpesvirus, could induce atherosclerosis in chickens[3-5].

MDV is an oncogenic herpesvirus that causes several syndromes in the chicken. It has been demonstrated that MDV infection can induce occlusive atherosclerotic lesions in large coronary arteries and aortas of infected chickens[5]. MDV infection also affected enzymatic activities in arterial smooth muscle cells, which resulted in aortic lipid accumulation in chicken, including cholesterol, cholesterol ester, triacylglycerol and phospholipids[4]. It has been suggested that the lipid accretion in aortas of MDV infected chickens was possibly due to alterations in cholesterol metabolism[4, 6].

Besides cholesterol metabolism, lipoprotein metabolism was involved in atherosclerosis[7, 8]. Progressive accumulation of cholesterol in the arterial wall causes atherosclerosis. Lipoproteins, as the key carriers of cholesterol through the circulating system, have been reported to be associated with the development of atherosclerosis. For instance, the low density lipoprotein (LDL) accumulated in the subendothelial matrix, resulting in a primary initiating event in atherosclerosis[1]. Moreover, epidemiologic evidence has revealed LDL cholesterol as a risk factor for atherosclerosis[9]. Conversely, the high density lipoprotein (HDL) is strongly protective against atherosclerosis. This protective effect results from the role of HDL in transporting excess cholesterol from foam cells to the liver, thus relieving the cholesterol accumulation in the circulating system[10].

Studies for the role of lipoprotein metabolism in atherosclerosis induced by MDV infection will give a better understanding for the initial process of atherosclerosis. Furthermore, it is important to note that, besides this animal model, studies in humans have also reported the epidemiological association between herpesviral infections and accelerated atherosclerosis in heart transplant patients[11, 12]. Multiple studies about herpesviral induced atherosclerosis has led to the hypothesis that herpesviruses initiate vascular disease processes in humans[13, 14]. Therefore, with this model of MDV infected chicken, elucidating the role of lipoprotein metabolism for herpesvirus induced atherosclerosis could enable us to demonstrate the etiology and pathogenesis of human arteriosclerosis.

Marek's disease and atherosclerosis

Marek's disease

Marek's disease (MD) was first recognized by József Marek in 1907 as a generalized polyneuritis in chickens. It was also shown that MD was associated with visceral lymphoma in the later 1920s[15,16]. The clinical disease is similar to the neoplastic (retrovirus-induced lymphatic leukosis) disease in chicken. A clear differentiation between retrovirus- and MDV-induced neoplasia was made in the later 1960s, when the herpesvirus aetiology of MD was identified[17]. It is important to note that in the 1960s, the lymphoid form of MD became a serious problem with the expansion and intensification of poultry production. It was only two years after the identification and cell-culture isolation of MDV[18,19] that the use of attenuated MDV was introduced as a highly effective vaccine[20,21]. The vaccines achieved unparalleled success in preventing the disease and provided a landmark: the first effective and widespread immune prophylaxis against a virus-induced cancer in any species. However, over the last 30 years, the virus has mutated to increasly virulent forms and vaccine breaks began to appear, which has led to the requirement of new strategies for controlling and preventing the disease. Importantly, it is well known that MD has a worldwide impact on the poultry industry. It has been demonstrated that MDV pathogenic strains are present in nearly every country from the multi-annual animal disease status report by the Office International des Epizooties. Furthermore, the economic impact of MD has been estimated to be US$ 1-2 billion annually. Therefore, investigation of MD has remained essential to this day.

Marek's disease virus (MDV)

MDV is an oncogenic herpesvirus that causes several syndromes in the chicken, its natural host. MDV was originally classified as a *Gammaherpesvirus* because its biological behavior and ability to infect lymphocytes are similar to Epstein-Barr virus (a γ-herpesvirus). However, electron-microscopy studies of the MDV genome found that MDV is a double-stranded linear DNA molecule[22] and possesses repeat structures, which are characteristic of *Alphaherpesvirinae* [23]. Therefore, on the basis of genomic properties, MDV is currently classified as an alphaherpesvirus.

The current model of MDV pathogenesis describes the disease in terms of two phases: the early cytolytic phase, and the latent and tumor phase of MDV infection[24]. In brief, the first step of MDV infection in the chicken is inhalation of the virus. Phagocytic cells in the respiratory route become infected[25], and within 24 hours of uptake of MDV-carrier cells, several tissues become infected, including the spleen, thymus and the bursa of Fabricius[26]. Here, the virus meets B cells and later activates $CD4^+$ as its main primary targets for the first phase of cytolytic replication[27,28]. After an early cytolytic phase, MDV enters the latent phase of infection from 6-7 days post infection. During MDV latency, the viral genomes present in the host cells, but there is no production of infectious progeny virus[29,30]. MDV latency is difficult to study since distinguishing latently infected cells from transformed cells is nearly impossible. Subsequently, there is transformation of latently infected cells to lymphoblastoid tumor cells[15, 31], which is generally considered as the ultimate consequence of interaction of MDV with the host cells. Indeed, pathogenesis of MD is complex and the life cycle of this fascinating herpesvirus remains to be elucidated,

such as the molecular details of T-cell transformation and the factors that control tumor formation.

Pathology of Marek's disease

MDV infection leads to pathological changes in its host, which involve lymphoid, nervous and other tissues of the chicken. The current pathology of MD is different from when it was first described in 1907, due to the increasing virulence of MDV. Currently, there are several pathological changes known to occur after MDV infection.

Acute cytolytic infection

It has been found that an acute cytolytic infection of lymphoid tissue appears at 3 days post infection, especially in the bursa, thymus and spleen[32]. B cells and $CD4^+$ and $CD8^+$ T cells are activated and infected in these organs, where the virus infection provokes an acute inflammatory response[27]. Moreover, the bursa and thymus exhibit severe regression of bursa lymphoid follicles and thymic cortex, respectfully, which result in weight loss in these organs[33]. However, inflammatory response in the spleen results in increase in weight.

Central nervous system and peripheral neuropathy

In 1959, Zander described a new encephalitic syndrome termed as 'transient paralysis' (TP). This clinical syndrome was related to brain lesions, including acute vasculitis, vasogenic oedema and perivascular cuffing[34, 35]. Subsequently, two further syndromes of central nervous system, 'persistent neurological disease' (PND) and 'late paralysis', were identified by Gimeno in 1999[36]. Additionally, in 1967, Payne

and Biggs demonstrated peripheral nerve lesions, which were considered inflammatory and possibly indicated regression of the neoplastic lymphoproliferation[37].

Lymphomatosis

Multifocal lymphoid proliferation appears one week after MDV infection and visceral lymphomas was involved in various tissues, such as the gonads, liver, kidney, spleen, heart, bursa and skeletal muscles. MD lymphoma is a cytological complex, which consists of transformed T cells and several other cells, including B cells, macrophages and natural killer cells[38, 39].

Atherosclerosis

Atherosclerosis is a complex process and involves the interaction of genetic and environmental factors. It has been demonstrated that injury to the vascular endothelium initiates the atherosclerotic lesions and herpesviruses have been described as potential initiators for arterial injury[5]. This theory was based on studies from Fabricant and colleagues in the 1970s, who found an association between coronary atherosclerosis and MDV infection, an avian herpesvirus. The results from this group were:

(1) MDV infection induced occlusive atherosclerotic lesions in large coronary arteries and aortas of infected normocholesterolemic and hypercholesterolemic chickens. In contrast, visible atherosclerotic lesions were not found in uninfected normocholesterolemic and hypercholesterolemic chickens. Histological evaluation demonstrated that arterial changes were characterized by fibromuscular intimal thickening in the infected animals[3, 5].

(2) At 4 and 8 months after MDV inoculation, MDV infected chickens had a significant increase ($P<0.05$) in total aortic lipid accumulation, including cholesterol, cholesterol ester, triacylglycerol and phospholipids, as compared with aortas in uninfected chickens. It has been demonstrated that the lipid accretion in aortas of MDV infected chickens was possibly due to alterations in cholesterol metabolism[4, 6].

Marek's disease resistant and susceptible lines

There are two main strategies for controlling MD: vaccination and genetic resistance, which have brought enormous benefits to the modern poultry industry[40]. However, aggressive increase in MD vaccinations drive MDV evolution to increased levels of virulence, which rendered renewed interest to study genetic selection to improve MD resistance. Differences in genetic resistance to MD were first investigated by Asmundson and Biely in 1930s[41]. These results were subsequently confirmed by other surveys and experiments[42, 43]. Currently, two distinct genetic resistances to MD have been identified: chicken major histocompatibility complex (MHC) associated resistance, and non-MHC associated resistance.

MHC associated resistance

It had been demonstrated that the inheritance of alleles in the *B* blood was associated with increased MD resistance[44]. Considering the *B* blood group locus as a marker for MHC, it has been indicated that genes within the MHC were responsible for MD

resistance. Moreover, the N and P lines were reported by Hutt and Cole in 1947[42], and the differences in susceptibility between two lines were associated with their MHC[45].

Non-MHC associated resistance

Besides the N and P lines, the resistant and susceptible lines (line 6 and 7) have been reported by Stone at East Lansing in 1975, who also provided the basis for studying genetic resistance. Importantly, line 6 and 7 have the same MHC haplotype[46], which indicated that differences in resistance must be related with non-MHC genes. There are dramatic differences in the size of primary lymphoid organs between line 6 and 7 (e.g., the larger lobes of the thymus in line 7)[47]. Furthermore, line 7 has higher lymphoproliferation traits than line 6 (e.g., the lymphocyte response to mitogens *in vitro*)[48]. The development of genomic mapping for chicken, together with DNA arrays and interference RNA (RNAi), makes it possible to identify the genes responsible for resistance and susceptibility to MD. Chicken genetic resistance possibly provides more sustainable means to prevent MD outbreaks in the future.

Lipoprotein and atherosclerosis

Cholesterol

Lipid organization in biological membranes is crucial for cell functions. Cholesterol is an important structural element of cell membranes and subcellular particles in higher eukaryotes[49], and acts as an essential determinant for membrane organization. For example, cholesterol helps to construct a barrier between cellular compartments, modulate the fluidity of the cell membrane, regulate the biological function of membrane proteins and is involved in transmembrane signaling processes[50]. In

addition to its structural role, cholesterol is an obligatory precursor for steroid hormones, vitamin D, and bile acids; it is necessary for the activation of several neuronal signaling molecules[51, 52]. In animals, only a small amount of circulating cholesterol comes from the diet, and more than 80 % is derived from endogenous synthesis[8]. The most actively synthesizing organs are the liver and intestine, where 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) catalyses acetyl-CoA to produce cholesterol. Additionally, most circulating cholesterol is esterified with long-chain fatty acids, and free cholesterol constitutes a minor component[49].

Since cholesterol plays an indispensible role for normal cell function, organisms have developed complex and sophisticated mechanisms to regulate cholesterol levels within a proper range[53]. Cholesterol, synthesized mainly in the liver and intestine or obtained from food, is delivered throughout the body to exert its biological functions. For long-distance transfer among cells through the aqueous environment, cholesterol and cholesterol ester, as hydrophobic molecules, must be shuttled by spheroidal macromolecules called lipoproteins. Therefore, it is not surprising that lipoprotein can profoundly influence cholesterol distribution or metabolism and eventually modulate its functions.

Lipoprotein structure and classification

Lipoproteins are the macromolecular vehicles that transport hydrophobic small molecules throughout the aqueous environment[54]. A lipoprotein is made up of two parts: a hydrophobic core and a hydrophilic coat[55, 56]. The core of a lipoprotein

contains apolar components, such as triacylglycerols and esterified cholesterol. The coat of a lipoprotein consists of a phospholipid monolayer with polar head groups facing the aqueous system. Several amphiphilic molecules, such as free cholesterol (unesterified) and apolipoproteins, embed in the phospholipid layer and confer functional properties to the lipoprotein[57].

Lipoproteins are assigned into four main classes according to size, particle density and composition: chylomicrons, very low density lipoproteins (VLDLs), low density lipoproteins (LDLs) and high density lipoproteins (HDLs). Chylomicrons and VLDLs are mainly used to carry triacylglycerols, whereas LDLs and HDLs are used to shuttle cholesterol.

(1) *Chylomicrons*

Chylomicrons are the lowest density molecules with the largest diameter (> 75 nm), and primarily consist of triacylglycerols (80-88% weight by weight (w/w)) and a minor fraction of protein (apolipoprotein B isoform B48)[54].

(2) *VLDLs*

VLDLs, similar to chylomicrons, are mainly composed of triacylglycerols (about 45-50% (w/w)) and also contain unesterified and esterified cholesterol. VLDLs have particle sizes ranging from 30-80 nm in diameter[54]. The main physiological role of VLDLs is to transport triacylglycerols from the liver to peripheral tissues for use. The VLDLs remnants are in part cleared by hepatic receptors, but the main fraction (70%) remaining in the plasma is subsequently converted to LDL through the removal of

triacylglycerols by lipoprotein lipase (LPL) and loss of apolipoprotein E by cholesteryl ester transfer protein (CEPT)[58, 59].

(3) *LDLs*

LDLs are the catabolism product of VLDLs and have particle sizes ranging from 18-25 nm in diameter[54]. Compared with chylomicrons and VLDLs, LDLs primarily consist of cholesterol and cholesterol esters, and contain apolipoprotein B-100. LDLs serve as the primary transport mechanism to deliver cholesterol from the liver to peripheral tissues, and transport 70-80% of the circulating cholesterol in humans[60]. LDLs are cleared from the circulating system by the LDL-receptor pathway[61].

(4) *HDLs*

HDL particles are a heterogeneous mixture with the smallest diameter (< 12 nm). In contrast to other lipoproteins, HDLs are of relatively high protein content (approximately 50% (w/w)), containing primarily apolipoprotein A1 and A2[54]. In addition to a high protein content, HDLs are composed of unesterified cholesterol and triacylglycerols[62]. HDLs serve as a shuttle that transports unesterified cholesterol from the peripheral tissues to the liver for excretion.

Lipoprotein metabolism

Lipoprotein metabolism is a complex and sophisticated network, including assembly, secretion, processing and catabolism[8]. In the small intestine, dietary fats are absorbed and converted into triacylglycerols (TGs). TGs are packaged with cholesterol ester and apolipoprotein B isoform B48 to form chylomicrons. Chylomicrons are secreted

via the lymphatic system, and circulate in the bloodstream until they interact with lipoprotein lipase (LPL). LPLs rapidly hydrolyze TGs in the chylomicrons into free fatty acid, leading to the formation of chylomicron remnants, which are cleared by the liver via an apolipoprotein E receptor[8, 54].

In VLDL and LDL cholesterol metabolism, VLDLs are synthesized in liver cells (hepatocytes) through packaging with TG, cholesterol and apolipoprotein B isoform B100. TG in VLDLs can be lipolyzed by LPL, resulting in the release of free fatty acids and monoacylglycerols from VLDLs to form VLDL remnants. A small amount of VLDL remnants are cleared by hepatic receptors, whereas the majority in the plasma are subsequently converted to LDL through CETP. LDL transports unesterified and esterified cholesterol from the liver to the periphery. During the circulation, LDL particles are endocytosed by peripheral cells and removed by LDL receptors[54, 63, 64].

In HDL cholesterol metabolism, apolipoprotein A1 (ApoA1) is synthesized in the liver and released to the periphery. These particles interact with ATP-binding cassette A1 (ABCA1) transport proteins expressed in the peripheral cells. ABCA1 catalyzes the transfer of unesterified cellular cholesterol. Subsequently, ApoA1 accumulates cholesterol and phospholipids, leading to the generation of nascent pre-HDL. The nascent pre-HDL is remodeled by lecithin-cholesterol acyltransferase (LCAT), phospholipid transfer protein and CETP, thus completing the transformation to mature HDL. These HDL particles continue to pick up free cholesterol from the

peripheral tissues with the help of ATP-binding cassette transporter G1, and then transport cholesterol to the liver. HDL particles are removed from the bloodstream via the scavenger receptor B1, which is expressed by the liver[8, 54, 65].

Lipoproteins, atherosclerosis and cardiovascular disease

Lipid metabolism is essential for sustaining normal cell function, and disturbance of this process can have serious consequences for organisms. Atherosclerosis and cardiovascular disease (CVD) are major diseases related with cholesterol and lipoprotein metabolism in human[7, 8]. Atherosclerosis is a progressive disease which is characterized by the accumulation of lipids in the large arteries. It has been demonstrated that atherosclerosis causes about 50% of all deaths in westernized societies[1]. Epidemiological and genetic disorder studies have revealed that several important environmental and genetic risk factors are associated with atherosclerosis and CVD, such as higher LDL/VLDL levels, reduced HDL levels, elevated blood pressure, diabetes, obesity, family history, systemic inflammatory, smoking and lack of exercise[1]. Of the factors, the relative abundance of several plasma lipoproteins seems to be of primary importance[1, 8].

Plasma lipoproteins and CVD risk

Clinical trials have shown that elevated concentrations of plasma LDL cholesterol are associated with increased in coronary heart disease[66, 67]. Plasma LDLs are normally catabolized through receptor-mediated endocytosis. Chronic excess of plasma LDL interrupts arterial relaxation. Moreover, LDL particles, which fail to be removed

through receptor-mediated pathway, can be recognized by scavenge receptors on arterial-wall macrophages[1, 68]. Once LDLs are engulfed by macrophages, they become oxidized and induce toxic intermediates, leading to cytokine production and taxis of inflammatory cells[68]. In addition, arterial-wall macrophages loaded with LDL become foam cells, which are components for atherogenic plaques, resulting in coronary heart disease[1].

Unlike LDLs, HDL cholesterol levels are inversely correlated with cardiovascular risk[69]. Epidemiological studies show that a 1 mg/dl increase in HDL cholesterol contributes to a 2-3% decrease in the risk of CVD[70]. It is well known that the primary physiological role of HDLs is reverse cholesterol transport (RCT) (i.e., the centripetal transport of cholesterol from the peripheral tissues to the liver). Cholesterol carried by HDLs particles might be cleared in the liver by the scavenger receptor B1 mediated pathway[71]. Therefore, HDLs help to maintain total circulating cholesterol within its correct range. Besides 'reverse cholesterol transport', HDLs have various protective properties, including anti-inflammatory, antioxidative, anticoagulant, and antiaggregatory function that provide potentially protective mechanisms against CVD[72, 73].

Plasma lipoproteins and atherosclerosis

Progressive accumulation of cholesterol in the arterial wall causes atherosclerosis. A classic study reported in 1913 by Anitschkow and Chalatow demonstrated that cholesterol-fed rabbits showed atherosclerotic plaques in their arteries, which

indicated a causal role of cholesterol in atherosclerotic plaque development[74]. Subsequently, epidemiological studies confirmed that increased blood cholesterol concentrations accelerated the development of coronary heart disease[75].

As the major carrier of cholesterol, LDLs have been associated with the development of atherosclerosis. It has been demonstrated that the early lesions of atherosclerosis are composed of subendothelial accumulation of cholesterol-engorged macrophages, termed 'foam cells', which have a key role in atherogenesis[1]. The accumulation of LDL in the subendothelial matrix initiates an atherosclerosis process. LDL retention in the subendothelium of vessel wall seems to be related to the interactions between LDL constituent apolipoprotein B (ApoB) and matrix proteoglycans[76]. Besides LDL, lipoprotein (a), a particle resembling LDL but containing ApoB and apolipoprotein(a), can retain in the intima and promote atherosclerosis[77]. Furthermore, LDLs undergo modification, including oxidation, aggregation and other means, to induce a series of biological responses that result in atherosclerosis[78]. Aggregated LDL is a major LDL modification in atherosclerotic lesions, which delivers enormous amounts of cholesterol to macrophages and results in the formation of foam cells[79].

However, HDL is very protective against atherosclerosis. The primary mechanism underlying this protective effect is the role of HDL in reverse cholesterol transport (carrying excess cholesterol from foam cells to the liver)[10]. In addition, HDL possesses antioxidant, anti-inflammatory, and antithrombotic properties, which

contribute to protection against atherosclerosis. For example, HDL interferes with an initial process of atherogenesis by inhibiting LDL oxidation. Recently, besides lowering LDL cholesterol levels, raising HDL cholesterol has emerged as a potential strategy to treat atherosclerosis[71].

Adiponectin and atherosclerosis

Adiponectin, one of adipocytokines

Adipose tissue is now considered to be not only a passive energy store, but also an important endocrine organ. Indeed, adipose tissue produces a range of bioactive cytokines called "adipocytokines"[80]. Leptin, resistin, visfatin and adiponectin are adipocytokines. Research conducted over the past several years has revealed that adipocytokines can affect lipid metabolism[81] and that absence or excess of individual adipocytokines causes obesity, potentially leading to diabetes and cardiovascular disease[82]. Leptin is primarily involved in regulating food intake and energy expenditure. Recent studies showed that leptin exerts many potentially atherogenic effects and plays an important role in cardiovascular diseases[83]. It has been found that increased levels of plasma resistin were positively correlated with cardiovascular risk[84]. Visfatin, a adipocytokine secreted by visceral fat, mimics insulin activity by binding to the insulin receptor[85]. Compared with the aforementioned factors, adiponectin has exhibited different biological properties. Unlike leptin and resistin which are pro-inflammatory cytokines, adiponectin is considered to be an anti-inflammatory cytokine[86] and has profound protective effects in the pathogenesis of

atherosclerosis[87]. Thus, adiponectin is perhaps the most promising of the adipocytokines for the development of therapeutic strategies.

Adiponectin

Adiponectin was identified independently by four research groups in 1995 and 1996. Subsequently it was also termed as AdipoQ[88], Acrp30 (adipocyte complement-related protein 30)[89], apM1 (adipose most abundant gene transcript 1)[90], and GBP28 (gelatin-binding protein 28)[91]. Adiponectin is a 30 kDa adipocytokine hormone synthesized mainly by adipose tissue in several animal species. It is a 244 amino acid protein and consists of a N-terminal collagen domain that is responsible for building tertiary structure and a C-terminal globular domain that is important for mediating adiponectin effects[92]. Adiponectin exists as a full-length protein as well as a cleavage fragment, which is known as globular adiponectin and consists only of the C-terminal globular domain[93]. Full-length adiponectin can oligomerize via the N-terminal collagen domain to form a trimer (low-molecular-weight adiponectin), a hexamer (middle-molecular-weight adiponectin), and a high-molecular-weight adiponectin[94].

Adiponectin is mainly synthesized by adipocytes. Recent studies have found that it is also expressed in skeletal muscle cells[95], cardiomyocytes[96] and endothelial cells[97]. Adiponectin cDNA in the chicken is 65-68 % homologous to different mammalian adiponectin and is expressed at the highest levels in adipose tissue, followed by the liver and anterior pituitary[98]. In humans, adiponectin circulates at high concentrations in serum ranging from 5-10 mg per ml and accounts for about 0.01 % of total plasma

proteins[99] (compared with leptin circulating at a concentration of a few ng per ml). It has been reported that plasma adiponectin levels are influenced by different factors, such as age, gender and lifestyle. Indeed, women have higher plasma adiponectin levels than men[100]. Some dietary factors, such as soy protein and fish oils, also increase plasma adiponectin levels[101].

Adiponectin receptors

Two adiponectin receptors have recently been identified (AdipoR1 and AdipoR2). Both AdipoR1 and AdipoR2 are seven transmembrane receptors, in which the N-terminus is internal and the C-terminus is external. These receptors are structurally distinct from G-protein-coupled receptors[102]. AdipoR1 is widely expressed, particularly in skeletal muscle, whereas AdipoR2 is most abundantly expressed in the liver[102]. AdipoR1 and AdipoR2 have been found to be structurally conserved in several animals, including the chicken[103]. Moreover, two types of adiponectin receptors have been demonstrated to have different affinities for binding globular and full-length adiponectin. AdipoR1 has a higher affinity for the globular adiponectin whereas AdipoR2 mainly engages with full-length adiponectin[102]. Globular and full-length adiponectin can bind to both receptors and mediate the activation of 5' adenosine monophosphate-activated protein kinase (AMPK) [104,105] and the peroxisome proliferator activated receptor (PPARα)[106], thus exerting its biological functions. Different functions between AdipoR1 and AdipoR2 in adiponectin signal pathways have been reported. AdipoR1 may be associated with the activation of

AMPK pathways whereas AdipoR2 seems to be more tightly linked to activation of the PPARα pathway[107].

Adiponectin as a key mediator of diseases

Adiponectin is a hormone that is secreted predominantly by adipocytes. Adiponectin acts as a major anti-diabetic, anti-atherogenic and anti-inflammatory adipocytokine. Adiponectin has been shown to play crucial roles in regulating glucose and lipid metabolism[104, 108] as well as oxidative stress[109] and inflammation[110]. Recent research has revealed that low plasma adiponectin levels, known as hypoadiponectinaemia, are associated with various disease conditions.

Adiponectin in atherosclerosis and cardiovascular disease

Adiponectin acts as a major anti-atherogenic and anti-inflammatory adipocytokine. Several clinical reports indicated that low levels of adiponectin are associated with the development of cardiovascular disease[111, 112, 113]. It has been demonstrated that inflammation is an essential factor in the initiation and development of atherosclerosis[114]. As previously mentioned, adiponectin inhibits the secretion of inflammatory cytokines, such as TNF in the atherogenic process, thus suppressing the expression of TNF-induced adhesion molecules in endothelial cells[115]. Adiponectin also inhibits foam cell formation[116] and reduces proliferation and migration of smooth muscle cells[117]. Considering that atherosclerosis is an inflammatory disease[114], the C-reactive protein (CRP), a proinflammatory marker, is one of the most reliable biomarkers to assess cardiovascular risk[118]. It has been found recently that levels of

adiponectin in plasma and adipose tissue were negatively correlated with CRP concentrations in patients with coronary artery disease[119].

It has been hypothesized that adiponectin plays both direct and indirect roles in protection against cardiovascular disease[120]. There is increasing evidence that the protection effect of adiponectin is due to its involvement in regulating both lipid and carbohydrate metabolism. It is important to note that in the Women's Health Study, cholesterol was the strongest predictor for future cardiovascular events, including low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and total cholesterol[121]. Low levels of plasma adiponectin have been reported to be related to small dense LDL, high triglyceride, and apolipoprotein B (APOB) levels[122]. It has also been found that adiponectin directly acts on vascular endothelium and protects against cardiovascular disease in part because of the reduction of lipid accumulation in macrophages[123]. Furthermore, administration of adiponectin prevents atherosclerosis in apolipoprotein E (APOE)-deficient mice[124].

Adiponectin in obesity, insulin resistance and diabetes

It has been demonstrated that plasma adiponectin levels are reduced in obesity, insulin resistance, and type 2 diabetes[125]. Adiponectin expression in adipose tissue and circulating levels in plasma are decreased in several animal models of obesity[126]. Studies in different human populations, including Japanese and Asian Indians, have also shown that low plasma adiponectin levels are predictors for future development of diabetes and insulin resistance[127,128,129]. It has also been found that simultaneous

disruption of AdipoR1 and AdipoR2 reduced adiponectin-specific binding and adiponectin sensitivity. Conversely, the increasing expression of either AdipoR1 or AdipoR2 in the liver ameliorated insulin resistance and diabetes[107]. Administration of adiponectin induced glucose-lowering effects and ameliorated insulin resistance in mice[93,126,130], whereas adiponectin-deficient mice exhibited insulin resistant and diabetic[108,131]. It has been reported that the adiponectin effect in insulin sensitivity may be regulated by increasing fatty acid oxidation via activation of the AMPK[104, 105,132] and PPARα pathways[106,133]. It is well known that AMPK has a crucial role in regulating body weight, food intake, and glucose and lipid homeostasis, thereby controlling the systemic energy balance[132]. Moreover, AdipoR1 and AdipoR2, the major receptors for adiponectin, serve as key physiological regulators for the glucose-lowering effect of adiponectin[107].

Adiponectin in inflammation

In metabolic diseases, such as obesity and diabetes, inflammation in the systemic microcirculation is widely known to lead to organ damage and other chronic complications. Among many proteins secreted by adipocytes, adiponectin serves a unique role as an anti-inflammatory and anti-atherogenic factor. It has been demonstrated that adiponectin exerts various anti-inflammatory effects, which include inhibition of pro-inflammatory cytokine production, induction of anti-inflammatory factors, and reduction of the expression of adhesion molecules[134]. Adiponectin-deficient mice have higher expression levels of tumor necrosis factor (TNF; a pro-inflammatory cytokine) mRNA in adipose tissue and higher plasma TNF

concentrations than adiponectin-sufficient mice[131]. In addition, adiponectin inhibits nuclear factor-κB (NF-κB) activation in endothelial cells and influences the function of macrophages[135]. Besides inhibiting the production of pro-inflammatory cytokines, adiponectin also induces the production of several important anti-inflammatory cytokines, including IL-10 and IL-1 receptor antagonists, via human monocytes and macrophages[136].

In the model of MDV-induced atherosclerosis, Fabricant et al. reported that atherosclerotic lesions were observed in large coronary arteries and aortas of both normocholesterolemic (relatively poor diet in cholesterol) and hypercholesterolemic (2 % cholesterol supplement) chickens by infection with MDV whereas no arterial diseases developed in uninfected chickens fed either cholesterol-poor or cholesterol-supplemented diets[3, 5]. These results provided direct evidence suggesting herpesviruses as potential initiators of arterial injury. The same group subsequently found that MDV infection caused atherosclerosis in the chicken while those animals that were previously vaccinated and later challenged with MDV did not exhibit total aortic lipid accumulation[4]. These results suggested that MD resistance induced by vaccination could prevent the development of atherosclerosis caused by MDV infection. Two main strategies for resistance of MD have been demonstrated: vaccination and genetic resistance to MD[40]. It has been demonstrated that prior vaccination with the turkey herpesvirus could prevent atherosclerosis in MDV-infected chickens[4, 6]. Therefore, it is very interesting to explore the role of genetic resistance in the progression of herpesvirus-induced atherosclerosis. Furthermore, the

role of lipoprotein metabolism in atherosclerosis induced by MDV infection remains elusive although the role of cholesterol metabolism has been demonstrated for decades. The present study was conducted to compare the effects on lipoprotein metabolism induced by MDV infection between MD resistant and susceptible chicken lines. We hypothesized that lipoprotein metabolisms is different in distinct MD susceptible and resistant lines. To test this hypothesis, two experiments were designed 1) to determine whether differences exist in the background of lipoprotein metabolism between MD resistant and susceptible lines and 2) to assess whether the changes of lipoprotein levels in response to MDV infection differ in the MD resistant and susceptible lines. Insights from these studies could provide us a better understanding of the role of lipoprotein metabolism in herpesvirus induced atherosclerosis, thus facilitating the elucidation of the etiology and pathogenesis of human arteriosclerosis.

Chapter 2: Comparison of lipoprotein metabolism in MD resistant and susceptible chicken lines

Introduction

Atherosclerosis is one of the main human diseases involving cholesterol and lipoprotein metabolism[7, 8]. Although a cholesterol accumulation in artery walls is the defining characteristic of atherosclerosis[91], lipoproteins also play an important role in the development of the disease. It has been demonstrated that LDL is a risk factor for atherosclerosis[9], whereas HDL is a protective factor against atherosclerosis[10]. Raising HDL cholesterol and lowering LDL cholesterol have, thus, emerged as potential strategies to lower the risk of developing atherosclerosis[10]. MDV infection can induce atherosclerosis in chickens[3-5]. It has been reported that MDV infection induced aortic lipid accumulation, including cholesterol, cholesterol ester, triacylglycerol and phospholipids[4]. The lipid accretion in aortas of MDV infected chickens may have been a result of alterations in cholesterol metabolism[4, 6].

Lipoproteins, the key carrier of cholesterol through the circulating system, fundamentally modulate cholesterol metabolism. Thus, lipoprotein metabolism may play an important role in MDV- induced atherosclerosis. We hypothesized that MDV influences lipoprotein metabolism differently in MD susceptible and resistant lines. Therefore, we investigated whether the changes of lipoprotein levels induced by MDV differ in the MD resistant and susceptible lines. First we determined whether basal lipoprotein metabolism is different between MD resistant and susceptible lines. We examined plasma lipoprotein levels and analyzed the phenotype in MD resistant

and susceptible lines. We also determined the expression levels of adiponectin and its receptors, since adiponectin, as a protective marker against atherosclerosis, fundamentally influences lipid metabolism[81].

Experimental design

To investigate whether any differences in plasma lipoprotein metabolism between line 6_3 and line 7_2 exist, 2-month-old (growing period) and 15-month-old (mature period) chickens from each line were used for analyzing phenotype, determining plasma lipoprotein levels, and detecting mRNA and protein expression levels of adiponectin and its receptors.

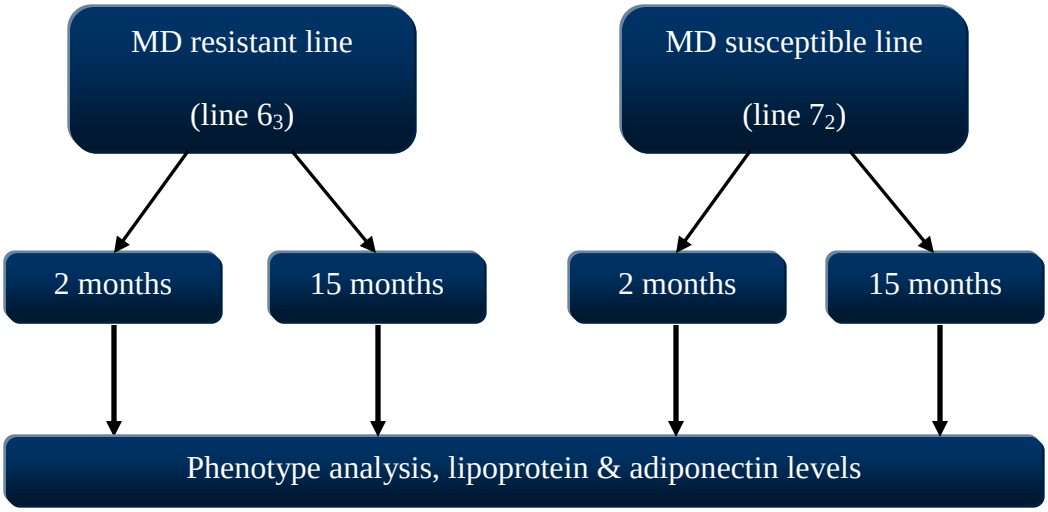


Figure 1. **Experimental design for determining the differences of lipoprotein metabolism between line 6_3 and 7_2.**

Methods and materials

Phenotype data

At the end of each study, animals were weighed and then sacrificed for blood and tissue collection. Tissue samples were weighed, and immediately frozen in liquid nitrogen and stored at -80 ℃. The weight percentage of each tissue was calculated by the equation:

$$\text{Tissue percentage} = \frac{\text{Tissue weight (g)}}{\text{Body weight (g)}} \times 100\%$$

Plasma HDL and LDL/VLDL cholesterol levels

Blood samples were drawn by cardiac puncture and placed in 10 ml tubes with EDTA. Plasma was separated from whole blood by centrifugation at 2000 X g for 20 min and kept at 4 ℃ until analysis. Plasma total cholesterol, HDL cholesterol and LDL cholesterol were measured using a HDL and LDL/VLDL cholesterol quantification kit (BioVision, Exton, PA).

Real-time quantitative PCR

Total RNA was extracted from abdominal fat and liver by using RNeasy mini kit (Qiagen Inc., Valencia, CA) following the manufacture's protocol. Total RNA was quantified according to UV absorbance (260/280 nm) using a spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Wilmington, DE). Complementary DNA (cDNA) was synthesized by reverse transcribing 800ng total RNA using an oligo-dT (12-18) primer and Superscript II reverse transcriptase (Invitrogen). Primers used to

amplify adiponectin, adipoR1, adipoR2[137] and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) were described as follows:

Adiponectin: Forward 5'-ACAGGTGCAGAAGGACCGAGAGGATT-3'

Reverse 5'-AAGACAGAGCCGCTTGCTTGGTCAAC-3'

AdipoR1: Forward 5'-GAATACACACCGAGACGGGCAACATCT-3'

Reverse 5'-GCCCAAGACGCAGACAATGGAGAGGTA-3'

AdipoR2: Forward 5'-GAGACTGGCAACATCTGGACGCATCTTC-3'

Reverse 5'-TGCGATGCCCAGGACACAAATCACAAT-3'

GAPDH: Forward 5'-TGACTTCAATGGTGACAGCC-3'

Reverse 5'-ACTCCTTGGATGCCATGTGG-3'

The products of the reverse transcription reaction were diluted (5 X) with ddH_2O, and 1 μl was used as a template in real-time polymerase chain reaction (RT-PCR). The RT-PCR was performed using a SYBR Green RT-PCR kit (Qiagen Inc.). The PCR reaction was carried out using the following program: 95 ℃ for 15 min, 40 cycles of 94 ℃ for 15 s, 57 ℃ for 30s and 70 ℃ for 30s. For a negative control, water was used as a template in place of single strand cDNA during RT-PCR. The quantitative PCR (qPCR) output provided the log-linear threshold values (C_T) for the threshold cycle. Samples from each animal were measured in duplicate to obtain average C_T values for each mRNA. Values were transformed to a ΔCt value by normalizing gene expression to the housekeeping gene GAPDH using the following equation: ΔCt = (Average Ct-target – Average Ct-GAPDH). The ΔΔCt value was then calculated by subtracting the sample Ct value from the sample with the highest expression level for controlling amplification efficiency. Results were then converted from log-linear to

linear terms by the function: $2^{-\Delta Ct}$. The relative mRNA expression of adiponectin, adipoR1 or adipoR2 in various groups was compared.

Western blot

Protein extraction

Protein extracts from chicken abdominal fat and liver were prepared as described previously[138], with slight modifications. Briefly, 0.2-0.3g of abdominal fat and liver were homogenized using a pestle in 2 ml lysis buffer (10mM Tris-HCl, 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Sigma). The homogenate was shaken in a thermomixer at 1000 rpm for 2 hour at 4 ℃, and the lysates were then centrifuged at 14,000 X g for 20 min at 4 ℃. The supernatant was collected, and total protein concentration was measured using a Victor 1420 multilable counter (PerkinElmer). Chicken plasma was used directly for western blot analysis to determine protein levels of adiponectin in the plasma.

Western blot

Protein samples were prepared for electrophoresis by heating with SDS-PAGE Laemmli buffer (50mM Tris-HCl, 10% glycerol, 2% SDS, 0.05% bromophenol blue, pH 6.8) containing 10% β-mercaptoethanol (Promega, Madison, WI) for 10 min at 100 ℃. Proteins were separated by SDS-PAGE in 10% (w/v) polyacrylamide gels[139] at 120v for 90 min. After separation by SDS-PAGE, proteins were transferred onto a PVDF membrane. The membranes were blocked with SuperBlock T20 blocking buffer (Pierce, Rockford, IL) for 1 h at room temperature. The membranes were then

incubated with primary antibodies (rabbit anti-chicken adiponectin, adipoR1 and adipoR2 from Dr. Ramesh Ramachandran) at 4 ℃ for 24 h. The antibodies were diluted with SuperBlock T20 (adiponectin in plasma and abdominal fat (1:40,000); adipoR1 and adipoR2 in liver (1:2,000) and abdominal fat (1:500)). The membranes were washed three times with 1 X Tris-Buffered Saline Tween-20 (TBST) for 5 min each and then incubated with secondary antibody donkey anti-rabbit IgG (Santa Cruz Biotechnology) (diluted by 1:5,000) for 1 h at room temperature. The membranes were then incubated with ECL chemiluminescense detection reagent (GE Healthcare, Piscataway, NJ).

Chemiluminescent signal was collected using a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA). The protein bands were quantified using Quantity One software (Bio-Rad). The molecular weights of proteins were estimated using Pageruler plus prestained protein ladder (Fermentas international Inc., Canada).

Stripping and reprobing β-actin

Primary and secondary antibodies were removed from the membranes by incubating with Western Blot Stripping Buffer (20 ml 10% SDS, 12.5 ml Tris-HCl, 0.8 ml β-mercaptoethanol, 67.5 ml ddH2O, pH 6.8) for 40 min at 50 ℃. The membranes were blocked with 5% nonfat dried milk for 1 h and then incubated with anti-β-actin antibody (Santa Cruz Biotechnology) (1:1,000 diluted with 1 X TBST) at 4 ℃ for 24 h. The membranes were incubated with donkey anti-mouse IgG (Santa Cruz Biotechnology) (1:10,000 diluted with 1 X TBST) for 1 h at room temperature. The expression of β-actin was detected in the same manner as above.

Statistical analysis

The data were analyzed with a two-way ANOVA followed by Tukey's test for comparison among different groups using Statistical Analysis System v.9.1 (SAS Institute, Cary, NC). Values shown are means ± S.D (Standard deviation).

The statistical model for 2 month- and 15 month-old chicken was:

$$Y = \mu + Li + Aj + Li*Aj + \varepsilon_{i,\,j,\,k}$$

The line, age and their interaction are treated as fixed factors in the model.

μ: grand mean;

Li: the effect of the ith line (6_3 or 7_2) on Y;

Aj: the effect of the jth age (2 months or 15 months) on Y;

$\varepsilon_{i,\,j,\,k}$: random error.

Results

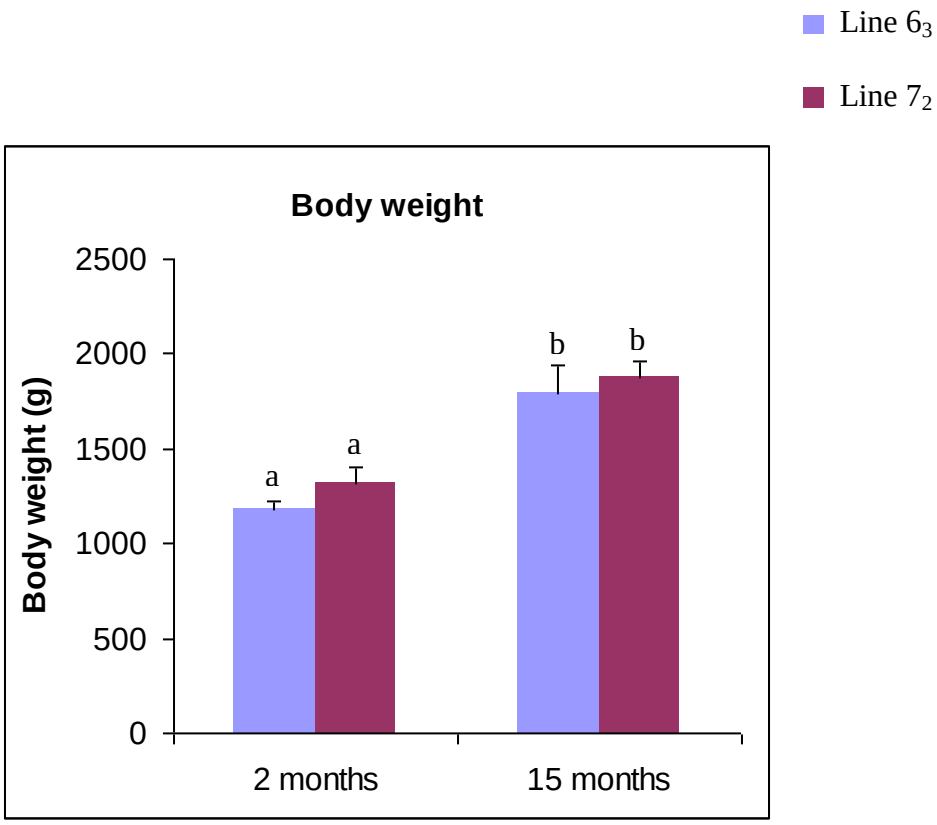
Phenotype analysis for 2 month- and 15 month-old chickens from line 6_3 and 7_2

To investigate whether there were differences in body weight and tissue weight percentage between line 6_3 ($L6_3$) and line 7_2 ($L7_2$), abdominal fat, breast muscle with bone, leg muscle with bone, liver, heart and spleen samples were collected from chickens at 2 months and 15 months of age. The statistical model used to analyze these phenotypes was $Y = \mu + Li + Aj + Li*Aj + \varepsilon_{i, j, k}$; including line, age and the interaction between line and age, all as fixed effects. The interactions between line and age were found to be non-significant for body weight, breast muscle with bone, leg muscle with bone and heart. The body weights were not statistically different between $L6_3$ and $L7_2$ in the two age chickens. However, an obvious increase in body weight was observed in 15 month-old chickens as compared to 2 month-old chickens in $L6_3$ (1788.11 $\pm$36.26 vs. 1184.75 $\pm$54.39 g) and $L7_2$ (1880.67 $\pm$36.26 vs. 1319.00 $\pm$54.39 g) (Figure 2a). We also observed a lower percentage of leg muscle with bone in $L7_2$ in compared to $L6_3$ in 15 month-old chickens ($p < 0.01$) (Figure 2d). $L7_2$ also had significantly higher heart percentage at 2 months and 15 months of age compared to $L6_3$ ($p < 0.001$) (Figure 2f). However, no difference between the lines was apparent for the percentage of breast muscle with bone (Figure 2c).

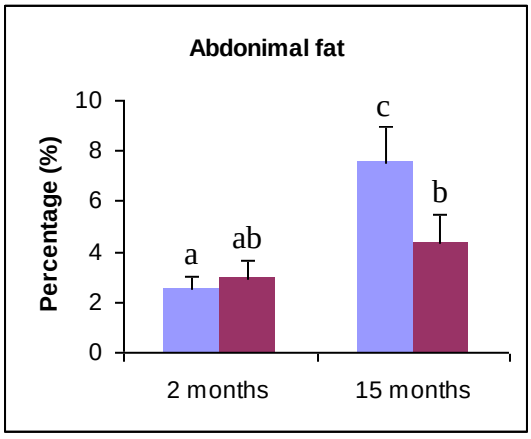
The interactions between line and age were significant for abdominal fat ($p<0.001$), liver ($p<0.001$) and spleen ($p<0.05$), so data for the three phenotypes were analyzed within each age. The abdominal fat percentage was not different between $L6_3$ and $L7_2$ at 2 months of age (2.51 $\pm$0.55 vs. 2.97 $\pm$0.55 %), but $L6_3$ had significantly higher

abdominal fat percentage than $L7_2$ (7.58 $\pm$0.37 vs. 4.36 $\pm$0.37 %, $p < 0.001$) (Figure 2b) at 15 months of age. $L7_2$ also had significantly higher liver weight percentage at 15 months of age and higher spleen weight percentage at 2 months of age compared to $L6_3$ ($p < 0.001$) (Figure 2e, 2g).

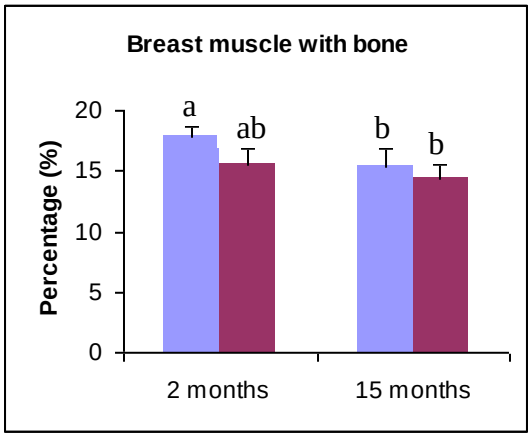
a


Line 6₃
Line 7₂
Body weight
Body weight (g)
2500
2000
1500
1000
500
0
a
a
b
b
2 months
15 months

b


Abdonimal fat
Percentage (%)
10
8
6
4
2
0
a
ab
c
b
2 months
15 months

c


Breast muscle with bone
Percentage (%)
20
15
10
5
0
a
ab
b
b
2 months
15 months

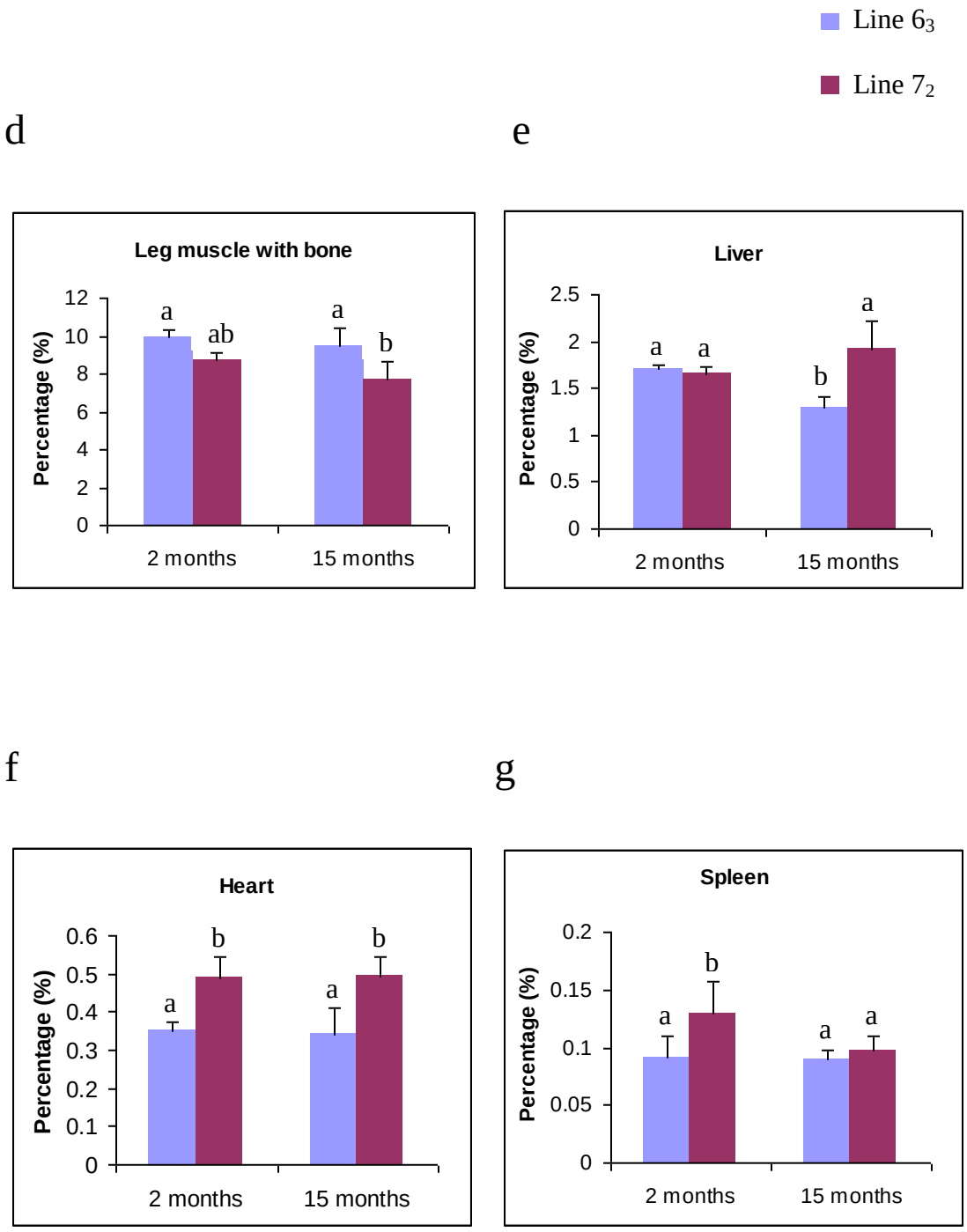

Line 6_3
Line 7_2
d
e
Leg muscle with bone
Percentage (%)
12
10
8
6
4
2
0
a
ab
a
b
2 months
15 months
Liver
Percentage (%)
2.5
2
1.5
1
0.5
0
a
a
b
a
2 months
15 months
f
g
Heart
Percentage (%)
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
a
b
2 months
15 months
Spleen
Percentage (%)
0.2
0.15
0.1
0.05
0
a
b
a
a
2 months
15 months

Figure 2. **Phenotype data for 2 month- and 15 month-old chickens.**

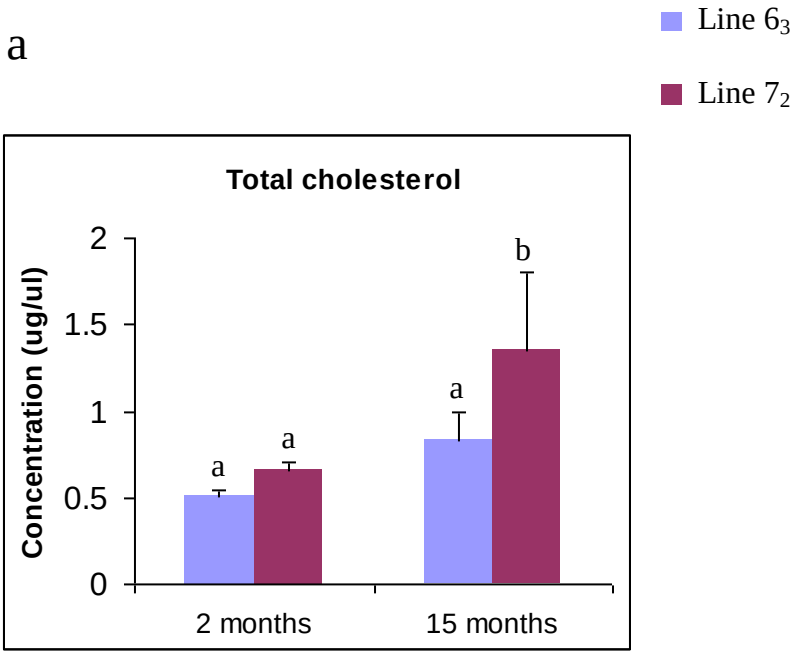
Body and tissue weight were measured for 2 month-old (n = 4) and 15 month-old (n = 9) chickens in line 6_3 (blue bar), and for 2 month-old (n = 4) and 15 month-old (n = 9) chickens in line 7_2 (purple bar). Tissue percentages were calculated by ((Tissue weight) / (Body weight)) *100%. (a), Body weight (g); (b), Abdominal fat percentage (%); (c), The percentage of breast muscle with bone (%); (d), The percentage of leg muscle with bone (%); (e), Liver percentage (%); (f), Heart percentage (%); (g), Spleen percentage (%). Values were shown as means ± S.D. (means ± standard deviation). Letters identify differences among groups; thus, two groups not sharing lowercase letters are significantly different (two-way ANOVA, Tukey's test, $p<0.05$).

Plasma lipoprotein levels for line 6_3 and line 7_2 at 2 months and 15 months of age

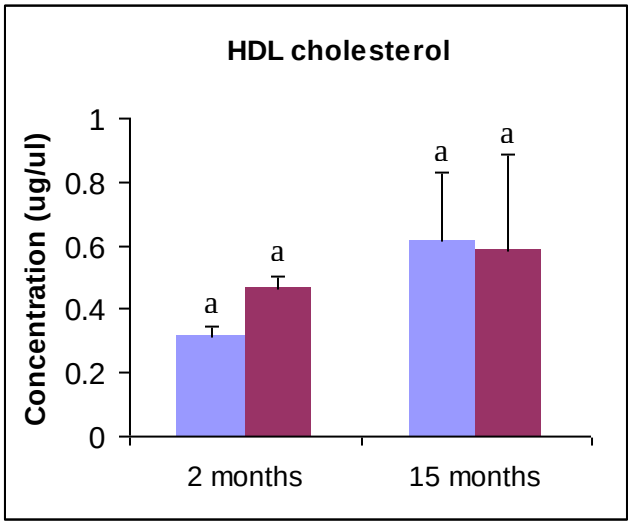
To determine whether there was a difference in lipoprotein metabolism between $L6_3$ and $L7_2$, plasma lipoprotein concentrations were measured in 2 month- and 15 month-old chickens (Figure 3). Results of total cholesterol revealed that $L6_3$ contained total cholesterol 0.50 ± 0.11 μg/μl at 2 months of age and 0.84 ± 0.09 μg/μl at 15 months of age, while $L7_2$ contained total cholesterol 0.66 ± 0.14 μg/μl and 1.36 ± 0.09 μg/μl, respectively (Figure 3a). With respect to individual lipoprotein classes, HDL cholesterol had a modest increase in both chicken lines during growth, but there were no significant differences between the lines ($p > 0.05$) (figure 3b). As for the (LDL+VLDL) cholesterol levels, the interaction between line and age was significant ($p<0.001$). We observed that (LDL+VLDL) cholesterol levels in $L6_3$ and $L7_2$ were not significantly different ($p > 0.05$) at 2 months of age (Figure 3c). However, at 15 months of age, $L7_2$ had 247% more (LDL+VLDL) than $L6_3$ ($p < 0.001$) (Figure 3c). Moreover, significant interactions between line and age were also observed in HDL ratio ($p<0.01$) and (LDL+VLDL) ratio ($p<0.01$).

Results of HDL ratio revealed no significant difference between 2 month- and 15 month-old chickens in $L6_3$ (0.62 ± 0.059 vs. 0.74 ± 0.048, $p > 0.05$), whereas there was a significant decrease in 15 month-old chickens (0.42 ± 0.05) compared to 2 month-old chickens (0.70 ± 0.07) in $L7_2$ ($p < 0.05$) (Figure 3d). Correspondingly, a significant increase in (LDL+VLDL) ratio was observed in 15 month-old chickens compared to 2 month-old chickens in $L7_2$ ($p < 0.05$).

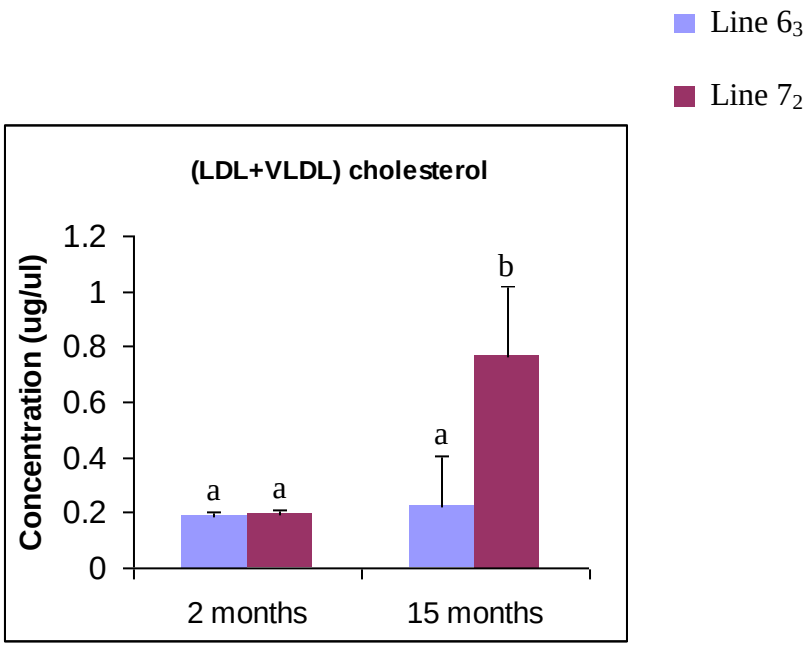
a


Line 6_3
Line 7_2
Total cholesterol
Concentration (ug/ul)
2
1.5
1
0.5
0
a
a
a
b
2 months
15 months

b


HDL cholesterol
Concentration (ug/ul)
1
0.8
0.6
0.4
0.2
0
a
a
a
a
2 months
15 months

c


Line 6₃
Line 7₂
(LDL+VLDL) cholesterol
Concentration (ug/ul)
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
b
2 months
15 months

d

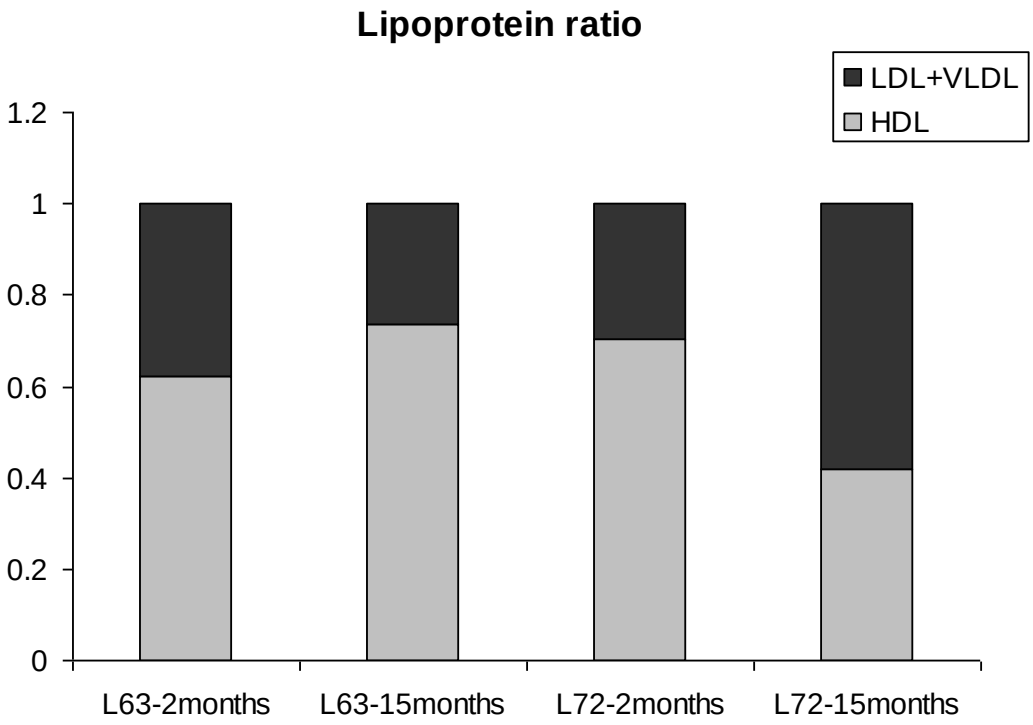

Lipoprotein ratio
LDL+VLDL
HDL
1.2
1
0.8
0.6
0.4
0.2
0
L63-2months
L63-15months
L72-2months
L72-15months

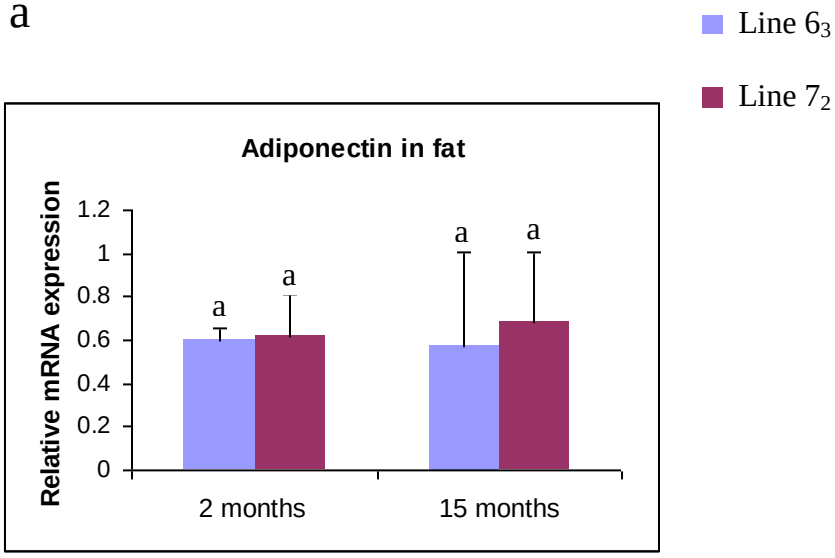
Figure 3. **Plasma lipoprotein levels for 2 month- and 15 month-old chickens.**

Lipoprotein concentrations were measured for 2 month-old (n = 6) and 15 month-old (n = 9) chickens in line 6_3 (blue bar), and for 2 month-old (n = 4) and 15 month-old (n = 9) chickens in line 7_2 (purple bar). (a), Total cholesterol (μg/μl); (b), HDL cholesterol (μg/μl); (c), LDL+VLDL cholesterol (μg/μl); (d), Lipoprotein ratio. In (a), (b), and (c), values were shown as means ± S.D. Letters identify differences among groups; thus, two groups not sharing lowercase letters are significantly different (two-way ANOVA, Tukey's test, $p<0.05$).

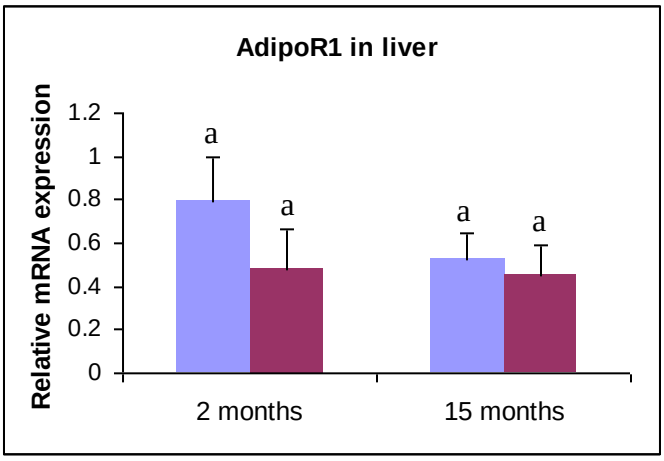
Adiponectin, adipoR1 and adipoR2 gene expression

Adiponectin has been associated with favorable effects on metabolism (i.e. reduced visceral adipose mass, decreased plasma LDL cholesterol, and increased HDL cholesterol)[140]. The biological effects of adiponectin are mainly mediated by two receptors (adipoR1 and adipoR2) [87]. As our biochemical experiments of plasma lipoprotein levels suggested that total cholesterol and LDL cholesterol levels were different between the two lines, we further tested whether the levels of adiponectin, adipoR1 and adipoR2 were different in $L6_3$ and $L7_2$. Using RT-PCR, we found no significant difference in adiponectin mRNA levels in abdominal fat among the four groups ($L6_3$-2months, $L7_2$-2months, $L6_3$-15months, $L7_2$-15months) (Figure 4a). AdipoR1 mRNA quantities in liver and abdominal fat were slightly lower in $L7_2$ compared with that in $L6_3$ (Figure 4b, 4c). Furthermore, adipoR2 mRNA quantities in liver and abdominal fat were not significantly different ($p > 0.05$) between $L6_3$ and $L7_2$ (Figure 4d, 4e).

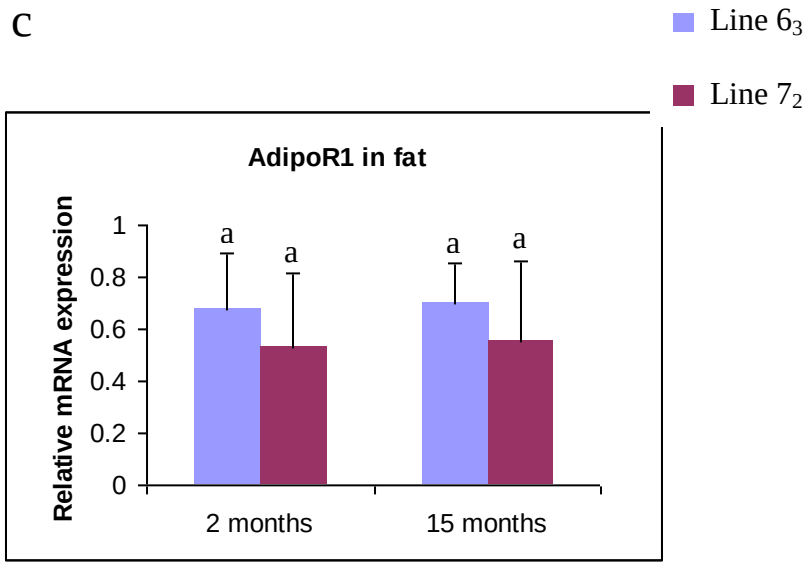
a


Line 6$_3$
Line 7$_2$
Adiponectin in fat
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
a
2 months
15 months

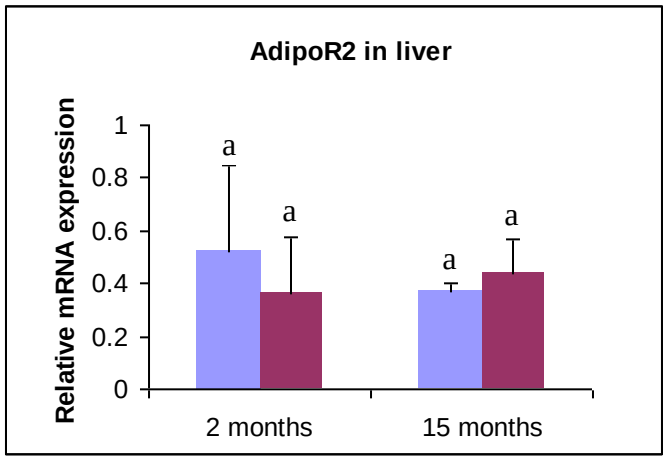
b


AdipoR1 in liver
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
a
2 months
15 months

c


Line 6₃
Line 7₂
AdipoR1 in fat
Relative mRNA expression
1
0.8
0.6
0.4
0.2
0
a
a
a
a
2 months
15 months

d


AdipoR2 in liver
Relative mRNA expression
1
0.8
0.6
0.4
0.2
0
a
a
a
a
2 months
15 months

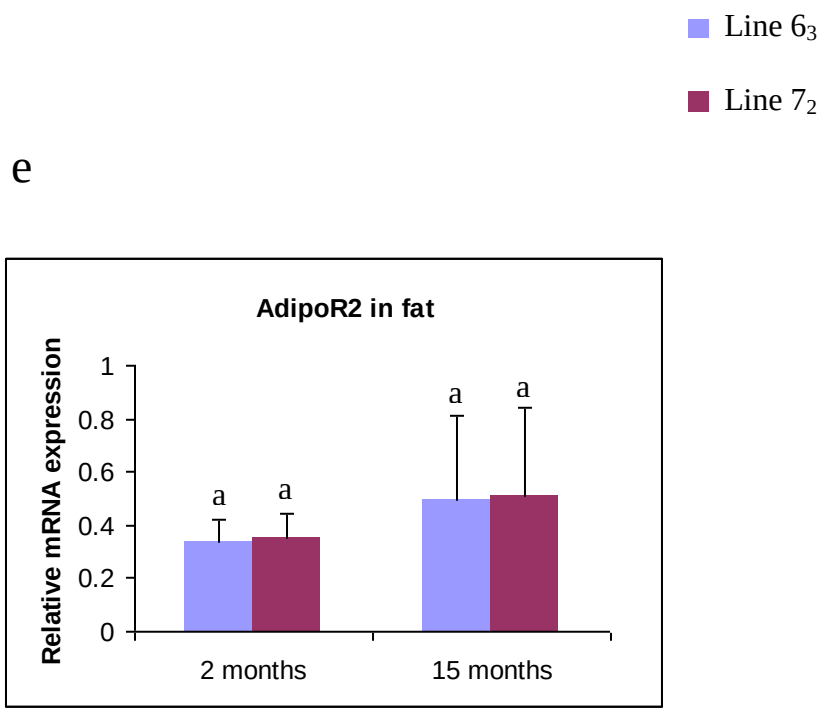


Figure 4. **mRNA levels of adiponectin, adipoR1 and adipoR2 by qPCR for line 6_3 (blue bar) and 7_2 (purple bar) at 2 months and 15 months of age (n=4).**

(a), Adiponectin in abdominal fat; (b), AdipoR1 in liver; (c), AdipoR1 in abdominal fat; (d), AdipoR2 in liver; (e), AdipoR2 in abdominal fat. GAPDH mRNA expression was used for normalization. Values were shown as means ± S.D. Letters identify differences among groups; thus, two groups not sharing lowercase letters are significantly different (two-way ANOVA, Tukey's test, $p<0.05$).

Adiponectin, adipoR1 and adipoR2 protein expression

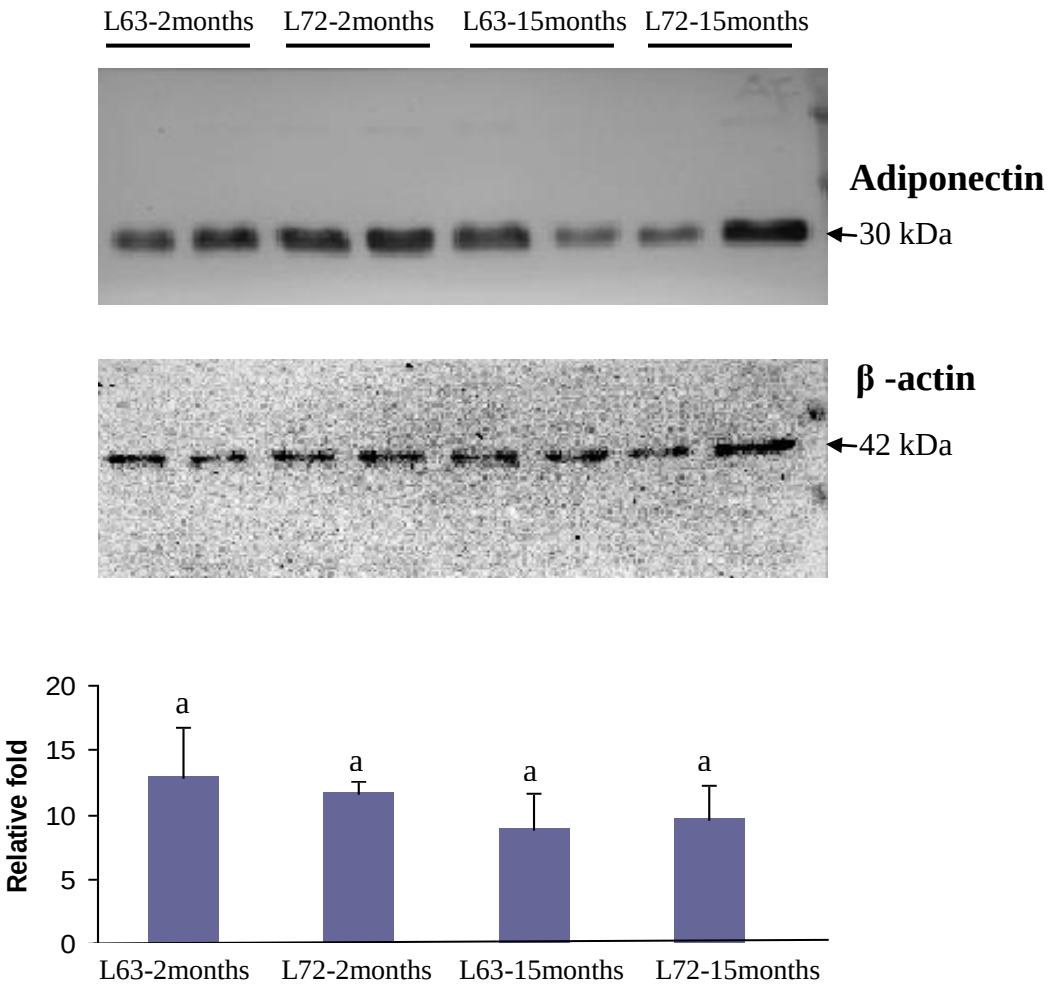
In light of the role identified for adiponectin in regulating plasma lipoprotein levels, we next examined protein expression levels for adiponectin and its receptors in $L6_3$ and $L7_2$ using western blots (Figure 5). Duplicates from each group were conducted in western blots. Lane 1 and 2 were designed for $L6_3$-2months; Lane 3 and 4 for $L7_2$-2months; Lane 5 and 6 for $L6_3$-15months; Lane 7 and 8 for $L7_2$-15months. Detection of β-actin in the same blot was used for normalization. Results were quantified by calculating the ratio of the density of the protein band and β-actin band. The results showed that adiponectin levels in abdominal fat were not significantly different among the four groups ($p > 0.05$) (Figure 5a), indicating that $L6_3$ and $L7_2$ may have synthesized a similar amount of adiponectin as adiponectin is primarily synthesized in abdominal fat[87]. However, there was a significant decrease in the level of adiponectin in plasma in $L7_2$ (5.91 ± 0.58) compared to that in $L6_3$ (11.41 ± 0.58) at 2 months of age ($p < 0.01$, Figure 5b).

Moreover, our results revealed that $L6_3$-2 month-old chickens had the highest level of adiponectin, followed by $L7_2$-2 months, $L6_3$-15months, and $L7_2$-15months. Interestingly, this trend seems to be inversely correlated to the change of total cholesterol concentration within four groups, which showed the highest level in $L7_2$-15months, followed by $L6_3$-15months, $L7_2$-2months, and $L6_3$-2months (Figure 3b). In order to determine the negative correlation, plasma adiponectin levels for four chickens from each group were detected. We then analyzed the correlation coefficient between plasma adiponectin levels and total cholesterol concentrations (Figure 3g).

The statistical analysis showed that correlation coefficient (r) was -0.800 when an outlier (for which total cholesterol concentration is 1.62 ug/ul) was removed from the data set. These results are consistent with others' report that plasma adiponectin levels is negatively correlated with total cholesterol[141].

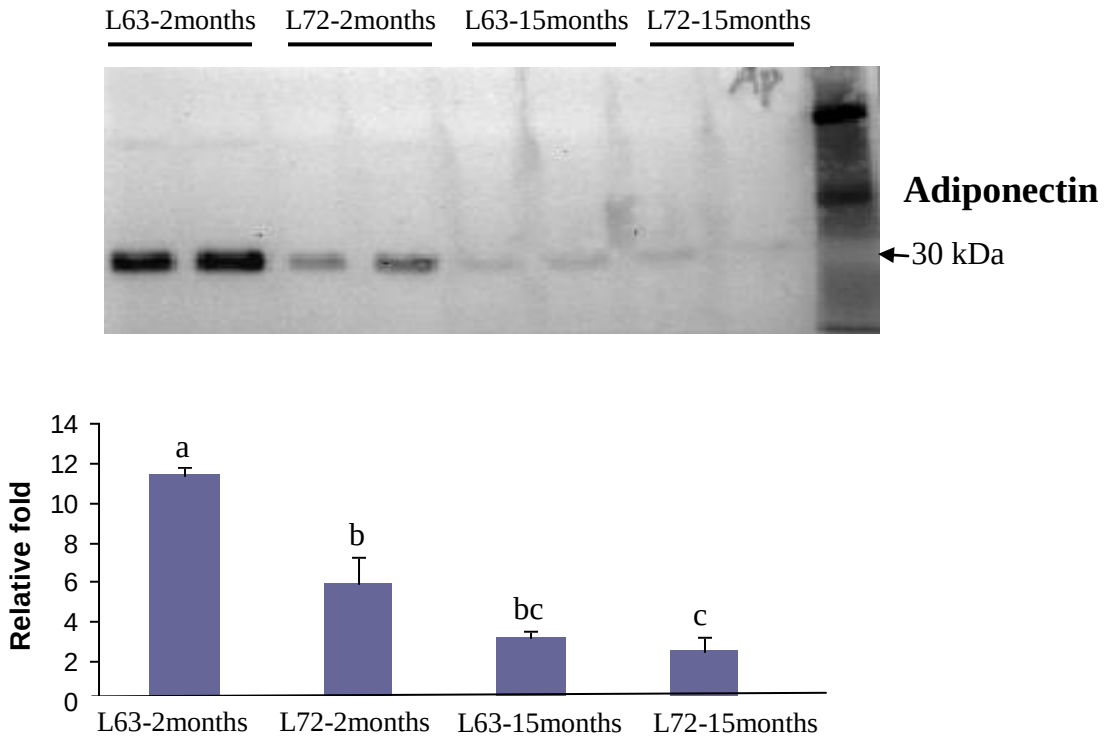
We also examined protein expression levels of adipoR1 and adipoR2 in liver and abdominal fat. Quantitative results revealed that there were no differences in adipoR1 in the liver between the two lines at 2 months and 15 months of age ($p > 0.05$, Figure 5c). It should be noted that no remarkable differences were observed between $L6_3$-2months and $L6_3$-15months groups, whereas a significant decrease in adipoR1 levels in the liver was detected in $L7_2$-15months (4.06 ± 0.64) compared to $L7_2$-2months (7.49 ± 0.64) ($p < 0.05$, Figure 5c). Quantitative results of adipoR1 in abdominal fat revealed no differences between the two lines in 2 month-old chickens ($p > 0.05$, Figure 5d). However, at 15 months of age, $L7_2$ had a significantly higher level of adipoR1 (6.21 ± 0.39) compared to $L6_3$ (3.54 ± 0.39) ($p < 0.05$, Figure 5d). Interestingly, $L7_2$-15months showed the lower level of adipoR1 in liver and the higher level of adipoR1 in abdominal fat. No significant differences in adipoR2 expression levels were observed among the four groups in both tissues (Figure 5e, 5f).

a



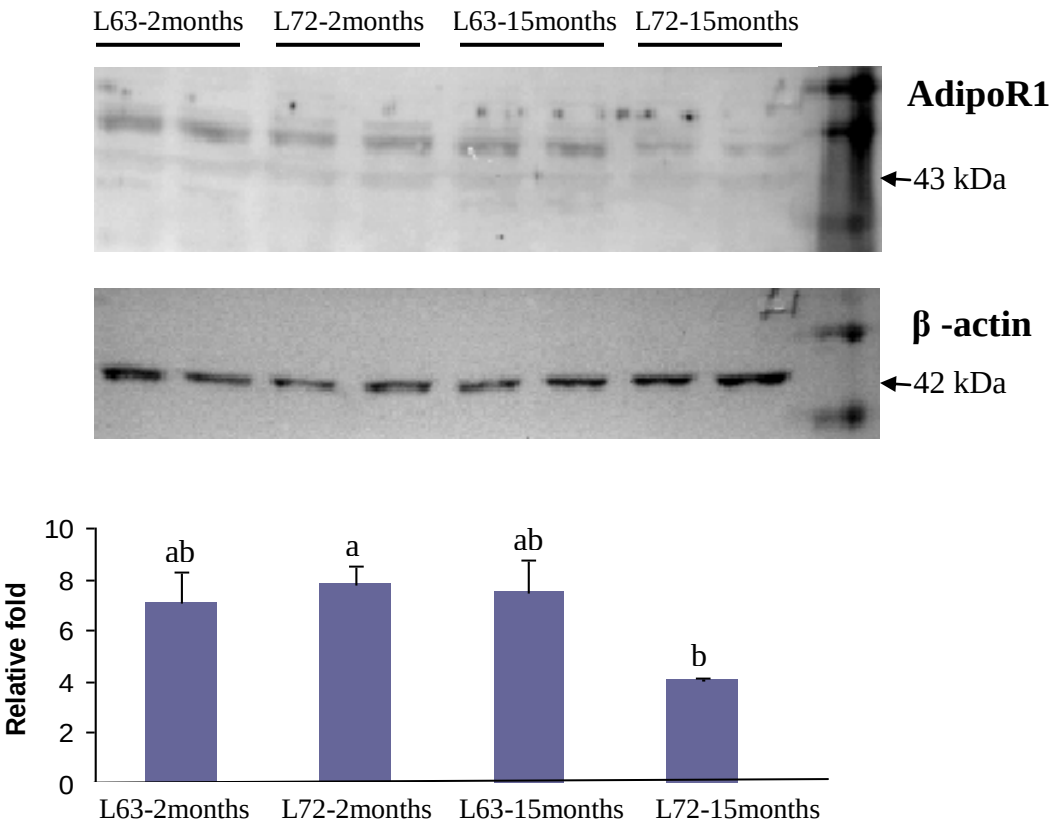
Adiponectin protein levels in abdominal fat

b



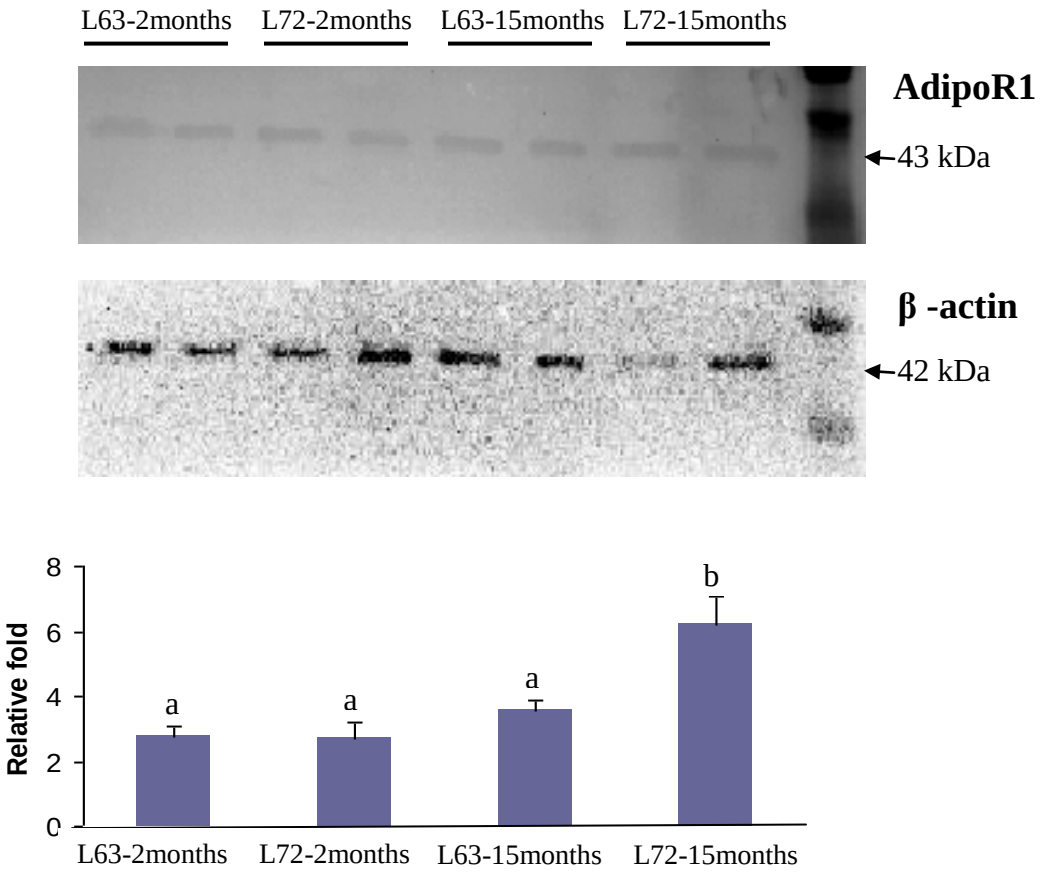
Adiponectin protein levels in plasma

C



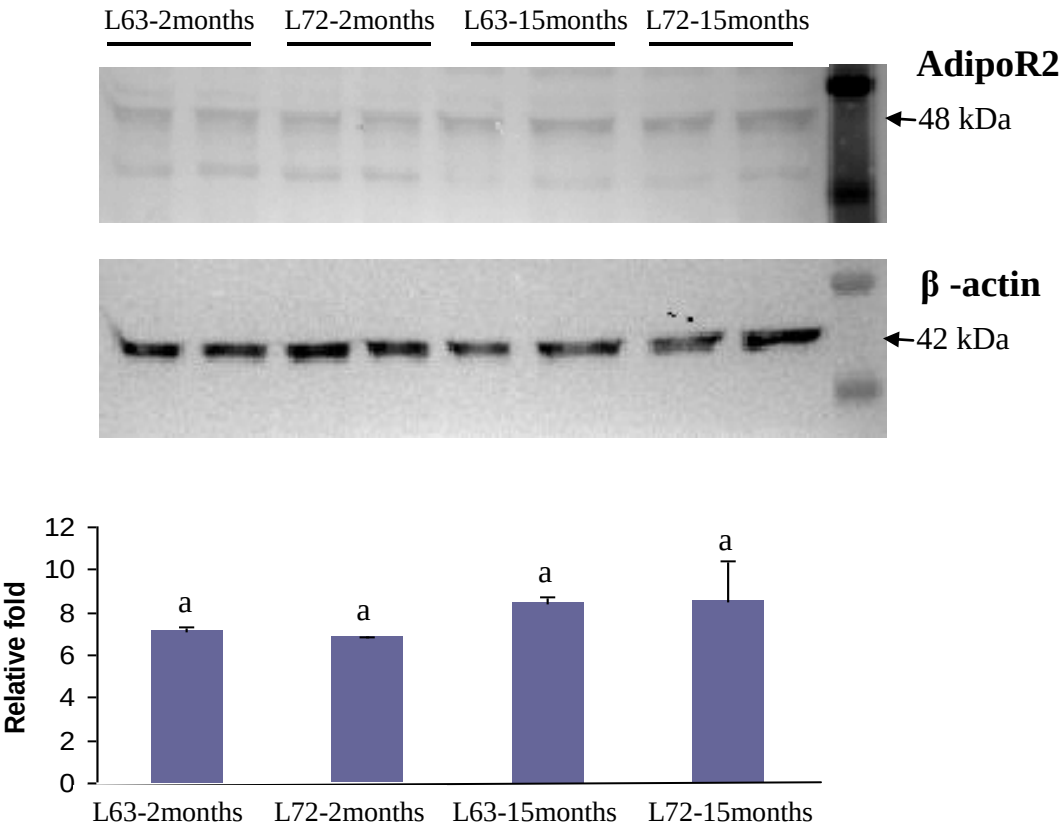
AdipoR1 protein levels in liver

d



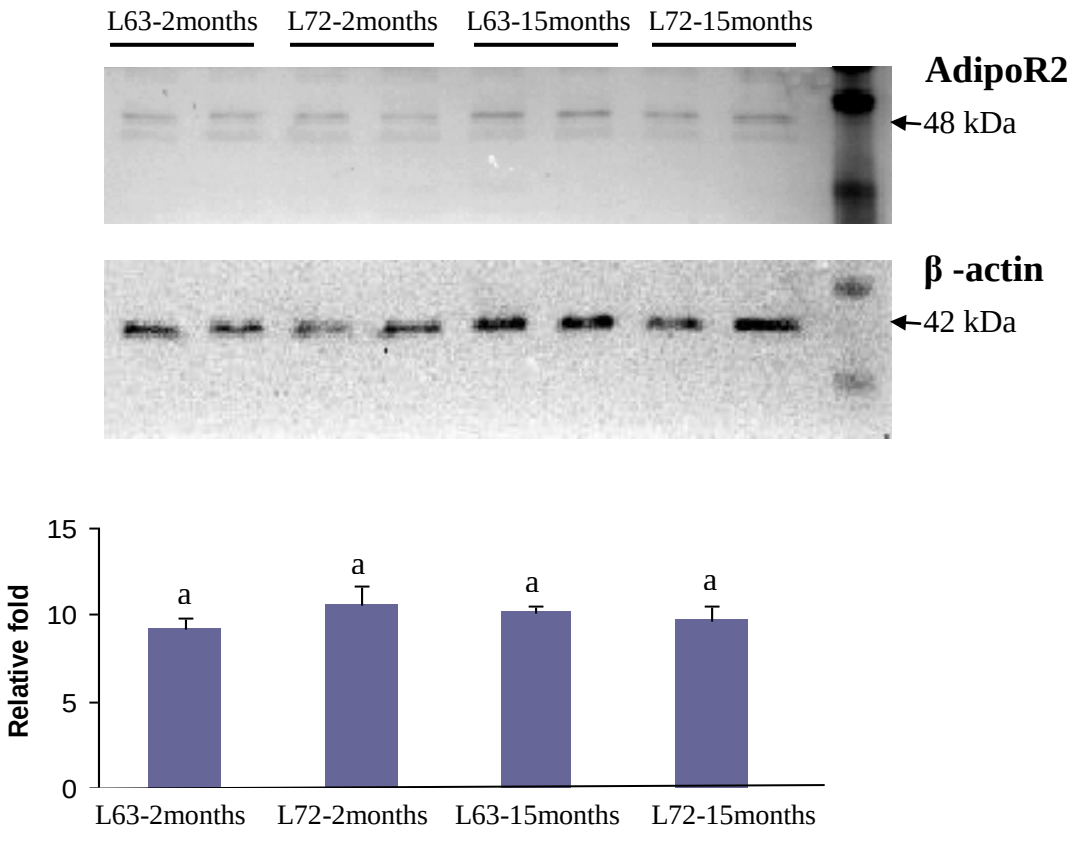
AdipoR1 protein levels in abdominal fat

e



AdipoR2 protein levels in liver

f



AdipoR2 protein levels in abdominal fat

g

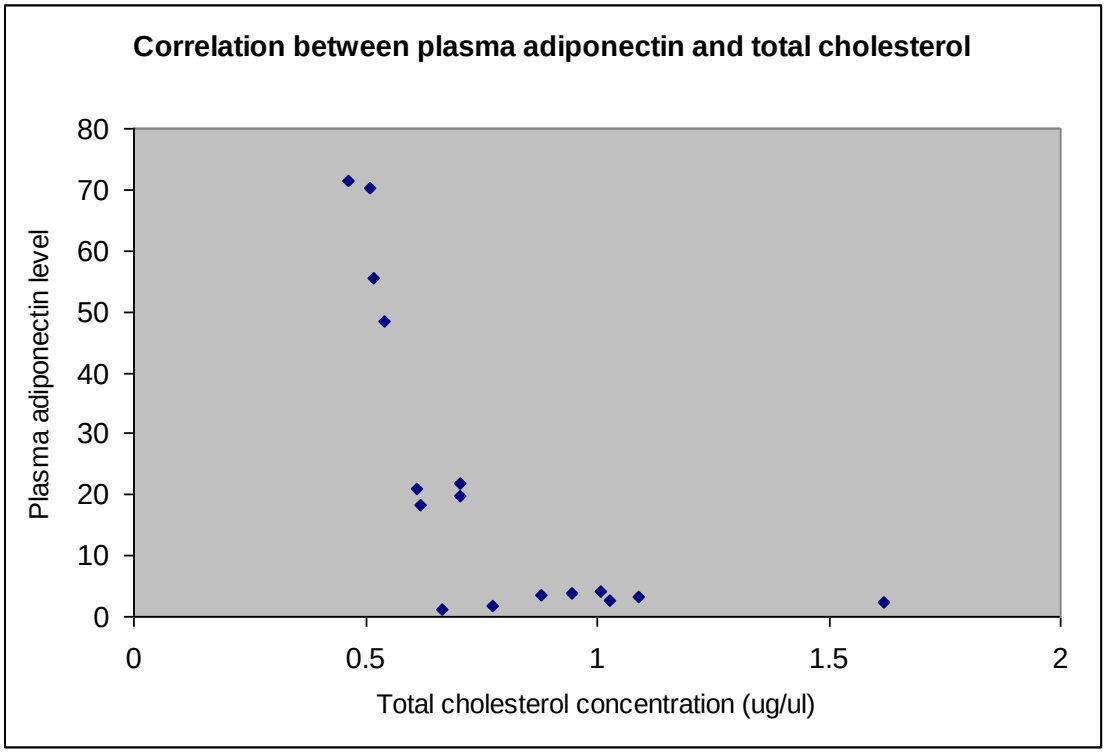

Correlation between plasma adiponectin and total cholesterol
Plasma adiponectin level
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
Total cholesterol concentration (ug/ul)

Figure 5. A**diponectin, adipoR1 and adipoR2 protein levels in 2 month- and 15 month-old chickens from line 6_3 and line 7_2.**

(a), Adiponectin in abdominal fat; (b), Adiponectin in plasma; (c), AdipoR1 in liver; (d), AdipoR1 in abdominal fat; (e), AdipoR2 in liver; (f), AdipoR2 in abdominal fat. For lane 1, 2, 3, 4, 5, 6, 7 and 8, every two lanes represent protein samples of two birds f one of the four groups (line 6_3 2 months of age, line 7_2 2 months of age, line 6_3 15 months of age, line 7_2 15 months of age), respectively. Detection of β-actin in the same blot was used for normalization. The level of protein expression is presented as relative fold in comparison with β-actin levels. With respect to chicken plasma, the same volume of plasma (1 μl) was used in each sample, and quantitative results were calculated by dividing the density of the protein band by 50. Values were shown as means ± S.D. Letters identify differences among groups; thus, two groups not sharing lowercase letters are significantly different (two-way ANOVA, Tukey's test, $p<0.05$). (g), Correlation analysis between plasma adiponectin levels and total cholesterol concentration for $L6_3$-2month, $L7_2$-2month, $L6_3$-15month and $L7_2$-15month groups (n=4).

Summary

The above experiments were designed to study the baseline lipoprotein metabolism in MD susceptible and resistant lines. Our results revealed no significant differences in HDL cholesterol between the two lines. However, (LDL+VLDL) levels were more than three-fold greater in $L7_2$ than in $L6_3$ at 15 months of age. During chicken growth from 2 months to 15 months of age, total cholesterol was only slightly increased in the MD resistant line ($L6_3$), but it was markedly enhanced in the MD susceptible line ($L7_2$). Notably, the increase in total cholesterol in the resistant line mainly resulted from an increase in HDL, whereas the increased total cholesterol in the MD susceptible line was due mostly to the elevation of (LDL+VLDL).

These findings led us to investigate possible mechanisms that are responsible for the changes in lipoprotein content during growth between MD susceptible and resistant lines. Adiponectin, a protective marker against atherosclerosis, is known to fundamentally influence lipoprotein metabolism[81]. Thus, we next studied whether or not the adiponectin pathway is responsible for the different lipoprotein changes between the two lines. Our results revealed no significant differences in the adiponectin levels synthesized from abdominal fat. However, $L7_2$ had significantly lower circulating levels of adiponectin compared to $L6_3$ in both 2 month- and 15 month-old chickens. Furthermore, the MD susceptible line at 15 months of age, with significantly higher level of LDL cholesterol, possessed a modestly lower level of adipoR1 in liver and an obviously higher level of adipoR1 in abdominal fat compared

to the other three groups. There were no significant differences in adipoR2 expression levels between $L6_3$ and $L7_2$ in both liver and abdominal fat.

We also studied the phenotype of MD susceptible and resistant lines, including body weight, the weight percentage of abdominal fat, breast muscle with bone, leg muscle with bone, liver, heart and spleen. The phenotype analysis revealed no significant difference in body weight between $L6_3$ and $L7_2$. However, $L7_2$ had a much lower percentage of abdominal fat and leg muscle with bone than $L6_3$ at 15 months of age. Conversely, $L7_2$ had a significantly higher percentage of liver weight at 15 months of age, of spleen weight at 2 months of age, and of heart weight in both ages compared to $L6_3$. There were no significant differences in the weight percentage of breast muscle with bone between the two lines. Taken together, our results demonstrated that the baseline lipoprotein metabolism differs in MD susceptible and resistant lines.

Chapter 3: lipoprotein levels in response to MDV infection in MD resistant and susceptible lines

Introduction

MDV infection can induce atherosclerosis in chickens[3-5]. It has been known that a cholesterol accumulation in artery walls is the defining characteristic of atherosclerosis[91]. Researches demonstrated that MDV infection induced aortic lipid accumulation, including cholesterol, cholesterol ester, triacylglycerol and phospholipids[4]. The lipid accretion in aortas of MDV infected chickens was possibly due to alterations in cholesterol metabolism[4, 6].

Lipoproteins are the key carriers of cholesterol throughout the circulation system, and fundamentally influence cholesterol accumulation in the aortas. In MDV induced atherosclerosis, it is reasonable to suspect that the cholesterol accretion in the aortas is caused by alterations in lipoprotein metabolism. Our results presented that the baseline lipoprotein metabolism differs in MD susceptible and resistant lines. We further investigated whether MDV infection would influence lipoprotein metabolism differently in MD susceptible and resistant chickens. In this study, phenotype analysis and plasma lipoprotein levels were conducted for non-infected and infected chickens from MD resistant and susceptible lines.

Experimental design

The MD resistant line ($L6_3$) and susceptible line ($L7_2$) were acquired from the Avian Disease and Oncology Laboratory (ADOL) in U.S. Department of Agriculture (USDA). To study the influence of plasma lipoprotein levels induced by MDV infection, 7-day-old chickens from each line were divided into two groups: one was not infected with MDV, one was infected with MDV. Phenotype and plasma lipoprotein levels were measured 21 days post infection (dpi).

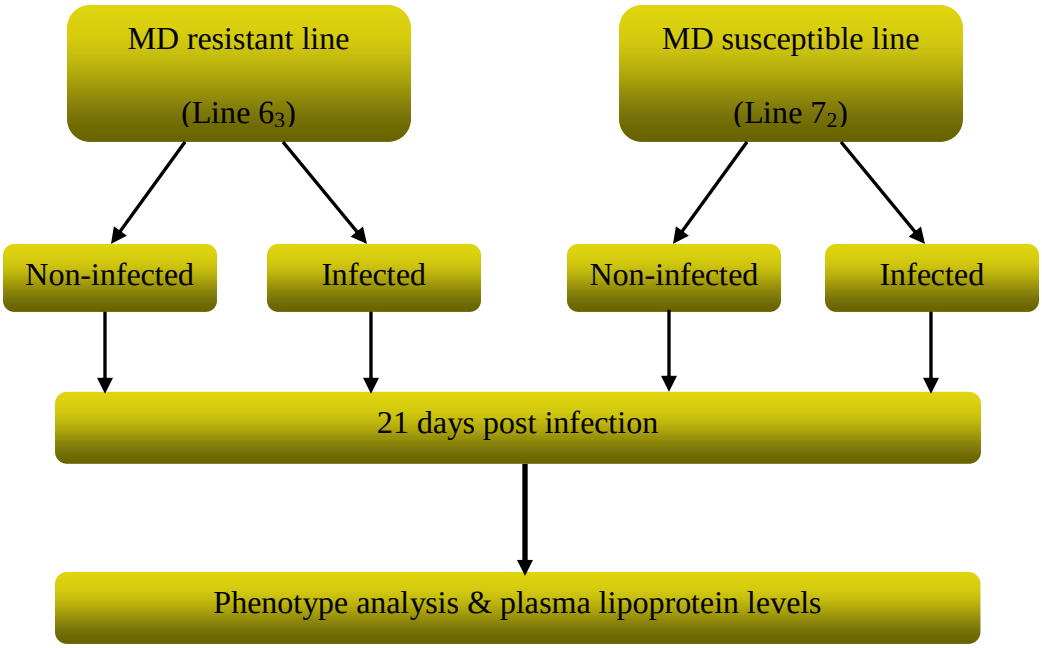


Figure 6. **Experimental design for the influence of MDV infection in plasma lipoprotein levels.**

Methods and Materials

Phenotype data

At the end of each study, animals were weighed and sacrificed for blood and tissue collection. Tissue samples were weighed, and immediately frozen in liquid nitrogen and stored at -80 ℃. The weight percentage of each tissue was calculated by the equation:

$$\text{Tissue percentage} = \frac{\text{Tissue weight (g)}}{\text{Body weight (g)}} \times 100\%$$

Plasma HDL and LDL/VLDL cholesterol levels

Blood samples were drawn by cardiac puncture and collected in 10 ml tubes with EDTA. Plasma was separated from whole blood by centrifugation at 2000 X g for 20 min and kept at 4 ℃ until analysis. Plasma total cholesterol, HDL cholesterol and LDL cholesterol were measured using a HDL and LDL/VLDL cholesterol quantification kit (BioVision, Exton, PA).

Statistical analysis

The data were statistically analyzed using a two-way ANOVA followed by Tukey's test for comparison among different groups using Statistical Analysis System v.9.1 (SAS Institute, Cary, NC). Values shown are means ± S.D.

The statistical model for before or after MDV infection was:

$$Y = \mu + Li + Tj + Li*Tj + \varepsilon_{i,\,j,\,k}$$

The line, treatment and their interaction are treated as fixed factors in the model.

μ: grand mean;

Li: the effect of the i^{th} line (6_3 or 7_2) on Y;

Tj: the effect of the j^{th} treatment (non-infected or infected by MDV) on Y;

$\varepsilon_{i, j, k}$: random error.

Results

Phenotype analysis for line 6_3 and line 7_2 after MDV infection

To determine the influence of MDV infection on body weight and various tissues, phenotype analysis was performed on $L6_3$ and $L7_2$ between the non-MDV-infected group and MDV-infected group (Figure 7). Although it appeared that body weight tended to decrease after MDV infection, no significant difference in body weight was detected between non-infected and infected chickens in $L6_3$ (358.40 ± 19.24 g vs. 286.38 ± 21.07 g). However, in $L7_2$, there was a significant ($p < 0.001$) decrease in body weight in infected chickens (194.26 ± 21.07 g) compared to non-infected chickens (353.30 ± 15.71 g) (Figure 7a). We also assessed whether MDV infection would affect tissue weight percentage. Phenotype analysis revealed no significant difference in abdominal fat percentage among $L6_3$-non-infected, $L6_3$-infected, $L7_2$-non-infected, and $L7_2$-infected chickens (Figure 7b). Similarly, there were no significant differences in the percentage of breast muscle with bone and of leg muscle with bone among the four groups (Figure 7c, 7d).

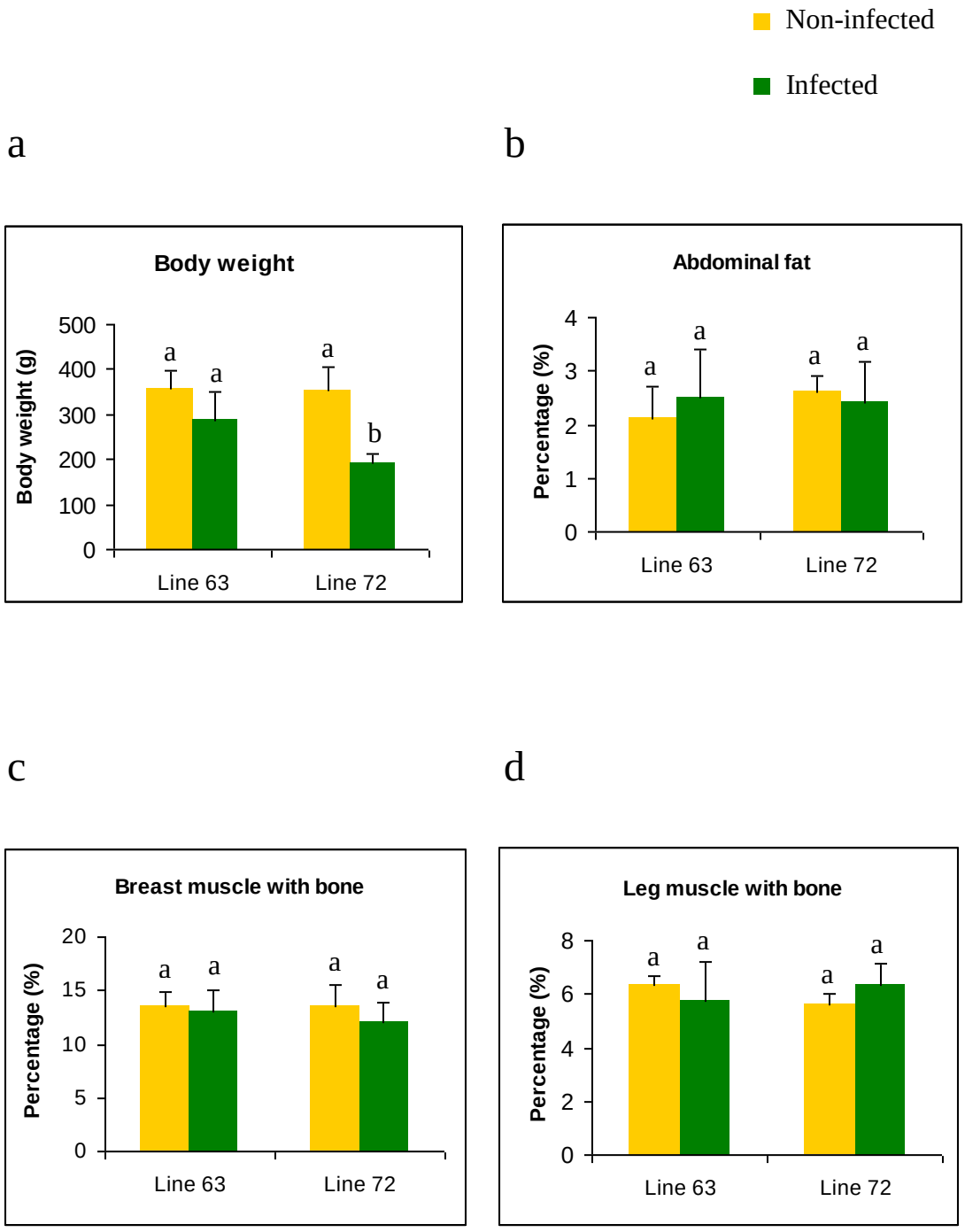

Non-infected
Infected
a
b
Body weight
Body weight (g)
500
400
300
200
100
0
a
a
a
b
Line 63
Line 72
Abdominal fat
Percentage (%)
4
3
2
1
0
a
a
a
a
Line 63
Line 72
c
d
Breast muscle with bone
Percentage (%)
20
15
10
5
0
a
a
a
a
Line 63
Line 72
Leg muscle with bone
Percentage (%)
8
6
4
2
0
a
a
a
a
Line 63
Line 72

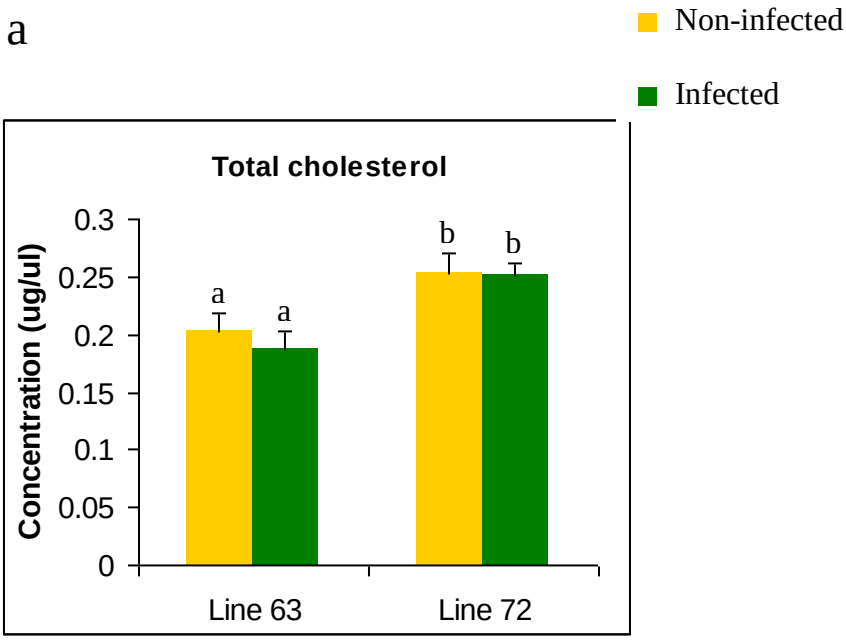
Figure 7. **Phenotype data for chicken without/with MDV infection.**

Body and tissue weight were measured for the non-infected groups (yellow bar) in line 6_3 (n = 6) and line 7_2 (n = 9), and the infected groups (green bar) in line 6_3 (n = 5) and line 7_2 (n = 5). Tissue percentages were calculated according to the equation ((Tissue weight) / (Body weight)) *100%. (a), Body weight (g); (b), Abdominal fat percentage (%); (c), The percentage of breast muscle with bone (%); (d), The percentage of leg muscle with bone (%). Values were shown as means ± S.D. Letters identify differences among groups; thus, two groups not sharing lowercase letters are significantly different (two-way ANOVA, Tukey's test, $p<0.05$).

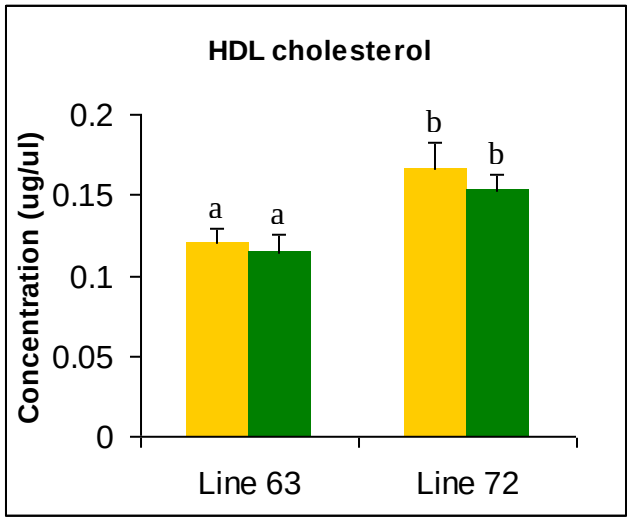
Plasma lipoprotein levels for line 6_3 and line 7_2 after MDV infection

Previous experiments suggested that cholesterol metabolism was altered in MDV infected chickens[4]. MDV infection is expected to possibly cause a change in lipoprotein levels. Thus, we measured plasma lipoprotein concentrations for non-infected and infected chickens in $L6_3$ and $L7_2$ (Figure 8). The interaction between line and treatment was not significant for total cholesterol, HDL cholesterol and (LDL+VLDL) cholesterol ($P>0.05$). Therefore, we analyzed the effects of line and treatment, respectively. In both lines, total cholesterol levels were not significantly different between chickens that were infected with MDV and those that were not (Figure 8a). However, it should be noted that $L7_2$ had higher total cholesterol concentration compared to $L6_3$ regardless of MDV infection ($p < 0.001$). Furthermore, a slight decrease in HDL cholesterol levels was observed in both lines after MDV infection (Figure 8b). Interestingly, MDV infection caused the decrease in (LDL+VLDL) levels in $L6_3$ (0.082 ± 0.005 μg/μl vs. 0.073 ± 0.005 μg/μl), but caused the increase in (LDL+VLDL) levels in $L7_2$ (0.087 ± 0.005 μg/μl vs. 0.098 ± 0.005 μg/μl) (Figure 8c). There was no significant difference between $L6_3$ and $L7_2$ in the non-infected groups. However, in MDV infected groups, $L7_2$ had significantly higher (LDL+VLDL) concentration compared to $L6_3$ ($p < 0.05$). Moreover, an analysis of lipoprotein ratios revealed that MDV infection also induced a slight increase in the HDL to total cholesterol ratio and a slight decrease in the (LDL+VLDL) to total cholesterol ratio in $L6_3$. The opposite was observed in $L7_2$ where the HDL ratio decreased and the (LDL+VLDL) ratio increased (Figure 8d).

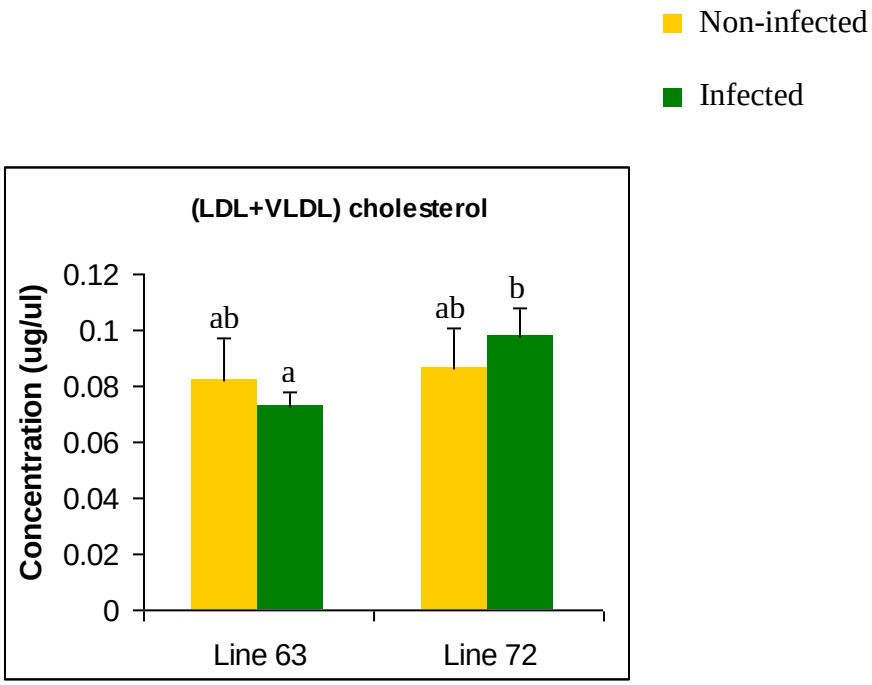
a


Non-infected
Infected
Total cholesterol
Concentration (ug/ul)
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
b
b
Line 63
Line 72

b


HDL cholesterol
Concentration (ug/ul)
0.2
0.15
0.1
0.05
0
a
a
b
b
Line 63
Line 72

c


Non-infected
Infected
(LDL+VLDL) cholesterol
Concentration (ug/ul)
0.12
0.1
0.08
0.06
0.04
0.02
0
ab
a
ab
b
Line 63
Line 72

d

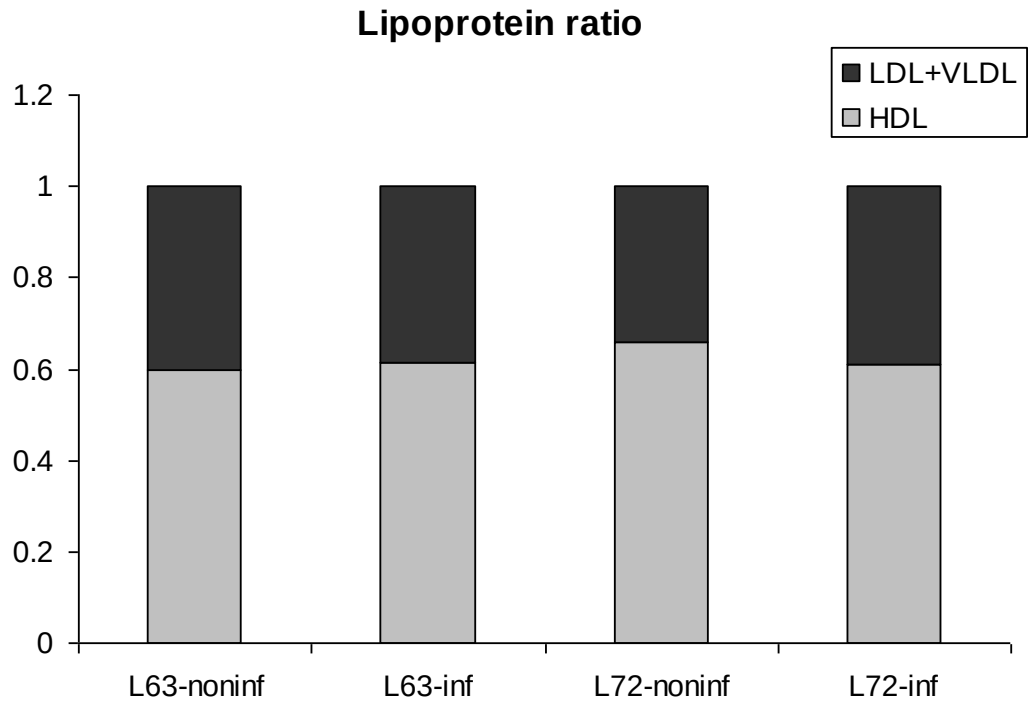

Lipoprotein ratio
LDL+VLDL
HDL
1.2
1
0.8
0.6
0.4
0.2
0
L63-noninf
L63-inf
L72-noninf
L72-inf

Figure 8. **Plasma lipoprotein levels for chickens without/with MDV infection (21 dpi).**

Lipoprotein levels were measured in the non-infected groups (yellow bar) and the infected groups (green bar) in line 6_3 (n = 5) and line 7_2 (n = 5). Cholesterol concentration of each sample was determined using the equation of the standard curve. Total cholesterol was calculated by the equation: Total cholesterol = HDL cholesterol + (LDL+VLDL) cholesterol. Lipoprotein ratio is represented by two different calculations: the ratio of HDL to total cholesterol, and the ratio of (LDL+VLDL) to total cholesterol.

(a), Total cholesterol (μg/μl); (b), HDL cholesterol (μg/μl); (c), LDL+VLDL cholesterol (μg/μl); (d), Lipoprotein ratio. In (a), (b), and (c), values were shown as means ± S.D. Letters identify differences among groups; thus, two groups not sharing lowercase letters are significantly different (two-way ANOVA, Tukey's test, $p<0.05$).

Summary

In the herpesvirus induced atherosclerosis model, we detected differences in body weight and the weight percentage of tissues associated with lipid metabolism such as abdominal fat, breast muscle with bone, and leg muscle with bone. A significant decrease in body weight was apparent in $L7_2$ after MDV infection compared to non-infected chickens, whereas no significant difference was observed in $L6_3$. It has been reported that the pathological changes in chickens after MDV infection include blindness, immunosuppression, skin lesions and weight loss[37]. Our results concerning body weight were consistent with this report. That is, the MD susceptible line ($L7_2$) experienced weight loss and there were no changes in body weight in the MD resistant line ($L6_3$). Phenotype analysis revealed that MDV infection did not change the percentage of abdominal fat, breast muscle with bone, and leg muscle with bone (21 dpi). In fact, MDV infection causes an acute cytolytic infection of lymphoid tissues, such as the bursa, thymus and spleen[32], which may be the underlying causes of change in weight percentage in these tissues. Although this study focused on tissues involved in lipid metabolism, future studies on lymphoid tissues may provide valuable insight for the changes of tissue phenotype caused by MDV infection.

Atherosclerosis is a prominent human diseases that involves cholesterol and lipoprotein metabolism[7, 8]. A study of the cholesterol and lipoprotein metabolism in a herpesvirus induced atherosclerosis model will help us better understand this disease. It has been reported that in MDV infected chickens total aortic lipid accumulation, including cholesterol, cholesterol ester, triacylglycerol and phospholipids, increased

compared to aortas in uninfected chickens[4, 6]. Lipoproteins are the key carriers of cholesterol through the circulating system and play an important role in the development of atherosclerosis. Thus, changes in lipoprotein levels would be expected in response to MDV infection. Our results revealed that there are no remarkable changes in total cholesterol and HDL cholesterol concentrations after MDV infection in both chicken lines. Interestingly, in chickens that were not infected with MDV, there was no obvious difference in (LDL+VLDL) cholesterol levels between line 6_3 and 7_2. However, after MDV infection $L7_2$ had a significantly higher level of (LDL+VLDL) cholesterol than $L6_3$. In summary, MDV infection induced a modest decrease of (LDL+VLDL) cholesterol level in resistant line 6_3 but induced an increase in susceptible line 7_2. It has been demonstrated that LDL is an important risk factor for atherosclerosis[9] and that elevated levels of (LDL+VLDL) are associated with the development of atherosclerosis[1]. The main physiological roles of VLDLs and LDLs are to transport triacylglycerols and cholesterols, respectively, from the liver to peripheral tissues. In turn, our findings suggest that the slightly increasing (LDL+VLDL) in the MD susceptible line possibly resulted in elevated levels of triacylglycerols and cholesterols in the circulating system, thus potentially promoting the progression of atherosclerosis.

Chapter 4: General discussions

The present study compared lipoprotein metabolism in MDV susceptible and resistant lines. First, we observed that baseline lipoprotein metabolism differed in MD susceptible and resistant chicken lines. While the animals were growing, total cholesterol lightly increased in the MD resistant line (6_3)whereas it doubled in the MD susceptible line (7_2). Interestingly, our findings demonstrate that the increase in total cholesterol is mainly attributable to the presence of HDL in the resistant line. In contrast, in the MD susceptible line, the increase in total cholesterol is mostly due to the elevation of (LDL+VLDL). It has been reported that LDLs serve as the primary transport mechanism for transporting cholesterol from the liver to peripheral tissues and transport 70-80% of circulating cholesterol[60]. The principal function of VLDLs is to transport triacylglycerols from the liver to peripheral tissues. Based on the biological functions of VLDLs and LDLs, the elevation of (LDL+VLDL) in $L7_2$ suggests that during chicken growth, the MD susceptible line is more likely to exhibit lipid accumulation in peripheral tissues compared to the MD resistant line.

As lipoprotein metabolism is essential to the development of atherosclerosis[7, 8], it is necessary to highlight the physiological significance of different baseline levels of lipoprotein between MD susceptible and resistant lines. LDL is a main risk factor for atherosclerosis whereas HDL is a protective factor against atherosclerosis[9]. Our

results strongly suggest that during growth, the MD susceptible line possessed a higher (LDL+VLDL) ratio and correspondingly lower HDL ratio, resulting in an increased possibility of developing atherosclerosis.

Adiponectin, a major anti-atherogenic adipocytokine, has characteristics that prevent the progression of atherosclerosis. It has been reported that adiponectin fundamentally influence lipid metabolism[81]. We next measured adiponectin levels in two chicken lines at 2 and 15 months of age. Our results displayed no significant differences in both mRNA and protein levels in abdominal fat between the two lines, whereas the MD susceptible line had lower levels of plasma adiponectin than the MD resistant line. As adiponectin is primarily synthesized in adipocytes[87], it is possible that the MD susceptible line synthesized adiponectin in abdominal fat similar to the resistant line, but had lower circulating levels. Adiponectin is a protective factor against atherosclerosis. Several reports indicated that lower levers of plasma adiponectin are associated with the development of atherosclerosis [111, 112]. Our results indicate that with less circulating adiponectin, as in the MD susceptible line, atherosclerosis may be more likely to develop.

Adiponectin exerts its biological functions via two receptors, adipoR1 and adipoR2[102]. AdipoR1 is expressed in various tissues and AdipoR2 is expressed most abundantly in the liver[102]. We next measured mRNA and protein expression levels of adipoR1 and adipoR2 in liver and abdominal fat. The results of Western blots demonstrated that the MD susceptible line at 15 months of age had a lower level of

adipoR1 in liver, but a higher level in abdominal fat was observed. With respect to adipoR2, the MD susceptible and resistant line displayed no significant differences in both liver and abdominal fat. AdipoR1 may be associated with the activation of AMPK pathways, whereas adipoR2 seems to be more tightly linked to activation of PPARα pathway[107]. Our results indicate that in the MD susceptible line, adipoR1 may activate the AMPK pathway, thus influencing fatty acid oxidation and subsequent lipoprotein metabolism. The results from studies of adiponectin and its receptors suggest one of the possible mechanisms that may explain the differences in lipoprotein levels in MD susceptible and resistant lines.

Second, we demonstrated changes in lipoprotein levels induced by MDV infection in MD susceptible and resistant chicken lines. Although total cholesterol and HDL cholesterol displayed no changes before and after MDV infection in both lines, we found that the (LDL+VLDL) level was inversely regulated in the two lines in response to MDV infection: (LDL+VLDL) was slightly elevated in the MD susceptible line while it was slightly reduced in the MD resistant line. The slight increase of (LDL+VLDL) level in MD susceptible chickens after MDV infection may provide a clue for MDV induced atherosclerosis as LDL is a main risk factor for atherosclerosis.

At this time, we cannot identify which mechanisms are responsible for the differences in lipoprotein metabolism induced by MDV infection in MD susceptible and resistant line. Regardless, increased (LDL+VLDL) cholesterol levels, primarily LDL

cholesterol, may be very important in the pathogenesis of MDV induced atherosclerosis. Results of experiments by other investigators also suggest that herpesvirus infection enhanced LDL binding and uptake in addition to transcription of the LDL receptor gene[142]. Fabricant et al. mentioned one mechanism that may be involved in cholesterol accumulation induced by MDV infection: MDV may enhance uptake of LDL via a LDL receptor mediated pathway[143]. However, the precise role of LDL in cholesterol accumulation following MDV infection remains to be resolved.

In conclusion, the results of our experiments support the hypothesis that different lipoprotein metabolisms exist in distinct MD susceptible and resistant lines.

References

1. Lusis, A.J. Atherosclerosis. *Nature* **407**, 233-241 (2000).
2. Nicholson, A.C. & Hajjar, D.P. Herpesvirus in atherosclerosis and thrombosis: etiologic agents or ubiquitous bystanders? *Arterioscler Thromb Vasc Biol* **18**, 339-348 (1998).
3. Fabricant, C.G., Fabricant, J., Litrenta, M.M. & Minick, C.R. Virus-induced atherosclerosis. *J Exp Med* **148**, 335-340 (1978).
4. Hajjar, D.P., Fabricant, C.G., Minick, C.R. & Fabricant, J. Virus-induced atherosclerosis. Herpesvirus infection alters aortic cholesterol metabolism and accumulation. *Am J Pathol* **122**, 62-70 (1986).
5. Minick, C.R., Fabricant, C.G., Fabricant, J. & Litrenta, M.M. Atheroarteriosclerosis induced by infection with a herpesvirus. *Am J Pathol* **96**, 673-706 (1979).
6. Hajjar, D.P., Falcone, D.J., Fabricant, C.G. & Fabricant, J. Altered cholesteryl ester cycle is associated with lipid accumulation in herpesvirus-infected arterial smooth muscle cells. *J Biol Chem* **260**, 6124-6128 (1985).
7. Maxfield, F.R. & Tabas, I. Role of cholesterol and lipid organization in disease. *Nature* **438**, 612-621 (2005).
8. Hegele, R.A. Plasma lipoproteins: genetic influences and clinical implications. *Nat Rev Genet* **10**, 109-121 (2009).
9. Libby, P., Aikawa, M. & Schonbeck, U. Cholesterol and atherosclerosis. *Biochim Biophys Acta* **1529**, 299-309 (2000).
10. Rader, D.J. Molecular regulation of HDL metabolism and function: implications for novel therapies. *J Clin Invest* **116**, 3090-3100 (2006).
11. Cunningham, M.J. & Pasternak, R.C. The potential role of viruses in the pathogenesis of atherosclerosis. *Circulation* **77**, 964-966 (1988).
12. Melnick, J.L., Adam, E. & Debakey, M.E. Cytomegalovirus and atherosclerosis. *Eur Heart J* **14 Suppl K**, 30-38 (1993).
13. Hajjar, D.P. Warner-Lambert/Parke-Davis Award Lecture. Viral pathogenesis of atherosclerosis. Impact of molecular mimicry and viral genes. *Am J Pathol* **139**, 1195-1211 (1991).
14. Visser, M.R. & Vercellotti, G.M. Herpes simplex virus and atherosclerosis. *Eur Heart J* **14 Suppl K**, 39-42 (1993).
15. Pappenheimer, A.M., Dunn, L.C. & Cone, V. Studies on Fowl Paralysis (Neurolymphomatosis Gallinarum) : I. Clinical Features and Pathology. *J Exp Med* **49**, 63-86 (1929).
16. Pappenheimer, A.M., Dunn, L.C. & Seidlin, S.M. Studies on Fowl Paralysis (Neurolymphomatosis Gallinarum) : Ii. Transmission Experiments. *J Exp Med* **49**, 87-102 (1929).
17. Churchill, A.E. & Biggs, P.M. Agent of Marek's disease in tissue culture. *Nature* **215**, 528-530 (1967).

18. Churchill, A.E. & Biggs, P.M. Herpes-type virus isolated in cell culture from tumors of chickens with Marek's disease. II. Studies in vivo. *J Natl Cancer Inst* **41**, 951-956 (1968).
19. Churchill, A.E. Herpes-type virus isolated in cell culture from tumors of chickens with Marek's disease. I. Studies in cell culture. *J Natl Cancer Inst* **41**, 939-950 (1968).
20. Churchill, A.E., Payne, L.N. & Chubb, R.C. Immunization against Marek's disease using a live attenuated virus. *Nature* **221**, 744-747 (1969).
21. Okazaki, W., Purchase, H.G. & Burmester, B.R. Protection against Marek's disease by vaccination with a herpesvirus of turkeys. *Avian Dis* **14**, 413-429 (1970).
22. Maotani, K. et al. Amplification of a tandem direct repeat within inverted repeats of Marek's disease virus DNA during serial in vitro passage. *J Virol* **58**, 657-660 (1986).
23. Cebrian, J., Kaschka-Dierich, C., Berthelot, N. & Sheldrick, P. Inverted repeat nucleotide sequences in the genomes of Marek disease virus and the herpesvirus of the turkey. *Proc Natl Acad Sci U S A* **79**, 555-558 (1982).
24. Osterrieder, N., Kamil, J.P., Schumacher, D., Tischer, B.K. & Trapp, S. Marek's disease virus: from miasma to model. *Nat Rev Microbiol* **4**, 283-294 (2006).
25. Addinger, H.K. & Calnek, B.W. Pathogenesis of Marek's disease: early distribution of virus and viral antigens in infected chickens. *J Natl Cancer Inst* **50**, 1287-1298 (1973).
26. Schat, K.A., Schinazi, R.F. & Calnek, B.W. Cell-specific antiviral activity of 1-(2-fluoro-2-deoxy-beta-D-arabinofuranosyl)-5-iodocytosine (FIAC) against Marek's disease herpesvirus and turkey herpesvirus. *Antiviral Res* **4**, 259-270 (1984).
27. Shek, W.R., Calnek, B.W., Schat, K.A. & Chen, C.H. Characterization of Marek's disease virus-infected lymphocytes: discrimination between cytolytically and latently infected cells. *J Natl Cancer Inst* **70**, 485-491 (1983).
28. Baigent, S.J., Ross, L.J. & Davison, T.F. Differential susceptibility to Marek's disease is associated with differences in number, but not phenotype or location, of pp38+ lymphocytes. *J Gen Virol* **79 (Pt 11)**, 2795-2802 (1998).
29. Calnek, B.W., Schat, K.A., Ross, L.J. & Chen, C.L. Further characterization of Marek's disease virus-infected lymphocytes. II. In vitro infection. *Int J Cancer* **33**, 399-406 (1984).
30. Morimura, T., Ohashi, K., Sugimoto, C. & Onuma, M. Pathogenesis of Marek's disease (MD) and possible mechanisms of immunity induced by MD vaccine. *J Vet Med Sci* **60**, 1-8 (1998).
31. Schat, K.A., Lucio, B. & Carlisle, J.C. Pathogenesis of infectious bursal disease in embryonally bursectomized chickens. *Avian Dis* **25**, 996-1004 (1981).
32. Payne, L.N. & Rennie, M. Pathogenesis of Marek's disease in chicks with and without maternal antibody. *J Natl Cancer Inst* **51**, 1559-1573 (1973).

33. Morimura, T., Hattori, M., Ohashi, K., Sugimoto, C. & Onuma, M. Immunomodulation of peripheral T cells in chickens infected with Marek's disease virus: involvement in immunosuppression. *J Gen Virol* **76 (Pt 12)**, 2979-2985 (1995).
34. Kornegay, J.N., Gorgacz, E.J., Parker, M.A., Duncan, J.R. & Schierman, L.W. Marek's disease virus-induced transient paralysis: a comparison of lesions in susceptible and resistant lines of chickens. *Acta Neuropathol* **61**, 263-269 (1983).
35. Swayne, D.E., Fletcher, O.J. & Schierman, L.W. Marek's disease virus-induced transient paralysis in chickens: demonstration of vasogenic brain oedema by an immunohistochemical method. *J Comp Pathol* **101**, 451-462 (1989).
36. Gimeno, I.M., Witter, R.L. & Reed, W.M. Four distinct neurologic syndromes in Marek's disease: effect of viral strain and pathotype. *Avian Dis* **43**, 721-737 (1999).
37. Payne, L.N. & Biggs, P.M. Studies on Marek's disease. II. Pathogenesis. *J Natl Cancer Inst* **39**, 281-302 (1967).
38. Sharma, J.M. In vitro suppression of T-cell mitogenic response and tumor cell proliferation by spleen macrophages from normal chickens. *Infect Immun* **28**, 914-922 (1980).
39. Burgess, S.C. & Davison, T.F. Identification of the neoplastically transformed cells in Marek's disease herpesvirus-induced lymphomas: recognition by the monoclonal antibody AV37. *J Virol* **76**, 7276-7292 (2002).
40. Venugopal, K. Marek's disease: an update on oncogenic mechanisms and control. *Res Vet Sci* **69**, 17-23 (2000).
41. Biely, J., Palmer, V.E., Lerner, I.M. & Asmundson, V.S. Inheritance of Resistance to Fowl Paralysis (Neurolymphomatosis Gallinarum). *Science* **78**, 42 (1933).
42. Hutt, F.B. & Cole, R.K. Genetic Control of Lymphomatosis in the Fowl. *Science* **106**, 379-384 (1947).
43. Cole, R.K. Studies on genetic resistance to Marek's disease. *Avian Dis* **12**, 9-28 (1968).
44. Gallatin, W.M. & Longenecker, B.M. Expression of genetic resistance to an oncogenic herpesvirus at the target cell level. *Nature* **280**, 587-589 (1979).
45. Longenecker, B.M. et al. Role of the major histocompatibility complex in resistance to Marek's disease: restriction of the growth of JMV-MD tumor cells in genetically resistant birds. *Adv Exp Med Biol* **88**, 287-298 (1977).
46. Lamont, S.J., Gerndt, B.M., Warner, C.M. & Bacon, L.D. Analysis of restriction fragment length polymorphisms of the major histocompatibility complex of 15I5-B-congenic chicken lines. *Poult Sci* **69**, 1195-1203 (1990).
47. Lee, L.F., Powell, P.C., Rennie, M., Ross, L.J. & Payne, L.N. Nature of genetic resistance to Marek's disease in chickens. *J Natl Cancer Inst* **66**, 789-796 (1981).
48. Lee, L.F. & Bacon, L.D. Ontogeny and line differences in the mitogenic response of chicken lymphocytes. *Poult Sci* **62**, 579-584 (1983).

49. Myant, N.B. Cholesterol metabolism. *J Clin Pathol Suppl (Assoc Clin Pathol)* **5**, 1-4 (1973).
50. Cherezov, V. et al. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318**, 1258-1265 (2007).
51. Brown, M.S. & Goldstein, J.L. A receptor-mediated pathway for cholesterol homeostasis. *Science* **232**, 34-47 (1986).
52. Incardona, J.P. & Roelink, H. The role of cholesterol in Shh signaling and teratogen-induced holoprosencephaly. *Cell Mol Life Sci* **57**, 1709-1719 (2000).
53. Goldstein, J.L. & Brown, M.S. Molecular medicine. The cholesterol quartet. *Science* **292**, 1310-1312 (2001).
54. Wasan, K.M., Brocks, D.R., Lee, S.D., Sachs-Barrable, K. & Thornton, S.J. Impact of lipoproteins on the biological activity and disposition of hydrophobic drugs: implications for drug discovery. *Nat Rev Drug Discov* **7**, 84-99 (2008).
55. Forte, T. & Nichols, A.V. Application of electron microscopy to the study of plasma lipoprotein structure. *Adv Lipid Res* **10**, 1-41 (1972).
56. Alaupovic, P., Lee, D.M. & McConathy, W.J. Studies on the composition and structure of plasma lipoproteins. Distribution of lipoprotein families in major density classes of normal human plasma lipoproteins. *Biochim Biophys Acta* **260**, 689-707 (1972).
57. Jackson, R.L., Morrisett, J.D. & Gotto, A.M., Jr. Lipoprotein structure and metabolism. *Physiol Rev* **56**, 259-316 (1976).
58. Morton, R.E. & Greene, D.J. Partial suppression of CETP activity beneficially modifies the lipid transfer profile of plasma. *Atherosclerosis* **192**, 100-107 (2007).
59. Qiu, X. et al. Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules. *Nat Struct Mol Biol* **14**, 106-113 (2007).
60. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *Jama* **285**, 2486-2497 (2001).
61. Jeon, H. & Blacklow, S.C. Structure and physiologic function of the low-density lipoprotein receptor. *Annu Rev Biochem* **74**, 535-562 (2005).
62. Joy, T. & Hegele, R.A. Is raising HDL a futile strategy for atheroprotection? *Nat Rev Drug Discov* **7**, 143-155 (2008).
63. Berglund, L. Lipoprotein metabolism: a well-tried tool to characterize dyslipidemic mechanisms. *Arterioscler Thromb Vasc Biol* **26**, 1201-1203 (2006).
64. Nissen, S.E. et al. Effect of torcetrapib on the progression of coronary atherosclerosis. *N Engl J Med* **356**, 1304-1316 (2007).
65. Lowenstein, C.J. & Cameron, S.J. High-density lipoprotein metabolism and endothelial function. *Curr Opin Endocrinol Diabetes Obes* **17**, 166-170.

66. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* **360**, 7-22 (2002).
67. Sever, P.S. et al. Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial--Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. *Lancet* **361**, 1149-1158 (2003).
68. Rader, D.J. & Daugherty, A. Translating molecular discoveries into new therapies for atherosclerosis. *Nature* **451**, 904-913 (2008).
69. Rhoads, G.G., Gulbrandsen, C.L. & Kagan, A. Serum lipoproteins and coronary heart disease in a population study of Hawaii Japanese men. *N Engl J Med* **294**, 293-298 (1976).
70. Assmann, G. & Schulte, H. The Prospective Cardiovascular Munster Study: prevalence and prognostic significance of hyperlipidemia in men with systemic hypertension. *Am J Cardiol* **59**, 9G-17G (1987).
71. Linsel-Nitschke, P. & Tall, A.R. HDL as a target in the treatment of atherosclerotic cardiovascular disease. *Nat Rev Drug Discov* **4**, 193-205 (2005).
72. Van Lenten, B.J., Navab, M., Shih, D., Fogelman, A.M. & Lusis, A.J. The role of high-density lipoproteins in oxidation and inflammation. *Trends Cardiovasc Med* **11**, 155-161 (2001).
73. Nofer, J.R. et al. HDL and arteriosclerosis: beyond reverse cholesterol transport. *Atherosclerosis* **161**, 1-16 (2002).
74. Classics in arteriosclerosis research: On experimental cholesterin steatosis and its significance in the origin of some pathological processes by N. Anitschkow and S. Chalatow, translated by Mary Z. Pelias, 1913. *Arteriosclerosis* **3**, 178-182 (1983).
75. Kannel, W.B., Castelli, W.P. & Gordon, T. Cholesterol in the prediction of atherosclerotic disease. New perspectives based on the Framingham study. *Ann Intern Med* **90**, 85-91 (1979).
76. Boren, J. et al. Identification of the principal proteoglycan-binding site in LDL. A single-point mutation in apo-B100 severely affects proteoglycan interaction without affecting LDL receptor binding. *J Clin Invest* **101**, 2658-2664 (1998).
77. Grainger, D.J., Kemp, P.R., Liu, A.C., Lawn, R.M. & Metcalfe, J.C. Activation of transforming growth factor-beta is inhibited in transgenic apolipoprotein(a) mice. *Nature* **370**, 460-462 (1994).
78. Williams, K.J. & Tabas, I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol* **15**, 551-561 (1995).
79. Tabas, I. Nonoxidative modifications of lipoproteins in atherogenesis. *Annu Rev Nutr* **19**, 123-139 (1999).
80. Funahashi, T. et al. Role of adipocytokines on the pathogenesis of atherosclerosis in visceral obesity. *Intern Med* **38**, 202-206 (1999).

81. Lago, F., Gomez, R., Gomez-Reino, J.J., Dieguez, C. & Gualillo, O. Adipokines as novel modulators of lipid metabolism. *Trends Biochem Sci* **34**, 500-510 (2009).
82. Ahima, R.S. & Flier, J.S. Adipose tissue as an endocrine organ. *Trends Endocrinol Metab* **11**, 327-332 (2000).
83. Sweeney, G. Cardiovascular effects of leptin. *Nat Rev Cardiol* **7**, 22-29.
84. Norata, G.D. et al. Plasma resistin levels correlate with determinants of the metabolic syndrome. *Eur J Endocrinol* **156**, 279-284 (2007).
85. Fukuhara, A. et al. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* **307**, 426-430 (2005).
86. Tilg, H. & Moschen, A.R. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol* **6**, 772-783 (2006).
87. Koerner, A., Kratzsch, J. & Kiess, W. Adipocytokines: leptin--the classical, resistin--the controversical, adiponectin--the promising, and more to come. *Best Pract Res Clin Endocrinol Metab* **19**, 525-546 (2005).
88. Scherer, P.E., Williams, S., Fogliano, M., Baldini, G. & Lodish, H.F. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* **270**, 26746-26749 (1995).
89. Hu, E., Liang, P. & Spiegelman, B.M. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* **271**, 10697-10703 (1996).
90. Maeda, K. et al. cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochem Biophys Res Commun* **221**, 286-289 (1996).
91. Nakano, Y., Tobe, T., Choi-Miura, N.H., Mazda, T. & Tomita, M. Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. *J Biochem* **120**, 803-812 (1996).
92. Pajvani, U.B. et al. Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. *J Biol Chem* **278**, 9073-9085 (2003).
93. Fruebis, J. et al. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci U S A* **98**, 2005-2010 (2001).
94. Waki, H. et al. Impaired multimerization of human adiponectin mutants associated with diabetes. Molecular structure and multimer formation of adiponectin. *J Biol Chem* **278**, 40352-40363 (2003).
95. Delaigle, A.M., Jonas, J.C., Bauche, I.B., Cornu, O. & Brichard, S.M. Induction of adiponectin in skeletal muscle by inflammatory cytokines: in vivo and in vitro studies. *Endocrinology* **145**, 5589-5597 (2004).
96. Pineiro, R. et al. Adiponectin is synthesized and secreted by human and murine cardiomyocytes. *FEBS Lett* **579**, 5163-5169 (2005).
97. Wolf, A.M. et al. Up-regulation of the anti-inflammatory adipokine adiponectin in acute liver failure in mice. *J Hepatol* **44**, 537-543 (2006).
98. Maddineni, S., Metzger, S., Ocon, O., Hendricks, G., 3rd & Ramachandran, R. Adiponectin gene is expressed in multiple tissues in the chicken: food

deprivation influences adiponectin messenger ribonucleic acid expression. *Endocrinology* **146**, 4250-4256 (2005).
99. Arita, Y. et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* **257**, 79-83 (1999).
100. Combs, T.P. et al. Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. *Diabetes* **52**, 268-276 (2003).
101. Nagasawa, A. et al. Effects of soy protein diet on the expression of adipose genes and plasma adiponectin. *Horm Metab Res* **34**, 635-639 (2002).
102. Yamauchi, T. et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* **423**, 762-769 (2003).
103. Ramachandran, R., Ocon-Grove, O.M. & Metzger, S.L. Molecular cloning and tissue expression of chicken AdipoR1 and AdipoR2 complementary deoxyribonucleic acids. *Domest Anim Endocrinol* **33**, 19-31 (2007).
104. Yamauchi, T. et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* **8**, 1288-1295 (2002).
105. Tomas, E. et al. Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proc Natl Acad Sci U S A* **99**, 16309-16313 (2002).
106. Kersten, S., Desvergne, B. & Wahli, W. Roles of PPARs in health and disease. *Nature* **405**, 421-424 (2000).
107. Yamauchi, T. et al. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. *Nat Med* **13**, 332-339 (2007).
108. Kubota, N. et al. Disruption of adiponectin causes insulin resistance and neointimal formation. *J Biol Chem* **277**, 25863-25866 (2002).
109. Brownlee, M. Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813-820 (2001).
110. Wellen, K.E. & Hotamisligil, G.S. Inflammation, stress, and diabetes. *J Clin Invest* **115**, 1111-1119 (2005).
111. Ouchi, N. et al. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* **100**, 2473-2476 (1999).
112. Kawano, T. et al. Close association of hypoadiponectinemia with arteriosclerosis obliterans and ischemic heart disease. *Metabolism* **54**, 653-656 (2005).
113. Maahs, D.M. et al. Low plasma adiponectin levels predict progression of coronary artery calcification. *Circulation* **111**, 747-753 (2005).
114. Ross, R. Atherosclerosis--an inflammatory disease. *N Engl J Med* **340**, 115-126 (1999).
115. Ouchi, N. et al. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation* **102**, 1296-1301 (2000).

116. Ouchi, N. et al. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* **103**, 1057-1063 (2001).
117. Arita, Y. et al. Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation* **105**, 2893-2898 (2002).
118. Blake, G.J. & Ridker, P.M. C-reactive protein and other inflammatory risk markers in acute coronary syndromes. *J Am Coll Cardiol* **41**, 37S-42S (2003).
119. Ouchi, N. et al. Reciprocal association of C-reactive protein with adiponectin in blood stream and adipose tissue. *Circulation* **107**, 671-674 (2003).
120. Gualillo, O., Gonzalez-Juanatey, J.R. & Lago, F. The emerging role of adipokines as mediators of cardiovascular function: physiologic and clinical perspectives. *Trends Cardiovasc Med* **17**, 275-283 (2007).
121. Ridker, P.M., Rifai, N., Rose, L., Buring, J.E. & Cook, N.R. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med* **347**, 1557-1565 (2002).
122. Kazumi, T., Kawaguchi, A., Sakai, K., Hirano, T. & Yoshino, G. Young men with high-normal blood pressure have lower serum adiponectin, smaller LDL size, and higher elevated heart rate than those with optimal blood pressure. *Diabetes Care* **25**, 971-976 (2002).
123. Tian, L. et al. Adiponectin reduces lipid accumulation in macrophage foam cells. *Atherosclerosis* **202**, 152-161 (2009).
124. Okamoto, Y. et al. Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. *Circulation* **106**, 2767-2770 (2002).
125. Hotta, K. et al. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* **20**, 1595-1599 (2000).
126. Yamauchi, T. et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* **7**, 941-946 (2001).
127. Daimon, M. et al. Decreased serum levels of adiponectin are a risk factor for the progression to type 2 diabetes in the Japanese Population: the Funagata study. *Diabetes Care* **26**, 2015-2020 (2003).
128. Yamamoto, Y., Hirose, H., Saito, I., Nishikai, K. & Saruta, T. Adiponectin, an adipocyte-derived protein, predicts future insulin resistance: two-year follow-up study in Japanese population. *J Clin Endocrinol Metab* **89**, 87-90 (2004).
129. Snehalatha, C. et al. Plasma adiponectin is an independent predictor of type 2 diabetes in Asian indians. *Diabetes Care* **26**, 3226-3229 (2003).
130. Berg, A.H., Combs, T.P., Du, X., Brownlee, M. & Scherer, P.E. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med* **7**, 947-953 (2001).
131. Maeda, N. et al. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* **8**, 731-737 (2002).

132. Kahn, B.B., Alquier, T., Carling, D. & Hardie, D.G. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* **1**, 15-25 (2005).
133. Yamauchi, T. et al. Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. *J Biol Chem* **278**, 2461-2468 (2003).
134. Ouchi, N. & Walsh, K. Adiponectin as an anti-inflammatory factor. *Clin Chim Acta* **380**, 24-30 (2007).
135. Yokota, T. et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* **96**, 1723-1732 (2000).
136. Wolf, A.M., Wolf, D., Rumpold, H., Enrich, B. & Tilg, H. Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes. *Biochem Biophys Res Commun* **323**, 630-635 (2004).
137. Ocon-Grove, O.M., Krzysik-Walker, S.M., Maddineni, S.R., Hendricks, G.L., 3rd & Ramachandran, R. Adiponectin and its receptors are expressed in the chicken testis: influence of sexual maturation on testicular ADIPOR1 and ADIPOR2 mRNA abundance. *Reproduction* **136**, 627-638 (2008).
138. Krzysik-Walker, S.M., Ocon-Grove, O.M., Maddineni, S.R., Hendricks, G.L., 3rd & Ramachandran, R. Is visfatin an adipokine or myokine? Evidence for greater visfatin expression in skeletal muscle than visceral fat in chickens. *Endocrinology* **149**, 1543-1550 (2008).
139. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685 (1970).
140. Lara-Castro, C., Fu, Y., Chung, B.H. & Garvey, W.T. Adiponectin and the metabolic syndrome: mechanisms mediating risk for metabolic and cardiovascular disease. *Curr Opin Lipidol* **18**, 263-270 (2007).
141. Dunajska, K. et al. Plasma adiponectin concentration in relation to severity of coronary atherosclerosis and cardiovascular risk factors in middle-aged men. *Endocrine* **25**, 215-221 (2004).
142. Hsu, H.Y., Nicholson, A.C., Pomerantz, K.B., Kaner, R.J. & Hajjar, D.P. Altered cholesterol trafficking in herpesvirus-infected arterial cells. Evidence for viral protein kinase-mediated cholesterol accumulation. *J Biol Chem* **270**, 19630-19637 (1995).
143. Fabricant, C.G., Hajjar, D.P., Minick, C.R. & Fabricant, J. Herpesvirus infection enhances cholesterol and cholesteryl ester accumulation in cultured arterial smooth muscle cells. *Am J Pathol* **105**, 176-184 (1981).