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Key Differences Between Hepatic and Intestinal apoC3 Regulation and Expression, and Implications for Cardiovascular Disease Risk

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Key Differences Between Hepatic and Intestinal *apoC3* Regulation and Expression, and Implications for Cardiovascular Disease Risk

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List of Abbreviations

apo= apolipoprotein
ASO= antisense oligonucleotide
CAD=coronary artery disease
CM= chylomicron
CVD= cardiovascular disease
DHA= Docosahexaenoic acid
EPA= eicosapentaenoic acid
FFA= free fatty acids
FoxO1= Forkhead box protein O1
HDL= high-density lipoproteins
IDL= intermediate-density lipoproteins
HMG-CoA= 3-hydroxy-3-methyl-glutaryl-coenzyme A
ISC= intestinal stem cells
LDL= low-density lipoproteins
MAG= monoacylglycerol
MTP= microsomal triglyceride transfer protein
PI3K= Phosphatidylinositol-4,5-bisphosphate 3-kinase
PUFAs= polyunsaturated fatty acids
TAG= triacylglycerol
TRLs= triglyceride-rich lipoproteins
VLDL= very-low-density lipoproteins

Abstract

In the intestine, apoC-III delays chylomicron secretion, and causes a decrease in chylomicron size. Once apoC-III is in circulation, apoC-III delays triglyceride-rich remnants clearance by inhibiting lipoprotein lipase and liver low-density lipoprotein receptor. In humans, high levels of plasma apoC-III directly result in hypertriglyceridemia. ApoC-III is a critical cardiovascular risk factor, and humans expressing null mutations in apoC-III are robustly protected from cardiovascular disease. Because of its critical role in elevating plasma lipids and CVD risk, the factors that regulate apoC-III expression in the liver have been determined, and include glucose, insulin (through FoxO1), and dietary fat. Considerably less is known about the factors that regulate intestinal apoC-III. This study utilizes primary murine enteroids, Caco-2 cells, and dietary studies in wild-type mice to show that intestinal apoC-III expression does not change in response to fatty acids, glucose, or insulin administration, in contrast to hepatic apoC-III. Intestinal apoC-III is not sensitive to changes in FoxO1 expression (which is itself very low in the intestine, as is FoxO1 target IGFBP-1), nor is intestinal apoC-III responsive to western diet, a significant contrast to hepatic apoC-III stimulation during western diet. These data strongly suggest that intestinal apoC-III is not a FoxO1 target. These data support the idea that apoC-III is not regulated coordinately with hepatic apoC-III, and establishes another key aspect of apoC-III that is unique in the intestine and different from the liver.

Introduction

Hyperlipidemia is central to pathophysiological development of atherosclerosis, the key component of coronary artery disease, a form of cardiovascular disease (CVD). CVD is the leading cause of death worldwide.[1] Because atherosclerosis develops with the uptake of LDL cholesterol (LDL-C) by the vascular endothelium,[2] strategies to prevent atherosclerosis have focused on lowering LDL-C in patients at risk for the disease. However, patients still have significant risk for developing CVD even when LDL-C is reduced to healthy levels.[3,4] Therefore, strategies that aim to reduce plasma triglycerides are also required to eliminate hyperlipidemia and CVD risk.

Apolipoproteins reside on the outside of lipoproteins and can act as signaling molecules and ligands, and are therefore important determinants of how lipoproteins and the lipids inside of them are metabolized.[5–7] Therefore, regulating the expression and abundance of specific apolipoproteins is a method by which pharmaceutical and dietary therapies can manage and direct lipid metabolism and moderate the risk of cardiovascular disease. Apolipoprotein C-III (apoC-III) is a small protein produced primarily by the liver and intestine.[8,9] ApoC-III is secreted from these tissues on triglyceride-rich lipoproteins (TRLs).[10–12] The presence of apoC-III on lipoproteins increases the resident time of these particles in circulation primarily by inhibiting uptake of TRLs and their remnants by the hepatic low-density lipoprotein receptor (LDLR).[13–15] In humans, plasma apoC-III levels are independently associated with both an increase in plasma triglycerides and CVD risk.[16,17] In the liver, the apoC-III protein promotes lipid loading onto nascent VLDL and enhances VLDL assembly and secretion.[18,19] In

contrast, intestinal apoC-III causes retention of unesterified dietary lipid within the enterocyte and a delay of triglyceride secretion into lymph.[20]

Factors that influence *apoC3* gene expression in the liver have been well researched, however, it is unknown how *apoC3* is regulated in the intestine. In the study presented herein, we aimed to determine if intestinal *apoC3* is responsive to the same dietary components and metabolites as hepatic *apoC3.* We use primary murine enteroids, Caco-2 cells, and dietary studies in wild-type mice to test the effect of glucose, insulin, and fat on hepatic and intestinal apoC-III expression.

Chapter 1: Review of Literature

1.1 DEVELOPMENT OF CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the leading cause of death in both the United States and globally.[1] CVD is an umbrella term for a number of conditions that affect the heart and blood vessels, such as coronary artery disease (CAD), myocardial infarction (MI), and stroke. CVD tremendously affects both industrialized and unindustrialized countries; approximately 17.7 million or 31% of deaths worldwide occur from CVD related causes.[1] Non-modifiable risk factors for CVD include genetic predisposition, age, gender, ethnicity, and socioeconomic status. However, the majority of risk factors for CVD, such as, hypertension, hyperlipidemia, type 2 diabetes, smoking, and stress are modifiable and can be improved with diet and lifestyle interventions.[1,21,22]

Hyperlipidemia is central to pathophysiological development of atherosclerosis, the key component of CAD. During the development and progression of atherosclerosis, arteries narrow and harden due to the accumulation of lipid and fibrous elements.[2] This lipid accumulation occurs when oxidized low-density lipoprotein (LDL) particles are taken up by the vascular endothelium and macrophages are recruited to the site of injury. These lipid-laden macrophages are known as ‘foam cells.’ Foam cells become problematic when smooth muscle cells (SMCs) of the media migrate into the intima and, with extracellular matrix, form a fibrous cap that encloses a necrotic core of lipid-rich cells.[2] These calcified arterial plaques cause stenosis that impedes oxygen-rich blood flow, and upon further injury to the luminal surface of the vessel, can rupture and cause compete occlusion resulting in myocardial infarction or stroke.

Given the central role of LDL-cholesterol in development atherosclerosis, lowering LDL-C has been a central objective in the battle against CVD. In 1984, The Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT) reported that use of the cholesterol-lowering, bile acid sequestrant, cholestyramine, in middle-aged men with primary hypercholesterolemia significantly reduced plasma cholesterol which resulted in a significant decrease in the incidence of coronary heart disease in the experimental group.[23] With the advent and widespread use of more potent cholesterol-reducing medications known as HMG-CoA reductase inhibitors, or statins, which lower plasma cholesterol by interfering with the rate-limiting step of endogenous cholesterol synthesis, research studies have been able to more effectively test the hypothesis that reducing cholesterol significantly reduces CVD occurrence. Indeed, several meta-analyses of randomized controlled trials have reported that statins significantly reduce all-cause mortality, combined fatal and non-fatal CVD and CHD events and stroke.[24–27]

Despite the impressive impact of cholesterol-lowering therapies on CVD risk, a troublingly large number of patients still have residual risk for CVD despite optimal LDL-C reduction.[28] For example, even one of the most important randomized control trials that demonstrated the *success* of statins in prevention of CVD-related events, The Scandinavian Simvastatin Survival Study (4S), reported that 19% of people in the simvastatin-treated group had one or more major coronary events in the 5.5 year follow-up period.[29] These data and others like them, suggest significant residual risk even for patients being treated with cholesterol-lowering drugs. Strategies that target the reduction of LDL-C as a means to reduce morbidity and mortality from CVD-related causes are not wholly effective, and hence, investigation into additional methods to

eliminate CVD occurrence is warranted.

1.2 ANOTHER TARGET FOR REDUCING CVD RISK

Elevated plasma triglyceride level is the other detrimental, component of hyperlipidemia. Nearly half a century ago JL Goldstein, AG Motulsky et al. asserted that hypertriglyceridemia might be as significant a risk factor for coronary atherosclerosis as hypercholesterolemia.[3] In their study of 500 fasting survivors 3 months post myocardial infarction, they reported that hypertriglyceridemia with (7.8%) or without (15.6%) accompanying elevation in cholesterol levels was three times more common in survivors than a high cholesterol level alone (7.6%).[3] This incidence of hyperlipidemia without hypercholesterolemia may account for the residual risk for CVD despite cholesterol-lowering interventions, and underlies the importance of studying TAG and the metabolism of triglyceride-rich lipoproteins that carry TAG throughout the body. Indeed, hypertriglyceridemia has since emerged as an independent risk factor for CVD. Two separate prospective studies, the Cholesterol and Recurrent Events (CARE) trial in the United States, and The Copenhagen City Heart Study in Denmark, reported that non-fasting plasma triglyceride level is a strong and independent predictor of future myocardial infarction, ischemic heart disease, and death in both normo- and hyper-cholesterolemic men and women. [4,30]

Apolipoproteins, with the exception of apo-B which provides more of a structural framework for the particle, are proteins that reside on the outside of lipoproteins. Lipoproteins like chylomicrons, VLDL, LDL, IDL, and HDL, carry lipids and cholesterol through the aqueous environment of the blood under their phospholipid coat.[7] Apolipoproteins can act as signaling molecules and ligands, and their placement on the

outside of lipoproteins makes them important determinants of how lipoproteins and their lipid cargo is metabolized.[5–7]

Apolipoprotein C-III links hypertriglyceridemia with increased CVD risk. ApoC-III is a small, 79 amino acid protein produced predominantly by the liver and small intestine, and is exported on the lipoproteins from these organs: VLDL and chylomicrons, respectively.[8,9] Apo C-III is a key determinant in how, and how quickly, triglyceride-rich lipoproteins are exported into plasma and catabolized by peripheral tissues. The protein is exchangeable in plasma, and therefore can migrate amongst different types and species of lipoproteins.[31] The presence of apoC-III on lipoprotein particles delays their catabolism and prolongs the period of time lipoproteins remain in circulation.[32] Hence, not surprisingly, plasma abundance of apoC-III directly correlates with plasma triglyceride levels.[33] Additionally, a high concentration of apoC-III on VLDL and LDL, independent of plasma triglyceride level, is a measure of coronary heart disease risk.[34] Given the increased risk of CVD incidence and adverse cardiovascular events that accompanies elevated plasma triglycerides, and the atherogenicity of apoC-III itself, the ability to strategically manipulate expression of apoC-III is a promising tactic to combat CVD occurrence.

1.3 FUNCTIONS OF APOC-III

Effect of apoC-III on circulating TAG levels

The level of apoC-III expression directly correlates to triglyceride levels in both rodent models and humans. During the development of a human apoC3 transgenic mouse model, Breslow et al. found that mice lines with 100 copies of the gene were severely hypertriglyceridemic, while other lines with only a one to two copies of the gene

produced less apoC-III protein and had only mildly elevated triglyceride levels.[35] This gene dose effect is similarly apparent in humans; humans heterozygous for a defective *apoC3* allele, such as the Lancaster Amish who are carriers of the null R19X mutation in the apoC3 gene, produce half as much apoC-III as those without the mutation.[36] These heterozygous carriers of the mutant gene also have a corresponding decrease in plasma triglycerides and coronary artery calcification, which is a marker of subclincal atherosclerosis.[36] Additionally, other genome-wide association studies (GWAS) have uncovered several distinct populations with rare mutations that affect *ApoC3* function. These loss-of-function mutations in *ApoC3* are associated with hypotriglyceridemia and confer nearly complete cardioprotection. [37–40]

Interference with TRL lipolysis and clearance

Several mechanisms by which apoC-III elevates plasma TAG have been established. In 1972, Brown and Baginsky first described apoC-III as an inhibitor of lipoprotein lipase (LPL),[9,41] which reduced the lipolysis and unloading of lipid into peripheral tissues, therefore keeping more TAG retained in large lipoproteins longer. Yet a small clinical study that tested the effect of the apoC-III specific antisense oligonucleotide (ASO), volanesorsen, in patients with a genetic LPL deficiency and resultant chylomicronemia, resulted in significant reductions of plasma TAG in these patients.[42] These patients do not have functioning LPL but their plasma TAG levels were still reduced when apoC-III expression was ablated, suggesting that the inhibition of LPL by apoC-III is not the mechanism, or at least not the central mechanism, by which apoC-III raises circulating triglyceride levels. This trial indicates that apoC-III

interferes with another mechanism by which lipid is cleared from plasma, independent of LPL.[15,42]

Likely the most significant mechanism by which apoC-III increases plasma TAG, is through its interference with hepatic receptors that clear TRLs and their remnants from circulation.[15] Gordts and Esko et al.[15] also used the ApoC-III ASO volanesorsen, which binds apoC-III mRNA and targets it for degradation, to reduce apoC-III levels in several distinct mutant mouse lines, including: low-density lipoprotein receptor (LDLR) knockouts, LDLR-related protein 1 (LRP1) knockouts, and heparan sulfate proteoglycan receptors (HSPGs) knockout mice, as well as additional sets of mice who were double knockouts for a pair of receptors. The results of their experiments show that apoC-III ASO treatment lowered plasma TAG in mice lacking hepatic HSPG receptors, LDLR, or LRP1. The drug also reduced TAG in animals with combined deletion of the genes encoding HSPG receptors and LDLRs or LRP1. However, apoC-III ASO treatment did not reduce triglycerides in mice who were knockouts of both LDLR and LRP1, suggesting that apoC-III primarily elevated plasma triglyceride levels by impeding hepatic TRL clearance via these receptors.[15]

There is also evidence that apoC-III inhibits hepatic TRL uptake by interfering with or displacing apoE, a major ligand by which liver receptors clear lipoproteins. Hypertriglyceridemic, human apoC-III transgenic mice accumulate triglyceride-rich, VLDL-like particles carrying abundant apoC-III and lacking apoE.[14] Furthermore, human lipoprotein kinetic studies conducted by the Zheng et al. have shown that patients with hypertriglyceridemia produce 3-times more VLDL without apoE but with apoC-III, than do normolipidemic controls.[43] Additionally, the apoC-III-containing apoB lipoproteins

devoid of apoE were removed significantly more slowly from circulation than those with both apo C and E.[43]

Promotion of VLDL assembly and secretion

In rat hepatoma McA-RH7777 cells transfected with human apoC-III and cultured in lipid-rich conditions, apoC-III increases VLDL-TAG secretion in a gene dose-dependent manner.[19] ApoC-III seems to promote lipid-loading into VLDL particles in the liver by functioning in conjunction with microsomal triglyceride transfer protein (MTP).[44] Transfection of human apoC-III into these hepatoma cells significantly increased the mRNA expression and activity of MTP.[18] In the initial stages of VLDL synthesis, lipidation of hepatic apoB begins immediately as the apoB protein is translated. [45] However, MTP is crucial for the continued lipidation of the nascent lipoprotein and is necessary to avoid degradation of lipid-poor apoB.[46] MTP is also required for later stages of lipid loading, in the recruitment of triglyceride into the endoplasmic reticulum and Golgi compartments for VLDL assembly.[44,47] Labeling of apoC-III protein with [^{35}S]methionine/cysteine in the same experiment using human apoC-III- expressing McA-RH7777 cells, showed that the majority of apoC-III was distributed within TAG-rich microsomal lumen fractions.[19] This data may suggest that apoC-III works in conjunction with MTP to partition TAG substrate into the endoplasmic reticulum and Golgi for VLDL assembly.[19,44] In support of the idea that apoC-III promotes VLDL lipid loading and secretion, is the finding that subjects with missense mutations in the *ApoC3* gene that code for its lipid-binding domain have unusually low plasma triglycerides.[36,48,49]

Atherogenicity of apoC-III

ApoC-III is a potent regulator of plasma triglyceride levels, but research has also demonstrated that apoC-III is proatherogenic aside from its role in elevating triglyceride concentration. In a prospective study of 2 populations: the female participants in the Nurses' Health Study (NHS) and the male participants in the Health Professionals Follow-up Study (HPFS), Mendivil & Sacks (2011)[50] sought to determine if increased concentrations of VLDL and LDL with apoC-III is associated with increased CVD risk versus VLDL and LDL devoid of apoC-III. The subjects in the two populations were free of CVD at the start of the study. Researchers found that concentrations of LDL with apoC-III positively correlate with risk of coronary heart disease in multivariable analysis that included the ratio of total cholesterol HDL cholesterol, LDL cholesterol, apoB, triglycerides, or HDL cholesterol and other risk factors.[50] While several other studies have implicated increased apoC-III concentrations with the development and progression of atherosclerosis [51–54] and the incidence of adverse cardiovascular events associated with CVD,[34,50,55,56] the molecular mechanisms by which apoC-III functions to promote atherosclerosis are still poorly understood.

Role of apoC-III in the intestine

Research has focused on the role of apoC-III in the liver and its role on plasma lipoproteins. Comparatively, little is known about the role of apoC-III in the small intestine. ApoC-III protein is present on chylomicrons secreted from the intestine. ApoC-III mRNA expression in the duodenum is approximately 30% of the amount produced in the liver, with the most proximal section of the small intestine producing the most and

the ileum the least.[20] Human apoC-III transgenic mice express about 5 times more apoC-III mRNA with the same tissue distribution of apoC-III as wild-type mice, hence this model can also be used to study apoC-III overexpression and its consequences in the intestine.[20]

Emerging evidence suggests that the role of apoC-III in intestine differs significantly from that in the liver. Contrary to apoC-III's function of increasing VLDL assembly and secretion in the liver, [19] intestinal apoC-III appears to inhibit TAG secretion into lymphatic circulation. Kohan and Wang[20] reported that human *apoC3* transgenic mice infused intraduodenally with radiolabeled lipid demonstrated delayed uptake of the lipid from the intestinal lumen, impaired esterification of FFA and MAG into TAG, and decreased secretion of lipid from the intestinal enterocytes into lymph.[20] In line with this finding, intestinal organoids prepared from intestinal stem cells from human apoC-III transgenic mice secrete smaller, less dense chylomicrons with less TAG. These data suggest that the function of intestinal apoC-III and the physiological outcome of expressing intestinal apoC-III, are both entirely opposite to their function in the liver.

1.4 REGULATION OF *APOC3* IN THE LIVER & INTESTINE

Scarce data exist regarding what factors regulate *apoC3* gene expression in the intestine, but a number of nutrients and metabolites have been shown to regulate hepatic *apoC3* via action on various transcription factors[51]. ApoC-III may contribute to the development of diabetic dyslipidemia in the insulin resistant state because its expression in the liver is upregulated by glucose and downregulated by insulin.[57] In primary rat hepatocytes and immortalized human hepatocytes, glucose increases apoC-

III transcription by activating transcription factors HNF-4α and ChREBP, which bind E-boxes found in the proximal C3P footprint in the *apoC3* promoter.[57] FXR and PPARα agonists decrease hepatic expression of *apoC3* because FXR and PPARα can displace HNF-4α from its location in the *apoC3* enhancer region or the CP3 footprint.[57,58]

Insulin's impact on hepatic *apoC3* expression is primarily attributed to the transcription factor Forkhead box O1 (FoxO1). When active, FoxO1 binds to the *apoC3* promoter and stimulates transcription.[59] FoxO1 is deactivated by insulin via signaling through the insulin receptor substrate (IRS) which causes phosphorylation of Foxo1 through the PI3K/Akt pathway and consequent nuclear exclusion.[60,61] Streptozotocin-treated mice, which model an insulin deficient diabetic state, have an approximately 1.5 fold increase in hepatic apoC-III mRNA levels, accompanied by elevated plasma glucose and triglycerides.[62] Hepatic apoC-III mRNA expression can be reduced by nearly 60%, and glucose and triglyceride levels normalized, when the animals are treated with insulin.[62] Similarly, HepG2 cells transfected with an apoC-III reporter construct demonstrate a dose-dependent downregulation of apoC-III transcription in response to insulin.[62] In primary rat hepatocytes transfected with FoxO1 cDNA by adenovirus, FoxO1 stimulates apoC-III mRNA expression and the hepatocytes exhibit a dose-responsive decrease in apoC-III transcription upon treatment with insulin.[59] Deletion or mutation of the FoxO1 binding site in the apoC-III promoter ablates this response to insulin and the corresponding FoxO1-mediated stimulation of apoC-III gene expression.[59]

FoxO1 has also been implicated in the regulation of apoC-III gene expression in the intestine, but only in one study. In the same study mentioned above, by Altomonte

and Dong et al,[59] in which researchers delivered FoxO1 cDNA to primary rat hepatocytes, they also conducted an experiment on Caco-2 cells overexpressing constitutively active FoxO1. Like the hepatocytes, Caco-2 cells overexpressing FoxO1 also produced more apoC-III mRNA and this expression was attenuated with insulin treatment. [59] However, the experiment did not examine the effect of insulin on Caco-2 cells that were not vector-transduced --those with only basal, physiological levels of FoxO1. Hence, the experiment did not determine if FoxO1 is a central regulator of apoC-III expression in the intestine.

Hepatic apoC-III gene expression appears to be moderated in part by the action of PPARα on FoxO1. Fibrates, which are PPARα agonists, are known to downregulate the expression of apoC-III in the liver and to reduce plasma concentration of apoC-III in both animal and human studies.[63] PPARα inhibits FoxO1 binding to the insulin-responsive element in the *apoC3* gene.[64] Long chain, n-3 polyunsaturated fatty acids (PUFAs) like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are found in fish and algal oils, are ligands that trigger PPARα activation.[65–67] In the liver, DHA suppresses the expression of FoxO1 and its target genes which include MTP, glucose-6-phosphatase, insulin-like growth factor binding-protein 1 (IGFBP1) and apoC-III.[68] Hence, this activation of PPARα, and subsequent deactivation of FoxO1 which results in less apoC-III expression in the liver, may contribute to the hypolipedemic effect of fish oil supplementation in humans.[69–73]

1.5 CONCLUSION

ApoC-III is a potent modulator of plasma triglyceride levels and abundance of this

apoprotein in human plasma is proatherogenic.[16,34,74] Given the widespread impact of CVD worldwide, new and effective therapies to reduce CVD risk are necessary. ApoC-III promotes hypertriglyceridemia by promoting VLDL assembly in the liver and decreasing clearance of TRLs from circulation.[15,19] Glucose, insulin, and dietary n-3 fatty acids have an impact on hepatic apoC-III expression.[57,59,68] However, considerably less is known about the role and regulation of apoC-III in the intestine. Interestingly, what is known about the function of intestinal apoC-III is paradoxical to its role in the liver; intestinal apoC-III delays secretion of chylomicrons and their TAG cargo.[20,75] ApoC-III can be a valuable target in reducing CVD risk, however given the differences between hepatic and intestinal apoC-III, more research regarding the tissue-specific function of this apoprotein is warranted.

Chapter 2: Key differences between apoC-III regulation and expression in intestine and liver.

This chapter is presented in its previously published format.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

INTRODUCTION

Apolipoprotein C-III (apoC-III) is expressed in both liver and the intestine, and is secreted from these tissues on triglyceride-rich lipoproteins (TRLs)[10–12]. ApoC-III was first established as an inhibitor of lipoprotein lipase (LPL), and it has been more recently established that the most robust role of apoC-III is likely its inhibition of hepatic low-density lipoprotein receptor (LDLR)[13–15]. These inhibitory actions of apoC-III increase the plasma residence time of TRLs and their remnants. In humans, plasma apoC-III levels are independently associated with both an increase in plasma triglycerides and CVD risk [16,17].

Elevated plasma triglycerides are an independent risk factor for cardiovascular disease (CVD), the leading cause of mortality in the United States. The importance of apoC-III in human CVD incidence has been well-established: in patients with the R19X null mutation in apoC-III, CVD incidence is significantly reduced, whereas in patients

with elevations in plasma apoC-III, CVD incidence in robustly increased [34,36–38,74,76].

We have recently established an intestinal role for apoC-III. We find that overexpression of apoC-III results in a delay in dietary lipid absorption and causes the secretion of smaller chylomicrons [20,75]. This is paradoxical to both the triglyceride-raising role apoC-III plays in the plasma, and cell culture data suggesting that intracellular apoC-III promotes the synthesis and secretion of larger, more triglyceride-rich VLDL from the liver [18,48].

Research on the mechanisms that control apoC-III gene expression has focused on hepatic apoC-III. In primary rat hepatocytes, glucose upregulates apoC-III transcription by activating transcription factors HNF-4α and ChREBP, which bind E-boxes found in the proximal C3P footprint in the apoC-III promoter [57]. Conversely, insulin down-regulates hepatic apoC-III through phosphorylation and nuclear exclusion of the transcription factor Forkhead box O1 (FoxO1)[77]. Furthermore, hepatic apoC-III mRNA is significantly elevated in mice with streptozotocin-induced insulin deficiency [62]. In liver, polyunsaturated fatty acids inhibit FoxO1 expression and its target genes in liver, including apoC-III [68].

While the regulation of hepatic apoC-III gene expression has been well studied, the factors that regulate apoC-III in the intestine are still largely unknown. In Caco-2 cells overexpressing constitutively active FoxO1, apoC-III mRNA is significantly increased [59]. However, little is known about the role of physiological levels of FoxO1 in apoC-III regulation in the intestine. Unlike hepatic apoC-III, which is inhibited by long chain polyunsaturated fatty acids [68], it is unknown how intestinal apoC-III expression responds to dietary fat.

Since we have previously established that the role of apoC-III in the intestine differs significantly from that in the liver, the purpose of this study is to establish the *in vivo* regulation of intestinal apoC-III. In this work, we establish that intestinal apoC-III expression is not responsive to dietary fat or glucose, and in primary murine enteroids and Caco-2 cells, apoC-III expression is not inhibited by insulin. We also show that in contrast to the liver, intestinal FoxO1 expression is very low and apoC-III is not regulated coordinately with FoxO1 target genes in the intestine. Finally, intestinal apoC-III expression does not change in response to a western diet challenge, whereas we confirm previous studies showing that hepatic apoC-III expression is stimulated by western diet. Our findings support the notion that though the liver and intestine share TRL synthesis and secretion, they do not share identical regulatory pathways for proteins that are involved TRL synthesis and secretion.

METHODS

Animals. Male and female C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME), 8-12 weeks old, were housed 3-4 per cage in a temperature-controlled (23 ±1°C) vivarium on a 12-h light-dark cycle. Mice received free access to water, and were maintained on either standard rodent chow (Teklad global cat.#2918) or western diet (42% kcal from fat, 0.2% cholesterol by weight) from Envigo TD.88137, for 12 weeks. Animals were sacrificed under isoflurane anesthesia following an overnight fast. For gavage studies, mice continued on chow diet and were gavaged at the same time daily for one week with saline, corn oil (60μL), or corn oil (60μL) with glucose (135μL of 5M glucose). Each gavage was made isovolumetric (195μL) using saline. Mice were fasted overnight on the last day before they were sacrificed. The next morning, plasma, liver, and small

intestine were collected, flash frozen in liquid nitrogen, and stored at -80°C until analysis. All animal procedures were performed in accordance with the University of Connecticut Internal Animal Care and Use Committee and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Caco-2 cells. Caco-2 cells were used for experiments at 17d post-confluence. As described by Nauli et al. [78], this protocol produces the most abundant apoB-lipoproteins. Cells were kept at 37°C with 5% CO_2 in Eagle's Minimum Essential Medium with L-Glutamine (Corning Cellgro #10-010-CM), 10% Fetal Bovine Serum (FBS), 1% L-glutamine, penicillin/streptomycin, sodium pyruvate, non-essential amino acids, and 1.5 g/L sodium bicarbonate. For experiments, cells were serum starved overnight prior to treatments. To measure the effect of varying concentrations of glucose and insulin, we incubated cells in low glucose DMEM (1g/L glucose) and high glucose DMEM (4.5g/L) with and without human insulin (final concentration 80nM, Sigma, St. Louis, MO. cat# I9278). Cells were treated with 2mL of treatment media in each well for 24 hours. After 24 hours, media was collected and cells were washed with ice cold 10% phosphate-buffered saline (PBS). Cells were incubated in fatty acid growth medium for 4 hours before they were washed with ice cold 10% PBS (phosphate-buffered saline). Fresh media was added to each well and plates were incubated for 24 hours before protein and RNA analysis.

Enteroid Culture. We isolated intestinal stem cells (ISCs) from crypts as described previously [75,79]. Primary crypts were isolated from WT mice, age 8-12 weeks. Crypts

were placed in Matrigel; following 30min polymerization, crypts were treated with 500µl of enteroid medium (Advanced DMEM/F12 (12634- 010; Life Technologies, Carlsbad, CA, USA) with 2mM L-Glutamine, 10mM HEPES, 100U/mL penicillin/100µg/mL streptomycin and 1× N2 and 1× B27 supplements,lus 1µL of R-spondin 1 (250µg/mL), 1µL of Noggin (50µg/mL) and 0.25µL of EGF (100µg/mL). Media was replaced every 3 days. For treatment with lipid, mature enteroids were dissociated from Matrigel by washing with ice cold DPBS, followed by a 150x g spin for 10 min. After removing the supernatant, the intact enteroids were then placed in 1ml of treatment media containing 400µM OA: BSA or BSA alone, or enteroid growth media containing glucose, or enteroid growth media containing insulin; all media contained Rho-kinase inhibitor. The enteroids were very gently opened by pipetting up and down with a p1000 pipette, followed by incubation with the lids open in a 37° 5% CO2 incubator for 2 hours. After 2 hours, the enteroids were centrifuged at 150 x g for 10 minutes and the supernatant collected. Following an additional wash and centrifuge with 1ml of DPBS (which was added to the media samples), the enteroids were resuspended in 1ml of enteroid growth media and placed back in the incubator for 6 hours, with the lid to their tubes left open for gas exchange. The media and cell pellet were then collected via centrifugation at 150 x g for 10 minutes.

Preparation of BSA-bound FFA. To test the effect of dietary fatty acids on apoC-III expression, we treated the enteroids and Caco-2 cells with BSA-bound FFA. Oleic acid (Nu-Check Prep) was prepared as 4mM stock solutions in complex with fatty acid-free bovine serum albumin (BSA) at a 1:4 molar ratio and the stock contained butylated-

hydroxytoluene 0.1% (19). Cells not receiving the 400μM OA: BSA complex were treated with an equivalent amount of BSA.

Immunofluorescence. Enteroids were grown in chamber glass wells and fixed with 4% PFA in PBS for 30 min, followed by 50mM NH_4Cl in PBS for 30min to quench autoflorescence. They were washed 5X with PBS, followed by 0.5% Triton X-100 in PBS for 30min, and then were blocked in 5%BSA in PBST overnight at 4°C. Fixed enteroids were incubated with primary antibody against apoB (1:100, Abcam #ab20737) for 3 days at 4°C. After 5 washes with PBS, samples were incubated with secondary Alexa 488 (1:500, Abcam #ab150065) overnight at 4°C. Enteriod were also stained with nuclei stain Hoechst 33342 (10μg/ml in PBST). Images were captured by confocal microscopy use NiKon A1R (20X and 60X water objective lens).

Gene Expression via RT-qPCR. RNA was isolated from washed cells (Caco-2 and enteroids) and isolated tissues (gavage and western diet mice) according to Trizol product instructions. RNA pellets were dissolved in nuclease-free dH_2O before concentration was determined using a BioTek Epoch spectrophotometer. cDNA was synthesized using 2 μg of RNA in a iScript cDNA synthesis kit (BioRad, #1708890). Quantitative real-time polymerase chain reaction (PCR) was conducted using the CFX Connect real time system (BioRad) and iTaq SYBR® Green Supermix (Bio-Rad). Human RPLPO was used as the reference gene in the Caco-2 studies. Mouse cyclophilin was used as the reference gene in dietary studies. Gene expression was calculated using the comparative threshold cycle method.

Plasma lipid and glucose analysis. To measure plasma lipids, we used the Randox (catalog # TR210) triglyceride assay and total cholesterol assay (catalog # CH200), in mice that had been fasted overnight (6pm-6am). Plasma glucose was also measured in the fasted state, using liquid glucose oxidase assay by Pointe Scientific, Inc (Canton, MI G7521-120).

Statistics*.* All data are presented as the mean ± the SEM. Statistics were performed using GraphPad Prism (version 6.0). The differences were analyzed by Student's t-test. Analyses of more than 1 experimental group, compared to control tissue, were analyzed by one-way ANOVA. Differences were considered significant at $P < 0.05$.

RESULTS

In murine intestinal enteroids, apoC-III is not regulated by glucose, insulin, or fatty acid.

Although previous studies have determined that insulin [77], glucose [57], and fatty acids [68] regulate apoC-III expression in the liver, little is known about intestinal apoC-III regulation. To determine how intestinal apoC-III is regulated, we utilized a primary intestinal enteroid culture system. Primary enteroids are derived from WT mouse duodenal and jejunal crypt stem cells, and we have previously established that this tissue culture system significantly improves upon Caco-2 cells as a model for dietary fat absorption and chylomicron secretion [75]. In Fig. 1A we show the 3-dimensional architecture of mature primary enteroids, which apoB-positive cells surrounding a central lumen (reflecting the *in vivo* architecture of the intestine). Because hepatic

apoC-III is regulated by both glucose and insulin, we tested the hypothesis that these conditions may also regulate intestinal apoC-III. Treatment of enteroids with glucose or insulin did not alter intestinal apoC-III expression in the enteroids (Fig. 1B, 1C). Since apoC-III is secreted from enterocytes on chylomicrons (a process that is stimulated by dietary fat), we hypothesized that intestinal apoC-III would also be responsive to dietary fatty acids[4]. After incubation of primary enteroids with oleic acid (Fig. 1D), we determined that intestinal apoC-III mRNA expression is not stimulated by this fatty acid. We confirmed these results in Caco-2 cells, since it has been previously shown that Caco-2 cells transfected with FoxO1, have decreased apoC-III expression in response to increasing insulin concentrations [77]. As in our enteroid culture studies, incubation with glucose, insulin, or oleic acid did not alter apoC-III expression in Caco-2 cells. Taken together, these results indicate in both primary intestinal enteroids and Caco-2 cells, apoC-III mRNA is uniquely non-responsive to changes in glucose, insulin, or fatty acids. This contrasts with the well-established regulation of hepatic apoC-III by these factors.

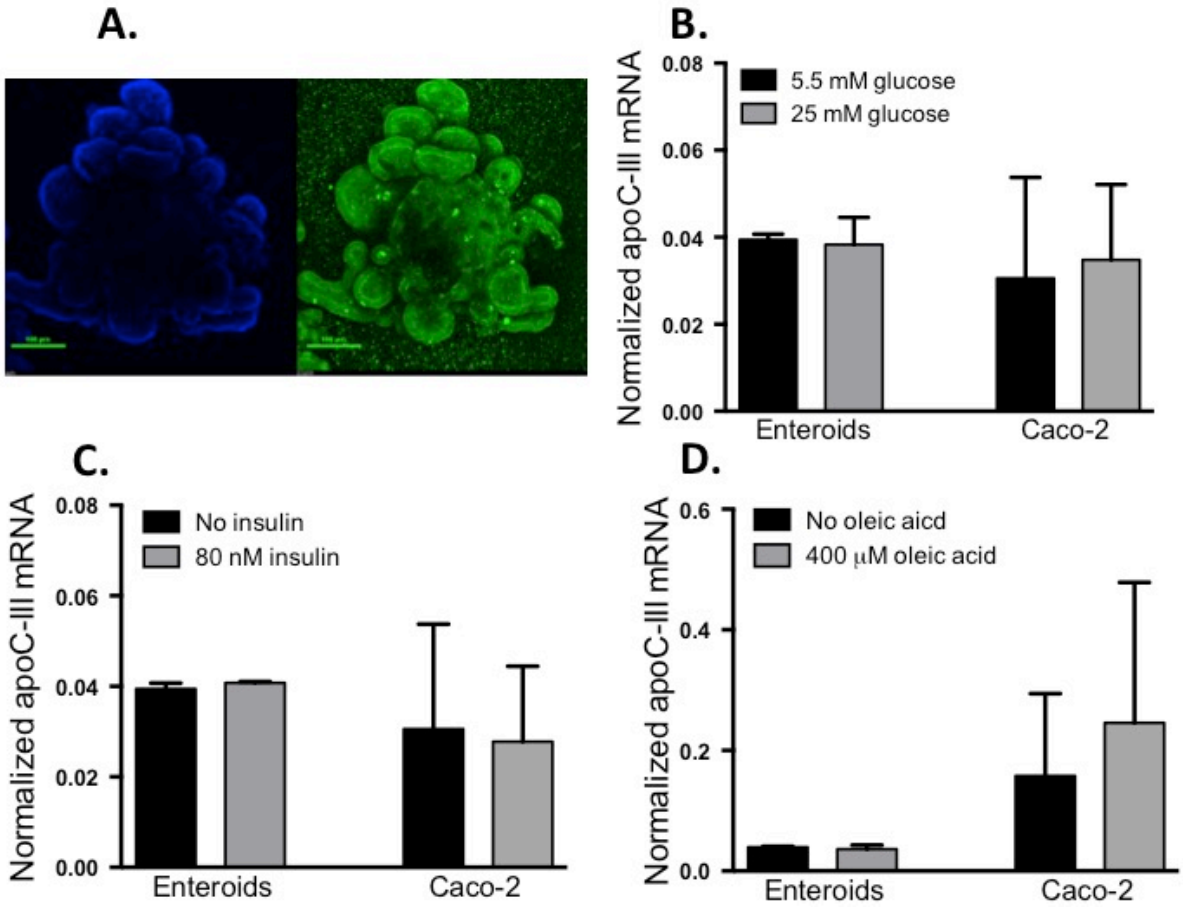

Fig. 1: ApoC-III mRNA expression in primary murine enteroids or Caco-2 cells is not affected by treatment with glucose, insulin, or oleic acid. (A) Primary duodenal murine enteroids were cultured for 10 days until they reach maturity with cells arranged around a central lumen, and a basolateral face towards the media. Enteroids were fixed and stained with Hoechst stain (blue: nuclei) and anti-apoB (green) to visualize the 3D architecture of the enteroid culture. Enteroid and Caco-2 cell (cultured for 17d post-confluence) apoC-III mRNA expression in response to **(B)** glucose, **(C)** insulin, **(D)** BSA bound oleic acid. Bars represent mean apoC-III expression ±SEM, n=3-7.

FoxO1 expression is low in the intestine

Since we saw no changes in intestinal apoC-III expression in response to glucose were observed, insulin, and fatty acid, we decided to investigate whether intestinal apoC-III is a target of FoxO1 inhibition in the intestine. We find that in comparison to hepatic FoxO1 mRNA expression, its expression in duodenal enteroids is low to undetectable (Fig 2A). We also find that treating primary enteroids with insulin, in the presence of either low or high glucose, does not change FoxO1 mRNA expression (Fig. 2B).

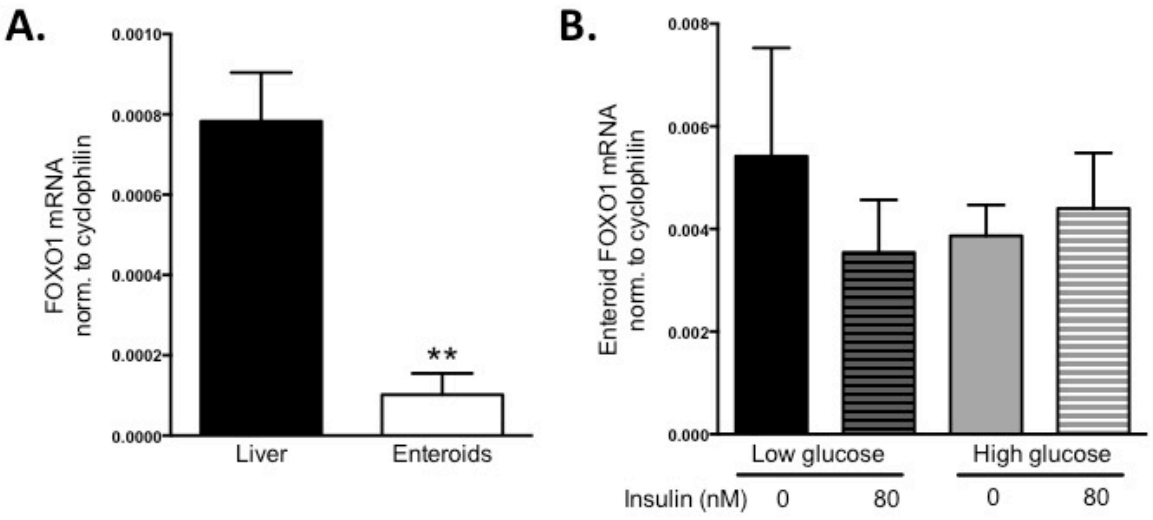

Fig. 2: FoxO1 mRNA expression in murine enteroids. (A) FoxO1 mRNA expression in WT mouse liver (fasted) and mature murine duodenal enteroids FOXO-1, normalized to cyclophilin. **(B)** FoxO1 mRNA expression in mature murine duodenal enteroids when treated for 6-h with insulin, in both low glucose (5.5 mM) and high glucose (25 mM) media. Bars represent mean apoC-III expression ±SEM, n=3-5.

In vivo, intestinal apoC-III mRNA expression is not regulated in response to gavaged lipid and glucose.

To further explore the differences in acute hepatic and intestinal apoC-III regulation in response to diet, we took an *in vivo* approach. We gavaged WT mice daily for one week with either saline, corn oil, or corn oil l plus glucose. After the treatment period, hepatic apoC-III expression increased with both the corn oil and corn oil + glucose treatment, confirming that hepatic apoC-III expression is regulated by these dietary nutrients (Figure 3A). In contrast, intestinal apoC-III expression did not change under any treatment condition, further corroborating the cell culture findings (Figure 3A). Since FoxO1 is regulated by both expression, phosphorylation, and nuclear exclusion, we measured the expression of canonical FoxO1 target gene, insulin-like growth factor binding protein 1 (IGFBP-1), in response to the gavaged nutrients (Fig. 3B). We find that in the liver, corn oil plus glucose robustly stimulate IGFBP-1, in parallel with apoC-III expression (and suggesting that FoxO1 is active under these conditions). In contrast, IGFBP-1 is non-detectable in the intestine in response to gavage.

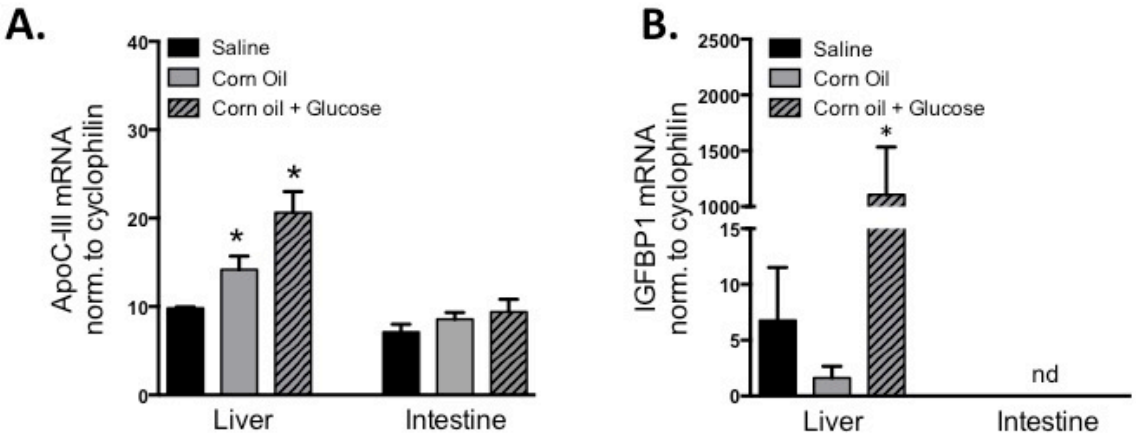

Fig 3. Duodenal mRNA expression of FoxO1 target genes, apoC-III and IGFBP-1, are not changed after a week-long daily gavage. (A) ApoC-III mRNA expression in liver or intestine in response to 1 week of daily gavages of isovolumetric saline, corn oil (60μL), or corn oil (60μL) with glucose (135μL of 5M glucose). **(B)** IGFBP-1 mRNA expression in liver or intestine of gavaged mice. Bars represent ±SEM of normalized mRNA expression, *P<0.05 versus saline, not detectable (nd), n=3-4 WT mice per gavage group.

Western diet stimulates hepatic apoC-III expression but does not alter intestinal apoC-III expression.

Western diet is known to increase plasma triglycerides, and the expression of genes in the liver that increase CVD risk. It is unknown to what extent the increase in plasma triglycerides are due to an increase in intestinal or hepatic apoC-III. We challenged WT mice for 12 weeks with western diet, consisting of 42% calories from butterfat and 0.2% total cholesterol, and compared these to mice provided standard chow diet. In response to western diet, mice have a significant increase in body weight compared to chow-fed controls (Fig. 4A). As expected, WT mice on the western diet had an approximately 2-fold increase in plasma TAG (53.15mg/dL versus 24.37mg/dL; p=0.01. Fig. 5B) and an approximately 3-fold increase in plasma cholesterol (230mg/dL

versus 77mg/dL; p=0.01. Fig.4C). Plasma glucose was not significantly increased in response to the western diet (Fig. 4D). Suggesting that the mice do not lose insulin-tolerance after the 12 week western diet.

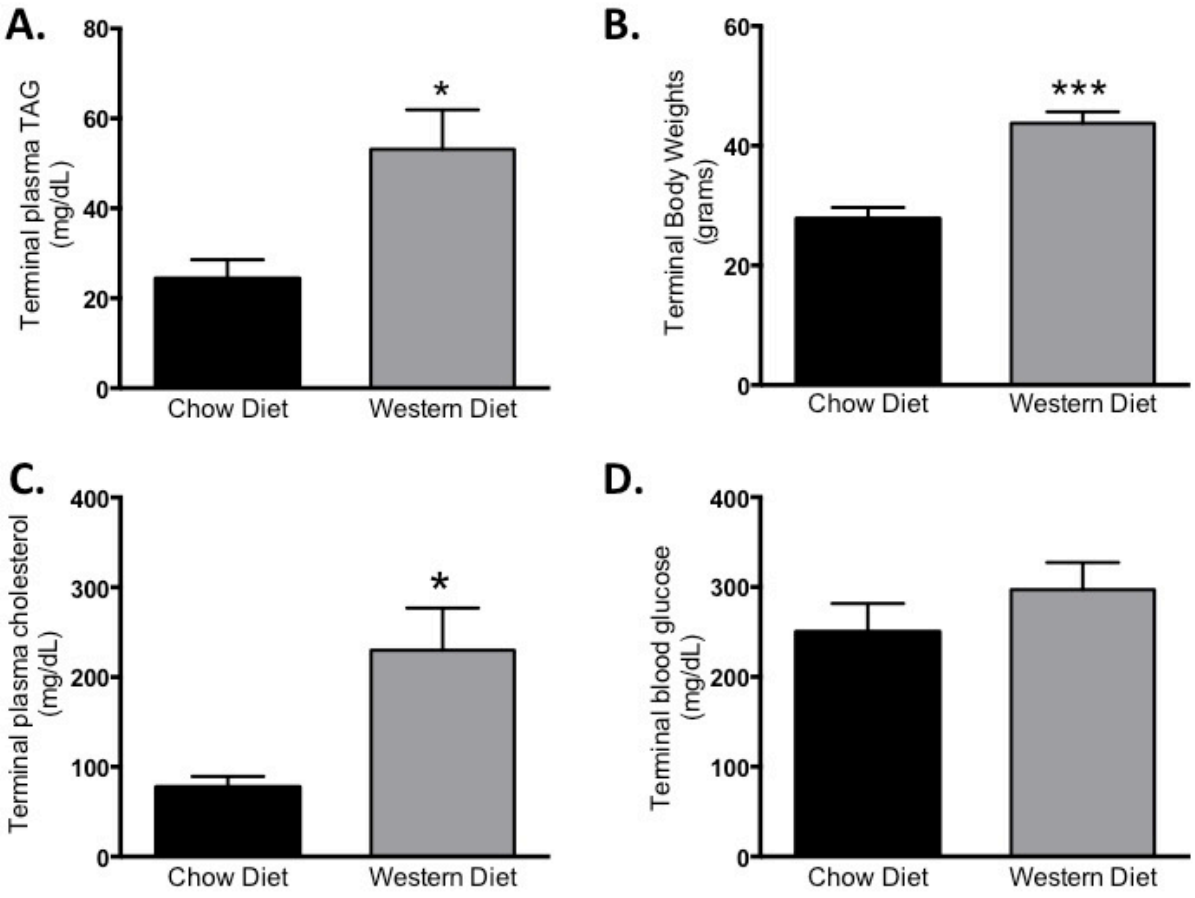

Fig. 4: A 12 week western type diet alters plasma lipids and increases body weight compared to a chow diet. (A) Fasting plasma triglycerides in chow and WTD-fed mice. **(B)** Terminal body weights in grams. **(C)** Fasting plasma total cholesterol. **(D)** Fasting blood glucose blood glucose levels after the diet concluded. Data represent mean ± SEM. **P<0.05*, ****P<0.001,* n=5 per diet.

In response to the western diet, hepatic apoC-III expression is significantly higher compared to chow-fed controls (Fig. 5A), and both hepatic FoxO1 mRNA and its canonical target gene, IGFBP1, are also increased in parallel to apoC-III (Fig. 5B, 5C).

In contrast to these hepatic changes, western diet did not alter the expression of intestinal apoC-III (Fig. 5D). Interestingly, intestinal FoxO1 expression was decreased in the intestine and IGFBP1 is not changed (Fig. 5E, 5F). These data confirm that apoC-III is not regulated in the intestine, as it is in the liver, and strongly suggests that FoxO1 is not responsible for intestinal apoC-III mRNA regulation, in contrast to its importance in regulating apoC-III in the liver.

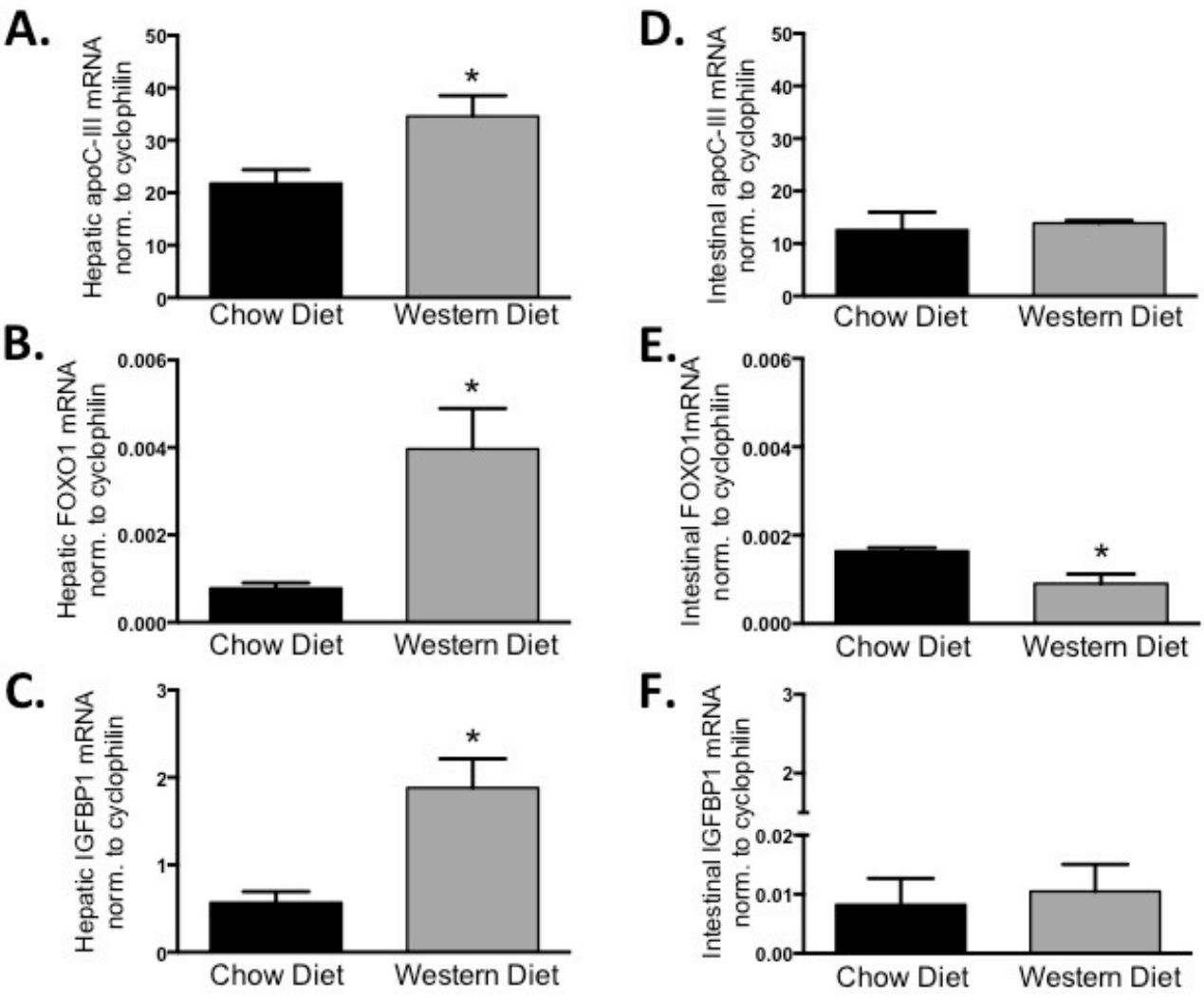

Fig. 5: Western diet increases hepatic but not intestinal apoC-III. Hepatic mRNA expression of **(A)** apoC-III, **(B)** FoxO1 and **(C)** IGFBP-1 expression in chow-fed versus WTD-fed mice. Intestinal expression of **(D)** apoC-III, **(E)** FoxO1 and **(F)** IGFBP-1. Bars represent ±SEM of normalized mRNA expression, *P<0.05 versus chow-fed, n=5/diet.

DISCUSSION

Hepatic apoC-III regulation has been studied at length because of its importance in triglyceride homeostasis and ultimately its role as an independent predictor of cardiovascular disease incidence [44,50,59]. Considerably less is known about the factors that regulate intestinal apoC-III. In this work, we show that intestinal apoC-III expression does not change in response to fatty acids, glucose, or insulin administration, in contrast to hepatic apoC-III. Intestinal apoC-III is not sensitive to changes in FoxO1 expression (which is itself very low in the intestine, as is FoxO1 target IGFBP-1), nor is intestinal apoC-III responsive to the western diet, a significant contrast to hepatic apoC-III stimulation during feeding with a western diet. These data strongly suggest that intestinal apoC-III is not a FoxO1 target. These data support the idea that apoC-III is not regulated coordinately with hepatic apoC-III, and establishes another key aspect of apoC-III that is unique in the intestine and different from the liver.

In these studies, we have focused on dietary lipid and glucose, since these were previously identified as major drivers of apoC-III expression in liver [59,77,80]. Because we have identified a unique consequence of apoC-III overexpression in the intestine (the inhibition of dietary fat absorption coupled with altered chylomicron secretion)[20,75], and because regulation of apoC-III in Caco-2 cell culture is so far removed from *in vivo* conditions that might regulate apoC-III, we wanted to establish the critical dietary factors in the regulation of intestinal apoC-III.

Dong et al previously established that hepatic apoC-III is a target of FoxO1, through the –498/–403 element in the *APOC3* promoter [59]. They showed that deletion of this consensus IRE causes unrestrained apoC-III expression during insulin-resistance

and diabetes, leading to hypertriglyceridemia. As part of their assessment of FoxO1 and apoC-III, Dong et al also provide evidence from Caco-2 cells overexpressing constitutively active FoxO1 that apoC-III expression is also under the control of FoxO1 in the intestine. They suggest that apoC-III is coordinately regulated in both intestine and liver.

Instead of Caco-2 cells, here we have used primary murine enteroids, isolated and cultured from duodenal and jejunal stem cells. We have previously established that these duodenal enteroids absorb fatty acid and form triglyceride-rich chylomicrons, which mirrors their origin in the small intestine. This primary culture system significantly improves upon Caco-2 cells because of their 3D architecture, with enterocytes arranged around a central lumen, and a basolateral surface facing the media. Therefore, the lack of apoC-III regulation in response to individual stimuli in this culture system strongly supports the idea that intestinal apoC-III, within the *in vivo* context, is not regulated by dietary factors in parallel with the liver.

We find that FoxO1 expression in mouse intestine is extremely low, and treatment with insulin changes this very slightly. This is in contrast to Dong et al. However, our data support recent work by Accili et al. [81], who have established a role of FoxO1 in the intestine in endocrine progenitor and serotonin-producing cells. Accili et al. use immunohistochemistry for a detailed analysis of FoxO1 expression in the human gut. They establish that FoxO1 is enriched in the crypt bottoms, and specifically co-localizes with serotonin (5HT)-positive endocrine (rather than absorptive enterocyte) cells. Our data in intact mouse intestine supports this finding because we see such low expression in whole tissue (endocrine cells are vastly outnumbered by enterocytes).

Primary enteroids also maintain a small population of enteroendocrine cells, which is likely the source of FoxO1 expression in our cultures. The restriction of FoxO1 to endocrine cells in the intestine would preclude it from directly regulating apoC-III expression in the absorptive epithelium, though this does not necessarily mean that endocrine cells don't play an important role in modulating the enterocyte function. Why does it matter that intestinal apoC-III in not regulated by western diet, or through the action of FoxO1? Our findings are additional support for the notion that while the liver and intestine share the role of triglyceride-rich lipoprotein synthesis and secretion, they do not share identical regulatory pathways for proteins that are involved. Our data also support the notion that apoC-III secretion from the intestine is under a unique set of pressures. Whereas the liver can quite significantly change VLDL secretion rates in response to the presence or absence of substrate and hormones, the intestine is less labile in the face of dietary lipid, which is quickly and efficiently secreted in chylomicrons. Therefore, our finding that apoC-III levels in the intestine do not change, suggests that apoC-III is important for this physiological action of the intestine, and that there is still room to identify factors that might inhibit apoC-III in this tissue for the purposes of ameliorating its stimulatory role in plasma hypertriglyceridemia and CVD.

Summary and Conclusions

Our work demonstrates that intestinal apoC-III is not regulated *in vivo* or *in vitro* by glucose, insulin, or fat and is therefore not coordinately regulated with hepatic apoC-III, which responds to those factors. Furthermore, this study determines that FoxO1 is not a regulator of intestinal apoC-III. Our lab has previously demonstrated that the physiological function of intestinal apoC-III differs drastically from hepatic apoC-III. Hence, both the regulation and role of intestinal apoC-III is distinctive from what we know about apoC-III in the liver.

Future Directions

Given the importance of the digestive system in mediating the body's interaction with exogenous nutrients, the intestine is likely still an important mediator of CVD risk. Since prior research by our lab has demonstrated that the physiological role of apoC-III in the intestine differs from that in the liver, it may be possible that intestinal apoC-III can favorably alter CVD risk. In the future, an organ-specific knockout of apoC-III will delineate the precise role of apoC-III in each tissue. We also have yet to uncover why apoC-III decreases TAG secretion from enterocytes and the precise machinery behind that effect. And finally, this work does not support the hypothesis that FoxO1 regulates apoC3 in the intestine. We did not uncover in this study the factors that regulate intestinal apoC-III. Continued exploration of factors that regulate apoC-III expression in the intestine are still necessary.

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