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Colon transcriptome is modified by a dietary pattern/atorvastatin interaction in the Ossabaw pig

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Abstract

Optimizing diet quality in conjunction with statin therapy is currently the most common approach for coronary artery disease (CAD) risk management. Although effects on the cardiovascular system have been extensively investigated, little is known about the effect of these interventions in the colon and subsequent associations with CAD progression. To address this gap, Ossabaw pigs were randomly allocated to receive, for a six-month period, isocaloric amounts of either a heart healthy-type diet (HHD; high in unrefined carbohydrate, unsaturated fat, fiber, supplemented with fish oil, and low in cholesterol) or a Western-type diet (WD; high in refined carbohydrate, saturated fat and cholesterol, and low in fiber), without or with atorvastatin therapy. At the end

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Author Statement

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Competing Interests statement

The authors declare no competing interests.

Supplementary materials

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of the intervention period, colon samples were harvested, mucosa fraction isolated, and RNA sequenced. Gene differential expression and enrichment analyses indicated that dietary patterns and atorvastatin therapy differentially altered gene expression, with diet-statin interactions. Atorvastatin had a more profound effect on differential gene expression than diet. In pigs not receiving atorvastatin, the WD upregulated “LXR/RXR Activation” pathway compared to pigs fed the HHD. Enrichment analysis indicated that atorvastatin therapy lowered inflammatory status in the HHD-fed pigs, whereas it induced a colitis-like gene expression phenotype in the WD-fed pigs. No significant association was identified between gene expression phenotypes and severity of atherosclerotic lesions in the left anterior descending-left circumflex bifurcation artery. These data suggested diet quality modulated the response to atorvastatin therapy in colonic mucosa, and these effects were unrelated to atherosclerotic lesion development.

Keywords

Dietary patterns; Statin; Colon; Atherosclerosis; Inflammation; Ossabaw pig

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death globally [1]. Approximately one-third of US adult deaths are attributable to CVD [2]. Coronary artery disease (CAD) is a type of CVD characterized by the development cholesterol laden plaques in coronary arteries, exacerbated by inflammation and dyslipidemia [2]. The colon contributes to the modulation of cholesterol homeostasis by regulating bile acids resorption and dietary cholesterol bioavailability [3]. Despite recent reports of a *heart-gut axis* [4,5], little is known about the influence of the gastrointestinal tract (GIT), particularly the colon, on CAD progression.

Evidence-based lifestyle recommendations for the prevention and management of CAD include adopting a heart-healthy dietary pattern [6-8], defined by the American Heart Association (AHA) and American College of Cardiology (ACC) as rich in fruits and vegetables, whole grains, healthy proteins, nuts, seeds and legumes, while limiting intake of sodium, saturated fat, processed meats and sugar-sweetened beverages [8]. Heart-healthy dietary patterns have been associated with optimal CVD risk factors, including plasma lipid and lipoprotein profiles, blood pressure and body weight, and higher life expectancy [9,10]. A cross-sectional analysis of gene expression signatures of peripheral blood mononuclear cells from healthy adults concluded that dietary patterns (Prudent *vs.* Western) were associated with altered gene networks related to the immune and/or inflammatory response, cancer and CVD, which may modulate the risk of chronic disease [11]. Additional work focusing on the relation between numerous dietary factors and gene expression signatures in human colon tissue concluded that dietary factors were associated with altered gene expression networks related to cancer, organismal injury, and cell death [12]. Neither study addressed issues concerning the relation between gene expression signatures and clinical endpoints. No evidence is currently available for the effect of dietary patterns on colonic gene expression signatures and subsequent association with CAD progression.

Statin therapy to lower low-density lipoprotein (LDL) cholesterol concentrations is frequently prescribed to individuals diagnosed with or at elevated CAD risk, and who fail to adopt or insufficiently respond to lifestyle modifications [6]. In addition to lower LDL cholesterol concentrations, statin therapy has been reported to increase nitric oxide production, and have antiproliferative and anti-inflammatory effects [13]. In the GIT, statin therapy has been associated with reduced risk of new onset inflammatory bowel disease and lower prevalence of gut microbiota dysbiosis [14,15].

The present study used a transcriptomic approach to assess the effect of two dietary patterns, a heart healthy-type diet (HHD) and Western-type diet (WD), with and without atorvastatin therapy, and their interaction, on colonic mucosa gene expression in the Ossabaw pigs. The heart and colon of the Ossabaw pigs and humans share similar anatomical structures and are comparable in size, making them a good experimental model to study the *heart-gut axis* [16]. This pig breed is a good experimental model of diet-induced metabolic syndrome [17] and CAD [18]. We hypothesized that in the colonic mucosa, unique gene expression signatures associated with atherosclerosis of Ossabaw pigs fed the WD relative to the HHD will be identified, and atorvastatin therapy will modulate these associations. Altered gene expression signatures will be largely involved in intestinal permeability, inflammation, and immune activation.

2. Materials and Methods

2.1. Study design and animals

Presented is an ancillary investigation of a previously reported study designed to determine the impact of two dietary patterns, WD and HHD, without or with atorvastatin therapy (−S or +S), on the progression of CAD in Ossabaw pigs [18]. Thirty-two 5–8 week old pigs (16 boars+16 gilts) were randomly allocated to one of four groups using a 2 × 2 factorial design: WD–S, WD+S, HHD–S, HHD+S. An equal number of boars and gilts was allocated in each group. After a one-month acclimation period the pigs were gradually shifted to their respective experimental diets for an addition 6 months, with incremental increases in energy to meet growth requirements. Two pigs died due to causes unrelated to the interventions, resulting in a final sample size of 30. The Beltsville Agricultural Research Center and Tufts Medical Center/Tufts University Institutional Animal Care and Use Committee approved the study protocol.

2.2. Diets and atorvastatin therapy

Diets were designed to be isocaloric and reflect typical human Western and heart healthy dietary patterns. The composition and ingredients have been previously described [18]. Briefly, both diets provided 47% of energy (E) as carbohydrate, 38% E as fat, and 15% E as protein. The diets differed in the types of carbohydrate and fat, quantity of cholesterol and fiber, and fish oil supplementation. The WD was high in refined carbohydrate (sugar, white flour), saturated fat (butter), and cholesterol, whereas the HHD was rich in unrefined carbohydrate (whole wheat flour, oats), unsaturated fat (canola, soybean and corn oils), and fiber (freeze-dried fruits and vegetables mix, Futureceuticals, Momence, IL). HHD-fed pigs also received fish oil supplements (Epanova 1000 mg [550 mg EPA+200 mg DHA as free

fatty acids], AstraZeneca, Cambridge, MA) three times per week. Pigs in the atorvastatin (Lipitor, Pfizer, New York, NY) therapy groups received 20 mg/day during months 1–3 and 40 mg/day during the months 4–6 of the intervention to accommodate increases in body weight.

2.3. Sample collection

At the end of the intervention period, pigs were euthanized by an intravenous injection of Euthasol (50 mg sodium pentobarbital/kg body weight; Virbac Animal Health, Inc., Fort Worth, TX). Proximal colon segments (2 cm in length) were harvested from an anatomically similar region, cleaned and rinsed with ice-cold PBS, flash-frozen in liquid nitrogen, and stored at −80°C. As previously described, blood samples were also collected at necropsy [18].

2.4. Sample processing

2.4.1. Blood samples—Serum cardiometabolic risk factors, including LDL cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, tumor necrosis factor-alpha (TNF-α), and high-sensitivity C-reactive protein (hsCRP) concentrations, were measured and reported as previously described [18].

2.4.2. Coronary artery histopathology—Histopathological assessment of atherosclerotic lesion severity in the left anterior descending-left circumflex bifurcation arteries, presented as Stary scores [19], were determined by a blinded board-certified veterinary cardiovascular pathologist, as previously reported [18].

2.4.3. Isolation of colonic mucosa and RNA extraction—Frozen colon segments were treated with prechilled RNA*later*-ICE (Invitrogen, Carlsbad, CA) at −20°C for 24 hours to preserve RNA quality and prepare samples for further dissection. Colon segments were opened longitudinally, and the mucosal layer was cleanly separated from the submucosal layer using a scalpel and tweezers. Total RNA from the mucosal layer was extracted using the TRI Reagent according to the manufacturer's instructions (Zymo Research, Irvine, CA). With the addition of RNAseOUT (Invitrogen, Carlsbad, CA) to minimize RNA degradation, residual DNA was removed using TURBO DNA-free kit (Invitrogen, Carlsbad, CA). The RNA quality and concentration were assessed using an Experion RNA StdSens Analysis kit (Bio-Rad, Hercules, CA). All samples had an RNA Quality Indicator greater than 8.

2.5. RNA sequencing

The sample libraries were prepared using Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA) and AMPure XP beads (Beckman Coulter, Hercules, CA). Libraries were quantified using a KAPA Library Quantification kit (KAPA Biosystems, Wilmington, MA) and Experion DNA 1K Analysis kit (Bio-Rad, Hercules, CA), for quality control per manufacture's protocol. Libraries were sequenced using NextSeq 500/550 Output kit v2.5 (Illumina, San Diego, CA) on NextSeq 500 platform (Illumina, San Diego, CA) with 100 base pair single end reads. Raw data in FASTQ format was trimmed for quality by CLC Bio Genomic Workbench (Qiagen, Valencia, CA).

The porcine translational research database (version NR 112918) [20], a manually curated pig genome, was used as reference to assemble and reconstruct the transcriptome. To further validate the results, a secondary analysis using the domestic pig (Ensembl *sus scrofa 11.1*, version 98.111) [21] as genome reference was conducted. The latter genome contained a wider range of annotated genes, but it also contained errors that were manually corrected using the former genome [20]. Comparison Analysis by Ingenuity Pathway Analysis (IPA; v 9.0, Mountain View, CA) was conducted to compare the results generated by these two genomes. All heatmaps presenting sequencing results were generated using Morpheus (Broad Institute, Cambridge, MA) [22].

2.6. Characterizing colonic mucosa cell types and sample homogeneity

To evaluate consistency of colonic mucosa sampling, the xCell tool [23] was used to analyze the RNA sequencing data (reads per kilobase million [RPKM]) that predicted enrichment of various cell types within each colon sample. One sample in the HHD-S group displayed low epithelial cell enrichment relative to all other samples (36% of the mean of other samples), suggesting low presence of colonic mucosa, and was therefore excluded from subsequent analyses, resulting in a final sample of n=29. The epithelial cell enrichment data among the four groups was analyzed by one-way ANOVA (Prism 8, GraphPad Software, La Jolla, CA). No significant differences were identified, suggesting similar enrichment of colonic mucosa among groups.

2.7. Differential expression analysis of RNA-seq data and gene enrichment analysis

Differential expression analysis was performed on a Bioconductor package “edgeR” [24] using a two-factor model design matrix (two-way ANOVA) in R (version 3.5.1; run on RStudio, version 1.0.153, Boston, MA). This model was constructed to determine differential gene expression attributable to dietary patterns, atorvastatin therapy and their interaction. Genes were considered differentially expressed based on a false discovery rate (FDR) ≤ 0.05 and absolute log fold change (logFC) ≥0.6 (absolute fold change ≥1.5). Fold change for genes were interpreted as diet effect (WD *vs.* HHD) and statin effect (+S *vs.* −S). An interaction of diet-statin with FDR<0.05 was considered significant.

To further assess potential interactions by dietary patterns or atorvastatin therapy, analyses adopting an exact test model were conducted in edgeR [24]. Comparison pairs included diet effect within statin groups (WD–S relative to HHD–S, and WD+S relative to HHD+S) and statin effect within diet groups (WD+S relative to WD–S, and HHD+S relative to HHD–S). Results were analyzed in a downstream gene enrichment analysis.

Following differential gene expression analysis, an exploratory gene enrichment analysis was conducted to determine relevant biological pathways and functional annotations (Diseases and Functions) altered by treatments. Genes with an absolute logFC ≥0.6 were uploaded to IPA. A *Z* score was calculated to determine up- or down-regulation of pathways or functional annotations. A term with an absolute *Z* score ≥2 and FDR ≤0.05 was considered statistically significant. In addition, Comparison Analysis in IPA was conducted to visualize interactions between dietary patterns and atorvastatin therapy.

2.8. Analysis Match with public gene expression datasets

To compare the derived biological interpretation of our dataset to other analyses, Analysis Match in IPA was used. The algorithm created a signature from the highest confidence predictions from our query analysis and compared it to the signatures of analyses generated from public gene expression datasets curated by OmicSoft (QIAGEN Mountain View, CA) from Gene Expression Omnubus (GEO), ArrayExpress, Sequency Read Archive (SRA), and other public data sources. This feature enables confirmation of our data interpretation and provides insights into underlying shared biological mechanisms. Matching results were filtered by sample types (colon, colonic mucosa) and ranked by matching Z scores (%) in descending order. Select matching results of interest were scrutinized.

2.9. Correlation analyses among gene expression and clinical traits

To determine the association of gene expression in colonic mucosa with atherosclerotic lesion severity and cardiometabolic risk factors, pigs from all groups were pooled (n=29). The differentially expressed genes and genes involved in pathways altered by dietary patterns and/or atorvastatin therapy were included in this analysis. In total, 95 genes were analyzed. Spearman's correlation coefficients were calculated (Prism 8, GraphPad Software, La Jolla, CA) between expression data of these genes (RPKM) and previously measured atherosclerotic lesion severity (Stary scores in the left anterior descending-left circumflex bifurcation arteries) and serum cardiometabolic risk factors (LDL cholesterol, HDL cholesterol, triglyceride, TNF-α, and hsCRP concentrations) [18]. Due to the exploratory nature of these analyses, an association was considered statistically significant when absolute correlation coefficient $r \geq 0.4$ with a P value $\leq .05$.

2.10. Sex difference

A descriptive secondary analysis was performed in colonic mucosa to determine whether boars and gilts differentially respond to the interventions, using the methods described in the Section "Differential expression analysis of RNA-seq data and gene enrichment analysis." Comparison Analysis in IPA was conducted to assess pathways altered by the main effects of dietary patterns and atorvastatin therapy on the basis of sex.

3. Results

3.1. Differential gene expression analysis

Thirty-one differentially expressed genes with FDR≤0.05 and absolute logFC≥0.6 were identified in colonic mucosa attributable to dietary patterns, atorvastatin therapy, and/or their interaction (Table 1). Of these genes, dietary patterns (WD *vs.* HHD) altered the expression of five genes, and atorvastatin therapy (atorvastatin *vs.* no atorvastatin) altered the expression of 29 genes. Note that all of the genes altered by dietary patterns were also altered by atorvastatin therapy. The expression of 10 genes demonstrated a significant diet-statin interaction.

3.2. Gene enrichment analysis

To assess the biological relevance of differential gene expression to dietary patterns and atorvastatin therapy, IPA was used to evaluate gene enrichment. Genes with absolute logFC ≥0.6 were included to extend our ability to explore potential pathways and biological functions altered by dietary patterns and atorvastatin therapy. Ten pathways were significantly affected by the main effect of dietary patterns (diet effect) and 11 by the main effect of atorvastatin therapy (statin effect; all absolute Z score≥2 and FDR≤0.05, Table 2). The trend of a diet-statin interaction was identified by IPA Comparison Analysis (Fig. 1). Results from the pathway analyses were similar regardless of the databased used; comparison of results between porcine translational research database and domestic pig genome database is presented in Supplemental Fig. 1.

To assess the main diet effect, 311 genes with absolute logFC≥0.6 that differed by dietary patterns were included in the gene enrichment analysis. The pigs fed the WD exhibited four upregulated pathways relative to HHD-fed pigs, including “LXR/RXR Activation” and “PPAR Signaling,” and six downregulated pathways including “Phospholipase,” “p38 MAPK Signaling,” and “TREM1 Signaling” (Table 2).

To assess the main statin effect, 312 genes with absolute logFC≥0.6 that differed by atorvastatin therapy were included in gene enrichment analysis. The pigs receiving atorvastatin therapy exhibited one upregulated pathway, “PPARα/RXRα Activation, and 10 downregulated pathways, including “p38 MAPK Signaling,” “TREM1 Signaling,” “Toll-like Receptor Signaling,” and “LPS/IL1 Mediated Inhibition of RXR Function,” than the pigs not receiving atorvastatin therapy (Table 2).

As results of the differential expression analysis indicated that a substantial portion of genes demonstrated significant diet-statin interaction, IPA Comparison Analysis was conducted to compare different core pathway analyses. To determine if atorvastatin therapy modified the effect of dietary patterns on colonic gene expression, we used the following comparisons (Fig. 1A, B): main effect (WD±S *vs.* HHD±S), pigs not receiving atorvastatin (WD–S vs. HHD–S), and pigs receiving atorvastatin (WD+S *vs.* HHD+S). Results from pathway analysis (Fig. 1A) were consistent between the main effect and pigs not receiving atorvastatin comparisons (4 upregulated, 4 downregulated, all Z score≥2 and FDR≤0.05). However, the diet effect was largely attenuated in pigs receiving atorvastatin. Further, results from functional annotation analysis (Fig. 1B) were consistent between the main effect and pigs not receiving atorvastatin therapy comparisons (1 upregulated, 39 downregulated, all Z score≥2 and FDR≤0.05). In contrast, the vast majority of these functional annotations in pigs receiving atorvastatin therapy responded in the opposite direction. The diet effect was more profound in the pigs not receiving atorvastatin.

To determine if dietary patterns modified the effect of atorvastatin therapy on colonic gene expression, we used the following comparisons (Fig. 1C, D): main effect (WD/HHD+S *vs.* WD/HHD–S), pigs fed the WD (WD+S *vs.* WD–S), and pigs fed the HHD (HHD+S *vs.* HHD–S). Results from pathway analysis (Fig. 1C) were consistent between the main effect and in pigs fed the HHD (1 upregulated, 11 downregulated, all Z score≥2 and FDR≤0.05). However, the statin effect was largely attenuated in the WD-fed pigs. Further, results from

functional annotation analysis (Fig. 1D) were consistent between the main effect and in pigs fed the HHD (40 downregulated, all Z score≥2 and FDR≤0.05). In contrast, the vast majority of these functional annotations in pigs fed the WD responded in the opposite direction. The statin effect was more profound in the HHD-fed pigs.

3.3. Analysis Match with public gene expression datasets

The IPA Analysis Match was conducted to further elucidate insights regarding how atorvastatin therapy affects colonic gene expression within different diet context. Results (Fig. 2A) indicated that the colonic mucosa gene expression pattern of WD+S relative to WD-S fed pigs was similar to that of a microbiota dysbiosis phenotype relative to normal control (mouse colon, Z score=77.96% on predicted Upstream Regulators) [25], and a ulcerative colitis phenotype relative to healthy control (mouse colon, Z score=70.01% on predicted Upstream Regulators) [26]. Results (Fig. 2B) also indicated that the colonic mucosa gene expression pattern of HHD+S relative to HHD-S fed pigs was similar to that of an anti-TNF treatment in Crohn's disease (human colon, Z score=65.57% on predicted Upstream Regulators) [27], and infliximab treatment in ulcerative colitis (human colon, Z score=56.57% on predicted Upstream Regulators) [28].

3.4. Association of gene expression with atherosclerotic lesion severity and cardiometabolic risk factors

3.4.1. Differentially expressed genes—Among the 31 differentially expressed genes altered by diet, statin and/or diet-statin interaction, the expression of *ASS1, CD274, GBP2*, and *SLC6A9* in the colonic mucosa were negatively associated with serum hsCRP concentrations (Table 3). *CLEC4G* expression was positively associated with serum HDL cholesterol concentrations. *CD5L* expression was positively associated with serum TNF-α concentrations. None of the differentially expressed genes were significantly associated with atherosclerotic lesion severity.

3.4.2. Genes in pathways altered by dietary patterns—Among genes expressed in "LXR/RXR Activation" pathway, *MMP9* was positively associated with atherosclerotic lesion severity, serum LDL cholesterol, HDL cholesterol, and triglyceride concentrations; *PTGS2* was negatively associated with serum LDL cholesterol, HDL cholesterol, and TNF-α concentrations; and *LYZ* was negatively associated with serum triglyceride concentrations (Table 4). *PLA2G3* expressed in both "Phospholipase" and "p38 MAPK Signaling" pathways were negatively associated with serum LDL cholesterol and HDL cholesterol concentrations. Among genes expressed in "TREM1 Signaling" pathway, *CD40* was negatively associated with atherosclerotic lesion severity, and *IL10* was negatively associated with serum LDL cholesterol concentration. No unique genes involved in "PPAR Signaling" and "PPARα/ RXRα Activation" pathways were associated with atherosclerotic lesion severity or serum cardiometabolic risk factors.

3.4.3. Genes in pathways altered by atorvastatin therapy—Among downregulated pathways altered by atorvastatin therapy, only the expression of *CR2* gene in "PI3K Signaling in B Lymphocytes" was positively associated with atherosclerotic lesion severity (Table 5). The gene expression of *CCR3, ICOS, CYBB, TNFSF11, ATF3,*

CD180 in various pathways were negatively associated with serum hsCRP concentrations; expression of *IL10* and *PLA2G3* in various pathways were negatively associated with serum LDL cholesterol concentrations; expression of *IRAK3* and *PLA2G3* in various pathways were negatively associated with serum HDL cholesterol concentrations; and expression of *LYZ* in "Production of Nitric Oxide and Reactive Oxygen Species in Macrophages" pathway was negatively associated with serum triglyceride concentrations. Of note, *APOD* gene in "Production of Nitric Oxide and Reactive Oxygen Species in Macrophages" pathway was positively associated with serum LDL cholesterol, HDL cholesterol and hsCRP concentrations. Among genes involved in the only upregulated pathway exhibited by atorvastatin therapy, "PPARα/RXRα Activation," none of them were significantly associated with atherosclerotic lesion severity or serum cardiometabolic risk factors. No genes involved in these pathways was significantly associated with serum TNF-α concentrations.

3.5. Sex difference

Although the study was under powered to assess sex-specific effect as previously reported [18], this variable was evaluated to identify possible trends. The impact of dietary patterns and atorvastatin therapy on pathways was similar in boars and gilts (Supplemental Fig. 2).

4. Discussion

Recent findings suggest there is an interplay between the gut and heart, referred to as the *heart-gut axis*, and that this relationship can be exploited for use as a therapeutic target for CAD risk reduction [5]. Yet, despite the widespread use of statins as a therapy to lower CAD risk, little is known about the potential pleotropic effects of statin therapy on the *heart-gut axis*, particularly in the colon or potential interactions with dietary modification [14,15]. The present study was designed to address these gaps by assessing the effect of two dietary patterns and atorvastatin therapy, and their interaction, on colonic mucosa gene expression and subsequent association with cardiometabolic risk factors and atherosclerotic lesion development.

Using the Ossabaw pig as a model of diet-induced atherosclerosis, we found that in colonic mucosa the WD compared to the HHD upregulated "LXR/RXR Activation" and "PPAR Signaling" pathways, and downregulated pathways related to proinflammatory immune response, including "TREM1 Signaling" and "p38 MAPK Signaling." We also found that atorvastatin therapy downregulated a number of pathways related to immune response, including "PI3K Signaling in B Lymphocytes," "LPS/IL-1 Mediated Inhibition of RXR Function," and "Toll-like Receptor Signaling." A diet-statin interaction in colonic mucosa was identified. Independent of treatment group, a small proportion of genes involved in these altered pathways were significantly associated with serum cardiometabolic risk factors (LDL cholesterol, HDL cholesterol, triglyceride, TNF-α, and hsCRP concentrations) or atherosclerotic lesion severity. Dietary pattern or atorvastatin therapy had no significant effect on expression of genes related to colonic permeability.

4.1. Diet effects

In colonic mucosa the “LXR/RXR Activation” pathway was upregulated in Ossabaw pigs fed the WD compared to the HHD. Induction of this pathway has been demonstrated to increase basolateral cholesterol efflux from intestinal epithelium into the circulation on HDL [29,30]. This upregulation was likely in response to the higher cholesterol content in the WD than HHD. When the diet effect was compared among the pigs receiving atorvastatin therapy, this effect was no longer significant, suggesting that atorvastatin therapy mitigated the differential diet effect on “LXR/RXR Activation.”

Compared to the HHD, the WD downregulated “p38 MAPK” and “TREM 1 Signaling” pathway in the colonic mucosa. These two pathways are activated by a diverse spectrum of stress stimuli including inflammatory cytokines, lipopolysaccharides (LPS) and reactive oxygen species, leading to proinflammatory immune responses [31-33]. The results were unexpected because the WD has been associated with a proinflammatory gene expression profile in coronary arteries and epicardial adipose tissues from the same pigs [34,35]. Also unexpected, among the genes involved in these pathways, the expression of *CD40* in “TREM1 Signaling” pathway was negatively associated with atherosclerotic lesion severity. The *CD40* gene encodes CD40 molecules, which are essential for mediating a broad variety of immune and inflammatory responses [36]. In the GIT, CD40 has been reported to contribute to proinflammatory functions, including NFkB activation, cytokine secretion, oxidative stress elevation and recruitment of leukocytes and platelets [37-40]. This observation awaits confirmation. Other genes involved in these two pathways (16 out of 17) were not significantly associated with atherosclerotic lesion severity, suggesting these diet-altered inflammation-related pathways in colonic mucosa have minimal association with atherosclerotic lesion development.

Among the diet-altered pathways, the *MMP9* gene expression in “LXR/RXR Activation” pathway was positively associated with atherosclerotic lesion severity, and serum LDL cholesterol and HDL cholesterol concentrations. *PLA2G3* gene expression in “p38 MAPK Signaling” and “Phospholipase” pathways was negatively associated with serum LDL cholesterol and HDL cholesterol concentrations. The *MMP9* gene encodes matrix metalloproteinase 9, and the *PLA2G3* gene encodes a protein that belongs to the secreted phospholipase A_2 family. MMP9 expression is induced in response to inflammation and contributes to atherosclerotic lesion development [41-44]. Prior work suggests MMP9 modulates cholesterol metabolism through inhibition of plasma secretory phospholipase A_2, which affects hepatic transcriptional responses to dietary cholesterol [45]. The significant association between the expression of *MMP9* in colonic mucosa, serum LDL cholesterol and HDL cholesterol concentrations, and atherosclerotic lesion severity suggested that the colon may be a target organ in modulating atherosclerosis progression via MMP9-cholesterol relation.

4.2. Statin effects

The vast majority of the differentially expressed genes were attributable to atorvastatin therapy, and about one-third of the genes had a significant diet-statin interaction. When atorvastatin-treated pigs were compared to pigs not receiving atorvastatin therapy, there

was a down regulation of pathways related to innate and adaptive immune response and inflammatory response. Some of these pathways, including “TREM1 Signaling,” “iNOS Signaling,” “Toll-like Receptor Signaling,” and “LPS/IL-1 Mediated Inhibition of RXR Function” are triggered by LPS, a luminal stimuli and major component of the outer membrane of Gram-negative bacteria [46]. Recently, statin medications have been reported to be associated with lower prevalence of gut microbiota dysbiosis [15]. These observations raise the possibility that atorvastatin therapy may have suppressed colonic inflammation by modifying the gut microbiome.

Interestingly, analyses showed that the pathways altered by atorvastatin therapy were only observed in the colonic mucosa of pigs fed the HHD, not the WD. The IPA Analysis Match found the gene expression pattern in response to atorvastatin therapy in the HHD-fed pigs was similar to that of anti-TNF treatment in humans diagnosed with Crohn’s disease, and that of infliximab treatment in humans diagnosed with ulcerative colitis. Crohn’s disease and ulcerative colitis are two main categories of inflammatory bowel disease, and the above stated treatments are used to lower inflammation in human colon [47,48]. Our results suggested that in Ossabaw pigs fed the HHD, but not WD, atorvastatin therapy lowered inflammatory status in colonic mucosa.

Although none of the pathways assessed were significantly altered by atorvastatin therapy in the WD-fed pigs, functional annotation analysis suggested that atorvastatin induced biological functions related to immune cell trafficking and activated colonic immune responses such as “Binding of leukocytes,” “Adhesion of immune cells,” and “Migration of lymphatic system cells.” The IPA Analysis Match indicated that the effect of atorvastatin on colon gene expression in the WD-fed pigs was similar to that previously reported in colonic tissue from mice with microbiota dysbiosis or ulcerative colitis. Hence, atorvastatin therapy in WD-fed pigs may have triggered colonic inflammation, suggesting a potential side-effect of atorvastatin therapy in this experimental model.

Among the genes involved in pathways altered by atorvastatin therapy, only one (*CR2*) out of 86 was significantly associated with atherosclerotic lesion severity. These findings suggested that the gene expression phenotype in colon induced by atorvastatin therapy had a minimal association with atherosclerotic lesions development in the Ossabaw Pig model.

4.3. Diet-statin interaction

Differential gene expression and pathway analyses identified diet-statin interaction. Among the differentially expressed genes, about one third demonstrated significant interactions. Based on pathway analysis, the main diet effect was only observed in the pigs not receiving atorvastatin, and the main statin effect was only observed in the HHD-fed pigs. Functional annotation analysis indicated that the diet effect in pigs receiving atorvastatin responded in the opposite direction to those pigs not receiving atorvastatin therapy. Additionally, the statin effect in the WD-fed pigs responded in the opposite direction to the HHD-fed pigs. Similar interaction patterns were not identified in our prior investigations in coronary arteries [34] or epicardial adipose tissue [49] of these same pigs. Reasons for these interactions may result from factors associated with changes in the gut microbiome.

4.4. Strengths and limitations

A study strength is that the diets were formulated to mimic those habitually consumed by humans, intending to simulate two dietary patterns, which allow for the study of diet from a holistic rather than individual food or nutrient perspective. The atorvastatin doses were chosen to mimic a dose typically prescribed for human [18].

A limitation of this work is that RNA was isolated from mucosal tissue homogenates that contained multiple cell types, hence, high sampling heterogeneity may have resulted in contamination of RNA from neurons and myocytes. To evaluate the extent of mucosa RNA contamination with other cell types, the xCell tool [23] was used to determine enrichment of different cell types. As a result of this analysis, one sample was excluded due to low epithelial enrichment, attributed to tissue sampling error. The parent study was not designed to determine causality between GIT physiology and development of atherosclerotic lesion severity. Given the exploratory nature of the enrichment analyses, the results should be interpreted with caution.

4.5. Conclusion

Our data indicate that dietary patterns and atorvastatin therapy differentially altered the colonic gene expression phenotype, with diet-statin interactions in Ossabaw pigs. Atorvastatin therapy had a more profound effect on gene expression than dietary patterns. Interactions suggested a potential side-effect of atorvastatin therapy on colonic mucosa within the context of a WD, emphasizing the critical role of diet quality in modulating response to atorvastatin therapy. Human studies are needed to confirm this finding. The specific gene expression phenotypes observed were not associated with the development of atherosclerotic lesions in the left anterior descending-left circumflex bifurcation artery. At the transcription level genes associated with colonic permeability were unaffected by dietary patterns or atorvastatin therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data statement

All raw RNA sequencing data from this manuscript will be available in the Gene Expression Omnibus (GEO) repository for public access (GSE163159).

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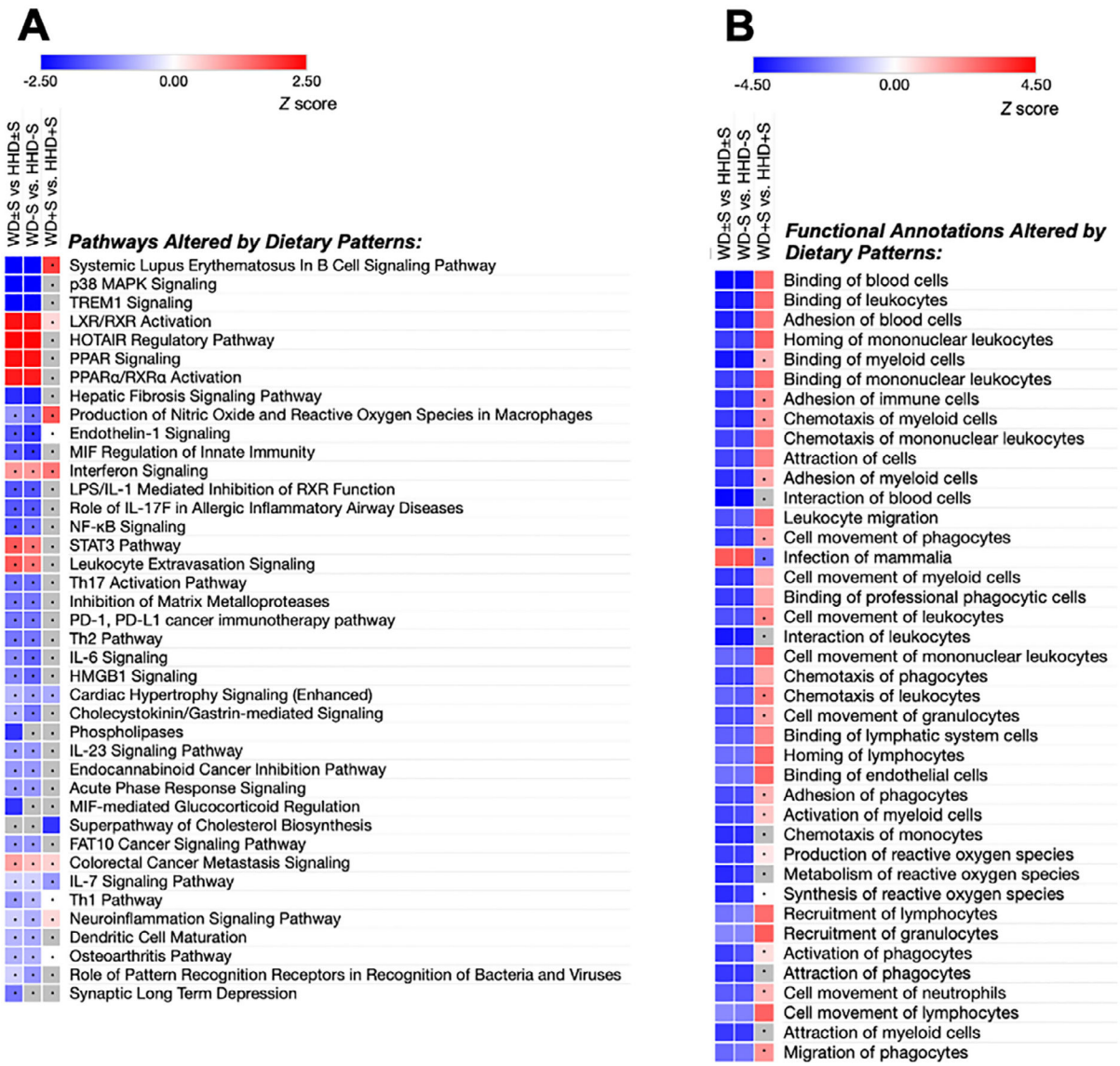

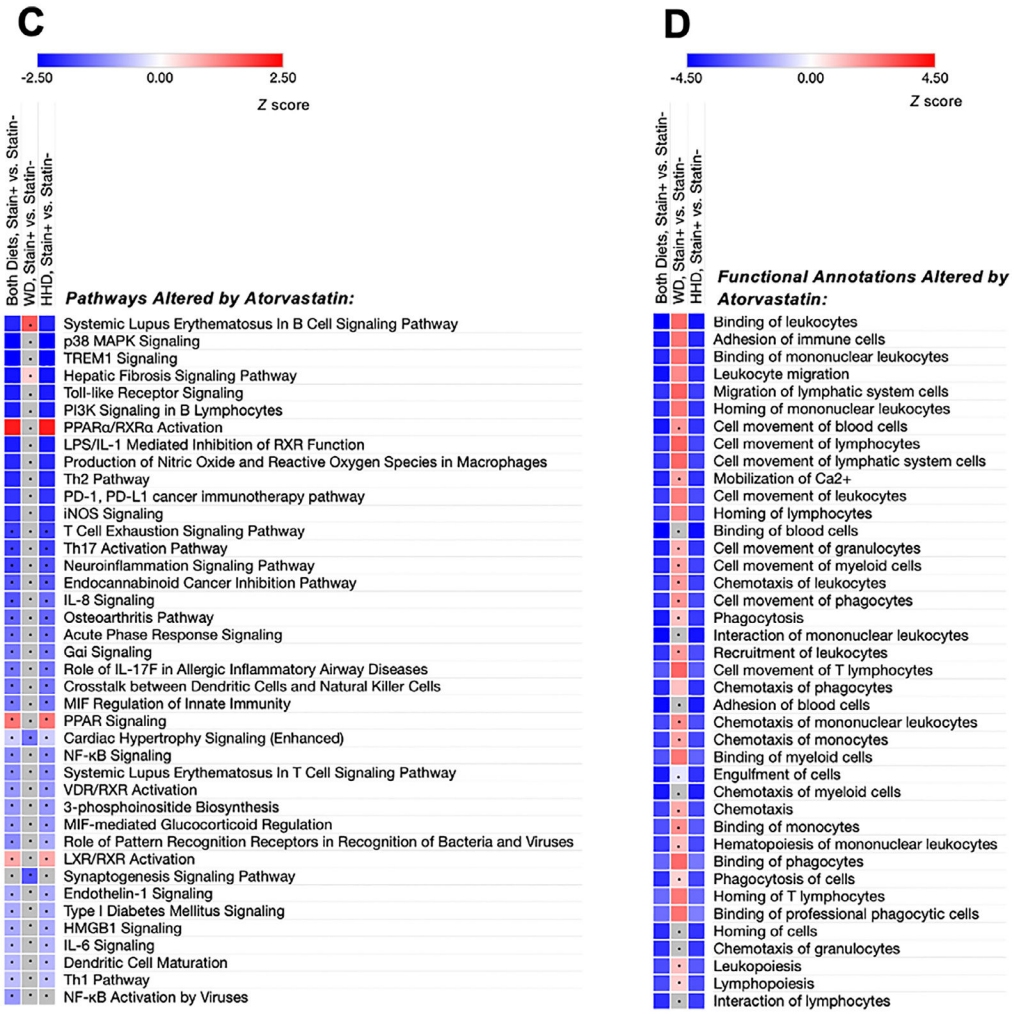

Fig. 1.

(A) Pathways and **(B)** Functional Annotations altered by dietary patterns; columns from left to right: main effect (WD±S *vs.* HHD±S), pigs not treated with atorvastatin (WD-S *vs.* HHD-S), and pigs treated with atorvastatin (WD+S *vs.* HHD+S). **(C)** Pathways and **(D)** Functional Annotations altered by atorvastatin therapy; columns from left to right: main effect (WD/HHD+S *vs.* WD/HHD–S), pigs fed the WD (WD+S *vs.* WD–S), and pigs fed the HHD (HHD+S *vs.* HHD–S). WD: Western-type diet; HHD: heart healthy-type diet; S: atorvastatin therapy. Squares with dot: not significant or no data available.

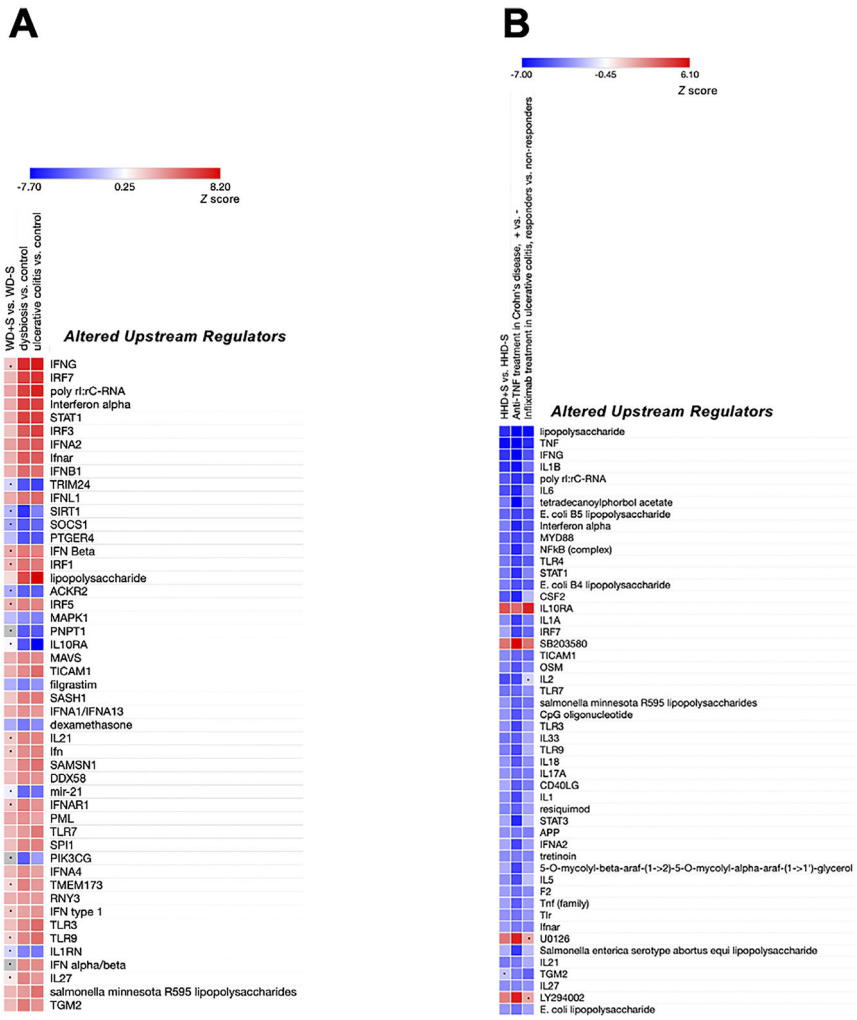

Fig. 2.

(A) Matched gene enrichment results (Upstream Regulators) to statin effect in WD-fed pigs. Columns from left to right: WD+S *vs.* WD–S of present study, a dysbiosis phenotype *vs.* normal control, an ulcerative colitis phenotype vs. healthy control. **(B)** Matched gene enrichment results (Upstream Regulators) to statin effect in HHD-fed pigs. Columns from left to right: HHD+S *vs.* HHD-S of present study, an anti-TNF treatment in Crohn's disease (with treatment *vs.* without treatment), an infliximab treatment in ulcerative colitis (responders vs. non-responders). WD: Western-type diet; HHD: heart healthy-type diet; S: atorvastatin therapy. Squares with dot: not significant or no data available.

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Table 1

Differentially expressed genes by dietary patterns, atorvastatin therapy and their interaction in the colonic mucosa*

		Diet effect† (WD *vs.* HHD)		Statin effect‡ (statin *vs.* nonstatin)		Interaction	Average expression (RPKM)			
Gene symbol	**Gene name**	**logFC**	**FDR**	**logFC**	**FDR**	**FDR**	**WD−S**	**WD+S**	**HHD−S**	**HHD+S**
							(n=7)	**(n=8)**	**(n=6)**	**(n=8)**
HEBP1	Heme binding protein 1	−2.4	0.01	−2.34	0.003	0.04	3.32	4.61	17.53	3.47
LOC110257199	-	−2.56	0.02	−2.52	0.004	0.005	0.15	0.32	0.87	0.15
PPP2R5E	Protein phosphatase 2 regulatory subunit B'epsilon	−5.81	0.005	−5.45	0.002	<0.001	30	381.5	1681.6	38.44
RN7SL1	RNA component of signal recognition particle 7SL1	−7.65	0.001	−7.26	<0.001	<0.001	0.53	18.97	107.4	0.7
SNORA53	Small nucleolar RNA, H/ACA box 53	−2.36	0.05	−2.1	0.03	0.18	0.12	0.17	0.63	0.14
CLEC4G	C-type lectin domain family 4 member G	−0.97	1	−3.66	0.005	<0.001	0.17	0.83	0.34	0.03
CXCL11	C-X-C motif chemokine ligand 11	−0.73	1	−1.54	0.1	0.03	1.6	3.52	2.65	0.91
SELL	Selectin L	−0.81	0.3	−1.1	0.003	0.05	5.28	6.05	9.22	4.29
STEAP4	STEAP4 metalloreductase	−1.83	0.17	−2.43	0.002	0.02	0.43	0.61	1.51	0.28
TREM1	Triggering receptor expressed on myeloid cells 1	−2.58	0.17	2.87	0.009	0.04	0.07	0.14	0.43	0.06
CD5L	CD5 molecule like	−1.14	1	−2.05	0.1	0.04	2.51	6.54	5.52	1.33
ACOD1	Aconitate decarboxylase 1	−2.32	0.46	−3.5	0.003	0.09	0.61	0.79	3.03	0.27
ANXA8	Annexin A8 like 1	−2.45	0.17	−2.68	0.01	0.66	1.09	0.68	5.96	0.93
ASS1	Argininosuccinate synthase 1	−1.45	0.96	−2.58	0.03	0.44	45.53	43.69	124.33	20.79
CCL19	C-C motif chemokine ligand 19	−0.61	0.83	−0.9	0.05	0.36	21.93	23.87	33.51	17.92
CD274	CD274 molecule	−1.16	0.83	−1.72	0.04	0.51	0.78	0.76	1.75	0.53
CHI3L2	Chitinase 3 like 2	−1.83	0.55	−2.77	0.004	0.18	0.28	0.31	1	0.15
CLEC4E	C-type lectin domain family 4 member E	−2.21	0.29	−2.6	0.02	0.06	0.09	0.16	0.39	0.07
CSF3R	Colony stimulating factor 3 receptor	−1.26	0.7	−2.05	0.003	0.08	1.16	1.42	2.77	0.67
FFAR2	Free fatty acid receptor 2	−0.99	0.46	−1.27	0.02	0.06	0.4	0.55	0.8	0.33
GBP2	Guanylate binding protein 2	−0.75	0.93	−1.28	0.03	0.31	33.14	37.86	55.84	22.93
HK3	Hexokinase 3	−0.95	0.7	−1.33	0.03	0.34	4.58	5.04	8.85	3.53
IDO1	Indoleamine 2,3-dioxygenase 1	−1.56	0.7	−2.19	0.02	0.36	1.15	1.25	3.39	0.75
NOS2	Nitric oxide synthase 2	−1.96	0.46	−2.73	0.007	0.34	12.85	12.43	49.94	7.49
PLA2G2D	Phospholipase A2 group IID	−0.67	1	−1.66	0.04	0.36	4.81	5.49	7.63	2.42

Gene symbol	Gene name	Diet effect† (WD *vs*. HHD)		Statin effect‡ (statin *vs*. nonstatin)		Interaction	Average expression (RPKM)			
		logFC	FDR	logFC	FDR	FDR	WD−S	WD+S	HHD−S	HHD+S
							(n=7)	(n=8)	(n=6)	(n=8)
SAA3	Serum amyloid A3, pseudogene	−1.6	0.83	−3.32	0.001	0.11	2.78	2.65	8.41	0.84
SLC6A9	Solute carrier family 6 member 9	−1.02	0.46	−1.52	0.003	0.34	4.03	3.69	8.19	2.86
SNORA73A	Small nucleolar RNA, H/ACA box 73A	−2.07	0.13	−2.11	0.02	0.18	0.24	0.31	1.04	0.24
TGM1	Transglutaminase 1	−1.95	0.38	−2.55	0.008	0.29	0.11	0.12	0.41	0.07
TRPM2	Transient receptor potential cation channel subfamily M member 2	−0.95	0.96	−1.8	0.02	0.16	0.74	0.96	1.43	0.41
WARS	Tryptophanyl-tRNA synthetase 1	−1.1	0.41	−1.57	0.003	0.32	26.42	24.86	56.75	19.08

*FDR, false discovery rate-adjusted *P* value; HHD, heart healthy-type diet; logFC, log fold change; WD, Western-type diet.

†Differential expression attribute to the main effect of dietary patterns (WD, n=15; HHD, n=14).

‡Differential expression attribute to the main effect of atorvastatin therapy (statin, n=16; nonstatin, n=13).

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Table 2

Biological pathways affected by dietary patterns and atorvastatin therapy in the colonic mucosa*

Pathways	FDR	*Z* score	Regulation	Genes involved
Diet effect (WD vs. HHD)†				
HOTAIR regulatory Pathway	0.02	2.4	Up	MMP1, MMP13, MMP3, MMP9, SPP1, TWIST1
PPAR signaling	<0.001	2.3	Up	IL18RAP, IL1B, IL1R2, IL1RAP, IL1RN, NGFR, PTGS2, TNF, TNFRSF11B
LXR/RXR activation	<0.001	2.3	Up	APOB, CCL2, IL18RAP, IL1B, IL1R2, IL1RAP, IL1RN, LBP, MMP9, NGFR, NOS2, NR1H4, PTGS2, TNF, TNFRSF11B
PPARα/RXRα activation	0.043	2.2	Up	ADIPOQ, CHD5, IL18RAP, IL1B, IL1R2, IL1RAP
MIF-mediated glucocorticoid regulation	0.001	−2.0	Down	PLA2G2D, PLA2G3, PLA2G5, PTGS2
Phospholipases	0.01	−2.0	Down	LIPG, PLA2G2D, PLA2G3, PLA2G5
Hepatic fibrosis signaling pathway	<0.001	−2.1	Down	CCL2, CD40, CXCL8, DIRAS3, IL18RAP, IL1B, IL1R2, IL1RAP, IL1RN, IRAK3, MAPK10, MMP1, MMP13, NCF1, NGFR, SPP1, TNF, TNFRSF11B, VEGFD
Systemic lupus erythematosus in B cell signaling pathway	0.001	−2.7	Down	CD40, CXCL8, IFNG, IGHD, IGHM, IL10, IL17B, IL17C, IL1B, TNF, TNFSF11
TREM1 signaling	<0.001	−2.8	Down	CCL2, CD40, CXCL8, IL10, IL1B, NOD1, TNF, TREM1
p38 MAPK signaling	<0.001	−3.3	Down	IL18RAP, IL1B, IL1R2, IL1RAP, IL1RN, IRAK3, PLA2G2D, PLA2G3, PLA2G5, TIFA, TNF
Statin effect (statin vs. nonstatin)‡				
PPARα/RXRα activation	0.04	2.2	Up	ADCY5, ADIPOQ, CHD5, IL1B, IL1R2, IL1RAP
iNOS signaling	<0.001	−2.0	Down	CD14, IFNG, IRAK3, LBP, NOS2
Th2 pathway	<0.001	−2.1	Down	CCR1, CCR3, CD86, ICOS, IFNG, IL10, ITGB2, PIK3R3, SOCS3, TIMD4, TNFRSF4
Production of nitric oxide and reactive oxygen species in macrophages	<0.001	−2.1	Down	APOD, CYBB, IFNG, LYZ, NCF1, NOS2, PIK3R3, PPP2R5E, RHOBTB2, TLR2
Systemic lupus erythematosus in B cell signaling pathway	<0.001	−2.1	Down	CD19, CXCL8, IFNG, IGHD, IGHM, IL10, IL17B, IL17C, IL1B, PDCD1, PIK3AP1, PIK3R3, TNFSF10, TNFSF11
LPS/IL-1 mediated inhibition of RXR function	<0.001	−2.2	Down	ACSBG1, ALDH1L1, CD14, FABP6, GSTA1, GSTA2, IL1B, IL1R2, IL1RAP, IL1RN, IL4I1, LBP, SLC27A6
Toll-like receptor signaling	<0.001	−2.2	Down	CD14, IL1B, IL1RN, IRAK3, LBP, TLR2
PI3K signaling in B lymphocytes	0.01	−2.2	Down	ATF3, C3, CD180, CD19, CR2, PIK3AP1
Hepatic fibrosis signaling pathway	0.004	−2.3	Down	CXCL8, CYBB, IL1B, IL1R2, IL1RAP, IL1RN, IRAK3, MMP13, NCF1, PIK3R3, RHOBTB2, TIMP1
p38 MAPK signaling	0.001	−2.6	Down	IL1B, IL1R2, IL1RAP, IL1RN, IRAK3, PLA2G2D, PLA2G3
TREM1 signaling	<0.001	−2.8	Down	CD86, CXCL8, IL10, IL1B, NLRP3, NOD1, TLR2, TREM1

*FDR, false discovery rate-adjusted *P* value; HHD, heart healthy-type diet; WD, Western-type diet.

[†]Altered pathways attribute to the main effect of dietary patterns based on 311 genes differentially expressed by the WD relative to HHD, with absolute log fold change of ≥ 0.6 (WD, n=15; HHD, n=14).

[‡]Altered pathways attribute to the main effect of atorvastatin therapy based on 312 genes differentially expressed by statin relative to nonstatin, with absolute log fold change of ≥ 0.6 (statin, n=16; nonstatin, n=13).

Table 3

Association of gene expression with atherosclerotic lesion and cardiometabolomic risk indicators: differentially expressed genes[*]

Gene symbol	Gene name	Atherosclerotic lesion severity *r* (*P* value)	LDL cholesterol *r* (*P* value)	HDL cholesterol *r* (*P* value)	triglyceride *r* (*P* value)	TNF-α *r* (*P* value)	hsCRP *r* (*P* value)
CLEC4G	C-type lectin domain family 4 member G	0.21 (.28)	0.35 (.06)	0.52 (<.01)[†]	−0.01 (.94)	0.11 (.59)	0.06 (.75)
CD5L	CD5 molecule like	−0.05 (.78)	0.05 (.79)	0.09 (.63)	−0.25 (.2)	0.56 (<.01)[†]	0.14 (.47)
ASS1	argininosuccinate synthase 1	0.09 (.64)	−0.05 (.79)	−0.03 (.87)	−0.1 (.62)	−0.04 (.83)	−0.47 (.01)[†]
CD274	CD274 molecule	0.08 (.69)	−0.04 (.83)	−0.16 (.41)	−0.06 (.78)	−0.09 (.66)	−0.43 (.02)[†]
GBP2	guanylate binding protein 2	0.05 (.78)	−0.01 (.95)	−0.07 (.72)	−0.13 (.5)	0 (1)	−0.48 (.01)[†]
SLC6A9	solute carrier family 6 member 9	0.09 (.66)	−0.13 (.49)	−0.24 (.21)	−0.21 (.27)	−0.07 (.73)	−0.49 (.01)[†]

[*]Analysis conducted independent of treatments (n=29, except for triglyceride [n=28] and TNF-α [n=25]). Atherosclerotic lesion severity was assessed by Stary score in left anterior descending-left circumflex bifurcation arteries. TNF-α: tumor necrosis factor- alpha; hsCRP: high-sensitivity C-reactive protein. Genes significantly associated with one or more of the clinical traits included.

[†]Absolute correlation coefficient $r \geq 0.4$, $P \leq .05$.

Table 4

Association of gene expression with atherosclerotic lesion and cardiometabolomic risk indicators: genes expressed in pathways altered by dietary patterns*

Gene symbol	Gene name	Atherosclerotic lesion severity *r* (*P* value)	LDL cholesterol *r* (*P* value)	HDL cholesterol *r* (*P* value)	triglyceride *r* (*P* value)	TNF-α *r* (*P* value)	hsCRP *r* (*P* value)
PPAR Signaling							
PTGS2	prostaglandin-endoperoxide synthase 2	−0.16 (.42)	−0.42 (.02)†	−0.4 (.03)†	−0.21 (.29)	0.41 (.03)†	−0.04 (.82)
LXR/RXR Activation							
LYZ	lysozyme	0.15 (.45)	−0.26 (.18)	−0.15 (.45)	−0.46 (.01)†	−0.13 (.53)	−0.13 (.49)
MMP9	matrix metallopeptidase 9	0.4 (.03)†	0.58 (<.01)†	0.41 (.03)†	0.42 (.03)†	−0.04 (.83)	0.09 (.65)
PTGS2	prostaglandin-endoperoxide synthase 2	−0.16 (.42)	−0.42 (.02)†	−0.4 (.03)†	−0.21 (.29)	0.41 (.03)†	−0.04 (.82)
Phospholipases							
PLA2G3	phospholipase A2 group III	0.05 (.79)	−0.45 (.01)†	−0.46 (.01)†	−0.22 (.27)	−0.11 (.6)	−0.11 (.57)
p38 MAPK Signaling							
IRAK3	interleukin 1 receptor associated kinase 3	−0.29 (.13)	−0.27 (.15)	−0.42 (.02)†	0.03 (.88)	−0.1 (.62)	−0.35 (.06)
PLA2G3	phospholipase A2 group	0.05 (.79)	−0.45 (.01)†	−.46 (0.01)†	−0.22 (.27)	−0.11 (.6)	−0.11 (.57)
TREM1 Signaling							
CD40	CD40 molecule	−0.44 (.02)†	−0.24 (.21)	−0.28 (.14)	0.16 (.41)	−0.26 (.19)	−0.31 (.1)
IL10	interleukin 10	−0.05 (.78)	−0.4 (.03)†	−0.34 (.07)	−0.21 (.29)	0.1 (.6)	−0.25 (.2)

*Analysis conducted independent of treatments (n=29, except for triglyceride [n=28] and TNF-α [n=25]). Atherosclerotic lesion severity was assessed by Stary score in left anterior descending-left circumflex bifurcation arteries. TNF-α: tumor necrosis factor- alpha; hsCRP: high-sensitivity C-reactive protein. Genes significantly associated with at least one of the clinical traits were included.

†Absolute correlation coefficient $r \geq 0.4$, $P \leq .05$.

Table 5

Association of gene expression with atherosclerotic lesion and cardiometabolomic risk indicators: genes expressed in pathways altered by atorvastatin therapy *

Gene symbol	Gene Name	Atherosclerotic lesion severity *r* (*P* value)	LDL cholesterol *r* (*P* value)	HDL cholesterol *r* (*P* value)	triglyceride *r* (*P* value)	TNF-α *r* (*P* value)	hsCRP *r* (*P* value)
iNOS Signaling							
IRAK3	interleukin 1 receptor associated kinase 3	−0.29 (.13)	−0.27 (.15)	−0.42 (.02) †	0.03 (.88)	−0.1 (.62)	−0.35 (.06)
Th2 Pathway							
CCR3	C-C motif chemokine receptor 3	0.1 (.61)	−0.16 (.4)	−0.11 (.56)	−0.07 (.73)	−0.1 (.62)	−0.43 (.02) †
ICOS	inducible T cell costimulator	−0.16 (.4)	−0.31 (.1)	−0.37 (.05)	−0.12 (.54)	−0.14 (.47)	−0.54 (<.01) †
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages							
APOD	apolipoprotein D	0.31 (.1)	0.42 (.02) †	0.49 (.01) *	−0.06 (.74)	−0.01 (.97)	0.4 (.03) †
CYBB	cytochrome b-245 beta chain	−0.01 (.95)	−0.18 (0.35)	−0.11 (0.57)	−0.09 (0.65)	−0.28 (0.15)	−0.43 (0.02) †
LYZ	lysozyme	0.15 (.45)	−0.26 (.18)	−0.15 (.45)	−0.46 (.01) *	−0.13 (.53)	−0.13 (.49)
Systemic Lupus Erythematosus In B Cell Signaling Pathway							
TNFSF11	TNF superfamily member 11	0.19 (.33)	0 (.99)	−0.02 (.91)	−0.12 (.55)	0.02 (.93)	−0.47 (.01) †
Toll-like Receptor Signaling							
CD14	CD14 molecule	0.25 (.19)	0.08 (.67)	0.15 (.43)	0.02 (.92)	−0.26 (.19)	0.04 (.85)
IL1B	interleukin 1 beta	−0.07 (.73)	−0.12 (.52)	−0.08 (.68)	−0.06 (.76)	0.12 (.54)	−0.12 (.52)
IL1RN	interleukin 1 receptor antagonist	0.3 (.11)	0.09 (.63)	−0.09 (.66)	−0.14 (.48)	−0.1 (.63)	0.05 (.79)
IRAK3	interleukin 1 receptor associated kinase 3	−0.29 (.13)	−0.27 (.15)	−0.42 (.02) †	0.03 (.88)	−0.1 (.62)	−0.35 (.06)
LBP	lipopolysaccharide binding protein	0.21 (.27)	−0.11 (.59)	−0.1 (.61)	−0.09 (.65)	0.01 (.94)	−0.29 (.13)
TLR2	toll like receptor 2	0.09 (.66)	0.05 (.81)	−0.02 (.93)	0.05 (.8)	−0.15 (.45)	−0.14 (.47)
PI3K Signaling in B Lymphocytes							
ATF3	activating transcription factor 3	−0.1 (.61)	−0.02 (.91)	−0.12 (.53)	0.12 (.54)	−0.1 (.63)	−0.51 (.01) †
C3	complement C3	0.19 (.32)	−0.03 (.89)	−0.07 (.71)	−0.04 (.85)	0.15 (.44)	−0.35 (.06)
CD180	CD180 molecule	0.07 (.72)	−0.07 (.73)	−0.1 (.62)	−0.16 (.42)	−0.26 (.19)	−0.4 (.03) †
CR2	complement C3d receptor 2	0.42 (.02) †	0.24 (.21)	0.16 (.4)	−0.08 (.68)	0.07 (.74)	−0.08 (.67)

Gene symbol	Gene Name	Atherosclerotic lesion severity *r* (*P* value)	LDL cholesterol *r* (*P* value)	HDL cholesterol *r* (*P* value)	triglyceride *r* (*P* value)	TNF-α *r* (*P* value)	hsCRP *r* (*P* value)
p38 MAPK Signaling							
IRAK3	interleukin 1 receptor associated kinase 3	−0.29 (.13)	−0.27 (.15)	−0.42 (.02)†	0.03 (.88)	−0.1 (.62)	−0.35 (.06)
PLA2G3	phospholipase A2 group III	0.05 (.79)	−0.45 (.01)†	−0.46 (.01)†	−0.22 (.27)	−0.11 (.6)	−0.11 (.57)
TREM1 Signaling							
IL10	interleukin 10	−0.05 (.78)	−0.4 (.03)†	−0.34 (.07)	−0.21 (.29)	0.1 (.6)	−0.25 (.2)

*Analysis conducted independent of treatments (n=29, except for triglyceride [n=28] and TNF-α [n=25]). Atherosclerotic lesion severity was assessed by Stary score in left anterior descending-left circumflex bifurcation arteries. TNF-α: tumor necrosis factor- alpha; hsCRP: high-sensitivity C-reactive protein. Genes significantly associated with at least one of the clinical traits were included.

†Absolute correlation coefficient $r \geq 0.4$, $P \leq .05$.