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Research Article

# Differential Liver Gene Expression in Atherosclerosis-resistant and –susceptible Japanese Quail Fed a Cholesterol Enriched Diet

Kimberly M. Cheng[1]*, Frederick G. Silversides[2], Shasha Liu[1,3], Ji Eun Kim[1], and Frederick C. Leung[4]

[1]Avian Research Centre, Faculty of Land and Food Systems, Canada

[2]Agriculture and Agri-Food Canada Research Centre, Agassiz, Canada

[3]The State Key Laboratory of Animal Nutrition, Chinese Academy of Agricultural Sciences, China

[4]School of Biological Sciences, Faculty of Science, Hong Kong, SAR, China

***Corresponding author**

Kimberly M, Cheng, Avian Research Centre, Faculty of Land and Food Systems, University of British Columbia, 2357 Main Mall, Vancouver, BC V6T 1Z4, Canada; Tel: 1-604-218-2468; Email: kmtc@mail.ubc.ca

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## Abstract

**Objectives:** (1) To use NGS to examine the differential liver gene expression in Japanese quail fed a cholesterol-enriched diet and to identify the gene systems that may be associated with the development of atherosclerosis, and

(2) To see how divergent genetic selection and diet has modified the expression of these gene systems.

**Methods:**

1. Liver tissue samples were obtained from Atherosclerosis – resistant (RES) and Atherosclerosis- susceptible (SUS) Japanese quail fed either a control or a cholesterol diet.
2. Total RNA was extracted from the tissue samples and processed for the construction of cDNA libraries.
3. Illumina Mi-Seq system was used for sequencing the quail liver transcriptome libraries. The raw reads were quality trimmed, assembled, and enriched for Differential Expression (DE) analysis.
4. Using the common assembly as reference, DE analysis was conducted using DESeq 2 – Bioconductor. DE transcripts were the blasted against the NCBI Nt database. For each comparison, LogFC was used to separate genes into up- or down-regulation. Pathway enrichment analysis was performed on each of the gene lists using g:Profiler and visualized using Cytoscape v3.6.0 using the Enrichment Map plugin v3.1.

**Results:** When faced with a high dietary cholesterol challenge, RES upregulated pathways that would be needed to maintain cholesterol and cellular homeostasis. SUS upregulated pathways for cholesterol metabolism and bile acid production, but more significantly upregulated pathways for fatty acid metabolism and attenuation of fatty liver conditions. SUS was not able to maintain lipid homeostasis and developed fatty liver and atherosclerosis.

**Conclusion:** Epigenetics plays an important role in atherosclerosis. Our previous study found RES and SUS hosting different gut microbiota that would differentially affect the processing of dietary cholesterol. We now have evidence that selective breeding has also changed the bird's ability to maintain lipid and cellular homeostasis in the liver.

## ABBREVIATIONS

COPD: Chronic Obstructive Pulmonary Disease; RES: Atherosclerosis-Resistant Quail; SUS: Atherosclerosis-Susceptible Quail; RES/CON: RES on Control Diet; RES/CHOL: RES on Cholesterol Diet; SUS/CON: SUS on Control Diet; EUS/CHOL: SUS on Cholesterol Diet; NGS: Next Generation Sequencing; DE: Differential Gene Expression; CE: Cholesteryl Ester; ACAT: Acyl-Coenzyme A (Coa):Cholesterol Acyltransferase; LCAT: Lecithin Cholesterol Acyltransferase; GO Terms: Gene Ontology Terms; ER: Endoplasmic Reticulum; ERAD: ER-Associated Degradation; PM: Plasma Membrane

## INTRODUCTION

Chronic obstructive pulmonary disease (COPD) claimed 3.0 million lives in 2016 and has remained the leading causes of death globally in the last 17 years [1]. Patients with COPD are at increased risk of atherosclerosis and other cardiovascular events. Both conditions are systemic disorders with overlapping mechanisms and pathophysiologic processes. Cardiovascular diseases are an important cause of morbidity and mortality in COPD [2]. Although environmental factors such as diet or smoking play an important role in atherosclerosis development, genetic factors represent consequential determinant of atherosclerotic

cardiovascular disease risk. Advances in techniques of molecular genetics have revealed that genetic disorders significantly influence susceptibility to atherosclerotic vascular diseases [3]. Although recent genome-wide association studies (GWAS), have confirmed that mediators of inflammation and perturbed lipid metabolism are major players in cardiovascular disease (CVD) development, much of individual disease heritability remains unexplained by the variants identified through GWAS [4]. There are also limitations in using clinical trials and human subjects for genetic studies involving the interaction between genetic and environment.

The atherosclerosis-resistant (RES) and -susceptible (SUS), Japanese quail is a proven animal model for studying atherosclerosis [5-9]. The two strains were developed by divergent selection from a common foundation population. When challenged with a high cholesterol diet, about 80% of the SUS males will develop atherosclerosis whereas only about 4% of the RES males will [10]. One reason why the Japanese quail is a good model to study atherosclerosis is that it can develop "complex" vascular lesions (focal hemorrhage, calcification and fibrosis) that are very similar to lesions in human [10-12]. Previous studies [10,13], have shown that after cholesterol feeding, plasma cholesterol levels remain high for a significantly longer time in the SUS than in the RES males. In addition, SUS males have fatty livers and higher amounts of liver cholesterol than RES males. Li *et al.* [8] used RT-PCR to examine the differential expression (DE), of 7 candidate genes (*HMGCR, FDFT1, SQLE, DHCR7, ABCG5, ABCG8,* and *APOA1*), in the liver of RES and SUS males with and without dietary cholesterol challenge and reported that RES metabolized and excreted cholesterol faster than SUS. However, the number of genes that they have sampled was small and thus could not draw any conclusion on how the gene system works or how the gene system has been affected by selective breeding. The objective of the present study was to conduct NGS analysis of DE in the liver of RES and SUS quail fed a control and a cholesterol-enriched diet.

## MATERIALS AND METHODS

All experiments were performed in accordance with protocols reviewed and approved by the UBC Animal Care Committee (Certificate # A12-0087). Liver tissue samples were collected from the same RES and SUS individuals whose intestinal contents were analyzed in Liu *et al.* [9,14]. The rearing and feeding protocol has been described in detail in Liu *et al.* [14]. Briefly, 80 RES and 80 SUS were fed a semi-synthetic diet [14], prepared according to the National Research Council (NRC) nutrient requirements standards recommended for Japanese quail (http://www.nap.edu/catalog/2114.html) from hatching to 6 weeks of age. At six weeks of age, they were divided into two dietary treatment groups and fed either the semi-synthetic diet (control) or the semi-synthetic diet with additional cholesterol (0.5% w/w) for another 6 weeks [8]. RES on control diet and on cholesterol diet were designated as RES/CON and RES/CHO, respectively. SUS on control diet and on cholesterol diet were designated as SUS/CON and SUS/CHO, respectively. Individual birds were identified by numbered wing bands. Both RES and SUS fed the same diet were kept in the same pen. Birds on the alternative diets were kept in a neighboring pen. The two side-by-side pens should have similar environment. At 12 weeks of age, four males from each of the four treatment groups with body weight closest to the mean of the population were euthanized by decapitation. Trunk blood was collected into Vacutainer tubes containing lithium heparin and centrifuged at 4 °C for 10 min at 3,000 × g. Plasma was stored at −20 °C until it was later used for lipid analysis [14]. Livers were then quickly removed and a tissue sample was dissected from the lower part of the right lobe and stored in RNAlater reagent (Cat# AM7023, Ambion, Austin, TX, USA) at -20$^0$C until use. The aortic tree (the brachycephalic arteries to their bifurcations and the aorta to the iliac branching) of each bird was dissected out, opened longitudinally and examined under a 10–30X dissecting microscope for a semi-quantitative scoring [12] of the seriousness of the atherosclerotic lesions on the interior wall. A score of 0 (normal) to 4 (presence of severe atherosclerotic lesions) was assigned by two independent scorers who were blind to the genetic and diet status of the bird.

### Total RNA extraction

The liver samples were homogenized in 10 - 20 volumes of TRI Reagent™ Solution (Cat#AM9738, Invitrogen, Carlsbad, CA, USA) and incubated for 5 -10 min at room temperature. After centrifugation at 12,000 x g for 15 min at 4$^o$C, the lower part of the sample was transferred to fresh tubes. Chloroform (Sigma-Aldrich, Cat# 02487, St. Louis, MO, USA) and 20% of TRI Reagent ™ solution were applied to the sample, mixed well and incubated for 15 min at room temperature. The samples we centrifuged at full speed for 15 min at 4$^o$C and the aqueous phase was transferred to fresh tubes. The supernatant was taken and same volume of pre-chilled isopropanol was applied. After incubation at -20 $^o$C, the sample was centrifuged again at 4$^o$C and the pellet saved. After washing with pre-chilled 75% ethanol, the pellet was briefly air-dried and RNA was dissolved in the RNAse free water (QIAgen Cat#129112, Toronto, ON, Canada), for the Illumina Mi-Seq platform (Illumina, San Diego, CA, USA). Total RNA content of each sample was measured by using NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA, USA ), and the quality of RNA samples was assessed by 1% agarose gel electrophoresis and Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA, USA) before proceeding to further downstream analyses.

### cDNA Library Preparation

The cDNA libraries were constructed with the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA) following the manufacturer's protocol using 1 µg of total RNA from each liver. The poly-A-containing mRNA molecules were purified using poly-T oligo-attached magnetic beads and fragmented using divalent cations under elevated temperature, then the first strand cDNA was synthesized using reverse transcriptase and random primers, followed by second-strand cDNA synthesis using DNA polymerase I and RNase H. The cDNA fragments then underwent end repair, addition of a single "A" base, and ligation of the adapters. The ligation products were purified and enriched with PCR to create a final cDNA library. The libraries were quantitated with Qubit 2.0 Fluorometer (Life Technologies, Foster City, CA, USA) and validated with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

### Sequencing on Mi-Seq and preprocessing of Raw Reads

Illumina Mi-Seq system (Illumina, SanDiego, CA), was used for sequencing the quail liver transcriptome library using Sequencing by Synthesis (SBS) technology. The 16 libraries were run in one go following manufacturer's instructions using the Mi-Seq Reagent Kit v2 (Illumina, SanDiego, CA), with 2 x 150 PE sequencing. Mi-Seq Control Software 2.2.0-RTA 1.17.28.0—CASAVA-1.8.2 was used to generate paired-end and single-end data in FastQ format [15]. Raw reads were filtered with Q20 quality trimming to remove low quality reads with average quality score <20 and trimming of low quality bases from the end of reads [14]. Sequence pre-processing software Trimmomatic v0.30 [16], was used to obtain clean paired-end and single-end Mi-Seq data in a FastQ format which was also subjected to quality control using FastQC. The high quality, filtered reads were used for downstream analyses. Velvet Assembler [17], was used for *de novo* assembly using chicken and turkey genome as reference.

### Differential Expression (DE) analysis

In order to perform transcript abundance and DE analysis, sequence assemblies from different samples were merged to serve as the common reference for groups being compared. Transcript abundance and differential expression was determined with RSEM / EBseq software tools (http://deweylab.biostat.wisc.edu/rsem/README.html). Using the common assembly as reference, DE analysis was done using DESeq 2 – Bioconductor [18]. Genes were considered differentially expressed if PPDE was greater than or equal to 0.95. DE transcripts were the blasted against the NCBI Nt database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Transcripts with a blastn coverage in the first quantile were removed, as these may be spurious hits. In order to perform pathway enrichment analysis, transcripts that did not have a corresponding *Gallus gallus* (chicken) gene name were removed. For each comparison, LogFC was used to separate genes into up- or down-regulation. Pathway enrichment analysis was performed on each of the gene lists using g:Profiler (PMID:27098042), version r1741_e90_eg37 against the *Gallus gallus* database with the query ordered by descending logFC for upregulated genes, and ascending logFC for downregulated genes. Significance threshold was set to 'Benjamini-Hochberg FDR' [19]. Results of the pathway enrichment was visualized using Cytoscape v3.6.0 using the Enrichment Map plugin v3.1 (PMID: 21085593).

## RESULTS AND DISCUSSION

### Atherosclerotic lesions on the intimal surface of the aortae

All SUS and RES fed the control diet scored 0. All four SUS on cholesterol diet scored 4, while two RES on the same diet scored 0 and two scored 1 [14].

### Plasma lipid parameters

There was a significant diet X genotype interaction in plasma Total Cholesterol (TC) (P<0.006; Table 1), LDL (P<0.004; Table 2) levels, and LDL/HDL ratio (P<0.004 Table 3). SUS fed the cholesterol-enriched diet was significantly higher in these parameters than the other three treatment groups [14].

**Table 1:** Significant (P<0.006) Diet X Genotype interaction in plasma total cholesterol level (mmol/L) [14].

| N=16 | Genotype* | |
|---|---|---|
| **Diet** | **RES** | **SUS** |
| **Control** | 4.75 ± 0.39[a] | 5.29 ± 0.46[a] |
| **Cholesterol** | 14.10 ± 1.07[a] | 36.65 ± 6.57[b] |

* RES = Atherosclerosis-resistant quail; SUS = Atherosclerosis-susceptible quail

**Table 2:** Significant (P<0.004) Diet X Genotype interaction in plasma LDL level (mmol/L) [14].

| N = 16 | Genotype* | |
|---|---|---|
| **Diet** | **RES** | **SUS** |
| **Control** | 1.04 ± 0.06[a] | 1.29 ± 0.10[a] |
| **Cholesterol** | 8.07 ± 2.09[a] | 32.03 ± 6.42[b] |

* RES = Atherosclerosis-resistant quail; SUS = Atherosclerosis-susceptible quail

**Table 3:** Significant (P<0.004) Diet X Genotype interaction in plasma LDL/HDL ratio [14].

| N = 16 | Genotype* | |
|---|---|---|
| **Diet** | **RES** | **SUS** |
| **Control** | 0.34 ± 0.02[a] | 0.38 ± 0.05[a] |
| **Cholesterol** | 2.45 ± 0.87[a] | 8.52 ± 1.48[b] |

* RES = Atherosclerosis-resistant quail; SUS = Atheros

### Differential Expression Analysis

**Comparison between RES/CON and RES/CHO:** Differentially expressed liver genes when comparing RES/CON and RES/CHO grouped by enriched pathways (GO terms), are listed in Supplementary Table S1. There were 63 genes more expressed and 18 genes less expressed by RES/CHO than RES/CON. As a result, there were 31 upregulated pathways and 5 downregulated pathways in RES/CHO when compared with RES/CON.

One of the most significantly upregulated pathways was *Catalytic Activity* (see lower left corner of Figure 1), which mediates steps in reverse transportation of cholesterol. Cholesterol is needed for cell growth and viability (Pathway *Cholesterol biosynthetic process*). The prevailing view is that mitochondrial NAD(H) pools tend to be oxidized and important for energy homeostasis, whereas cytosolic NADP(H) pools tend to be highly reduced for reductive biosynthesis (Pathway *Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor*). During fasting, acetyl-CoA is used as a precursor for the formation of ketone bodies within mitochondria using $NAD^+$, but following feeding, it is used as a precursor for cholesterol and lipid synthesis using NADPH as a co-factor [20]. Excess cellular cholesterol is stored as cholesteryl esters (CE). The conversion of cholesterol to CE is catalyzed by the enzyme acyl-coenzyme A (CoA): cholesterol acyltransferase (ACAT). However, chronic accumulation of CE in macrophages

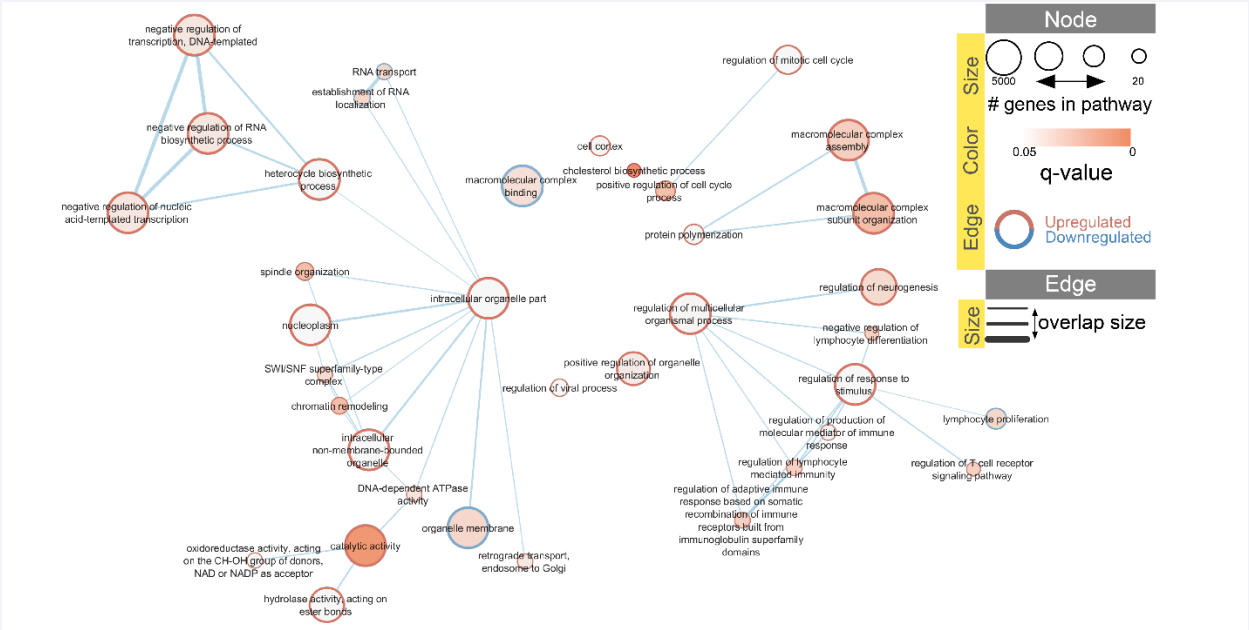


**Figure 1** Upregulation/downregulation of enriched pathways (GO terms) when RES was challenged with a high cholesterol diet.

causes these cells to appear foamy and is a hallmark of early stages in atherosclerosis. Heterocyclic amides potently inhibited liver ACAT (Pathway *heterocycle biosynthetic process*) [21, 22]. In addition, lecithin cholesterol acyltransferase (LCAT) catalyzes the esterification of HDL cholesterol causing it to relocate from the surface of the lipoprotein to the core of the particle. The surface of HDL is therefore available to accept more free cholesterol for transportation. HDL was found to also transport endogenous miRNAs, and deliver them to recipient cells [23]. However, HDL-carried miRNAs contribute to altering HDL functions and HDL effect on endothelial cells and macrophages. Down regulation of *RNA transport* may help maintain the normal properties of HDL cholesterol [24]. In the liver, the scavenger receptor class B1 (SR-B1) modulates the selective uptake of HDL CE by hepatocytes. HDL complexes with SR-B1 is endocytosed (Pathway *Retrograde transport, endosome to Golgi*). CE is hydrolyzed by cholesterol ester esterase (Pathway *Hydrolase activity, acting on ester bonds*), and secreted as biliary cholesterol or utilized to produce steroid hormones [25]. However, SR-B1 also binds modified LDL and maleylated BSA (M-BSA), and contribute to the development of foam cells [26]. Downregulation of Pathway *Macromolecular complex binding* may be necessary in cholesterol-filled cells.

Three major apolipoproteins are synthesized in the liver (Pathway *Macromolecular complex assembly*): apoB, apoA-I, and apoE. ApoB is a large apolipoprotein involved in the transport of triglyceride and cholesterol from the liver to the peripheral tissues [27]. Cholesterol transport into mitochondria is the rate-determining and hormone-sensitive step in steroid biosynthesis. Mitochondrial translocator protein and the steroidogenic acute regulatory protein were shown to be critical for this process. These two proteins functionally interact to facilitate cholesterol transport and may be part of a larger multimeric mitochondrial complex of proteins assembled (Pathway *Macromolecular complex subunit organization*) to facilitate the hormone- induced cholesterol transfer into mitochondria [28]. Cholesterol-loaded livers are highly susceptible to TNF-a and Fas-mediated apoptosis by a mechanism that involves mitochondrial oxidative stress [29]. Upregulating *DNA-dependent ATPase activity* may counter oxidative damages [30]. Na (+)-K (+)-ATPase is involved with the maintenance of ion gradients across the plasma membrane (PM) [31]. Dietary cholesterol can induce genes involved in acute inflammation, including three genes of the serum amyloid A family, three major histocompatibility class II antigen genes, and various cytokine-related genes. Atherosclerosis-resistant C57BL/6ByJ mouse strain was largely resistant to dietary induction of these inflammatory and fibrotic response genes, indicating a negative regulation of gene-specific transcription (Pathway *Negative regulation of nucleic acid-templated transcription*) by the liver [32].

When challenged with high dietary cholesterol, RES upregulated several pathways related to *Lymphocyte mediated immunity* (See lower right hand corner of Fig 1). Lymphocytes can be classified into different subtypes: CD4+ TH1 cells; CD4+ Tregs; CD8+ T cells; B cells; NKT cells; NK cells. Microenvironmental cues in the form of cytokines and co-stimulatory triggers guide T cells down different functional routes. Studies have found that subtypes like Tregs and B cells are athero-protective, but other subtypes such as CD4+ T cells, CD8+ T cells, and NKT cells have been shown to accelerate atherosclerosis. Preventing lymphocytes to differentiate (Pathway *Negative regulation of lymphocyte differentiation*), into and minimizing the proliferation (downregulated Pathway *Lymphocyte proliferation*) of these subtypes may play a crucial role in slowing atherosclerosis development [33].

In summary, when faced with a high dietary cholesterol challenge, RES upregulated pathways that would maximize lipid metabolism, cholesterol transport and bile acids production to excrete excess cholesterol, upregulate pathways to optimize

lymphocyte functions, minimize oxidative stress and damage, and down regulate pathways that would promote foam cell formation.

**Comparison between SUS/CON and SUS/CHO**: Differentially expressed liver genes when comparing SUS/CON and SUS/CHO grouped by enriched pathways (GO terms) are listed in Supplementary Table S2. There were 103 more expressed genes and no less expressed genes by SUS/CHO than SUS/CON. As a result, there were 28 upregulated pathways in SUS/CHO when compared with SUS/CON. Surprisingly, only 2 genes, *FDFT1* and *MLLT4*, were similarly more expressed in RES challenged with dietary cholesterol.

One of the most significantly upregulated pathways was *cellular catabolic process* (Figure 2). Triglycerides from *de novo* lipogenesis (Pathway *Small molecule binding*) [34], and from internalized remnant lipoproteins and chylomicrons are packaged in VLDL (Pathway *ATP binding*) and delivered to peripheral tissues such as adipose and muscle for storage and energy utilization. The liver can also package significant quantities of triglycerides in the form of cytosolic lipid droplets that can be harvested to supplement energy reserves during times of fasting or nutrient deprivation [35]. However, when exposed to a diet rich in lipids, hepatocytes will be overloaded with these fat-storage organelle and develop non-alcoholic fatty liver disease that may result in liver fibrosis.

Cholesterol is oxidized in the liver into a variety of bile acids (Pathway *cholesterol metabolic process*). High levels of *STARD4* (Pathway *Cholesterol Binding*), increases the synthesis of bile acids and CE in liver hepatocytes. *STARD5* (Pathway *Cholesterol Binding*), binds both cholesterol and 25-hydroxycholesterol and appears to function to redistribute cholesterol to the endoplasmic reticulum (ER) and/or the PM [36]. The PDZ1 domain of the four PDZ domain-containing protein PDZK1 has been reported to bind the C terminus of the HDL SR-BI (Pathway *Protein C-terminus binding*), and to control hepatic SR-BI expression and function [37]. SR-B1 modulates the selective uptake of HDL CE by hepatocytes.

Mitochondria are capable of both the accumulation of Mg2+ and the release of Mg2+ (Pathway *Magnesium ion binding*). Magnesium is necessary for the activity of LCAT and lipoprotein lipase (LPL), which lower triglyceride levels and elevate the carrying capacity of HDL [38].

Several redox and oxidant signaling pathways involving cholesterol are at play in fatty liver disease development. These include impairment of the mitochondrial (Pathway *Regulation of autophagy of mitochondrion*), and lysosomal function (Pathway *Lysosomal transport*), by cholesterol loading of the inner-cell membranes (Pathway *Whole Membrane*); formation of cholesterol crystals and hepatocyte degradation; and crown-like structures surrounding degrading hepatocytes, activating

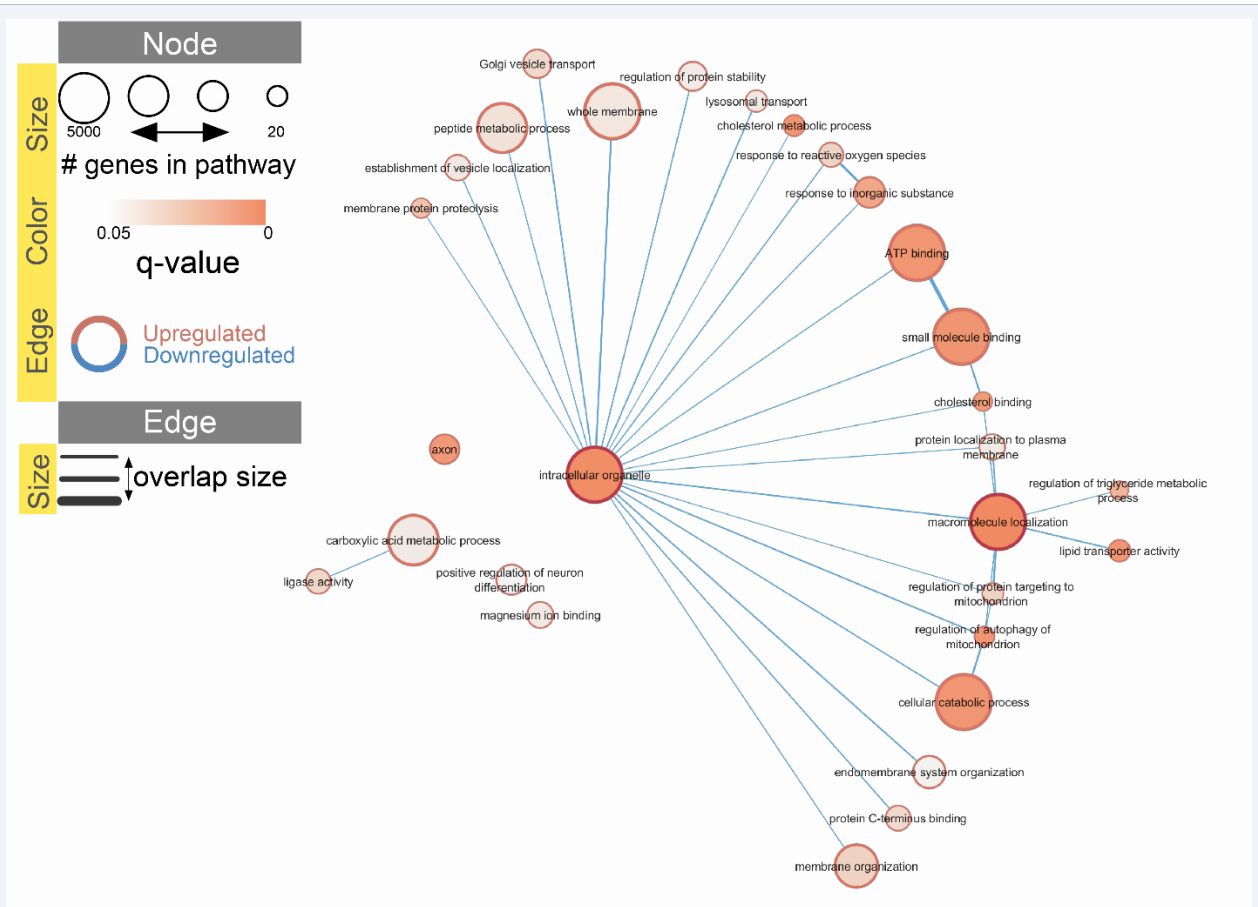


**Figure 2** Upregulation/downregulation of enriched Pathways (GO terms) when SUS was challenged with a high cholesterol diet.

Kupffer cells, and evoking inflammation. Cholesterol loading in hepatocytes can result in chronic HIF-1α activity because of the decreased oxygen availability and excessive production of nitric oxide and mitochondrial reactive oxygen species (Pathway *Response to reactive oxygen species*). The failure to respond to ROS is associated with reduced levels of antioxidants, mitochondrial damage, hepatocyte cell death, activation of the immune system and induction of pro-fibrotic mediators [39,40]

While the ER produces the bulk of the structural phospholipids and cholesterol, together with significant levels of triglyceride and CE with non-structural roles, the PM is a scaffold involving in cell-to-cell communication and the initiation of intracellular signals among other functions (Pathway *Endomembrane system organization*) [41]. Critical to this function is the PM compartmentalization in lipid rafts that control the localization (Pathway *Macromolecule localization*), and productive interactions of proteins involved in cell signal propagation (Pathway *Protein localization to plasma membrane*). The appropriate localization of proteins is crucial because it provides the physiological context for their function. Aberrant localization of proteins contributes to the pathogenesis of many human metabolic and cardiovascular diseases. In addition, cells are divided into compartments limited by other membranes whose integrity and homeostasis are finely controlled (Pathway *Establishment of vesicle localization*), and which determine the identity and function of the different organelles (Pathway *Intracellular Organelle*) [42]. Lysosome is a key coordinator in the sorting and delivery of lipids to several membrane compartments. Endogenous lipids are also sorted by the lysosome when it fuses with double-membraned autophagosomes. The role of lysosomes is well established in lipid catabolism and in dietary lipid overload, when cellular lipid content or composition induces changes to alter autophagic activity in a tissue-specific manner.

A growing body of evidence indicates a causal link between lysosomal cholesterol accumulation and inflammation leading to cardiovascular disease and liver steatosis [43]. Disruption of a Golgi system interferes with the modification, sorting and delivery of proteins, and with larger cellular processes such as cell migration, stress responses and apoptosis [44]. C-terminal-binding protein 1 (CTBP1), has dehydrogenase activity and is involved in controlling the equilibrium between tubular and stacked structures in the Golgi complex (Pathway *Protein C-terminus binding*) [45].

Triglycerides represent the major form of storage and transport fatty acids within cells and in the plasma. The liver is the central organ for fatty acid metabolism (Pathway *Regulation of triglyceride metabolic process*). Fatty acids accrue in liver by hepatocellular uptake from the plasma (Pathway *Lipid transporter activity*), and by *de novo* biosynthesis. Fatty acids are eliminated by oxidation within the cell (Pathway *carboxylic acid metabolic process*), or by secretion into the plasma within triglyceride-rich VLDLs (Pathway *Lipid transporter activity*). Under normal circumstances, the liver stores only small amounts of fatty acids as triglycerides. When exposed to a high dietary lipid, hepatic fatty acid metabolism is altered (Pathway *Peptide metabolic process*) [46]. Dysregulated lipid transport and metabolism may lead to the accumulation of triglycerides within hepatocytes, resulting in liver steatosis.

Studies have demonstrated that the autonomic nervous system innervating the liver (Pathway *Positive regulation of neuron differentiation*), plays a crucial role in regulation of hepatic lipid homeostasis, inflammation and fibrosis. Some of the upregulated pathways identified in individuals with Fatty Liver Syndrome have originally been identified from neural tissues. Additionally, there is growing evidence that neurotrophic factors (Pathway *Small molecule binding*), can modulate all stages of non-alcoholic fatty liver disease [47]. The synthesis of phosphatidylcholine, the major membrane phospholipid, occurs in axons (Pathway *Axons*), and its synthesis at this location is required for axonal elongation. Cholesterol is delivered from cell bodies to axons by anterograde transport [48].

Liver fibrosis is characterized by the accumulation of collagen-rich extracellular matrix. E3 ubiquitin ligase (Pathway *ligase activity*), is involved in collagen synthesis in liver fibrosis. Collagen I is a major component of the extracellular matrix essential for supporting and organizing most tissues. Malformed collagen I chains are removed by retrotranslocation into the cytosol, followed by ER-associated degradation (ERAD), a process that reduces the burden of excess unfolded proteins (Pathway *Peptide metabolic process*) on the ER. E3 ubiquitin ligases catalyze ubiquitination, which can tag specific proteins for degradation [49].

In summary, SUS upregulated pathways in cholesterol metabolism and bile acid production when challenged by high dietary cholesterol, but the more significant upregulated pathways were for fatty acid metabolism and attenuation of fatty liver conditions.

**Comparison between RES/CHO and SUS/CHO:** Differentially expressed liver genes grouped by enriched pathways (GO terms) when comparing RES/CHO and SUS/CHO are listed in Supplementary Table S3. There were 216 genes in 32 pathways more expressed in RES/CHO than SUS/CHO. There were 94 genes in 20 pathways more expressed by SUS/CHO than RES/CHO.

In comparison with SUS/CHOL, RES/CHOL pathways for maintaining cholesterol homeostasis and cellular homeostasis [50], were significantly more expressed. One of the highly significantly upregulated pathways was *Intracellular membrane-bounded organelle* (Figure 3). Intracellular organelle includes nucleus, mitochondria, plastids, vacuoles, and vesicles (Pathway *Cytoplasmic part*). The endomembrane system (Pathways *Whole Membrane, Protein kinase C binding, Coated membrane*), includes the ER (Pathways *ubiquitin-dependent ERAD pathway, 'de novo' posttranslational protein folding*), Golgi apparatus (Pathway *Golgi vesicle transport*), and lysosomes (Pathways *Catalytic activity, Protein glycosylation*). Vesicles also allow the exchange of membrane components with a cell's PM (Pathways *Phosphatidylinositol binding, Protein N-linked glycosylation via asparagine*). Intracellular trafficking plays a major role in the proper disposition of internalized cholesterol and in the regulation of cholesterol efflux (Pathways *Catabolic process*) [41,51].

The nucleus contains the majority of the cellular genome and keeps this information physically separated from other cellular functions (Pathway *Nucleoside triphosphate metabolic*

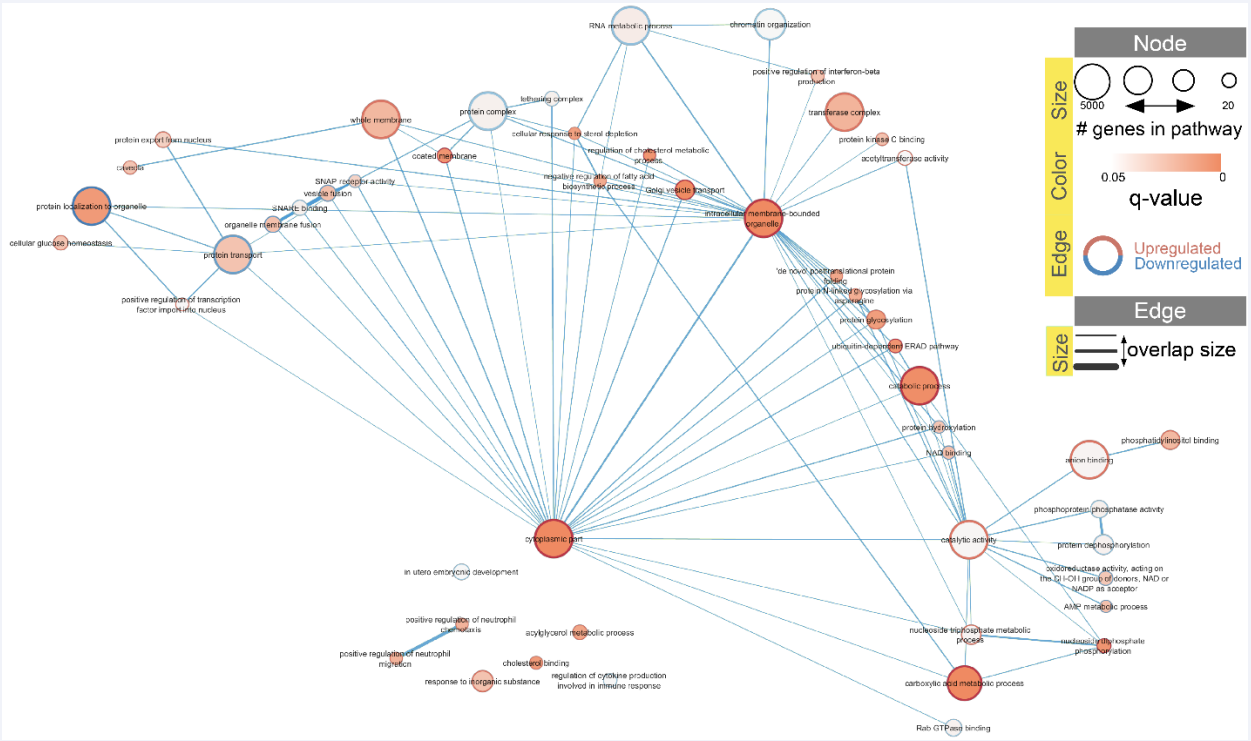


**Figure 3** Upregulation/downregulation of enriched Pathways (GO terms) when comparing RES and SUS, both on a high cholesterol diet.

*process*). The nucleoplasm is separated from the cytoplasm by the nuclear envelope, a double-bilayer membrane contiguous with the ER. Transport through the envelope is controlled by large multiprotein complexes called nuclear pore complexes (NPCs) (Pathway *Protein export from nucleus*). One of the most common methods by which cells control protein distribution is by modifying the binding affinity of the localization signal for its corresponding receptor. The nuclear localization of many cell cycle–related proteins is controlled by phosphorylation events in or near the Nuclear Localization Sequence (NLS) [52] (Pathway *Positive regulation of protein localization to nucleus*).

In order to maintain cholesterol homeostasis, RES/CHOL also upregulated the following pathways in comparison with SUS/CHOL: *Regulation of cholesterol metabolic process; Cholesterol binding; Transferase complex; Caveola; Response to inorganic substance.* Lipid homeostasis pathways: *negative regulation of fatty acid biosynthetic process; carboxylic acid metabolic process; Acetyltransferase activity; Nucleoside diphosphate phosphorylation.* Anti-atherosclerosis pathways: *Positive regulation of neutrophil migration; Positive regulation of neutrophil chemotaxis; Positive regulation of interferon-beta production; Anion binding.*

In comparison with RES/CHOL, the inadequacy of SUS/CHOL to maintain lipid homeostasis became obvious. Among the many pathways that were more expressed by RES/CHOL, there were 6 pathways that SUS has already upregulated when challenged with high dietary cholesterol. Never the less, pathways that were more expressed by SUS/CHOL than RES/CHOL can be grouped into 3 related areas,

1. Maintaining cholesterol homeostasis: *RNA metabolic process; SNARE binding; Protein dephosphorylation; Tethering complex; Protein complex; Protein hydroxylation; AMP metabolic process* [53].
2. Promoting lypogenesis and Increasing cellular triglycerides level: *Chromatin organization; Organelle membrane fusion; Protein localization to organelle; Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor; NAD binding.*
3. Attenuation of steatosis or atherosclerosis: *Rab GTPase binding; Intracellular membrane-bounded organelle; Phosphoprotein phosphatase activity; Regulation of cytokine production involved in immune response; Protein transport; in utero embryonic development* (This GO term may be misleading. The genes in this pathway are involved with repressing polymerase II transcription and suppressing cell death) [54].

In summary, RES/CHOL was able to maintain lipid and cellular homeostasis to avoid the development of atherosclerosis, whereas SUS/CHOL was unable to maintain lipid homeostasis and resulting in development of Fatty Liver and atherosclerosis [55].

**Comparison between RES/CON and SUS/CON:** Differentially expressed liver genes when comparing RES/CON and SUS/CON grouped by enriched pathways (GO terms) are listed in Supplementary Table S4. There were 162 genes in 11 pathways that were more expressed in RES/CON than SUS/CON. There were 33 genes in 1 pathway that were more expressed by SUS/CON than RES/CON (Figure 4).

The SUS and RES were developed by divergent selection from the same foundation population. The selection criterion

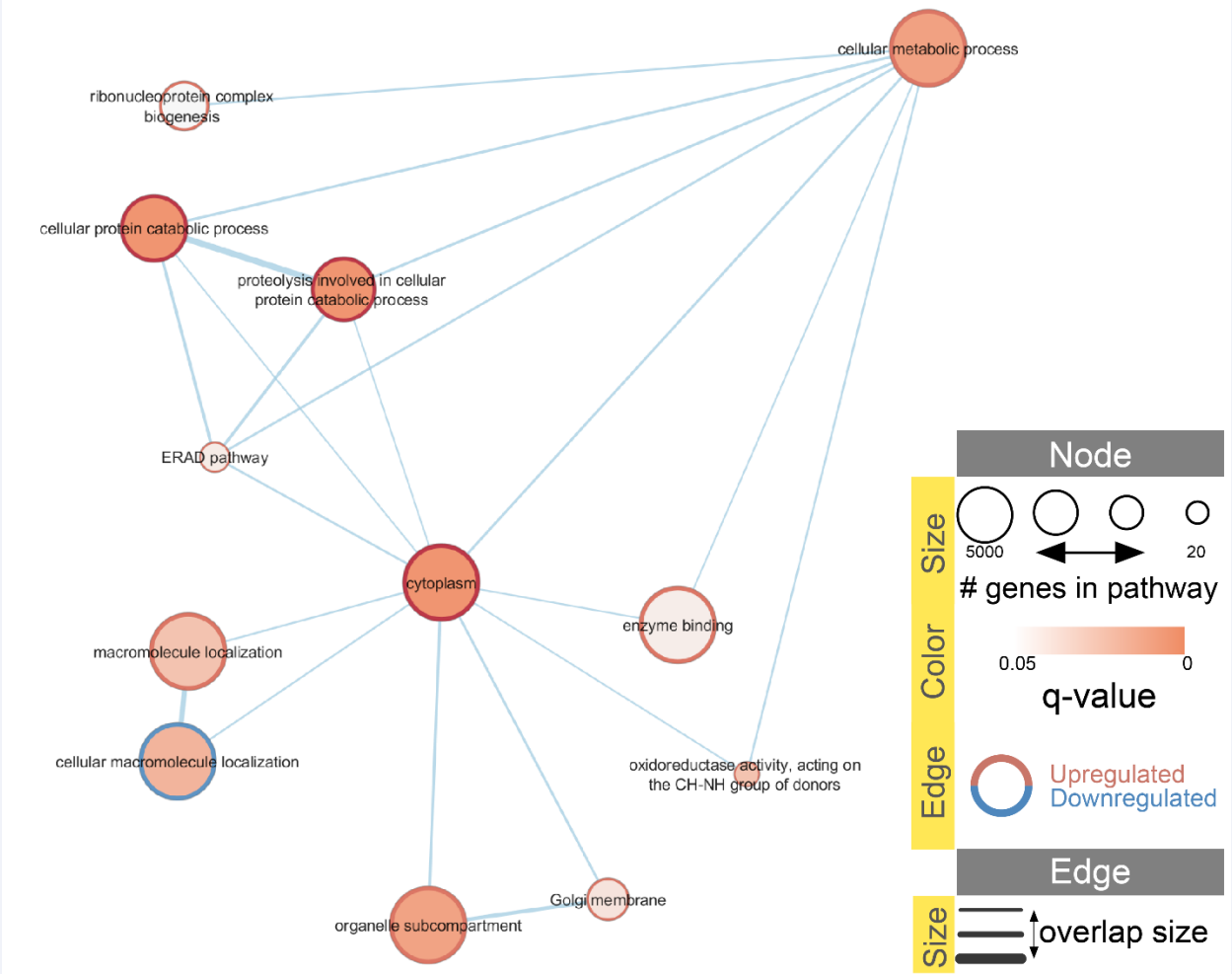


**Figure 4** Upregulation/downregulation of enriched Pathways (GO terms) when comparing RES and SUS, both on control diet.

was atherosclerotic plaque score after the birds were exposed to dietary cholesterol. The DE when the birds were on control diet was therefore a correlated response to the selection. Pathways that were upregulated by RES/CON in comparison with SUS/CON can be grouped into two related areas:

1. Regulation of lipid metabolism – *Cytoplasm; Enzyme binding; Golgi membrane.*
2. Tumor suppression, disease or toxin resistance – *Cellular metabolic process; Oxidoreductase activity, acting on CH-NH group of donars;*

ER maintenance --*Cellular protein catabolic process; ERAD pathway; Macromolecule localization; Proteolysis involve in cellular protein catabolic process.*

DNA damage repair -- *Ribonucleoprotein complex biogenesis;*

Modulation of inflammatory response -- *Organelle subcompartment; Enzyme binding.*

It is well known that wild type Japanese quail has high disease resistance and high tolerance of toxic chemicals [56- 58]. It is more likely that selection for susceptibility to atherosclerosis has suppressed the expression of the genes in these pathways in SUS/CON rather than the selection for resistance to atherosclerosis has upregulated these pathways in RES/CON.

Only one pathway, *Cellular macromolecule localization,* was upregulated in SUS/CON compared with RES/CON. An examination of the genes in this pathway revealed that they were mostly associated with Fatty Liver Syndrome, cancer cell adhesion, migration and metastasis, essential hypertension, and response to mycotoxin.

## CONCLUSION

Epigenetics plays an important role in atherosclerosis [59,60]. Liu *et al.* [9,14], examined the gut microbiota of the same birds used in this study and found that RES and SUS fed the same diets and reared in the same environment were hosting different gut microbiome. They have provided evidence that the microbiome in the ileum and ceca of RES contributed significantly towards the resistance to diet induced atherosclerosis. The epigenetics of the intestinal wall probably created intestinal environment that favors different microbes. The intestinal wall also affects the absorption of dietary cholesterol [61], and the selection for resistance to atherosclerosis is likely to have affected, directly or indirectly, the intestinal pathways involving in the absorption of

cholesterol [62]. As such, there may be less dietary cholesterol reaching the RES liver than expected and made it easier for RES to maintain lipid and cellular homeostasis in the liver.

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