The Cholesterol Transporter ABCA1 and Cholesterol Accumulation are Key Regulators of Adipose Function in Obesity

By

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List of Abbreviations

ABCA1	ATP Binding Casette Transporter A1
ABCG1	ATP Binding Casette Transporter A1
ASKO	Adipose Specific ABCA1 KO mouse
FC	Free Cholesterol
TC	Total cholesterol
TG	Triglyceride
EAT	Epididymal Adipose Tissue
BAT	Brown Adipose Tissue
SAT	Subcutaneous Adipose Tissue
RRAT	Retrorenal Adipose Tissue
WAT	White Adipose Tissue
VAT	Visceral Adipose Tissue
UCP-1	Uncoupling Protein 1
SRB-1	Scavenger Receptor Class B -1
LDLr	Low Density Lipoprotein Receptor
VLDLr	Very Low Density Lipoprotein Receptor

CD36/FAT	Fatty Acid Translocase
SREBP1/2	Sterol Response Element Binding Protein 1/2
SCAP	SREBP Cleavage Activating Protein
INSIG	Insulin Induced Gene
FAS	Fatty Acid Synthase
FA	Fatty Acid
NEFA	Non-Esterified Fatty Acid
LDL	Low Density Lipoprotein
HDL	High Density Lipoprotein
VLDL	Very Low Density Lipoprotein

ABSTRACT

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The Cholesterol Transporter ABCA1 and Cholesterol Accumulation are Key Regulators of Adipose Function in Obesity

Dissertation under the direction of John S. Parks, Ph.D., Professor of Internal Medicine and Biochemistry

Obesity now affects over 1/3 of the U.S. adult population, and this number is predicted to grow in the next decades. In addition, obesity, and it's related comorbidities, cost the U.S. healthcare system roughly $150 billion per year. Central to the development of obesity is adipose tissue mass expansion. During nutritional excess, adipose stores excess energy as triglycerides (TG), which results in adipocyte hypertrophy and fat pad expansion. In addition, adipose is a dynamic endocrine organ that releases a variety of adipokines that affect whole body metabolism. While much research has focused on adipose storage of TG in the past, this dissertation focuses on cholesterol. Adipocytes can account for up to 50% of the body's free cholesterol storage, but the effect of cholesterol on adipose tissue, and the role of adipose tissue in whole body cholesterol metabolism are relatively understudied. This thesis presents two models to study adipose cholesterol. The first used a non-human primate model to determine the effect of additional dietary cholesterol on adipose tissue depots, and the second uses an adipose specific knock out of ATP Binding Cassette Transporter A1 (ABCA1) to study adipose cholesterol metabolism in mice.

In the first study, non-human primates (African Green Monkeys) were fed low, medium or high cholesterol diets for 10 weeks. Upon examination of the adipose tissue, we observed that adipocytes from the high cholesterol fed group were larger in the visceral, but not subcutaneous, adipose depots. In addition, visceral adipose from the high cholesterol group showed significantly higher levels of inflammation than the low cholesterol group. These results suggest that high levels of dietary cholesterol are detrimental to adipose tissue, and that cholesterol accumulation in adipocytes may contribute to adipocyte hypertrophy.

In the second study, we used an adipocyte specific mouse Cre promoter (AdiponectinCre) to knock down the cholesterol transporter ABCA1 in adipocytes. We hypothesized that this would result in lower levels of plasma cholesterol, and an accumulation of cholesterol in adipocytes. Adipocyte ABCA1 was determined to have minor effects on plasma cholesterol levels, but significant effects on adipose cholesterol content. In fact, ASKO adipose had roughly 3-fold the cholesterol content of control mice. The cholesterol accumulation also led to smaller adipocytes, a decrease in adipose tissue TG accumulation on an obesogenic diet, and resistance to diet-induced weight gain. This study suggests that: 1) adipocyte ABCA1 does contribute to whole body cholesterol metabolism, 2) ABCA1 is the major cholesterol efflux protein in adipocytes and is therefore responsible to determining cholesterol content, and 3) cholesterol in adipocytes can signficantly effect normal adipose (TG accumulation) function.

Overall, these studies provide strong evidence that adipose cholesterol is important for adipose function. Our results suggest that dietary cholesterol is detrimental to adipose function, and may contribute to adipocyte hypertrophy and inflammation, and that cholesterol, under certain circumstances, can regulate TG accumulation in adipocytes.

Chapter 1

Introduction

Helen Cuffe prepared this chapter with John Parks acting in an advisory and editorial capacity

In 2014, obesity prevalence among US adults was 34.9%, representing a 100% increase from 1994 [1]. The obesity epidemic was predicted to cost the US health system over $147 billion in 2008 [2]. Development of obesity is associated with comorbidities, including dyslipidemia, cardiovascular disease, the metabolic syndrome, type 2 diabetes, and, more recently, cancer [3]. Central to the development of obesity is adipose tissue expansion [4]. Although adipose tissue functions to store excess energy as triglyceride (TG), it is also a dynamic endocrine organ that affects whole body metabolism [5]. As the obesity epidemic continues, it becomes important to understand at a molecular level the contribution of adipose tissue to whole body metabolic health, and to identify and define the mechanisms that cause metabolic dysfunction. This thesis attempts to clarify the effects and consequences of cholesterol overload on adipose tissue, and the role that cholesterol accumulation has on obesity progression and its comorbidities.

1. Adipose Tissue

1.1 Adipose tissue types and locations

Adipose tissue can be categorized into two different subsets, brown and white, although some intermediate (beige) adipose has also been observed [6].

Brown adipose tissue (BAT) expends energy as heat by non-shivering thermogenesis through the uncoupling of mitochondrial oxidative phosphorylation. Non-shivering thermogenesis increases during cold exposure.

The uncoupling of BAT oxidative phosphorylation is due to the high activity of Uncoupling Protein 1 (UCP-1), which creates a proton leak at the mitochondrial membrane, thus eliminating the electrochemical gradient needed for oxidative phosphorylation and ATP production [7]. BAT tissue contains fewer fat cells, a high mitochondrial content, and increased vascularity compared to white adipose tissue (WAT). BAT is involved in fever production, hibernation, and cold-induced thermogenesis [8]. In humans, BAT is abundant pre-natally, but less so in adults. Recent studies have found metabolically active brown adipocytes in multiple adipose depots in adult humans [7]. Brown/beige adipocytes are visibly distinct from white adipocytes because they have smaller multilocular lipid droplets, and a higher mitochondrial content [9]. The bieging of white adipocytes is currently an area of extensive research as a potential therapy for the treatment of obesity.

WAT is the predominant whole body adipose depot, the major site for TG storage, and the source of free (i.e., unesterified) fatty acids (FFAs) that are used as substrates for ATP generation during fasting [8]. White adipose is subdivided into two main categories; visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). VAT surrounds the inner organs and can be further divided into mesenteric (attached to the intestines), retroperitoneal (surrounding the kidneys), gonadal (attached to uterus and ovaries or epididymus and testis), and pericardial (surrounding the heart) depots [10]. Monkeys and humans have an additional omental depot in the upper abdominal cavity that surrounds the liver, intestines and stomach [10].

WAT was initially thought to only function in insulation and energy storage; however, it is now known that adipose has a multitude of functions than can vary considerably by location in the body. VAT secretes pro-inflammatory adipokines [11] and is involved in local and systemic inflammatory processes. WAT located within skeletal muscle secretes free fatty acids, interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα) and, as a consequence, plays a significant role in the development of insulin resistance [12]. Pericardial adipose tissue secretes cytokines resulting in local inflammatory events and chemotaxis that can result in the development of atherosclerosis and systolic hypertension [13]. Perirenal adipose tissue plays a role in sodium reabsorption, affecting intravascular volume and hypertension [14]. Subcutaneous adipose tissue contributes less to the comorbidities associated with obesity, but it is responsible for the majority of adipokine (leptin) secretion [15] and does release proinflammatory cytokines in obesity [16]. Despite known differences between the functionality of WAT depots, the mechanisms by which they differ are not fully understood. The amount of white fat (in particular abdominal) is directly related to morbidity, as well as the incidence, of type 2 diabetes, cardiovascular disease, and the metabolic syndrome [3].

1.2 Adipocytes

The main cell type in adipose tissue is the adipocyte or fat cell [17]. These cells store TG in a large, central lipid droplet that can occupy over 90% of the cell volume. The lipid droplet is surrounded by a small rim of cytoplasm, which separates it from the plasma membrane and contains all the other organelles

[18]. Adipocytes, unlike other cells, have a relatively low number of mitochondria, allowing them to specialize in TG storage, with minimal FA oxidation [19]. WAT is composed of adipocytes held together by a loose connective tissue that is highly vascularized and innervated, and includes other cells, such as macrophages, preadipocytes, endothelial cells, fibroblasts and other immune cells [20]. Adipocytes have the ability to expand depending on lipid storage requirements; their size increases dramatically during weight gain, and adipocyte size strongly correlates with TG content [21]. Adipocytes are unique in that they can store a large amount of lipid without the lipotoxicity experienced by other cells types [17]. .

Adipocytes differentiate from precursor cells present in adipose tissue and differentiation occurs relatively early in development. The extent of adipocyte differentiation in adult mammals is unknown [22]. Adipocyte precursor cells emerge from mesenchymal stem cells (MSCs) that are themselves derived from the mesodermal layer of the embryo. Two key transcription factors initiate differentiation; CCAAT/enhancer binding protein alpha (C/EBPα) and peroxisome proliferator-activated receptor gamma (PPARγ). These transcription factors promote lipid storage and lipid droplet formation, as well as the gene expression profile of a mature adipocyte. Other transcription factors influence the differentiation process; however, PPARγ is the only factor that has been shown to be essential [22].

1.3 Adipose TG metabolism

WAT TG represents the major energy reserves of the body. The pool of TG is in a constant state of flux that is regulated by food intake and fasting, and the consequences of those dietary states on the levels of pancreatic hormones (reviewed in [23]). In addition, adipose tissue fat pools change as a result of other hormonal fluctuations, inflammatory processes, and pathophysiology [8].

The majority of TGs initially enter the body through food consumption. Initial processing begins in the stomach and small intestine where a series (mostly pancreatic lipase) of lipases hydrolyze TGs to mono-acylyglycerol (MG) and FFAs. These molecules then move into the enterocytes through diffusion, or transport (CD36, FATP). Inside the enterocytes, FFAs and MG are re-esterified to form TG at the ER by the monoacylglycerol acetyltransferase (MGAT) and diacyglycerol acetyltransferase enzymes (DGAT). TGs are then packaged with other lipids (phospholipids, cholesterol) into chylomicrons, which enter the blood stream through the lymphatic system [24]. Chylomicrons deliver TGs to extrahepatic tissues, such as muscle and adipose, by binding to lipoprotein lipase, which hydrolyzes TG to free FAs and glycerol, which are then taken up into the tissues. Lipoprotein lipase (LPL) is synthesized by adipocytes, secreted into the surrounding tissue, and transported to capillary endothelial cells [25], where is binds to glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1(GPIHBP1), which anchors LPL to the capillary wall, allowing it to hydrolyze plasma lipoprotein TGs [26]. Ten-16% of dietary TGs end up in the liver, where they can be re-packaged into VLDL particles, secreted, and once

again interact with LPL to deliver FAs to the tissues [27]. In times of starvation, FAs are primarily delivered to the muscle for use, whereas in times of dietary excess, TGs are primarily taken up by the adipose tissue for storage. The liver also produces TG through *de novo* lipogenesis, and these molecules are also transported to peripheral tissues via VLDL particles [28].

In times of nutrient excess, adipocytes take up excess energy through the action of LPL, which catalyzes the release of FAs from TGs contained in circulating VLDL, LDL and chylomicrons. Fatty acids are then taken up into the cell through transporters (CD36, FATP1) or diffusion. Once inside the cell, FAs are acylated, and re-esterified to glycerol-3-phosphate (G3P) to form TGs. G3P is produced from glucose through the glycolysis pathway. Glycerol-3-phosphate acyltransferase (GPAT) performs the first step, which commits G-3-P to the TG synthesis pathway, and adds one FA. A second FA is then added by 1-acylglycerol-3-phosphate acyltransferase (AGPAT) to produce phosphatidate. Phosphatidate is then converted by phosphatidate phosphatase-1 (PAP) to diacylglycerol. Lastly, the diacylglycerol acetyltransferase (DGAT) enzymes add a final fatty acid to produce TG [29, 30]. The DGAT enzymes are thought to be rate limiting for this pathway [31]. The completed TG is then stored in the lipid droplet until needed.

Although adipocytes do have the ability to synthesize fatty acids *de novo*, very little seems to occur under physiological conditions. Therefore, the major sources of adipocyte FAs are derived from hepatic *de novo* lipogenesis or diet [8]. Deuterated water incorporation studies have shown that adipose tissue, no

matter the state of obesity, has extremely low levels of *de novo* lipogenesis [32]; however, increased rates of adipocyte *de novo* lipogenesis have been shown to prevent or slow weight loss [33], and may increase during over feeding [34]. Despite limited lipogenesis in mature adipocytes, *de novo* lipogenesis is essential for adipocyte differentiation [35].

During times of metabolic stress (fasting or prolonged exercise), multiple signals (epinephrine, norepinephrine, and glucagon) stimulate the hydrolysis of TG to FAs in adipocytes. This process is termed lipolysis. When lipolytic stimuli bind to their cognate adipocyte receptors, they activate adenylate cyclase, which results in increased cAMP levels [36]. This, in turn, through the action of protein kinase A (PKA), causes the phosphorylation, activation, and translocation of hormone sensitive lipase (HSL) to the lipid droplet [36]. At the lipid droplet surface, lipolytic enzymes are responsible for cleaving FAs from TGs. ATGL has triacyglycerol hydrolase activity, which forms DG, and is also likely responsible for the majority of basal lipolysis. HSL is thought to be the rate limiting step in lipolysis, and has been shown to have both DG and TG hydrolysis activity. Lastly, monoacylglycerol lipase (MGL) catalyzes the final step in the process, and releases the final FA. Additional proteins at the lipid droplet surface, including perilipin and CGI-58, act to stabilize and activate lipolytic proteins and provide access to TG stores [37]. Since FAs have minimal solubility in the aqueous cytoplasm, adipocytes use FABPs to bind and sequester FAs before they are released from the cell. Once released from the cell, FAs are rapidly bound to and transported by albumin. This process also releases glycerol resulting from

the TG backbone. FFAs and glycerol leave the adipocyte and are transported via the bloodstream to other tissues, including the liver, skeletal muscle and heart where they are used to produce ATP, or in the case of the liver, can be re-packaged into TG and secreted in VLDL particles.

Several transcription factors orchestrate adipocyte differentiation, including PPARγ [38, 39], the family of CCAAT enhancer binding proteins (C/EBP) [40], and Sterol Response Element Binding Protein 1 (SREBP1) [41]. PPARγ is the major transcription factor responsible for TG accumulation during adipocyte differentiation, and continues to be important in mature adipocytes. PPARγ is a nuclear transcription factor that along with co-activators, such as PPARγ co-activator 1 (PCG1), induces the transcription of multiple genes, including LPL, adipocyte fatty acid binding protein (aP2), acyl-CoA synthase, and fatty acid transport protein (FATP). PPARγ is upregulated by insulin signalling [42] and downregulated by cytokine signalling, in particular TNFα [43]. SREBP1 and C/EBP proteins also upregulate the transcription of PPARγ [44]. The C/EBP proteins induce the transcription of PPARγ during differentiation and in mature cells, and upregulate genes involved in insulin signalling (IRS, GLUT4), and lipid metabolism (FABP4) [45]. Lastly, activated nuclear SREBP1 (a and c) induce transcription of genes involved in lipogenesis (FAS, SCD-1, PPARγ) [46]. SREBP1 is highly regulated by both insulin (through the upregulation of Insulin induced gene INSIG) and ER cholesterol content through SREBP cleavage activating protein (SCAP) [47].

1.4 Adipose and insulin regulation

Adipocyte metabolism is highly regulated by insulin, which is secreted by the pancreas after feeding. Insulin simultaneously promotes lipogenesis and inhibits lipolysis in adipocytes. Insulin binds to the insulin receptor (IR), which then binds and phosphorylates insulin receptor substrates (IRS-1 and 3 in adipocytes). The majority of downstream insulin signalling occurs through the PI3K pathway, which binds to p-IRS. PI3K can then, through several additional steps, phosphorylate and activate AKT, which facilitates glucose uptake by causing the translocation of glucose transporter 4 (GLUT4) to the cell surface, and prevents lipolysis by dephosphorylating and inhibiting both HSL and perilipin. In the liver, insulin signalling also downregulates INSIG-1, which leads to the activation of SREBP1c and increased lipogenesis [48]; however, recent studies have reported that this may not be the case in adipocytes, and that insulin may act to decrease SREBP1c activation in adipose tissue [49].

1.5 Adipose tissue as an endocrine organ

Besides its primary role as a lipid storage organ, adipose tissue functions as an endocrine organ [20, 50]. Adipokines, such as leptin, adiponectin and resistin, affect adipose tissue and whole body energy metabolism. Adipose tissue secretes more than 50 hormones and signaling molecules, collectively called adipokines, which exert their biological roles in an autocrine, paracrine, or systemic manner, influencing energy and glucose metabolism, and immunity [51].

Of particular importance is the adipokine leptin. Leptin is 16kDa peptide whose central function is the regulation of overall body weight by limiting food intake and increasing energy expenditure. However, leptin also regulates the neuroendocrine axis, inflammatory responses, blood pressure, and bone mass. Adipocytes secrete leptin in direct proportion to adipose tissue mass as well as nutritional status, and secretion is greater for SAT relative to VAT [15, 52]. Leptin effects on energy homeostasis are well-documented [53]. Many of these effects, particularly on energy intake and expenditure, are mediated via hypothalamic pathways, whereas others are mediated via direct action on peripheral tissues, including muscle, pancreatic cells, and adipocytes [54].

Other adipokines, such as adiponectin and resistin, alter whole body metabolism. Adiponectin-mediated AMPK activation results in increased glucose uptake, increased fatty acid oxidation, increased phosphorylation and inhibition of acetylCoA carboxylase (ACC) in muscle and liver, and reduced activity of gluconeogenic enzymes and glucose output. A strong and consistent inverse association between adiponectin and both insulin resistance and inflammatory states has been established [55, 56]. Serum resistin is elevated in rodent obesity, and infusion or sustained expression of resistin produces insulin resistance [57-59]. In addition to these two adipokines, others have been described, included pro-inflamatory adipokines, such as IL-6, TNF-α, and MCP-1.

In addition to adipocytes, WAT contains macrophages, leukocytes, fibroblasts, adipocyte progenitor cells, and endothelial cells. The presence of the fibroblasts, macrophages, and other leukocytes along with adipocytes accounts for the vast

array of proteins that are secreted from WAT under varying physiological conditions. In particular, resident immune cells seem to be primarily responsible for the production and release of inflammatory adipokines from adipose tissue. Adipose tissue macrophages release TNF-α, IL-6, IL-8, IL-10, SAA, MCP-1, and other cytokines that affect not only adipose tissue, but the whole body [60].

2. Adipose tissue in obesity

Obesity is associated with marked changes in the secretory function of adipocytes and macrophages, chronic low-grade inflammation, and an increased risk of developing insulin resistance, diabetes, and vascular disease [3]. Obesity is characterized by excess body fat, due to adipocyte TG accumulation and the subsequent adipose tissue mass expansion. In the obese state, adipocytes undergo enlargement, resulting in multiple functional consequences. Hypertrophied adipocytes exhibit modified properties including insulin resistance, increased release of inflammatory cytokines and leptin, decreased release of other adipokines, including adiponectin, and altered lipid metabolism [61-63]. Indeed, within an obese population, adipocyte size is signficantly corollated with the metabolic syndrome, which is a disorder of energy utilization characterized by abdominal (central) obesity, elevated blood pressure, elevated fasting plasma glucose, high serum triglycerides, and low high-density lipoprotein (HDL) concentrations [64]. Adipocyte enlargement during weight gain plays an important role in adipose dysfunction that occurs in obesity; however, the exact mechanisms by which this occurs are not fully understood. What is clear is that: 1) adipocyte dysfunction plays an important role in obesity and comorbidity

development, and 2) adipocyte dysfunction is a complicated process that is influenced by insulin signalling, inflammatory signalling, and lipid flux. To prevent obesity, it is essential to understand the underling mechanisms by which adipocyte dysfunction occurs.

2.1 TG metabolism in obesity

Hypertrophied adipocytes are more lipolytic (mainly due to insulin resistance), and as a result, release more FFAs into the tissue [65]. Increased FFAs have several consequences both in adipose tissue and in other tissues. In adipose, FFAs signal to adipocytes and resident immune cells to promote inflammation. Increased FFA flux also results in lipid deposition in other tissues, which can lead to liver and muscle insulin resistance and hepatic steatosis [66]. In the obese state, hypertrophied adipocytes are defective in storing excess lipid, resulting in dyslipidemia, inflammation, and systemic insulin resistance [8]. Although adipocyte dysfunction is complex, adipocyte hypertrophy alone is enough to promote both insulin resistance and inflammation [67]. Recent evidence also shows that the obesity-associated insulin resistance, systemic inflammation, hepatic steatosis, and fibrosis can be prevented by manipulating energy metabolism in the adipocyte to promote lipid droplet expansion and TG storage [68, 69]. This suggests that in the obese or hypertrophied state, adipocytes are not capable of incorporating and storing the excess lipid load, and that this defect is central to the dysfunction encountered during obesity.

2.2 Adipocyte insulin resistance

The most common known metabolic consequence of obesity is insulin resistance, which manifests itself as hyperinsulinemia in the presence of dyslipidemia and hyperglycemia [8]. In systemic insulin resistance, the attenuation of downstream insulin signalling results in uncontrolled adipose tissue lipolysis, due to the fact that insulin can no longer inactivate key lipolytic enzymes (mostly HSL), resulting in ectopic lipid deposition in skeletal and cardiac muscle. This, in turn, further exacerbates systemic insulin resistance. Dysregulated lipolysis is the primary source of FFA flux to the liver, which significantly contributes to NAFLD and hepatic insulin resistance [70]. Hypertrophied adipocytes are less sensitive to insulin [71], and have decreased expression of key TG esterification genes, and lipogenic transcription factors, most notably PPARγ. Interestingly, PPARγ agonists alleviate adipose tissue insulin resistance and, in turn, promote lipid storage [72]. While the exact mechanisms of adipocyte insulin resistance remain unclear, adipose tissue plays a central and important role in the development of insulin resistance during obesity.

2.3 Adipose inflammation in obesity

Obesity is a proinflammatory condition in which hypertrophied adipocytes and adipose tissue-resident immune cells (primarily lymphocytes and macrophages) contribute to increased circulating proinflammatory cytokines levels. The obesity-associated state of chronic low-grade systemic inflammation, termed “metabolic inflammation,” is considered a focal point in the pathogenesis of insulin resistance and T2D in humans and rodent animal models [63, 73-75]. Although

liver and muscle show obesity-induced mild inflammatory responses, WAT is the key site mediating systemic inflammation [76]. Adipose tissue from lean individuals preferentially secretes anti-inflammatory adipokines, such as adiponectin, interleukin (IL)-10, and IL-4. In contrast, obese adipose tissue mainly releases proinflammatory cytokines, among which are TNF-α, IL-6, leptin, and resistin, [75]. In lean individuals, anti-inflammatory adipokines mediate physiological functions, whilst in states of metabolic disease, the proinflammatory adipokines modulate insulin resistance either directly by affecting the insulin signaling pathways or indirectly via stimulation of inflammatory pathways.

Lean adipose tissue is characterized by healthy adipocytes and has mostly anti-inflammatory (M2) macrophages. During the development of obesity, there is a switch from anti-inflammatory macrophages to pro-inflammatory macrophages, although there is some debate about the function and type of these macrophages [77]. Recent evidence suggests that obesity-initiated inflammation is due to T cell rather than adipose tissue macrophages [78]. Although immune cells are responsible for the majority of adipose inflammation, the initiating signals, which include MCP-1 (Macrophage Chemoattractant protein -1), LTB4 (leukotriene B4), and others, for immune cell invasion come from adipocytes [79]. Several papers now report that ablating immune cells, including NK cells [80] and macrophages [81], as well as receptors that activate an inflammatory response (e.g., TLR9 [82], and GPR105 [83]) in adipose tissue improves insulin resistance. Although this is a complicated and understudied area, adipose tissue immune cells clearly play an important role in the development of obesity-related symptoms.

Diet-induced obesity also results in altered release of adipocyte-specific adipokines, particularly leptin. Diet-induced obesity simultaneously leads to increased leptin secretion, and reduced autocrine signalling in adipocytes [84]. Leptin tends to be anti-adipogenic, since mice that overexpress the leptin receptor in adipocytes are protected from diet-induced obesity [66]. Higher leptin levels generally indicate a more obese state and have a significant impact on food intake and adipocytes (in particular reducing response to insulin [85]). Obese individuals are also known to be leptin resistent; a situation in which leptin is less able to suppress appetite [86]. In contrast, adiponectin is expressed at high levels in lean adipose tissue, but drops during obesity, and this decrease contributes significantly to obesity and insulin resistance [55]. The expression and release of other adipokines is altered during obesity, and currently over 50 adipokines have been identified [51]. These results suggest that adipose tissue can play both a protective and a detrimental role in the development of obesity, and that the specific regulation of adipose tissue adipokines is essential for normal energy homeostasis.

3. Cholesterol

3.1 Whole body cholesterol metabolism

Cholesterol is an extremely important biological molecule that plays a role in membrane structure [87] as well as being a precursor for the synthesis of the steroid hormones and bile acids [88]. Both dietary cholesterol and cholesterol synthesized *de novo* are transported by circulating lipoprotein particles. Plasma

cholesterol concentrations are maintained by biosynthesis through the endogenous pathway and absorption of dietary and biliary cholesterol through the exogenous pathway [89].

In the intestinal lumen, cholesterol along with other lipids are taken up into the enterocytes. This process is facilitated by bile salts, which form micelles [90]. The mechanism by which cholesterol is taken up into the enterocytes was once thought to be an energy-independent, passive diffusion process; however, recent evidence contradicts this as transporter proteins ABCG5/8 and Niemann-Pick C1 like 1 (NPC1L1) have been identified. NPC1L1 is primarily responsible for cholesterol uptake from the intestine lumen into enterocytes, whereas the ABCG5/8 heterodimer is responsible for shuttling cholesterol back into the intestinal lumen [24]. Once in the enterocytes, cholesterol is esterified with FA and packaged into chylomicrons and released into the blood through the lymphatic system. In circulation, chylomicrons deliver TGs to peripheral tissues as previously described. Once the TG is hydrolyzed and the released FA has been taken up into tissues, the chylomicrons remnants, which include cholesterol, are taken up by the liver [91]. This uptake is facilitated by the interaction of apoE with hepatic cell surface receptors, including the LDL receptor (LDLr) and through hepatic lipase [92]. Cholesterol from the liver can be secreted into the bile or can be incorporated, as free or esterified cholesterol, into lipoproteins, namely very low density lipoprotein (VLDL) and LDL, which are then secreted into plasma. Elevated liver cholesterol leads to increased VLDL and/or LDL production, as well as down-regulation of the LDL receptor [93]. LDL and

VLDL move through the circulation, interacting with receptors on the cell surface of peripheral tissues, including LDLr, VLDLr, and LRP, which facilitates cholesterol uptake [94].

When peripheral cells (macrophages, adipocytes etc.) contain too much cholesterol (as discussed below), cellular efflux to HDL is increased. In species containing cholesterol ester transfer protein (CETP), HDL cholesterol can either be transferred by CETP to LDL and VLDL, or be taken up by the liver. Cholesterol can again be secreted through the bile or re-secreted in VLDL particles. This process of cholesterol efflux from peripheral tissues and macrophages back to the liver, and then cholesterol secretion through the bile, is the only quantitative way the body has to remove excess cholesterol [95].

3.2 Cellular cholesterol regulation

Cellular cholesterol levels are tightly controlled, mainly through the action of the liver X receptors (LXR) and sterol response element binding protein 2 (SREBP2). LXRs are nuclear receptors that are activated by oxidized derivatives of cholesterol (i.e., oxysterols). LXRs act as cholesterol sensors; when cellular oxysterols accumulate as a result of increasing cholesterol concentrations, LXR induces the transcription of genes that protect cells from cholesterol overload [96]. This includes upregulating cholesterol efflux through ABCA1, ABCG1 and apoE, and downregulating cholesterol uptake, mainly acting by upregulating IDOL (inducible degrader of LDLr), which subsequently leads to LDLr degradation [97]. SREBP2 is a transcription factor whose activation is highly

dependent on cholesterol content in the ER membrane, where it is bound to SCAP (SREBP Cleavage activating protein). SCAP, through its sterol sensing domain, is able to detect ER cholesterol levels. When low levels of cholesterol exist, SCAP facilitates SREBP2 movement to the Golgi where two proteases, site1 and site 2, generate a soluble N-terminal fragment of SREBP2 containing a basic helix-loop-helix leucine zipper containing transcription factor. The N terminal SREBP2 then translocates to the nucleus where it functions as a transcription factor for every gene involved in cholesterol biosynthesis. In addition, SREBP2 upregulates genes involved in cholesterol uptake (LDLr) and downregulates genes involved in cholesterol efflux [98], acting together with cholesterol biosynthetic pathways to normalize and maintain appropriate cellular cholesterol levels.

3.3 Cellular cholesterol distribution

Cholesterol is an essential component of mammalian cell membranes but the overall cholesterol:protein ratio can vary signficantly depending on cell density. Cholesterol is enriched in the plasma membrane where it typically accounts for 40-45%of the lipid molecules, with phospholipids, sphingolipids, and glycolipids making up the remainder [99, 100]. Cholesterol has many functions in the plasma membrane, affecting protein binding, lipid ordering, and permeability [101].

Cholesterol is abundant in the Golgi, with enrichment towards the trans-golgi compartments, and the endocytic recycling compartment [102]. In contrast, the

ER has a low cholesterol content, although cholesterol in the ER does appear to have several functions, most importantly interacting with SCAP to regulate the SREBP transcription factors [103]. Cholesterol content in the ER is essential for the movement of membrane secretory proteins to the Golgi [104]. Specifically, cholesterol depletion inhibits the movement of secretory proteins [104], but induces the movement of SCAP to the Golgi [105]. As discussed previously, ER sterol content functions as a sensor for the regulation of SREBP activity, and can therefore regulate lipogenesis and cellular cholesterol content.

Adipocyte Cholesterol

4.1 Adipose tissue is a major cholesterol depot

Although TG is the major lipid in adipose tissue, sterols account for 1-5 mg/g of total lipid content [106]. In normal weight individuals, adipose tissue can account for 15-20% of total cholesterol body stores, which increases to ~50% in obese individuals [107]. Unlike other cells, adipocytes store cholesterol almost exclusively as free cholesterol (FC) (>95%), rather than esterified cholesterol [108]. Due to the biophysical properties of FC, cholesterol in adipocytes is not located within the core of the lipid droplet, but instead accumulates in the plasma membrane or in the lipid droplet phospholipid monolayer [109]. There is a strong correlation between adipocyte size and cholesterol content [107], with larger fat cells typically containing proportionately higher amounts of cholesterol. TG to cholesterol ratio remains constant over a wide body mass index (BMI) range;

however, as discussed below, intracellular FC distribution significantly affects adipocyte function.

4.2 Regulation of adipocyte cholesterol content

The relative rate of cholesterol synthesis in adipocytes is low with incorporation of radiolabeled acetate into adipocyte cholesterol only 4% that of liver [110]. Likewise, in studies using radiolabelled mevalonate, only 10% of the radiolabel was found in cholesterol after 3 hours of incubation [111]. Since the synthesis rate is relatively low, the majority of adipose cholesterol likely originates from circulating lipoproteins. Adipocytes, like other cell types, express LDL and VLDL receptor proteins, and cultured adipocytes rapidly accumulate cholesterol when incubated with LDL [112, 113]. Adipocytes also express LPL, which is involved in lipolysis of TG-rich lipoproteins and subsequent fatty acid uptake, and it is likely that during this process some cholesterol is also taken up. A large amount of adipocyte cholesterol appears to come from selective uptake from HDL [114] where cholesterol, but not the intact lipoprotein, is internalized. This process occurs through both scavenger receptor B1 (SR-B1) dependent [115] and independent mechanisms that involve LDL receptor-related protein (LRP) and CETP [116, 117]. SR-B 1 dependent uptake occurs at caveolae, small cholesterol rich invaginations on the cell surface, where CE is extracted and incorporated into the membrane compartment. SR-B1 is highly expressed on adipocytes [118]; however, relatively little is known about its function and regulation.

Cholesterol is effluxed from adipocytes through several mechanisms. Although adipocytes express high levels of ATP-binding cassette transporters, there is still some controversy about their ability to efflux cholesterol from adipocytes. In cultured 3T3L1 cells, cholesterol efflux through ABCA1 only occurred during prolonged TG lipolysis [119]; however, in other studies, LDL [120] and serum amyloid A (A-SAA) stimulate ABCA1-dependent efflux [121]. Recent *in vivo* work by our lab and others have confirmed the ability of adipose tissue to efflux cholesterol through ABCA1 [122, 123] and that this protein may have additional functions within adipocytes, such as regulating inflammation [124] and insulin resistance [123, 125]. Adipocytes also express ABCG1; however, its function may be restricted to cholesterol transport between the lipid droplet and the plasma membrane. The first studies regarding adipose ABGC1 have shown that silencing it in 3T3L1 cell culture results in reduced TG storage, and that high levels of expression positively corollate with both obesity and degree of insulin resistance in humans [126]. Recent evidence has shown that adipocyte cholesterol efflux, in particular ABCA1-mediated efflux, contributes signficantly to whole body cholesterol homeostasis [122, 123].

4.3. Cholesterol and adipocyte dysfunction

Few studies have focused on the effects of cholesterol on adipocyte function, such as lipid storage, insulin resistance, and inflammation. Although adipocyte cholesterol content has not yet been directly correlated with human disease, there are several potential mechanisms by which cholesterol accumulation and imbalance might affect normal adipocyte function.

Cholesterol distribution is altered in hypertrophied adipocytes. For example, plasma membrane FC content is decreased in hypertrophied adipocytes obtained from obese vs. normal weight rats [127]. Adipocyte cholesterol may act as a sensor for adipocyte size through transcriptional regulation of SREBP2 and SREBP1 [125]. In hypertrophied adipocytes, low levels of ER cholesterol would result in the activation and nuclear localization of SREBP1 and 2. These transcription factors would then promote the transcription of genes involved in lipogenesis (PPARγ, FAS, SCD-1) through SREBP1, and genes involved in cholesterol content (LDLr, HMGCoA reducatase and synthase). This concept has been demonstrated for SREBP2; hypertrophied adipocytes, despite having increased total cholesterol, actually activate SREBP2 and show increased expression of cholesterol synthesis genes, HMGCoA reductase and synthase [125]. As previously discussed, the SREBP1 pathway regulates a number of lipid storage genes and transcription factors, in particular PPARγ [44]. In theory, adipocyte hypertrophy causes a relative depletion of membrane and ER cholesterol, leading to the activation of SREBP1 and 2, and promotion of lipid (both TG and cholesterol) storage.

Cellular FC distribution may also affect insulin signalling, which in turn, affects *de novo* lipogenesis. For example, acute removal of adipocyte membrane FC by incubation with methyl-beta-cyclodextrin results in decreased glucose utilization and reduce FA uptake in response to insulin stimulation, suggesting membrane FC content affects insulin signaling [125] [128]. Thus, larger adipocytes that

accompany obesity would have relatively lower amounts of membrane FC, resulting in adipocyte insulin resistance.

Cholesterol also plays an important role in adipose tissue inflammation. For example, adding cholesterol alone to the diet leads to increased adipose inflammation in mice and nonhuman primates [129, 130]. In macrophages and the artery wall, increases in cholesterol content are associated with increased inflammation. HDL also has known anti-inflammatory effects on adipose tissue. When ABCA1, ABCG1 and SR-B1 were sequentially deleted from adipocytes, response to inflammatory stimuli increased [124]. Likewise, the lectin-like oxidized LDL receptor 1 is essential for the release of pro-inflammatory adipocyte cytokines and is also upregulated during obesity [131].

Overall, current evidence suggests that adipocyte cholesterol content has a significant influence of adipocyte function. In particular, cholesterol location, rather than total amount, seems to be important to maintain normal adipocyte function.

Research Intent

Adipose tissue is the main TG storage organ in mammals and plays an essential role in the development of obesity. During adipocyte expansion and TG accretion that accompanies obesity, adipose tissue becomes inflamed and dysfunctional, which affects whole body energy metabolism and homeostasis. While much is known about the pathways regulating TG storage in adipose tissue, relatively little is known about cholesterol. Adipose tissue in the obese state can account

for up to 50% of the body's cholesterol stores, making this a significant depot. Early research suggests that cholesterol can, and does, have significant effects of adipose tissue metabolism. The intent of this research is to further explore the mechanisms by which cholesterol affects adipocyte function and relate these pathways back to the obese state.

In the work described here, we have taken two approaches. The first uses a non-human primate model to study the effect of dietary cholesterol on adipose tissue, and the second uses an adipose-specific ABCA1 knockout mouse model to determine the adipose specific effects of cholesterol overload. In our non-human primate model, dietary cholesterol overload led to increased adipocyte size and increased inflammation. Our mouse model, however, was in stark contrast to the non-human primate results; adipocyte cholesterol overload resulted in a resistance to diet-induced weight gain, smaller adipocytes and reduced TG storage, with no induction of inflammation. These models highlight the importance of adipocyte cholesterol, and support the idea that cholesterol balance, particularly membrane cholesterol content, signficantly affects adipocyte function.

References

1. Ogden, C.L., et al., *PRevalence of childhood and adult obesity in the united states, 2011-2012.* JAMA, 2014. **311**(8): p. 806-814.

2. Finkelstein, E.A., et al., *Annual Medical Spending Attributable To Obesity: Payer-And Service-Specific Estimates.* Health Affairs, 2009. **28**(5): p. w822-w831.

3. Iacobellis, G., et al., *Prevalence of uncomplicated obesity in an Italian obese population.* Obes Res, 2005. **13**(6): p. 1116-1122.

4. Guilherme, A., et al., *Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes.* Nat Rev Mol Cell Biol, 2008. **9**(5): p. 367-377.

5. Goossens, G.H., *The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance.* Physiology & Behavior, 2008. **94**(2): p. 206-218.

6. Harms, M. and P. Seale, *Brown and beige fat: development, function and therapeutic potential.* Nat Med, 2013. **19**(10): p. 1252-1263.

7. Redinger, R.N., *Fat storage and the biology of energy expenditure.* Transl Res, 2009. **154**(2): p. 52-60.

8. Rutkowski, J.M., J.H. Stern, and P.E. Scherer, *The cell biology of fat expansion.* J Cell Biol, 2015. **208**(5): p. 501-512.

9. Schulz, T.J. and Y.H. Tseng, *Brown adipose tissue: development, metabolism and beyond.* Biochem J, 2013. **453**(2): p. 167-78.

10. Bjørndal, B., et al. (2011). "Different Adipose Depots: Their Role in the Development of Metabolic Syndrome and Mitochondrial Response to Hypolipidemic Agents." Journal of Obesity **2011**: 490650.

11. Batra, A. and B. Siegmund, *The Role of Visceral Fat.* Digestive Diseases, 2012. **30**(1): p. 70-74.

12. Argilés, J.M., et al., *Cross-talk between skeletal muscle and adipose tissue: A link with obesity?* Medicinal Research Reviews, 2005. **25**(1): p. 49-65.

13. Fitzgibbons, T.P. and M.P. Czech, *Epicardial and Perivascular Adipose Tissues and Their Influence on Cardiovascular Disease: Basic*

Mechanisms and Clinical Associations. Journal of the American Heart Association, 2014. **3**(2).

14. Naumnik, B. and M. Mysliwiec, *Renal consequences of obesity.* Med Sci Monit, 2010. **16**(8): p. Ra163-170.

15. Fain, J.N., et al., *Comparison of the Release of Adipokines by Adipose Tissue, Adipose Tissue Matrix, and Adipocytes from Visceral and Subcutaneous Abdominal Adipose Tissues of Obese Humans.* Endocrinology, 2004. **145**(5): p. 2273-2282.

16. Wajchenberg, B.L., et al., *Depot-Specific Hormonal Characteristics of Subcutaneous and Visceral Adipose Tissue and their Relation to the Metabolic Syndrome.* Horm Metab Res, 2002. **34**(11/12): p. 616-621.

17. Konige, M., H. Wang, and C. Sztalryd, *Role of adipose specific lipid droplet proteins in maintaining whole body energy homeostasis.* Biochim Biophys Acta, 2014. **1842**(3): p. 393-401.

18. Napolitano, L., *THE DIFFERENTIATION OF WHITE ADIPOSE CELLS. AN ELECTRON MICROSCOPE STUDY.* J Cell Biol, 1963. **18**: p. 663-679.

19. Kopecky, J., et al., *Mitochondrial uncoupling and lipid metabolism in adipocytes.* Biochem Soc Trans, 2001. **29**(Pt 6): p. 791-797.

20. Fruhbeck, G., et al., *The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation.* Am J Physiol Endocrinol Metab, 2001. **280**(6): p. E827-847.

21. Chen, H.C. and R.V. Farese, *Determination of adipocyte size by computer image analysis.* Journal of Lipid Research, 2002. **43**(6): p. 986-989.

22. Avram, M.M., A.S. Avram, and W.D. James, *Subcutaneous fat in normal and diseased states - 3. Adipogenesis: From stem cell to fat cell.* Journal of the American Academy of Dermatology, 2007. **56**(3): p. 472-492.

23. Frayn, K.N., et al., *Integrative physiology of human adipose tissue.* Int J Obes Relat Metab Disord, 2003. **27**(8): p. 875-888.

24. Iqbal, J. and M.M. Hussain, *Intestinal lipid absorption.* American Journal of Physiology - Endocrinology and Metabolism, 2009. **296**(6): p. E1183-E1194.

25. Young, S.G., et al., *GPIHBP1, an endothelial cell transporter for lipoprotein lipase.* Journal of Lipid Research, 2011. **52**(11): p. 1869-1884.

26. Young, S.G., et al., *GPIHBP1: an endothelial cell molecule important for the lipolytic processing of chylomicrons.* Curr Opin Lipidol, 2007. **18**(4): p. 389-396.

27. Lambert, J.E. and E.J. Parks, *Postprandial metabolism of meal triglyceride in humans.* Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 2012. **1821**(5): p. 721-726.

28. Hegele, R.A., *Plasma lipoproteins: genetic influences and clinical implications.* Nat Rev Genet, 2009. **10**(2): p. 109-121.

29. Takeuchi, K. and K. Reue, *Biochemistry, physiology, and genetics of GPAT, AGPAT, and lipin enzymes in triglyceride synthesis.* American Journal of Physiology - Endocrinology and Metabolism, 2009. **296**(6): p. E1195-E1209.

30. Ahmadian, M., et al., *Triacylglycerol metabolism in adipose tissue.* Future lipidology, 2007. **2**(2): p. 229-237.

31. Yen, C.-L.E., et al., *DGAT enzymes and triacylglycerol biosynthesis.* Journal of Lipid Research, 2008. **49**(11): p. 2283-2301.

32. Guo, Z.K., et al., *De novo lipogenesis in adipose tissue of lean and obese women: application of deuterated water and isotope ratio mass spectrometry.* Int J Obes Relat Metab Disord, 2000. **24**(7): p. 932-937.

33. Garrido-Sánchez, L., et al., *De Novo Lipogenesis in Adipose Tissue Is Associated with Course of Morbid Obesity after Bariatric Surgery.* PLoS ONE, 2012. **7**(2): p. e31280.

34. McDevitt, R.M., et al., *De novo lipogenesis during controlled overfeeding with sucrose or glucose in lean and obese women.* The American Journal of Clinical Nutrition, 2001. **74**(6): p. 737-746.

35. Collins, J.M., et al., *De novo lipogenesis in the differentiating human adipocyte can provide all fatty acids necessary for maturation.* Journal of Lipid Research, 2011. **52**(9): p. 1683-1692.

36. Egan, J.J., et al., *Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(18): p. 8537-8541.

37. Londos, C., et al., *Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells.* Biochem Soc Trans, 1995. **23**(3): p. 611-615.

38. Tontonoz, P., et al., *mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer.* Genes Dev, 1994. **8**(10): p. 1224-1234.

39. Tontonoz, P., E. Hu, and B.M. Spiegelman, *Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor.* Cell, 1994. **79**(7): p. 1147-1156.

40. Wu, Z., N.L. Bucher, and S.R. Farmer, *Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids.* Mol Cell Biol, 1996. **16**(8): p. 4128-4136.

41. Tontonoz, P., et al., *ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation.* Mol Cell Biol, 1993. **13**(8): p. 4753-4759.

42. Rieusset, J., et al., *Insulin acutely regulates the expression of the peroxisome proliferator-activated receptor-gamma in human adipocytes.* Diabetes, 1999. **48**(4): p. 699-705.

43. Chinetti, G., J.C. Fruchart, and B. Staels, *Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation.* Inflamm Res, 2000. **49**(10): p. 497-505.

44. Fajas, L., et al., *Regulation of Peroxisome Proliferator-Activated Receptor γ Expression by Adipocyte Differentiation and Determination Factor 1/Sterol Regulatory Element Binding Protein 1: Implications for Adipocyte Differentiation and Metabolism.* Molecular and Cellular Biology, 1999. **19**(8): p. 5495-5503.

45. Lefterova, M.I., et al., *PPARγ and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale.* Genes & Development, 2008. **22**(21): p. 2941-2952.

46. Shimano, H., et al., *Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and in cultured cells.* Journal of Clinical Investigation, 1997. **99**(5): p. 846-854.

47. Sun, L.-P., et al., *Insig Required for Sterol-mediated Inhibition of Scap/SREBP Binding to COPII Proteins in Vitro.* Journal of Biological Chemistry, 2005. **280**(28): p. 26483-26490.

48. Owen, J.L., et al., *Insulin stimulation of SREBP-1c processing in transgenic rat hepatocytes requires p70 S6-kinase.* Proceedings of the National Academy of Sciences, 2012. **109**(40): p. 16184-16189.

49. Boden, G., et al., *Comparison of in vivo effects of insulin on SREBP-1c activation and INSIG-1/2 in rat liver and human and rat adipose tissue.* Obesity, 2013. **21**(6): p. 1208-1214.

50. Kershaw, E.E. and J.S. Flier, *Adipose Tissue as an Endocrine Organ.* Journal of Clinical Endocrinology & Metabolism, 2004. **89**(6): p. 2548-2556.

51. Waki, H. and P. Tontonoz, *Endocrine Functions of Adipose Tissue.* Annual Review of Pathology: Mechanisms of Disease, 2007. **2**(1): p. 31-56.

52. Wajchenberg, B.L., *Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome.* Endocr Rev, 2000. **21**(6): p. 697-738.

53. Friedman, J.M. and J.L. Halaas, *Leptin and the regulation of body weight in mammals.* Nature, 1998. **395**(6704): p. 763-770.

54. Bjorbaek, C. and B.B. Kahn, *Leptin signaling in the central nervous system and the periphery.* Recent Prog Horm Res, 2004. **59**: p. 305-331.

55. Chandran, M., et al., *Adiponectin: more than just another fat cell hormone?* Diabetes Care, 2003. **26**(8): p. 2442-2450.

56. Diez, J.J. and P. Iglesias, *The role of the novel adipocyte-derived hormone adiponectin in human disease.* Eur J Endocrinol, 2003. **148**(3): p. 293-300.

57. Rangwala, S.M. and M.A. Lazar, *Peroxisome proliferator-activated receptor γ in diabetes and metabolism.* Trends in Pharmacological Sciences, 2004. **25**(6): p. 331-336.

58. Qi, Y., et al., *Loss of Resistin Improves Glucose Homeostasis in Leptin Deficiency.* Diabetes, 2006. **55**(11): p. 3083-3090.

59. Rajala, M.W., et al., *Adipose-derived resistin and gut-derived resistin-like molecule–β selectively impair insulin action on glucose production.* Journal of Clinical Investigation, 2003. **111**(2): p. 225-230.

60. Greenberg, A.S. and M.S. Obin, *Obesity and the role of adipose tissue in inflammation and metabolism.* The American Journal of Clinical Nutrition, 2006. **83**(2): p. 461S-465S.

61. Qatanani, M. and M.A. Lazar, *Mechanisms of obesity-associated insulin resistance: many choices on the menu.* Genes & Development, 2007. **21**(12): p. 1443-1455.

62. Savage, D.B., K.F. Petersen, and G.I. Shulman, *Disordered Lipid Metabolism and the Pathogenesis of Insulin Resistance*. Vol. 87. 2007. 507-520.

63. Shoelson, S.E., L. Herrero, and A. Naaz, *Obesity, Inflammation, and Insulin Resistance.* Gastroenterology, 2007. **132**(6): p. 2169-2180.

64. O'Connell, J., et al., *The Relationship of Omental and Subcutaneous Adipocyte Size to Metabolic Disease in Severe Obesity.* PLoS ONE, 2010. **5**(4): p. e9997.

65. Novak, C.C., et al., *Metal ion levels in maternal and placental blood after metal-on-metal total hip arthroplasty.* Am J Orthop (Belle Mead NJ), 2014. **43**(12): p. E304-308.

66. Wang, M.Y., et al., *Fat storage in adipocytes requires inactivation of leptin's paracrine activity: implications for treatment of human obesity.* Proc Natl Acad Sci U S A, 2005. **102**(50): p. 18011-18016.

67. Kim, J. I., et al. (2015). "Lipid-Overloaded Enlarged Adipocytes Provoke Insulin Resistance Independent of Inflammation." Molecular and Cellular Biology **35**(10): 1686-1699.

68. Franckhauser, S., et al., *Increased Fatty Acid Re-esterification by PEPCK Overexpression in Adipose Tissue Leads to Obesity Without Insulin Resistance.* Diabetes, 2002. **51**(3): p. 624-630.

69. Stark, R., et al., *A role for mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) in the regulation of hepatic gluconeogenesis.* J Biol Chem, 2014. **289**(11): p. 7257-7263.

70. Samuel, V.T., et al., *Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease.* J Biol Chem, 2004. **279**(31): p. 32345-32353.

71. Czech, M.P., *Cellular basis of insulin insensitivity in large rat adipocytes.* The Journal of Clinical Investigation, 1976. **57**(6): p. 1523-1532.

72. Albrektsen, T., et al., *Novel genes regulated by the insulin sensitizer rosiglitazone during adipocyte differentiation.* Diabetes, 2002. **51**(4): p. 1042-1051.

73. Gregor, M.F. and G.S. Hotamisligil, *Inflammatory Mechanisms in Obesity.* Annual Review of Immunology, 2011. **29**(1): p. 415-445.

74. Hotamisligil, G.S., *Inflammation and metabolic disorders.* Nature, 2006. **444**(7121): p. 860-867.

75. Ouchi, N., et al., *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol, 2011. **11**(2): p. 85-97.

76. Odegaard, J.I. and A. Chawla, *Pleiotropic actions of insulin resistance and inflammation in metabolic homeostasis.* Science, 2013. **339**(6116): p. 172-177.

77. Kratz, M., et al., *Metabolic dysfunction drives a mechanistically distinct proinflammatory phenotype in adipose tissue macrophages.* Cell Metab, 2014. **20**(4): p. 614-625.

78. Deng, T., et al., *Class II Major Histocompatibility Complex Plays an Essential Role in Obesity-Induced Adipose Inflammation.* Cell Metabolism, 2013. **17**(3): p. 411-422.

79. Curat, C.A., et al., *From Blood Monocytes to Adipose Tissue-Resident Macrophages: Induction of Diapedesis by Human Mature Adipocytes.* Diabetes, 2004. **53**(5): p. 1285-1292.

80. O'Rourke, R.W., et al., *Systemic NK cell ablation attenuates intra-abdominal adipose tissue macrophage infiltration in murine obesity.* Obesity, 2014. **22**(10): p. 2109-2114.

81. Feng, B., et al., *Clodronate Liposomes Improve Metabolic Profile and Reduce Visceral Adipose Macrophage Content in Diet-Induced Obese Mice.* PLoS ONE, 2011. **6**(9): p. e24358.

82. Nishimoto, S., et al., *Genetic ablation of TLR9 improves insulin resistance through macrophage accumulation in adipose tissue*. Vol. 34. 2013.

83. Xu, J., et al., *GPR105 Ablation Prevents Inflammation and Improves Insulin Sensitivity in Mice with Diet-Induced Obesity.* The Journal of Immunology, 2012. **189**(4): p. 1992-1999.

84. Balistreri, C.R., C. Caruso, and G. Candore, *The Role of Adipose Tissue and Adipokines in Obesity-Related Inflammatory Diseases.* Mediators of Inflammation, 2010. **2010**: p. 19.

85. Kim, S. and N. Moustaid-Moussa, *Secretory, Endocrine and Autocrine/Paracrine Function of the Adipocyte.* The Journal of Nutrition, 2000. **130**(12): p. 3110S-3115S.

86. Myers, M.G., et al., *Obesity and Leptin Resistance: Distinguishing Cause from Effect.* Trends in endocrinology and metabolism: TEM, 2010. **21**(11): p. 643-651.

87. Maxfield, F.R. and I. Tabas, *Role of cholesterol and lipid organization in disease.* Nature, 2005. **438**(7068): p. 612-621.

88. Rezen, T., et al., *Interplay between cholesterol and drug metabolism.* Biochim Biophys Acta, 2011. **1814**(1): p. 146-160.

89. van der Wulp, M.Y.M., H.J. Verkade, and A.K. Groen, *Regulation of cholesterol homeostasis.* Molecular and Cellular Endocrinology, 2013. **368**(1–2): p. 1-16.

90. Woollett, L.A., et al., *Micellar solubilisation of cholesterol is essential for absorption in humans.* Gut, 2006. **55**(2): p. 197-204.

91. Cooper, A.D., *Hepatic uptake of chylomicron remnants.* J Lipid Res, 1997. **38**(11): p. 2173-2192.

92. Havel, R.J., *Receptor and non-receptor mediated uptake of chylomicron remnants by the liver.* Atherosclerosis, 1998. **141 Suppl 1**: p. S1-7.

93. Chen, J., W. Song, and R.N. Redinger, *Effects of dietary cholesterol on hepatic production of lipids and lipoproteins in isolated hamster liver.* Hepatology, 1996. **24**(2): p. 424-434.

94. Takahashi, S., et al., *The very low-density lipoprotein (VLDL) receptor: characterization and functions as a peripheral lipoprotein receptor.* J Atheroscler Thromb, 2004. **11**(4): p. 200-208.

95. Tall, A.R., *An overview of reverse cholesterol transport.* European heart journal, 1998. **19 Suppl A**: p. A31-35.

96. Zhao, C. and K. Dahlman-Wright, *Liver X receptor in cholesterol metabolism.* J Endocrinol, 2010. **204**(3): p. 233-240.

97. Zelcer, N., et al., *LXR Regulates Cholesterol Uptake Through Idol-Dependent Ubiquitination of the LDL Receptor.* Science, 2009. **325**(5936): p. 100-104.

98. Eberle, D., et al., *SREBP transcription factors: master regulators of lipid homeostasis.* Biochimie, 2004. **86**(11): p. 839-848.

99. Ray, T.K., et al., *Lipid Composition of Rat Liver Plasma Membranes.* Journal of Biological Chemistry, 1969. **244**(20): p. 5528-5536.

100. van Meer, G., D.R. Voelker, and G.W. Feigenson, *Membrane lipids: where they are and how they behave.* Nat Rev Mol Cell Biol, 2008. **9**(2): p. 112-24.

101. Ikonen, E., *Cellular cholesterol trafficking and compartmentalization.* Nat Rev Mol Cell Biol, 2008. **9**(2): p. 125-38.

102. Mukherjee, S., R.N. Ghosh, and F.R. Maxfield, *Endocytosis.* Physiol Rev, 1997. **77**(3): p. 759-803.

103. Lange, Y., *Disposition of intracellular cholesterol in human fibroblasts.* J Lipid Res, 1991. **32**(2): p. 329-39.

104. Ridsdale, A., et al., *Cholesterol is required for efficient endoplasmic reticulum-to-Golgi transport of secretory membrane proteins.* Mol Biol Cell, 2006. **17**(4): p. 1593-605.

105. Nohturfft, A., M.S. Brown, and J.L. Goldstein, *Sterols regulate processing of carbohydrate chains of wild-type SREBP cleavage-activating protein (SCAP), but not sterol-resistant mutants Y298C or D443N.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 12848-53.

106. Angel, A. and J. Farkas, *Regulation of cholesterol storage in adipose tissue.* Journal of Lipid Research, 1974. **15**(5): p. 491-499.

107. Krause, B.R. and A.D. Hartman, *Adipose tissue and cholesterol metabolism.* Journal of Lipid Research, 1984. **25**(2): p. 97-110.

108. R. H. Schreibman, R.B.D., *Human adipocyte Cholesterol. Concentration, localization, synthesis, and turnover.* Journal of clinical investigation, 1975. **55**(5): p. 986-993.

109. Prattes, S., et al., *Intracellular distribution and mobilization of unesterified cholesterol in adipocytes: triglyceride droplets are surrounded by cholesterol-rich ER-like surface layer structures.* Journal of Cell Science, 2000. **113**(17): p. 2977-2989.

110. Kovanen, P.T., E.A. Nikkilä, and T.A. Miettinen, *Regulation of cholesterol synthesis and storage in fat cells.* Journal of Lipid Research, 1975. **16**(3): p. 211-223.

111. Tilvis, R.S., P.T. Kovanen, and T.A. Miettinen, *Release of newly synthesized squalene, methyl sterols and cholesterol from human adipocytes in the presence of lipoproteins.* Scandinavian Journal of Clinical & Laboratory Investigation, 1978. **38**(1): p. 83-87.

112. Barbaras, R., et al., *Binding of lipoproteins and regulation of cholesterol synthesis in cultured mouse adipose cells.* Biochim Biophys Acta, 1985. **845**(3): p. 492-501.

113. Fong, B.S., P.O. Rodrigues, and A. Angel, *Characterization of low density lipoprotein binding to human adipocytes and adipocyte membranes.* Journal of Biological Chemistry, 1984. **259**(16): p. 10168-10174.

114. Fazio, S. and M.F. Linton, *Unique pathway for cholesterol uptake in fat cells.* Arterioscler Thromb Vasc Biol, 2004. **24**(9): p. 1538-1539.

115. Yvan-Charvet, L., et al., *In vivo evidence for a role of adipose tissue SR-BI in the nutritional and hormonal regulation of adiposity and cholesterol homeostasis.* Arterioscler Thromb Vasc Biol, 2007. **27**(6): p. 1340-1345.

116. Vassiliou, G. and R. McPherson, *A novel efflux-recapture process underlies the mechanism of high-density lipoprotein cholesteryl ester-selective uptake mediated by the low-density lipoprotein receptor-related protein.* Arterioscler Thromb Vasc Biol, 2004. **24**(9): p. 1669-1675.

117. Vassiliou, G., et al., *The low density lipoprotein receptor-related protein contributes to selective uptake of high density lipoprotein cholesteryl esters by SW872 liposarcoma cells and primary human adipocytes.* J Biol Chem, 2001. **276**(52): p. 48823-48830.

118. Acton, S.L., et al., *Expression cloning of SR-BI, a CD36-related class B scavenger receptor.* J Biol Chem, 1994. **269**(33): p. 21003-21009.

119. Le Lay, S., et al., *Regulation of ABCA1 expression and cholesterol efflux during adipose differentiation of 3T3-L1 cells.* Journal of Lipid Research, 2003. **44**(8): p. 1499-1507.

120. Zhao, S.P., et al., *Dual effects of oxidized low-density lipoprotein on LXR-ABCA1-apoA-I pathway in 3T3-L1 cells.* Int J Cardiol, 2008. **128**(1): p. 42-47.

121. Poitou, C., et al., *Role of serum amyloid a in adipocyte-macrophage cross talk and adipocyte cholesterol efflux.* J Clin Endocrinol Metab, 2009. **94**(5): p. 1810-1817.

122. Chung, S., et al. (2011). *Adipose tissue ATP binding cassette transporter A1 contributes to high-density lipoprotein biogenesis in vivo.* Circulation **124**(15): 1663-1672.

123. de Haan, W., et al., *ABCA1 in adipocytes regulates adipose tissue lipid content, glucose tolerance, and insulin sensitivity.* J Lipid Res, 2014. **55**(3): p. 516-523.

124. Umemoto, T., et al. (2013). *Apolipoprotein A-I and HDL Have Anti-Inflammatory Effects on Adipocytes via Cholesterol Transporters: ATP-Binding Cassette (ABC) A-1, ABCG-1 and Scavenger Receptor B-1(SRB-1)*. Circulation Research **112**(10): 1345-1354.

125. Le Lay, S., et al., *Cholesterol, a Cell Size-dependent Signal That Regulates Glucose Metabolism and Gene Expression in Adipocytes.* Journal of Biological Chemistry, 2001. **276**(20): p. 16904-16910.

126. Frisdal, E., et al. (2015). "*Adipocyte ATP-Binding Cassette G1 Promotes Triglyceride Storage, Fat Mass Growth, and Human Obesity*." Diabetes **64**(3): 840-855.

127. Guerre-Millo, M., et al., *Alteration in membrane lipid order and composition in metabolically hyperactive fatty rat adipocytes.* Lipids, 1994. **29**(3): p. 205-209.

128. Covey, S.D., et al., *Cholesterol depletion inhibits fatty acid uptake without affecting CD36 or caveolin-1 distribution in adipocytes.* Biochemical and Biophysical Research Communications, 2007. **355**(1): p. 67-71.

129. Subramanian, S., et al., *Dietary Cholesterol Worsens Adipose Tissue Macrophage Accumulation and Atherosclerosis in Obese LDL Receptor–Deficient Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2008. **28**(4): p. 685-691.

130. Aguilar, D. and M.L. Fernandez, *Hypercholesterolemia induces adipose dysfunction in conditions of obesity and nonobesity.* Adv Nutr, 2014. **5**(5): p. 497-502.

131. Takanabe-Mori, R., et al., *Lectin-like oxidized low-density lipoprotein receptor-1 is required for the adipose tissue expression of proinflammatory cytokines in high-fat diet-induced obese mice.* Biochem Biophys Res Commun, 2010. **398**(3): p. 576-80.

Chapter II

Dietary Cholesterol Promotes Adipocyte Hypertrophy and Adipose Tissue Inflammation in Visceral, But Not Subcutaneous, Fat in Monkeys

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Abstract

Objective: Excessive caloric intake is associated with obesity and adipose tissue dysfunction. However, the role of dietary cholesterol in this process is unknown. The aim of this study was to determine whether increasing dietary cholesterol intake alters adipose tissue cholesterol content, adipocyte size, and endocrine function in nonhuman primates.

Approach and Results: Age-matched, male African Green monkeys (n=5 per group) were assigned to one of three diets containing 0.002 (Lo), 0.2 (Med) or 0.4 (Hi) mg cholesterol/Kcal. After 10 weeks of diet feeding, animals were euthanized for adipose tissue, liver, and plasma collection. With increasing dietary cholesterol, free cholesterol (FC) content and adipocyte size increased in a step-wise manner in visceral, but not subcutaneous fat, with a significant association between visceral adipocyte size and FC content (r^2=0.298; n=15; p=0.035). In visceral fat, dietary cholesterol intake was associated with: 1) increased pro-inflammatory gene expression and macrophage recruitment, 2) decreased expression of genes involved in cholesterol biosynthesis and lipoprotein uptake, and 3) increased expression of proteins involved in FC efflux.

Conclusions: Increasing dietary cholesterol selectively increases visceral fat adipocyte size, FC and macrophage content, and proinflammatory gene expression in nonhuman primates. Visceral fat cells appear to compensate for

increased dietary cholesterol by limiting cholesterol uptake/synthesis and increasing FC efflux pathways.

SIGNIFICANCE

Obesity has reached epidemic proportions; 67% of US adults are either overweight or obese, increasing the risk of other chronic diseases. During progression of obesity, fat cell size increases, resulting in adipocyte dysfunction and adipose tissue inflammation. Caloric-rich diets containing saturated fat and cholesterol (i.e., Western diets) are sufficient to cause obesity in humans and experimental animals. However, the role of individual dietary constituents, particularly dietary cholesterol, in the development of obesity is poorly understood. In this study, we provide the first evidence that increasing dietary cholesterol alone in a typical North American diet selectively induces visceral adipocyte enlargement, adipose tissue cholesterol accumulation, and inflammatory responses in nonhuman primates in the absence of significant body weight differences among animals. Our results establish new metabolic interrelationships between dietary cholesterol and adipocyte hypertrophy, adipose tissue cholesterol accumulation, and inflammation that may explain part of the metabolic dysfunction that accompanies diet-induced obesity.

Introduction

Obesity has reached epidemic proportions worldwide. In the US, 67% of adults (>20 yr old) are overweight (≥25 kg/m^2 body mass index (BMI)) or obese (≥30 kg/m^2 BMI), resulting in medical care cost of $147 billion in 2008 [1, 2]. Obesity increases the risk of other chronic diseases, such as type 2 diabetes, coronary heart disease, insulin resistance, and non-alcoholic fatty liver disease [3]. Adipocytes are active endocrine cells that secrete adipokines, store excess energy as triglyceride (TG), release fatty acids during fasting states, and control systemic glucose disposal [4]. During progression of obesity, fat cell size increases due to TG accretion [5]. Larger adipocytes exhibit decreased responsiveness to insulin, decreased glucose uptake, and increased secretion of proinflammatory adipokines [6-9]. Enlarged adipocytes are associated with macrophage infiltration into adipose tissue, resulting in increased production of proinflammatory cytokines and insulin resistance [10]. Diets high in calories, saturated fat, and cholesterol (i.e., Western diets) are sufficient to cause obesity in humans and experimental animals [3, 5]. However, the role of individual dietary constituents, particularly dietary cholesterol, in the development of obesity is poorly understood.

Adipose tissue is a major site for cholesterol storage in man [11]. In obese states, approximately half of the whole body pool of cholesterol is stored in adipose depots. As adipocyte hypertrophy occurs during development of obesity, cholesterol content also increases [12]. Adipocytes are unique among cells in that nearly all cholesterol (>93%) is stored in the unesterified form (i.e., free

cholesterol, FC) and nearly all of the adipocyte FC (~88%) is distributed on the TG droplet surface, with the remainder located in the plasma membrane [13, 14]. Dietary cholesterol worsened adipose tissue inflammation in a mouse model of diet-induced obesity, suggesting that high dietary cholesterol can lead to adipocyte dysfunction[5]. Whether dietary cholesterol leads to adipocyte hypertrophy and dysfunction in humans is unknown and is a difficult question to address experimentally.

To address this gap in knowledge, we studied the effect of dietary cholesterol on adipocyte size, adipose tissue cholesterol content, and gene/protein expression in a nonhuman primate atherosclerosis model. We chose this model because of its close phylogenetic relationship to humans, and because lipid and lipoprotein responses to a Western-type atherogenic diet in nonhuman primates mimic human responses better than rodent models [15]. In addition, dietary composition is more easily controlled than in humans, and tissues are readily available for subsequent analyses. Here, we demonstrate that increasing dietary cholesterol alone was sufficient to drive adipocyte hypertrophy specifically in visceral fat, with increased adipose tissue FC and inflammatory gene expression in nonhuman primates. Results from this study suggest a role for dietary cholesterol in increasing adipocyte dysfunction, a characteristic feature of the pathogenesis of obesity.

Methods

Animal Studies

Adult male African green monkeys (*Chlorocebus aethiops*) (n=15, age 5–10 years) were obtained from St. Kitts island. Monkeys were housed in an AAALAC-accredited facility under the direct care of the Wake Forest School of Medicine Animal Resources Program and euthanized at the termination of the study. All experiments were approved by the Institutional Animal Care and Use Committees of Wake Forest School of Medicine and University of Florida. Monkeys were singly housed in climate-controlled conditions with a 12 h light and dark cycle. The monkeys were provided water ad libitum and were initially fed a weighed amount of a chow diet (Monkey Diet 5038, Lab Diet) twice daily, such that their daily caloric intake was 70 kcal day/kg body weight. During the 10-week experimental diet feeding phase, the monkeys were fed twice daily with a weighed amount of semi-synthetic diet containing 0.002 (Lo), 0.2 (Med) or 0.4 (Hi) mg cholesterol/Kcal, which was prepared at the Wake Forest Primate Center Diet Laboratory. Daily caloric intake was 90 kcal/day/kg body weight (see Supplemental Table 1 for detailed diet compositions).

Plasma Lipid and Lipoprotein Concentrations

Following an overnight fast, monkeys were sedated with ketamine (10 mg/kg intramuscularly), and blood was collected in EDTA-containing Vacutainer® tubes. Plasma was isolated by centrifugation at 1,500 x g for 30 min at 4°C. Total plasma cholesterol and triglyceride concentrations were measured using reagent

kits from Sigma. Cholesterol distribution among lipoproteins was determined after fractionation of plasma by gel filtration chromatography using a Superose 6 10/300 GL column (GE Healthcare). An aliquot of plasma containing 15 µg total cholesterol was injected onto the column and eluted with 0.9% saline containing 0.01% EDTA and 0.01% sodium azide at a flow rate of 0.4 ml/min. The column effluent was mixed with a commercially available enzymatic total cholesterol reagent (Pointe Scientific, Inc., Canton, MI) delivered at 0.125 ml/min. After passing the mixed effluent through a knitted reaction coil maintained at 37°C, the absorbance of the reaction mixture was read at 500 nm using a ultraviolet-visible detector. The area under the VLDL, LDL and HDL peaks was calculated using Chromperfect Spirit (Justice Laboratory Software) chromatography software. To calculate the cholesterol concentration in each lipoprotein fraction, the ratio of each peak area (VLDL, LDL, HDL) to total peak area was multiplied by the total plasma cholesterol concentration.

Adipose Tissue Cholesterol Content

Adipose cholesterol content was analyzed by gas chromatography after lipid extraction using chloroform:methanol (2:1) [16]. Cholesterol values were normalized to protein content, after quantification by BCA protein assay (Thermo Scientific).

Histology Analysis of Adipose Tissue

Freshly isolated omental and subcutaneous (subQ) adipose tissue was fixed in 10% buffered formalin, and then embedded in paraffin, before sectioning (5 µm) and staining with hematoxylin and eosin. From each adipose depot, three sections were prepared and stained, and 100x magnification images were captured using a Nikon eclipse 50i microscope and camera. Using Image Pro software, a 20-square grid was placed over each image and one cell was measured from each grid square, taking the longest apparent diameter of each cell. A total of 200 diameter measurements were made for each adipose tissue sample by ImagePro.

Immunoassays

Insulin and adiponectin concentrations in plasma were measured by ELISA (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions.

Quantitative Real-Time PCR of Adipose Tissue and Liver

Total RNA was isolated using TRIzol (Invitrogen) from subcutaneous and omental adipose tissue and liver that was snap frozen in liquid nitrogen. To remove potential genomic DNA contamination, RNA was treated with DNase (Mediatech) before 2 µg of RNA was reverse transcribed into cDNA in a total volume of 20 µl (iScript cDNA synthesis kit, Bio-Rad). Gene expression was determined by quantitative real-time PCR (CFX96, Bio-Rad) and relative gene expression was normalized by the average of two reference genes, 36B4 and

GAPDH, as described previously [1] (see Supplemental Table II for primer sequences).

Immunoblot Analysis

Total protein lysates from adipose tissue were prepared in RIPA buffer (Boston BioProducts) supplemented with protease inhibitors (Roche Molecular Biochemicals) and PMSF. Lysates were cleared by centrifugation at 4° for 10 min at 10,000 x g. Protein concentration was determined using the Bradford assay (Biorad) with BSA as reference. Samples (10–40µg) were separated on NuPAGE Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (Amersham), which were then probed with the following antibodies: ABCA1 (Novus, 1:1000), beta-actin (Sigma, 1:10000), apoE (1:1000; [17]), and SR-BI (Novus, 1:1000). Primary and secondary antibodies were diluted in 5% milk solution and visualized using ECL (Amersham) and GE ImageQuant LAS4000 Imager. Blots were quantified by densitometry using ImageJ software.

Statistical Analysis

Results are presented as mean ± SEM. The data were analyzed using Student's *t*-test or one-way ANOVA, with post-hoc analysis using Tukey's multiple comparison test. For the analysis of adipocyte size, Gaussian curve fitting and linear regression were performed using GraphPad Prism 5® software.

Immunofluorescence

Paraffin embedded adipose tissue sections were deparaffinized and stained using TNF-α (AbCam 1:300) and CD 68 (AbD Serotec 1:300) primary antibodies and goat-anti mouse alexafluor 405 (1:500) and goat anti-rat alexafluor 647 (1:500) secondary antibodies (Biotium). Both primary and secondary antibodies were diluted in 5% goat serum and visualized using Olympus VS-110 imaging system or Olympus DD71fluorescent microscopes.

Results

Dietary cholesterol increases plasma VLDL and LDL cholesterol

To examine the effect of increasing levels of dietary cholesterol on plasma lipids and lipoprotein concentrations in African green monkeys, three isocaloric Western-type diets containing 35% fat with 0.002 (Lo), 0.2 (Med) or 0.4 (Hi) mg cholesterol/kcal were fed to monkeys. During the 10-week diet feeding period, no significant difference was observed in food consumption (data not shown), body weight (Figure 1A) or plasma TG levels (Figure 1B). However, there was a striking increase in plasma total cholesterol concentrations (TC) for the Hi cholesterol group ($P<0.0001$) relative to the Med and Lo groups, which was apparent after 3 weeks (Figure 1C). The increase in plasma TC was due, in part, to a significant increase in VLDL cholesterol (Figure 1D), but was primarily due to large increases in LDL cholesterol (Figure 1E). Plasma HDL cholesterol concentrations were not affected by increasing dietary cholesterol (Figure 1F).

The effects of increasing dietary cholesterol on intestinal cholesterol absorption, cholesterol excretion, and hepatic lipid content were also examined (Table 1). The percentage dietary cholesterol absorption was similar among all three diet groups (average of 52-56%). Fecal cholesterol excretion increased in a stepwise manner with increasing dietary cholesterol. In addition, there was a significant increase of hepatic FC ($p=0.0001$) and CE ($p=0.001$) content with increasing dietary cholesterol, caused primarily by large increases in the Hi cholesterol group. Hepatic TG content trended higher with increasing dietary cholesterol, but

there were no significant differences among the diet groups, there was also no visible evidence of hepatic steatosis by histological examination of liver sections (Supplemental figure I). We also observed changes in hepatic gene expression that were compatible with the increase in hepatic cholesterol content (Table 2). Specifically, SREBP2 and HMGCR mRNA expression decreased, whereas ABCG1 expression increased (ABCA1 also trended upward).

Dietary cholesterol increases cholesterol content and adipocyte size in omental, but not subcutaneous, adipose tissue

Several studies suggest that adipose cholesterol accumulation correlates with an increased risk for metabolic syndrome and cardiovascular diseases[16, 17]. However, it is unclear whether dietary cholesterol directly affects adipose tissue cholesterol accumulation and, if so, whether dietary cholesterol accumulation differs among adipose depots. To address this question, we measured cholesterol levels in omental and subQ adipose tissue. Dietary cholesterol intake up to 0.4 mg cholesterol/Kcal had no significant effect on subQ adipose tissue total, free, or esterified cholesterol content (Figure 2A-C, respectively). By contrast, omental fat total and free cholesterol content was significantly increased in monkeys fed the Hi vs. Lo cholesterol diet (Figure 2D-E). Omental fat cholesteryl ester (CE) content was low (<10% of total cholesterol) and did not vary among diet groups (Figure 2F). To determine whether dietary cholesterol altered adipocyte hypertrophy in a depot-specific manner, we examined adipocyte size by analyzing digital images of hematoxylin and eosin-stained

paraffin sections from omental and subQ adipose tissue. Consistent with cholesterol accumulation, adipocyte size increased with increasing dietary cholesterol in omental fat (Figure 3A; 42.3 ± 2.4, 49.0 ± 2.7 and 58.9 ± 3.1 μm for Lo, Med, and Hi, respectively), but not in subQ fat (Figure 3B; p=0.49). Histograms of adipocyte size distribution also demonstrated a clear shift towards larger size for omental, but not subQ fat. Linear regression analysis showed a significant correlation (r^2 =0.298, p=0.035) between omental adipose tissue cholesterol content and adipocyte size, but not subQ adipose tissue (r^2 =0.048, p=0.44) (Figure 3C). Collectively, these data demonstrated that increasing dietary cholesterol in monkeys selectively drives increases in adipose tissue cholesterol content and adipocyte size in omental fat. There was also a significant (P=.0246, r^2=.331) correlation between plasma LDL cholesterol concentrations and omental adipocyte diameter, suggesting that elevations in plasma LDL were a primary determinant of increased adipocyte size (Supplemental Figure II). There was no correlation between VLDL cholesterol concentrations and adipocyte size.

Dietary cholesterol is associated with omental adipose tissue inflammation

To determine whether the increase of omental fat cholesterol content induced by dietary cholesterol altered endocrine function, we examined adipokine gene expression by quantitative real-time PCR. There was a significant increase in expression of the pro-inflammatory genes interleukin (IL)-6 and IL-8 in the Hi cholesterol diet group relative to the other groups (Figure 4A). TNFα gene

expression trended upward with increasing dietary cholesterol, but did not reach statistical significance (p=0.065). Next, we examined macrophage gene expression in omental adipose tissue. There was a significant upregulation of macrophage recruitment-associated genes, including monocyte chemoattractant protein-1 (MCP-1), C-C chemokine receptor type 2 (CCR2), and the macrophage marker CD68, in Hi vs. Lo cholesterol diet groups (Figure 4B). We observed no increase in M2 type macrophage gene expression (Supplemental Figure III A and B) suggesting increasing macrophage accumulation was primarily M1 type macrophages. Immunofluorescence images of representative omental adipose tissue suggested increased expression of TNF-α and CD68 in the Hi vs. Lo cholesterol group (Supplemental Figure IV), but not subcutaneous adipose tissue (Supplemental Figure V). Cytokine protein expression (TNF-α and IL-6) primarily co-localized with tissue macrophages (Supplemental figure VI). Despite increased inflammation, expression of adiponectin and leptin were unaffected by dietary cholesterol (Figure 4C). In addition, plasma levels of glucose, insulin, or adiponectin were similar among diet groups (Supplemental figure VII) and TNF-α levels were undetectable, indicating that 10 weeks of atherogenic diet feeding did not impair insulin sensitivity or systemic inflammation. These data suggest that increasing dietary cholesterol intake promotes adipose tissue inflammation and recruitment of macrophages into omental fat, but in the absence of body weight differences among diet groups, does not lead to systemic insulin resistance.

Next, we determined whether an increase in omental fat FC content altered expression of genes involved in cholesterol biosynthesis, and cellular cholesterol uptake and efflux (Figure 5). SREBP2, a master regulator of cholesterol biosynthesis, was unaffected by dietary cholesterol (Figure 5A). However, expression of HMG-CoA reductase, the rate-limiting enzyme for cholesterol biosynthesis, was significantly reduced in Med and Hi cholesterol diet groups compare to the Lo cholesterol group (Figure 5A). In addition, VLDLr and LDLr gene expression were significantly lower in the Med and Hi groups compared to the Lo cholesterol group (Figure 5B). Scavenger receptor class B type I (SR-BI) showed a decreasing trend with increasing dietary cholesterol ($p=0.076$). LDLr-related protein (LRP) gene expression level did not differ among diet groups.

To determine the effect of dietary cholesterol on cholesterol efflux pathways, we analyzed expression of several genes and proteins involved in adipocyte cholesterol efflux [18, 19]. Adipose tissue ABCA1 and ABCG1 mRNA expression was similar among diet groups, but apoE expression levels tended to increase with increasing dietary cholesterol ($p=0.12$; Figure 5C). However, Western blot analysis revealed an increase in ABCA1 ($p=0.053$ by ANOVA; $p=0.025$ by t-test, Lo vs. Hi) and apoE ($p=0.016$ by ANOVA) protein expression with increasing dietary cholesterol (Figure 5D), suggesting an upregulation of cellular cholesterol efflux pathways. Finally, we also measured expression of several lipogenic genes and lipoprotein lipase (LPL). Despite increased adipocyte size in omental adipose tissue, expression of lipogenic genes, including transcription SREBP1c,

fatty acid synthase (FAS), acetyl coA carboxylase (ACC), and LPL, was similar among diet groups (Supplemental figure IIIC-F). Expression of fatty acid oxidation genes, PPARα and ACOX, was significantly lower in the Hi vs. Lo cholesterol diet groups (Supplemental figure IIIG-I), suggesting a decrease in adipose tissue fatty acid oxidation with increasing dietary cholesterol.

Discussion

Adipose tissue is the largest free cholesterol storage site in the body, and its cholesterol content affects gene expression and cellular function [20, 21]. However, it is unclear whether dietary cholesterol has a direct impact on adipocyte size, cholesterol content, and metabolic function. The fundamental question addressed in our study was whether increasing dietary cholesterol drives adipocyte hypertrophy and inflammation and, if so, whether it occurs in a fat depot-specific manner. Increasing dietary cholesterol (from 0.002 to 0.4 mg/kcal) was associated with a selective increase in cholesterol content and adipocyte size in omental adipose tissue; these relationships were not observed in subQ fat (Figures 1-3). Our data also revealed that increased visceral fat cholesterol content induced by Hi cholesterol diet feeding promoted increased expression of genes associated with adipose tissue inflammation and macrophage recruitment (Figure 4), with cytokine expression primarily associated with CD68 positive cells (Supplemental figures IV-VI). These changes occurred in the absence of significant differences in body weight among monkeys consuming the three levels of dietary cholesterol. Collectively, our work shows that increasing dietary cholesterol alone is sufficient to induce adipocyte hypertrophy and adipose tissue inflammation selectively in visceral fat of nonhuman primates.

Whether dietary cholesterol, *per se*, can affect adipocyte hypertrophy and inflammation is unknown. Obesity results from excess caloric consumption that often includes increased dietary cholesterol intake and is associated with adipocyte hypertrophy and increased recruitment of macrophages into adipose

tissue [10]. Enlarged adipocytes are insulin resistant, secrete more proinflammatory cytokines, and have greater basal and catecholamine-stimulated lipolysis than smaller adipocytes [6-9, 22]. Fat cell hypertrophy is associated with increased adipocyte cholesterol in humans [13, 23] and rodents [24], but whether this occurs passively with adipocyte TG accumulation during progression of obesity or actively induces adipocyte hypertrophy is unknown.

Past studies have yielded conflicting results regarding the role of dietary cholesterol on adipocyte size and cholesterol content. Angel and Farkas documented an increase in rat adipocyte cholesterol with increasing dietary cholesterol load (0.05% to 5%); however, rats fed a cholesterol-free diet had more cholesterol in adipocytes than those fed diets containing 0.05% or 0.1% cholesterol, suggesting a complex relationship between dietary cholesterol intake and adipocyte sterol homeostasis [24]. In a recent study, Subramanian et al fed LDLr knockout mice a high fat (60% cal), high carbohydrate (26% cal) diet with (0.15%) or without added cholesterol (0.03% from lard in the diet) and showed that added dietary cholesterol resulted in macrophage infiltration into epididymal fat (i.e., visceral fat depot), but did not increase adipocyte size. On the other hand, inguinal (i.e., subQ fat depot) fat cells were increased in size with added dietary cholesterol, but macrophage infiltration was not observed. Kovanen et al reported that a high cholesterol diet failed to increase adipocyte cholesterol in rats, but did increase plasma and hepatic cholesterol concentrations [12]. No study to date has examined the interrelationships between dietary cholesterol intake and adipocyte size, adipose tissue cholesterol content, and inflammatory status.

In this study, we demonstrate that feeding non-human primates diets that were identical except for increasing level of cholesterol was sufficient to increase visceral fat cell size and adipose tissue cholesterol and macrophage content. Furthermore, these increases were not observed in subQ fat and occurred in the absence of body weight differences among diet groups. The difference in our outcomes (increased visceral adipocyte size, cholesterol and macrophage content with increasing dietary cholesterol) from those of Subramanian et al (increased visceral fat macrophage content, but not size) are likely related to the animal model (monkey vs. LDLr knockout mice) and/or diet composition. Our diets are typical of the North American diet in total fat content (35% cal) and fatty acid composition (enriched in saturated and monounsaturated fat). The Hi cholesterol diet is equivalent to consumption of 3-4 eggs/day for a 2000-2500 calorie/day diet, which might be consumed by individuals at risk of developing obesity and coronary heart disease. To our knowledge, this is the first study to show that elevated dietary cholesterol in a typical North American diet can induce adipocyte hypertrophy and inflammation in nonhuman primates. Thus, high dietary cholesterol intake may be one causal factor in development of adipose tissue hypertrophy and inflammation that accompanies obesity, insulin resistance, and type 2 diabetes. Although we did not observe systemic insulin resistance in our study, likely due to similar body weights among our groups of nonhuman primates, a longer feeding period may have resulted in hyperglycemia and/or hyperinsulinemia.

The increased visceral fat cholesterol content in monkeys fed the Hi cholesterol diet likely occurred through increased uptake of cholesterol from plasma VLDL and LDL. Supporting this idea, the Hi cholesterol diet significantly elevated plasma VLDL and LDL cholesterol concentrations (Figure 1) and we observed a significant correlation between plasma LDL cholesterol concentrations and omental adipocyte size (Supplemental figure II). Most cells respond to an increase in FC by converting excess FC to cholesteryl ester via acyl CoA:cholesterol acyltransferase (ACAT), decreasing *de novo* cholesterol biosynthesis, decreasing lipoprotein uptake via downregulation of the LDLr, and increasing efflux of cholesterol via ABCA1, ABCG1, and SR-BI [25]. Adipocytes are unusual in that >95% of cellular cholesterol is FC and most of their FC is located on the surface of the lipid droplet [12-14]. All of these cellular responses to excess FC are mediated by two master transcriptional factors, SREBP2 (sterol regulatory element binding protein 2) [26] and LXR (liver X receptor) [27], which prevent cholesterol uptake/synthesis and stimulate FC efflux, respectively. In our study, visceral fat from Hi cholesterol-fed monkeys had 50% more FC compared to monkeys fed the Lo cholesterol diet. Based on visceral fat gene expression (Figure 5 and Supplemental figure IIIG-I), cholesterol biosynthetic (HMGCR), lipoprotein receptor (LDLr and VLDLr), and fatty acid oxidation (PPARα and ACOX) gene expression were down-regulated, suggesting the visceral fat tissue was attempting to compensate for increased FC content. We [18] and others [19] have shown that ABCA1 expression is critical in the regulation of adipocyte FC content in mice. Although gene expression of LXR-responsive genes involved in

FC efflux was unaltered by diet (Figure 5C), ABCA1 and apoE protein expression was significantly higher in Hi vs. Lo cholesterol groups (Figure 5D), suggesting efflux of FC likely plays a critical role in regulating adipocyte sterol homeostasis in nonhuman primates as well. Thus, although increased plasma cholesterol has not been associated with increased adipocyte cholesterol in all studies [24, 28], it is the most likely explanation for increased visceral fat cholesterol in this one.

Visceral fat is more metabolically active and susceptible to dysfunction relative to subQ fat [29]. In our study, subQ fat did not respond to increased dietary cholesterol intake with a change in fat cell size, adipose tissue FC content, or inflammatory cytokine expression. Interestingly, adipocyte size and adipose tissue cholesterol content were 50% greater for subQ vs. visceral fat in monkeys consuming the Lo cholesterol diet (Figures 2 and 3). Feeding monkeys the Hi cholesterol diet increased visceral fat cell size and adipose tissue cholesterol content to levels observed for subQ fat. These results suggest that large fat cell size or adipose tissue cholesterol content, *per se*, does not result in inflammation and dysfunction. The reason for the metabolic differences between visceral vs. subQ fat that lead to divergent responses to increased dietary cholesterol are unknown and will require further investigation.

In conclusion, we provide the first evidence that addition of cholesterol to a typical North American diet selectively induces visceral adipocyte enlargement, adipose tissue cholesterol accumulation, and inflammatory responses in

nonhuman primates. Visceral fat cells appear to compensate for increased dietary cholesterol by limiting cholesterol uptake/synthesis and increasing FC efflux pathways. Our results establish new metabolic interrelationships between dietary cholesterol and adipocyte hypertrophy, adipose tissue cholesterol accumulation, and inflammation that may explain part of the metabolic dysfunction that accompanies diet-induced obesity. Although our study did not specifically address reducing dietary cholesterol as a means of lowering the risk of adipocyte hypertrophy and dysfunction, we speculate that this might be the case. If so, recommending a prudent intake of dietary cholesterol could potentially be a simple strategy to reduce adipocyte hypertrophy and dysfunction in humans.

References

(1) Finkelstein EA, Trogdon JG, Cohen JW, Dietz W. Annual medical spending attributable to obesity: payer-and service-specific estimates. *Health Affairs* 2009;28:w822-w831.

(2) Flegal KM, Carroll MD, Ogden CL, Curtin LR. Prevalence and trends in obesity among US adults, 1999-2008. *JAMA* 2010;303:235-241.

(3) Roger VL, Go AS, Lloyd-Jones DM et al. Heart Disease and Stroke Statistics - 2012 Update. *Circulation* 2012;125:e2-e220.

(4) Spiegelman BM, Flier JS. Adipogenesis and obesity: rounding out the big picture. *Cell* 1996;87:377-389.

(5) Subramanian S, Han CY, Chiba T, McMillen TS, Wang SA, Haw A, III, Kirk EA, O'Brien KD, Chait A. Dietary cholesterol worsens adipose tissue macrophage accumulation and atherosclerosis in obese LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol* 2008;28:685-691.

(6) Salans LB, Knittle JL, Hirsch J. The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity. *The Journal of Clinical Investigation* 1968;47:153-165.

(7) Smith U. Studies of human adipose tissue in culture. I. Incorporation of glucose and release of glycerol. *Anat Rec* 1972;172:597-602.

(8) Winkler G, Kiss S, Keszthelyi L et al. Expression of tumor necrosis factor (TNF)-alpha protein in the subcutaneous and visceral adipose tissue in correlation with adipocyte cell volume, serum TNF-alpha, soluble serum TNF-receptor-2 concentrations and C-peptide level. *European Journal of Endocrinology* 2003;149:129-135.

(9) Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 2007;92:1023-1033.

(10) Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003;112:1796-1808.

(11) Krause BR, Hartman AD. Adipose tissue and cholesterol metabolism. *J Lipid Res* 1984;25:97-110.

(12) Kovanen PT, Nikkila EA, Miettinen TA. Regulation of cholesterol synthesis and storage in fat cells. *J Lipid Res* 1975;16:211-223.

(13) Schreibman PH, Dell RB. Human adipocyte cholesterol. Concentration, localization, synthesis, and turnover. *J Clin Invest* 1975;55:986-993.

(14) Prattes S, Horl G, Hammer A, Blaschitz A, Graier WF, Sattler W, Zechner R, Steyrer E. Intracellular distribution and mobilization of unesterified cholesterol in adipocytes: triglyceride droplets are surrounded by cholesterol-rich ER-like surface layer structures. *J Cell Sci* 2000;113 (Pt 17):2977-2989.

(15) Parks JS, Kaduck-Sawyer J, Bullock BC, Rudel LL. Effect of dietary fish oil on coronary artery and aortic atherosclerosis in African green monkeys. *Arteriosclerosis* 1990;10:1102-1112.

(16) Berg AH, Scherer PE. Adipose tissue, inflammation, and cardiovascular disease. *Circ Res* 2005;96:939-949.

(17) Subramanian S, Chait A. The effect of dietary cholesterol on macrophage accumulation in adipose tissue: implications for systemic inflammation and atherosclerosis. *Curr Opin Lipidol* 2009;20:39-44.

(18) Chung S, Sawyer JK, Gebre AK, Maeda N, Parks JS. Adipose tissue ATP binding cassette transporter A1 contributes to high-density lipoprotein biogenesis in vivo. *Circulation* 2011;124:1663-1672.

(19) Zhang Y, McGillicuddy FC, Hinkle CC, O'Neill S, Glick JM, Rothblat GH, Reilly MP. Adipocyte modulation of high-density lipoprotein cholesterol. *Circulation* 2010;121:1347-1355.

(20) Le LS, Krief S, Farnier C, Lefrere I, Le L, X, Bazin R, Ferre P, Dugail I. Cholesterol, a cell size-dependent signal that regulates glucose metabolism and gene expression in adipocytes. *J Biol Chem* 2001;276:16904-16910.

(21) Parpal S, Karlsson M, Thorn H, Stralfors P. Cholesterol depletion disrupts caveolae and insulin receptor signaling for metabolic control via insulin receptor substrate-1, but not for mitogen-activated protein kinase control. *J Biol Chem* 2001;276:9670-9678.

(22) Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 2007;117:175-184.

(23) Bjorntorp P, Sjostrom L. The composition and metabolism in vitro of adipose tissue fat cells of different sizes. *Eur J Clin Invest* 1972;2:78-84.

(24) Angel A, Farkas J. Regulation of cholesterol storage in adipose tissue. *J Lipid Res* 1974;15:491-499.

(25) Tabas I. Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J Clin Invest* 2002;110:905-911.

(26) Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002;109:1125-1131.

(27) Zelcer N, Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. *J Clin Invest* 2006;116:607-614.

(28) Krause BR, Phares F, Serbin V, Krause L, Hartman AD. Adipocyte cholesterol storage: effect of experimental hypercholesterolemia in the rat. *J Nutr* 1979;109:2213-2225.

(29) Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev* 2000;21:697-738.

Table 1. Cholesterol absorption, fecal cholesterol excretion, and hepatic lipid content in African green monkeys fed different amounts of dietary cholesterol

	Lo-chol	Med-chol	Hi-chol	p value
Cholesterol content (mg/Kcal diet)	0.002	0.2	0.4	-
Cholesterol absorption (%)	56.0 ± 5.3	52.0 ± 5.8	52.4 ± 2.2	0.81
Fecal excretion (mg/day/kg BW)	5.5 ± 0.4^{a}	11.40 ± 1.9^{ab}	15.62 ± 2.1^{b}	0.003
Hepatic FC (mg/g protein)	14.33 ± 0.3^{a}	17.4 ± 0.7^{a}	31.2 ± 1.8^{b}	0.0001
Hepatic CE (mg/g protein)	11.36 ± 1.6^{a}	25.0 ±5.1^{a}	158 ± 40.9^{b}	0.001
Hepatic TG (mg/g protein)	39.92 ± 10.3^{a}	57.0 ± 12.6^{a}	67.6 ± 9.4^{a}	0.22

Mean ± SEM, n=5 per diet group. Values with different superscript letter are statistically different ($p<0.05$) by one-way ANOVA with Tukey's multiple comparison test.
BW = body weight; CE = cholesterol ester; FC = fecal cholesterol; TG = triglycerides.

Table 2. Hepatic gene expression in African green monkeys fed different amounts of dietary cholesterol

Gene	Lo-chol	Hi-chol	p value
SREBP2	1.03 ± 0.13	0.51 ± 0.13	0.02
HMGCR	1.07 ± 0.20	0.48 ± 0.15	0.05
apoE	1.06 ± 0.17	1.22 ± 0.24	n.s
ABCA1	1.13 ± 0.31	1.76 ± 0.55	n.s.
ABCG1	1.17 ± 0.31	3.93 ± 0.84	0.01
SRB1	1.18 ± 0.29	0.72 ± 0.22	n.s.
LDLr	1.18 ± 0.29	3.38 ± 1.90	n.s.
FAS	1.15 ± 0.26	3.72 ± 1.81	n.s
CD36	1.22 ± 0.23	1.47 ± 0.25	n.s

Gene expression was determined by quantitative real time PCR and results were normalized to GAPDH. Results were then normalized to the mean value for each gene in the Lo-chol group, which was set to a value of 1. Mean ± SEM, n=5 per diet group.

Figure 1: Supplementation of Hi cholesterol diet increases plasma cholesterol in African green monkeys

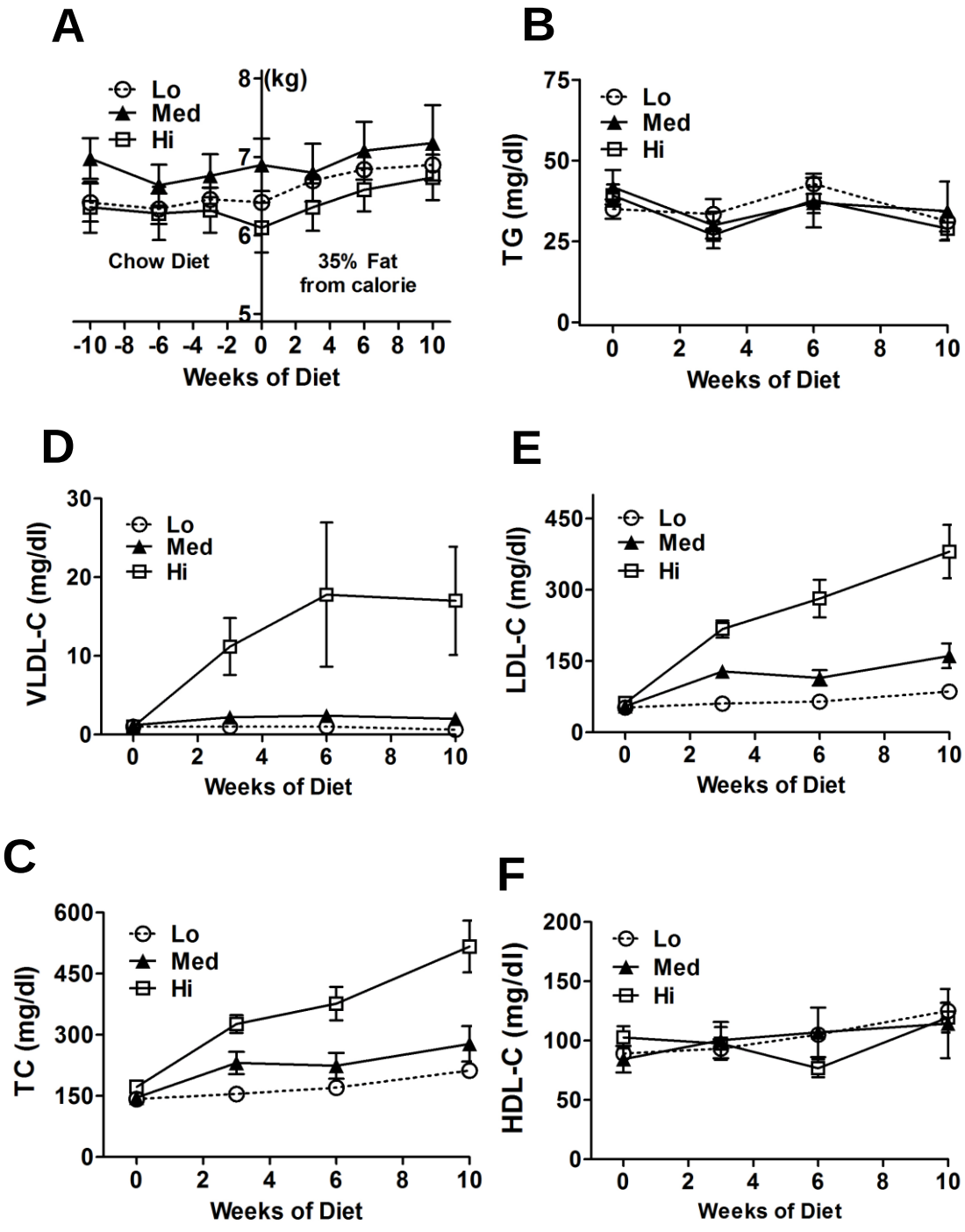


Figure 1: African green monkeys were fed atherogenic diets containing 35% calories as fat and 0.002 (Lo, ○), 0.2 (Med, ▲), or 0.4 (Hi, □) mg cholesterol/kcal for 10 wks. Periodic blood samples were taken to measure plasma lipid and lipoprotein cholesterol concentrations, after fractionation of plasma by fast protein liquid chromatography. **A**. Body weight of monkeys before (chow diet) and after diet consumption, **B**. Plasma triglyceride concentrations, **C**. Plasma total cholesterol concentrations; Hi cholesterol group was significantly greater than Med or Lo groups by repeated-measures ANOVA, **D**. Very low density lipoprotein cholesterol concentrations, **E**. Low density lipoprotein cholesterol concentrations; Hi cholesterol group was significantly greater than Med or Lo groups by repeated-measures ANOVA, and **F**. High density lipoprotein cholesterol concentrations. Each data point represents mean ± SEM; n=5/group.

Figure 2: Dietary cholesterol consumption selectively increases cholesterol content in visceral, but not subQ, adipose tissue

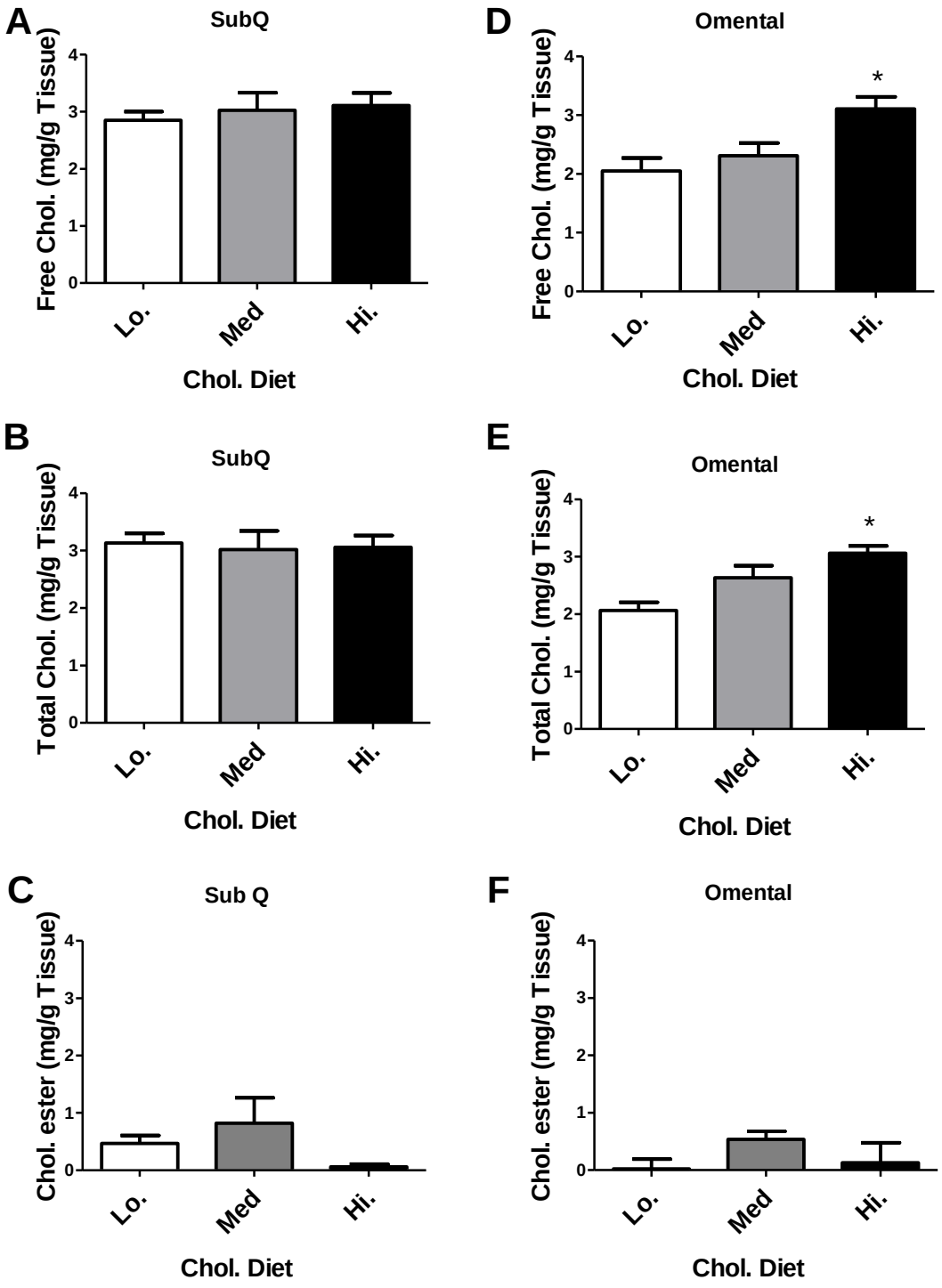


Figure 2. After 10 wks of diet consumption, monkeys were euthanized, adipose tissue was collected and lipid extracted, and cholesterol content was quantified by gas-liquid chromatography and normalized to protein content. **A-C**, Subcutaneous (subQ) total cholesterol (TC), free cholesterol (FC), and cholesteryl ester (CE). **D-F**, Omental TC, FC, and CE. Mean ± SEM; n=5/diet group. *****$p<0.05$ by one-way ANOVA.

Figure 3: Dietary cholesterol is associated with increased omental adipose tissue cholesterol content and size

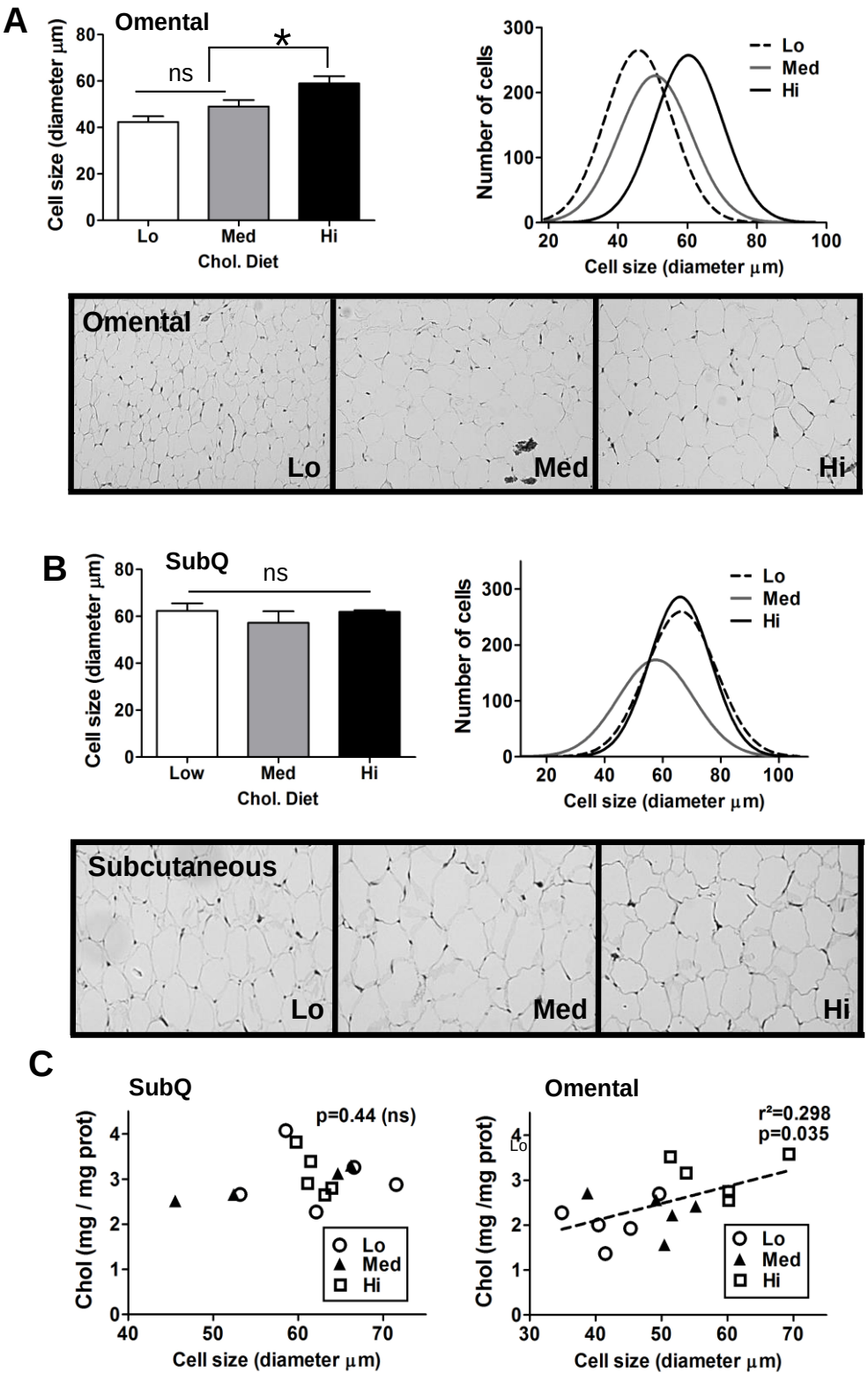


Figure 3. Adipocyte cell size (n=200/animal) was determined from digital images of paraffin-embedded, hematoxylin and eosin-stained omental (**A**) and subQ (**B**) adipose tissue sections using Image Pro® software. Mean ± SEM, n=5/diet group. Adipocyte cell size histograms for all animals within each diet group are shown in the right panel. Representative images from omental and subQ fat for each diet are shown below the plots in panels A and B. (**C)**. Correlation of subQ and visceral adipocyte size with cholesterol content using linear regression analysis. The line of best fit is shown for each plot.

Figure 4: An increase in dietary fat is associated with adipose tissue inflammation.

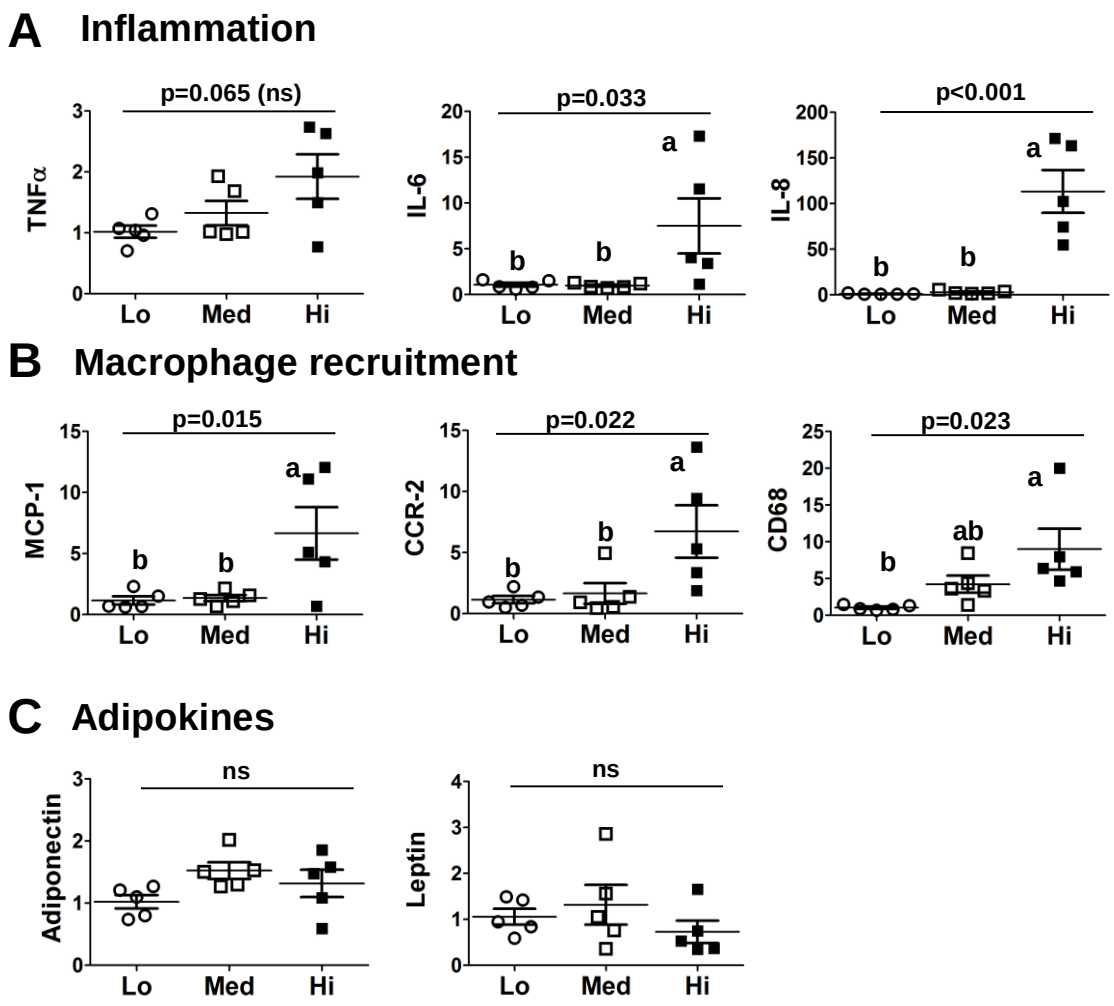


Figure 4. (**A**) Expression of the proinflammatory genes TNFα, IL-6, and IL-8; (**B**) expression of the macrophage recruitment genes MCP-1, CCR2, and CD68; and (**C**) expression of the adipokine genes adiponectin and leptin. Data from individual animals are shown with mean ± SEM denoted by the horizontal lines, n=5/diet group. P values were obtained by one-way ANOVA with Tukey's multiple comparison test. Diet groups with different letters are statistically different, $p<0.05$.

Figure 5: An increase in dietary cholesterol is inversely associated with adipose tissue lipoprotein receptor gene expression.

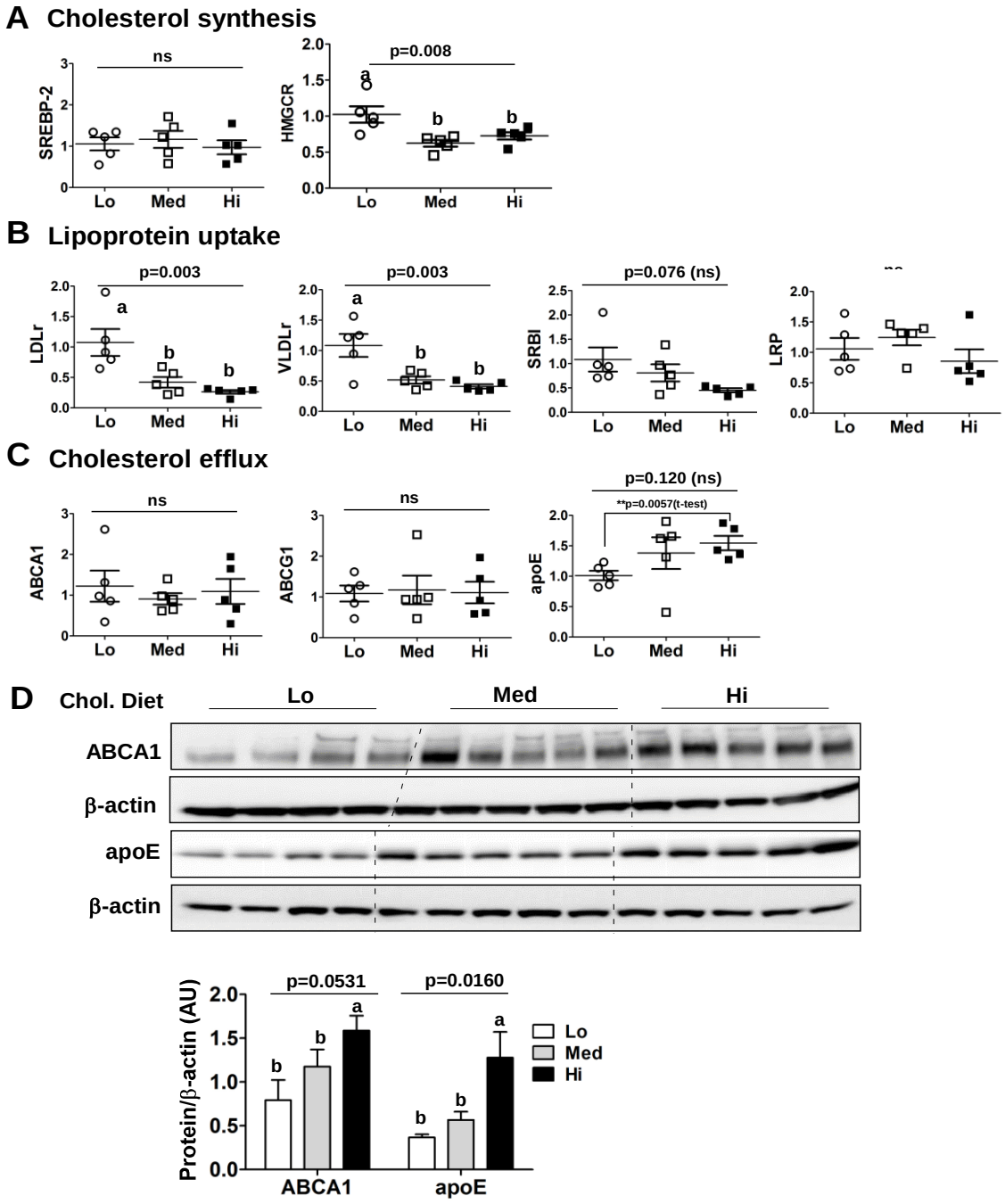


Figure 5. Omental fat from monkeys fed Lo, Med, or Hi cholesterol diets (n=5 per group) was analyzed for gene expression by quantitative real-time PCR. Expression of genes involved in cholesterol biosynthesis (**A**), lipoprotein/cholesterol uptake (**B**), and cholesterol efflux (**C**). Western blot analysis of ABCA1, apoE, and β-actin from omental adipose tissue (n=4-5). Relative band intensity quantified by ImageJ software (**D**). Results are mean ± SEM. P value was obtained by one-way ANOVA with Tukey's multiple comparison test. Diet groups with different letters are statistically different, $p<0.05$.

Supplemental Table I. Detailed diet compositions

Ingredient	g/100g	g Lipid	g Carb	g Protein
		Same for all diets		
ACHumko Oleic Blend*	16.4000	16.40	--	--
Fish Oil (Omega Protein)	0.2000	0.20	--	--
Casein, USP	9.0000	--	--	9.00
Lactalbumin	5.0000	--	--	5.00
Dextrin	9.6000	--	9.60	--
Sucrose	10.0000	--	10.00	--
Wheat Flour, self-rising	35.0000	0.35	25.9	3.15
Alphacel	7.2690	--	--	--
Vitamin Mixture, Teklad	2.5000	--	1.03	--
Hegsted Salt Mixture	5.0000	--	--	--
β-sitosterol (ICN)	0.0068	--	--	--
Tenox 20A	0.0080	--	--	--
MTS-50	0.0122	--	--	--
Vit E 5-67	0.0040	--	--	--
TOTAL	100	17.0	46.5	17.2
(calories)		150	186	68.6
% of Calories		37	46	17

Crystalline cholesterol		**mg chol/Kcal**
Lo chol diet	0.00	0.002
Med chol diet	0.08	0.200
Hi chol diet	0.17	0.400

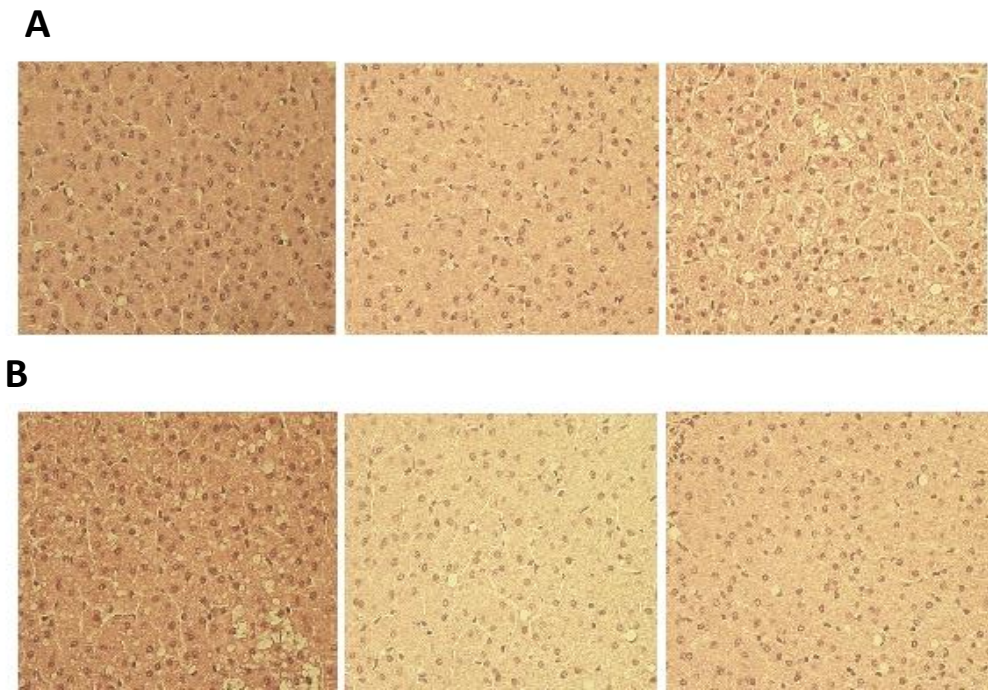
* Oleic Blend Composition: 34% Regular RBD Palm Oil, 53% Hi Oleic sunflower RBD, 2% Regular Safflower oil, 5% Soybean oil RBD, 6% regular cocoa butter

Supplemental Table II. Primer sequences for quantitative real-time PCR

Gene	Forward/Reverse	Sequence (5′-3’)
ABCA1	F	CCTGTTTCCGTTACCCGACTC
	R	ACAGGCGAGCCACAATGG
ABCG1	F	ACACCATCCCCACGTACCTA
	R	GATGACCCCTTCGAACCCA
HMGcoAR	F	GACGCAACCTTTATATCCGTTT
	R	TTGAAAGTGCTTTCTCTGTACCC
SRBI	F	GGCACACTCCTTGTTCCTGG
	R	GTCCAATGCCTGCGACAGAT
LRP	F	TGCTGGGGGACTTCATCTAC
	R	TGCTGGGGGACTTCATCTAC
VLDLr	F	ACAACCTGAATGATGCCCAAGA
	R	GTGGTCACATTGATCCTTTGACAGT
LDLr	F	TGCTCTGATGGAAACTGCATCC
	R	AGAGTGTCACATTAACGCAGCC
SREBP2	F	GACGCCAAGATGCACAAGT
	R	GCCTAGGTCGATGCCCTTTA
apoE	F	GCAACTGGCCAATCACAGGC
	R	CTGGCTCCACCGGTTGCTC
IL-6	F	CTTCTCCACAAGCGCCTTC
	R	CAGGCAACACCAGGAGCA
IL-8	F	GCTCTGTGTGAAGGTGCAGTT
	R	AATTTCTGTGTTGGCGCAGT
TNFα	F	GGCAGTCAGATCATCTTCTCG
	R	GGTTTGCTACAACATGGGCTA
CCR2	F	GAGAGGAGAAGGAGGGAGACA
	R	AGATGGGGCTAAGGACCAGTA
MCP-1	F	CCCCAGTCACCTGCTGTTAT
	R	AGATCTCCTTGGCCACAATG
CD68	F	GCTGGCTGTGCTTTTCTCG
	R	GTCACCGTGAAGGATGGCA
Adiponectin	F	TTGAGGCTGGGCCATCTCCT
	R	AGCTCCCAGCAACAGCATCC
Leptin	F	GGCTTTGGCCCTATCTTTTC
	R	TGGAGGAGACTGACTGCGTGT
SREBP1c	F	TGCATTTTCTAGCACGCTTC
	R	GATGTTCCCGGAATAGCTGA
FAS	F	GGCAAGCTGAAGGACCTGTCTA
	R	AATCTGGGTTGATGCCTCCGT
LPL	F	CAGCAAAACCTTCATGGTGAT
	R	CAAGTTTTGGCACCCAACTC

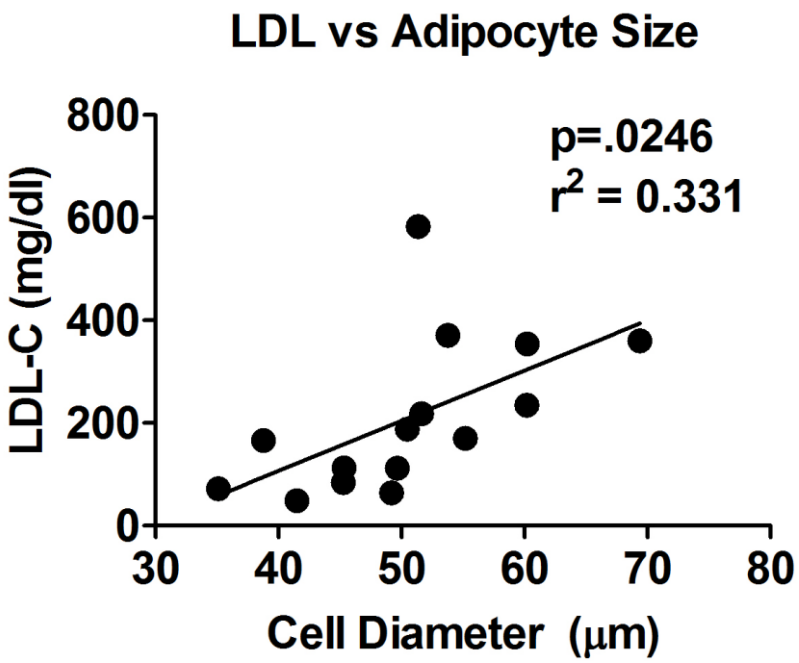
ACC	F	CATCAAATGCATCAGCAGAGACT
	R	CTGCGTCATATGGATGATGGAAT
PPARα	F	GACACGCTTTCACCAGCTT
	R	GCAGATTCTACATTCGATGTTC
CPT1	F	CTTTGGCCCTGTAGCAGATGA
	R	TCGTCTCTGAGCTTGAGAACTT
ACOX1	F	TGCTTTGGTTGATGCATTTGA
	R	CATAGCGGCCAAGCACAGA
GAPDH	F	GGCCTCCAAGGAGTAAGACC
	R	AGGGGAGATTCAGTGTGGTG
36B4	F	GAAGGCTGTGGTGCTGATG
	R	GTGAGGTCCTCCTTGGTGAA
Mannose Receptor	F	AGCGTGCAAAGTCCAAGAT
	R	CCAGAAGGCTAAGTGGGGAA
Arginase -1	F	TTCTCAAAGGGACAGCCACG
	R	TCAAGCAGACCAGCCTTTCT
CD36	F	AAAATGGGCTGTGACCGGAA
	R	TCTTCGAGGACAACTTGCTTT

Supplemental Figure I. Hemotoxylin and Eosin stained liver sections (400x magnification)



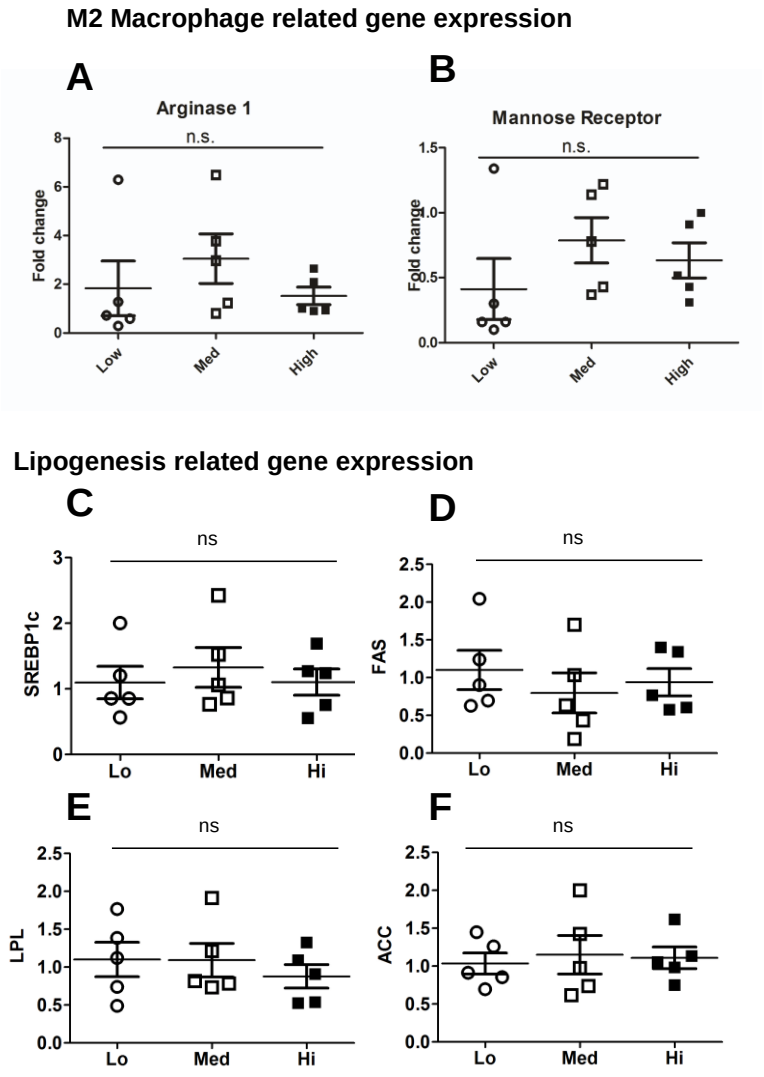
Supplemental Figure I. Representative sections from three monkeys consuming the low cholesterol (**A**) or high cholesterol diet (**B**).

Supplemental Figure II. Association between plasma LDL cholesterol concentrations and omental fat adipocyte cell diameter.



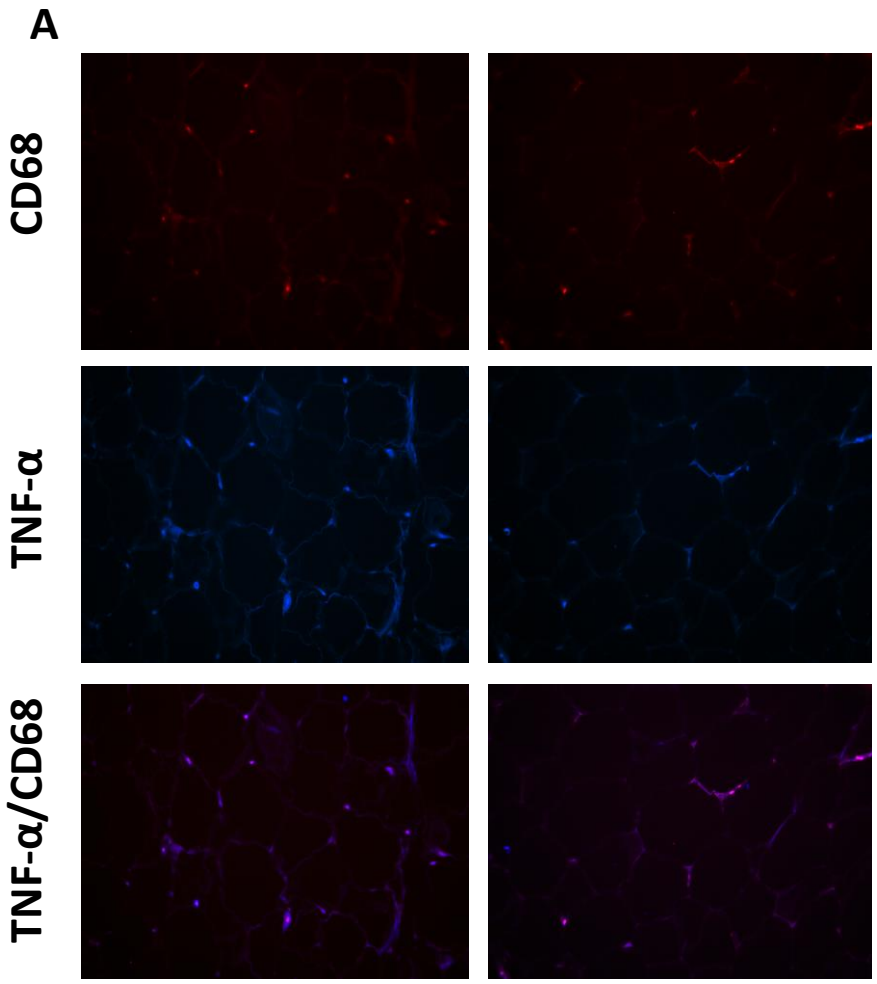
Supplemental Figure II. Each point represents data from an individual monkey consuming either the low, medium, or high cholesterol diet for 10 wks. The line of best fit, determined by linear regression analysis, is shown along with the correlation coefficient.

Supplemental Figure III: Quantitative real-time PCR was used to analyze omental fat gene expression in monkeys fed diets containing low, medium, or high dietary cholesterol.

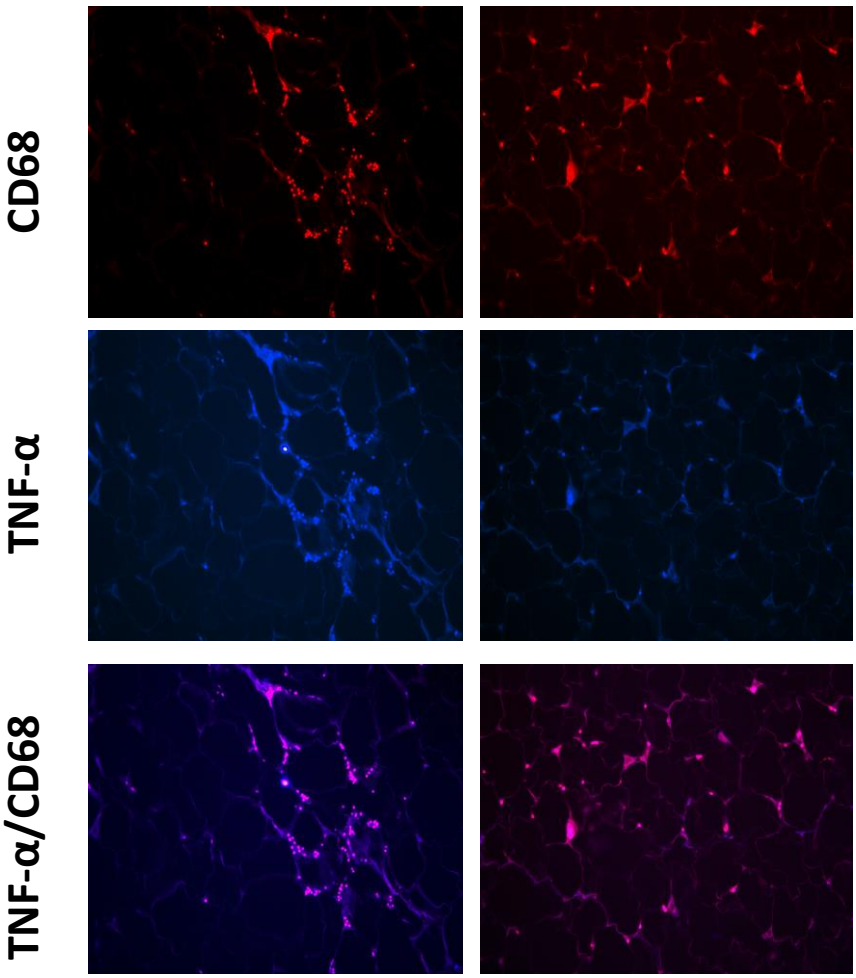


Supplemental Figure III: A. Arginase 1, **B.** Mannose receptor, **C.** Sterol regulatory element binding protein 1c (SREBP1c), **D.** Fatty acid synthase (FAS), **E.** Lipoprotein lipase (LPL), **F.** Acetyl-CoA carboxylase (ACC), **G.** PPAR-α, **H**. Carnitine palmitoyltransferase -1 (CPT-1), and **I**. Acyl-CoA oxidase (ACOX). Data from individual animals are shown with mean ± SEM denoted by the horizontal lines, n=5/diet group. ns= not statistically different by one-way ANOVA, p=0.05.

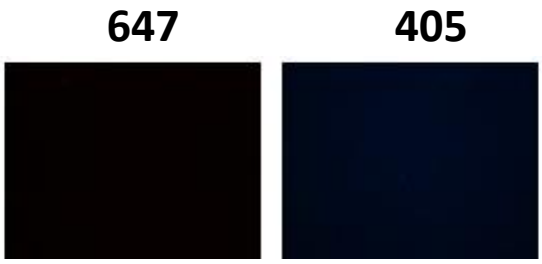
Supplemental Figure IV: Immunofluorescence visualization of macrophages and inflammatory protein expression in omental adipose tissue.



B

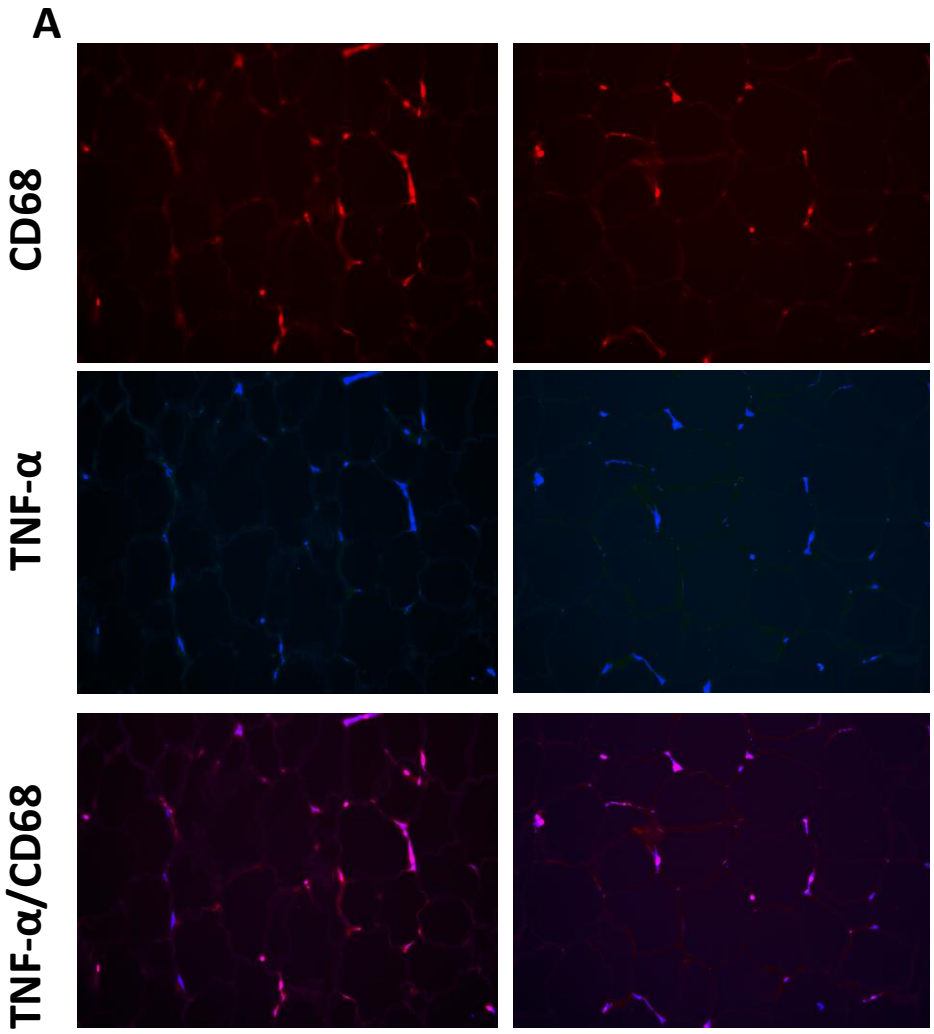


C

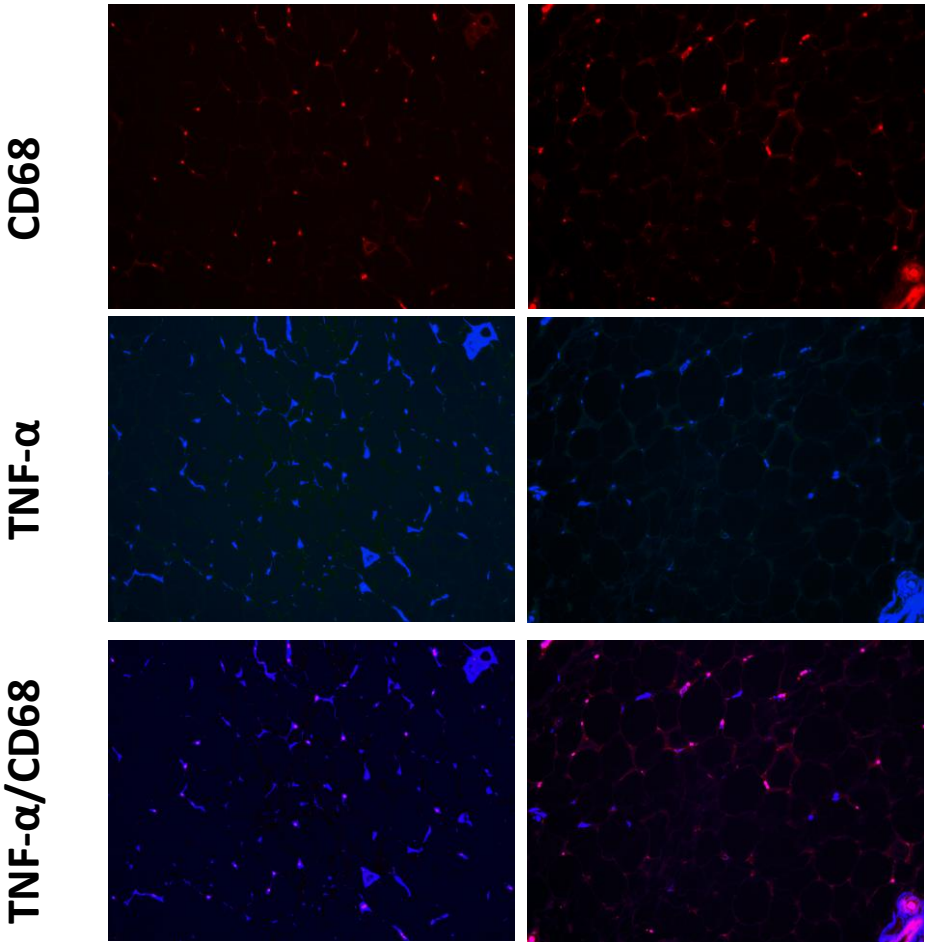


Supplemental Figure IV: Fixed adipose tissue sections were incubated with antibodies against TNF-α and CD68, and visualized using fluorescent-labeled secondary antibodies. **A.** Representative sections are shown for two monkeys in the low cholesterol (**A**) or high cholesterol (**B**) group. Images were taken at 200X magnification. Control slides (**C**) were stained with secondary antibody only.

Supplemental Figure V: Immunofluorescence visualization of macrophages and inflammatory protein expression in subcutaneous adipose tissue.



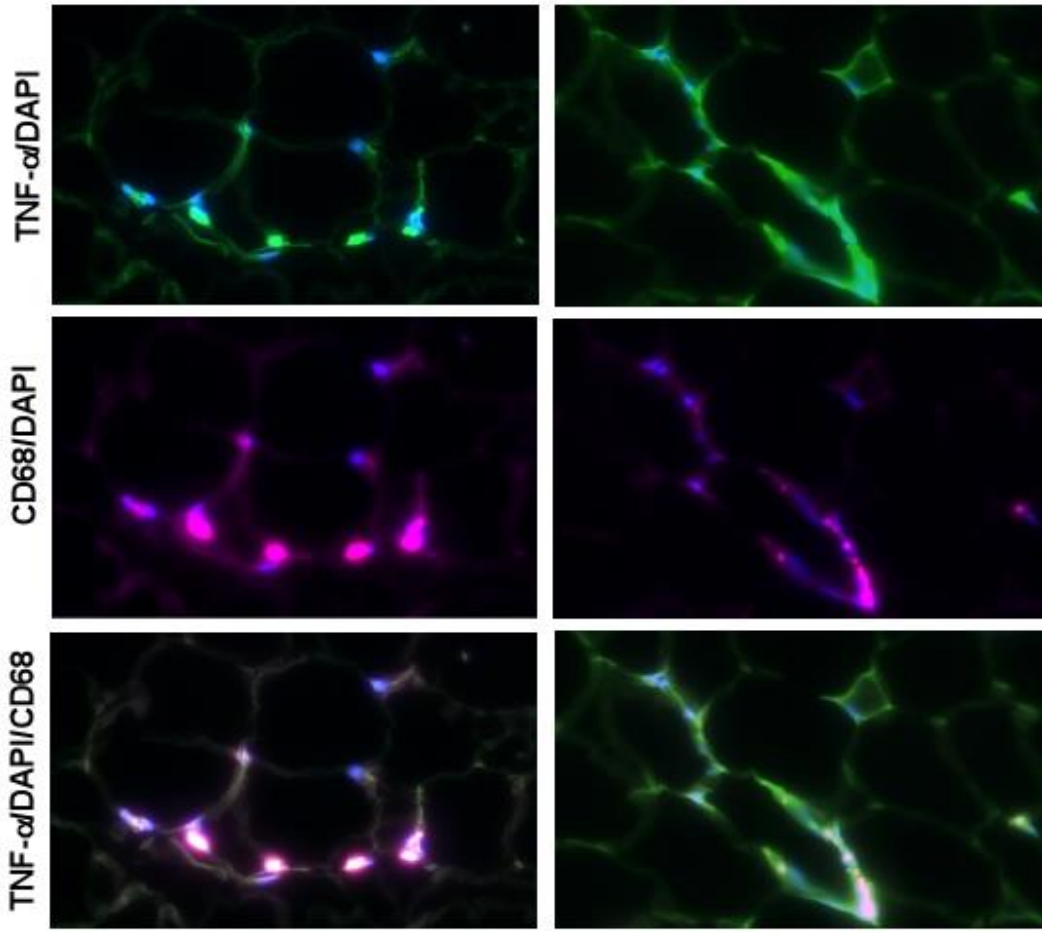
B



C

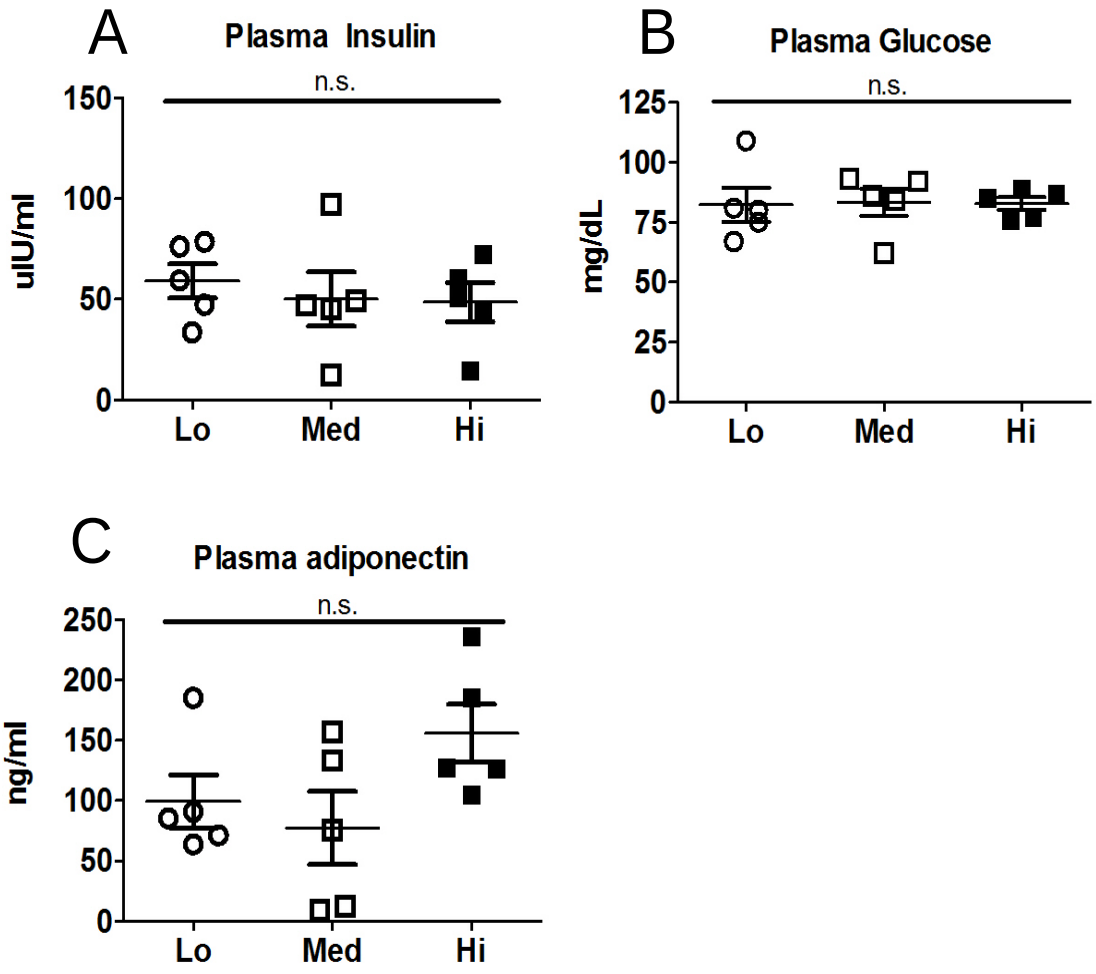
Supplemental Figure V: Fixed adipose tissue sections were incubated with antibodies against TNF-α and CD68, and visualized using fluorescent-labeled secondary antibodies. Representative sections are shown for two monkeys in the low cholesterol (**A**) or high cholesterol (**B**) group. Images were taken at 200X magnification. Control slides (**C**) were stained with secondary antibody only.

Supplemental Figure VI: Immunofluorescent colocalization of omental adipose tissue macrophages and TNFa protein expression.



Supplemental Figure VI: Fixed adipose tissue sections were incubated with antibodies against TNF-α and CD68, and visualized using fluorescent labeled secondary antibodies; nuclei were visualized with DAPI. Images were captured using an Olympus VS-110 imaging system.

Supplemental Figure VII: Increasing dietary cholesterol is not associated with increased systemic insulin resistance or plasma adiponectin levels.



Supplemental Figure VII: ELISAs were used to determine plasma concentrations of insulin and adiponectin in monkeys fed diets containing Lo, Med, or Hi dietary cholesterol. **A.** Insulin, **B.** Glucose, and **C.** Adiponectin. Date from individual animals are shown with mean ± SEM denoted by the horizontal lines, n=5/diet group. ns= not statistically different by one-way ANOVA, p=0.05.

Chapter III

Adipose ABCA1 is a critical regulator of adipose cholesterol content and adipocyte function

Helen Cuffe, Pam Clough, Alex Bashore, Allison Weckerle, Mingxia Liu, Elena Boudigyuina, and John S Parks

Helen Cuffe performed the experiments and prepared the manuscript. Dr. John S. Parks acted in an advisory and editorial capacity.

Introduction

Obesity has reached epidemic proportions worldwide. In the US, 67% of adults (>20 yrs old) are overweight (≥25 kg/m^2 body mass index) or obese (≥30 kg/m^2 body mass index), resulting in medical care costs exceeding $147 billion dollars in 2008 [1, 2]. Obesity also increases the risk of other chronic diseases, such as type 2 diabetes, coronary heart disease, insulin resistance, and non-alcoholic fatty liver disease [3].

Adipocytes are active endocrine cells that secrete adipokines, store excess energy as triglyceride (TG), release fatty acids during fasting states, and control systemic glucose disposal [4]. During the progression of obesity, fat cell size increases due to accretion of TG [5]. Larger adipocytes exhibit decreased responsiveness to insulin, decreased glucose uptake, and increased secretion of proinflammatory adipokines [6-9]. Enlarged adipocytes are also associated with macrophage infiltration into adipose tissue, resulting in increased production of proinflammatory cytokines and insulin resistance [10]. Diets high in calories, saturated fat, and cholesterol (i.e., Western diets) are sufficient to cause obesity in humans and experimental animals [3, 5]. However, the role of individual dietary constituents, particularly dietary cholesterol, in the development of obesity is poorly understood.

Several studies have now shown that cholesterol and TG accumulate proportionately in adipocytes during weight gain [11]. While much research has focused on effects of adipose tissue TG accumulation, little is known about how

cholesterol accumulation affects adipocyte function. Unlike other tissues, adipocytes store nearly all cholesterol as free cholesterol (>93% of total cholesterol) and, in the obese state, can contain up to 50% of the body's free cholesterol, making it the largest FC pool in the body [12]. Adipocytes also exhibit very low levels of cholesterol synthesis compared to other tissues, suggesting that the majority of adipocyte cholesterol comes from dietary sources [13, 14]. Several studies suggest a link between cholesterol content and adipose inflammation [5, 15] and insulin resistance [16]; however, mechanistic explanations are lacking. To determine the mechanisms by which cholesterol accumulation affects adipocyte function, our lab [17] and others [18] have used *in* vivo mouse models to delete the cholesterol transporter ABCA1 from adipocytes. Both of these studies have used the AP2Cre mouse to delete ABCA1 from adipocytes. Findings from these studies [17, 18] suggest that adipocyte ABCA1 contributes significantly to plasma cholesterol levels, and that a deletion results in significantly higher levels of cholesterol in adipose tissue, suggesting that ABCA1 is the major cholesterol efflux mechanism in adipocytes. Despite these findings, these models are complicated by the fact that the AP2 Cre mouse also results in partial and variable ABCA1 deletion from macrophages [17, 19]. To address this, we created the first adipocyte specific ABCA1 mouse model using the adiponectin Cre mouse [19]. This model allows us to study the results of cholesterol accumulation in adipocytes, and allows us to accurately determine the role of adipocyte ABCA1 in the regulation of both plasma, and adipose cholesterol levels for the first time.

Based on our results, we can conclude that adipose ABCA1 is critical for the regulation of cholesterol content in adipose tissue, and that disruption of normal adipose cholesterol metabolism has dramatic effects on adipose tissue. Our adipose-specific ABCA1 KO (ASKO) animals were less susceptible to weight gain on a high fat high cholesterol diet, and exhibited a 3-fold increase in adipose cholesterol. The results of this study not only highlight the importance of ABCA1 in adipose tissue, but also the consequences of cholesterol accumulation in adipocytes.

Methods

Mice

ASKO mice were generated by crossing ABCA1 fl/fl mice with C57BL/6 adiponectin Cre mice (from Evan Rosen). PCR of genomic tail DNA was used to determine the genotype of offspring using previously described primers and conditions for ABCA1 [5] and adiponectinCre [20]. Female and male mice were fed either a high fat (45% calories from fat), high cholesterol (0.2%) (HFHC) diet (Harlan Teklad 88137) for 16 weeks starting at 8 weeks of age, or fed chow for the equivalent time period. All animals were maintained in a specific pathogen-free facility on a 12 h light/dark cycle and all protocols were approved by the Institutional Animal care and Use Committee. Animal weights were recorded every 4 weeks during HFHC diet feeding, and plasma and glucose measures were taken after a 12 h overnight fast.

Lipid and Lipoprotein Analysis

Plasma was collected from 12 h fasted mice by tail bleeding or cardiac puncture at study termination. Total plasma cholesterol and TG concentrations were measured with commercial enzymatic assays (Wako). Plasma cholesterol distribution among lipoproteins was determined after fast protein liquid chromatography (FPLC) fractionation using a Superose 6 10/300 column (GE Healthcare) and calculated by multiplying the fractional lipoprotein distribution by plasma total cholesterol concentration [21]. Tissue lipid extractions were performed using chloroform and methanol as previously described [22]. Briefly,

adipose tissue (50-60 mg) was finely diced and placed in 9ml of 2:1 chloroform:methanol. The samples were mixed thoroughly and placed in a 60°C heating block overnight. For total cholesterol (TC) measurement, lipid extracts were saponified and TC was measured by gas-liquid chromatography [23]. TG was measured from non-saponified samples after solubilization in Triton-X-100, using a commercially available kit (WAKO). All tissue lipid measurements were normalized to wet weight or protein content (Lowry assay; [24]).

Real-Time PCR

RNA was extracted using TriZol reagent from snap-frozen tissue. cDNA synthesis was performed with an Omniscript kit using 1µg of RNA . Real-time PCR (rtPCR) was performed using Syber Green reagent (Roche) was described previously. Data was analyzed using the ΔΔCt method [25], and all data are expressed as fold change over WT values.

Cell Sizing

Adipose samples were harvested at sacrifice and put directly into 10% formalin for 48 h. Samples were then embedded in paraffin. Cell sizing was performed on hemotoxylin and Eosin (H&E) stained 5 µm sections using Adiposoft add on to Image J [26] and converted from pixels to μm^2.

Adipose and Adipocyte Fractionation

Adipose tissue was first digested to isolate adipocytes from the stromal vascular fraction as previously described using collagenase type 1 (Worthington) [27].

Adipocytes were then put in lysis buffer (10 mm Tris at pH 7.5, 10 mm NaCl, 60 mm KCl, 14 mm β-mercaptoethanol, 2 mm EDTA, 0.5% NP-40, and 1 mm DTT; supplemented with protease inhibitors (Roche), lysed with 10 strokes of a dounce homogenizer, and spun at 2,000 rpm for 15 min in an Eppendorf 5810 R rotor to remove nuclei. Nuclei were then washed and suspended in RIPA buffer for protein extraction. For membrane isolation, an additional spin was performed on the supernatant at 100,000 rpm for 4 h at 4°C in a Beckman TLA100.2 ultracentrifuge. These protocols were adapted from previously described experiments [16, 28]. Lipid and protein from the lipid droplet and membrane fractions were isolated and evaluated as described above for whole tissue.

Western Blot Analysis

Protein lysates for all Western blots were obtained using RIPA buffer and performed as described previously using 4-16% SDS gels [29]. Blots were incubated with SuperSignal West Pico chemiluminescence substrate (Pierce) and visualized with a Fujifilm LAS-3000 camera. Band intensities were quantified using Image J software. A list of antibodies can be found in table 1.

Immunofluorescent and IHC Staining

Paraffin embedded adipose tissue was cut into 5 µm sections and placed on slides. Sections were then deparaffinized using a xylene to 50% ethanol method after baking for roughly 60 min at 60°C [30]. H&E staining or immunostaining was performed as previously described [23] using antibodies listed in Table 2.

In Vivo Insulin Stimulation Experiments

Insulin (1 U/kg body weight) was injected directly into the portal vein of ketamine sedated mice. After 5 min, the liver, adipose, and thigh muscle were snap frozen in liquid nitrogen and stored at -80°C. Protein was then extracted from tissue using RIPA buffer containing protease and phosphatase inhibitors (Roche) and insulin signaling was analyzed by Western blot.

Stimulation of Lipolysis

To study β-adrenergic receptor-stimulated lipolysis, a β_3-specific agonist, CL 316243 (Sigma C5976), was administered to 16-week-old mice on HFHC diet for 8 weeks (0.1 µg/gram body weight by intraperitoneal injection [31]). Blood was collected before and 20 min after injection through a tail bleed. Non-esterified fatty acid (NEFA) analysis was performed using the Wako NEFA-C kit per the manufacturer's instructions.

Glucose, Leptin and Insulin Measurements

Blood glucose was measured after a 12 h overnight fast using a Contour Glucometer and test strips (Bayer). Plasma insulin and leptin concentrations were determined in mice fed a HFHC diet for 16 weeks using mouse Insulin and Leptin ELISA kits (Crystal Chem) according to the manufacturer's instructions.

Metabolic Cages and Food Intake Studies

Mice were housed in single cages for four days prior to placement into the comprehensive lab animal monitoring systems (CLAMS) from Columbus

Instruments. Mice were maintained in the metabolic cages for two days to acclimate before two days of measurements were taken. All data were averaged over five consecutive timepoints, and are shown for two consecutive days, beginning at 3 pm. In a separate experiment, manual measurement of food intake was also performed to supplement the automated CLAMS food intake measurements. Daily food intake of single-housed mice in wire-bottomed cages was measured for a week and then daily food intake was averaged.

Radiolabeled Fatty Acid and Acetate Incorporation into Adipose Tissue TG

Adipose explants were taken from ketamine-sedated mice at termination of 16 weeks of HFHC diet feeding. Explants were dissected from the mice, blood vessels were removed, and adipose tissue was weighed. The tissue was then minced into 1-2 mm pieces and placed into 1 ml of low glucose DMEM medium without Fetal Bovine Serum (FBS). For ^{3}H oleate uptake, sodium oleate and [^{3}H]-sodium oleate (a total of 1µCi/well) were complexed to BSA (10.5 %) at a final concentration of 4mM, in a similar manner to previous studies [32]. Explants were cultured for 2 hours at 37°C. For [^{14}C]-acetate studies, 5 µCi of [^{14}C]-sodium acetate were added directly to the explant in 1ml medium, and incubated for 24h at 37°C. At the end of the incubation time, explants were washed 3 times with cold PBS and placed in 2:1 chloroform:methanol for lipid extraction as described above. The lipid extract was loaded onto a TLC plate and developed in 80:20:2, hexane:ethyl ether:acetic acid. Individual lipid fractions were obtained from the TLC plates (TG, CE, FC, PL and FA) by scraping after visualization with iodine stain. The fractions were placed in scintillation cocktail and radiolabel was

quantified using a liquid scintillation counter. All values were normalized back to wet weight.

Statistics

All results are presented as mean ± SEM. Statistical analyses were performed with an unpaired Student's t-test or ANOVA using GraphPad Prism 7 software. Statistical significance was set at $p<0.05$. Adipocyte cell size distribution was analyzed using a chi-squared test.

Results

ABCA1 is Selectively Deleted from Adipose Tissue

Previous reports on *in vivo* adipocyte ABCA1 deletion used an *Abca1*$^{flox/flox}$ mouse crossed with an aP2 Cre recombinase mouse [17, 18]; however, the resulting knockout mice also demonstrated partial and variable macrophage *Abca1* deletion, complicating interpretation of results. In this study, we created an adipocyte-specific *Abca1* knock out mouse model by crossing *Abca1*$^{flox/flox}$ mice with an adiponectin-Cre recombinase mice obtained from Dr. Evan Rosen [33]. Using an adiponectin Cre recombinase mouse restricts recombination of floxed alleles to adipocytes [19]. Figure 1A demonstrates that ABCA1 protein expression was reduced in WAT and BAT and the reduction exhibited the expected gene dosage effect (i.e, homozygous<heterozygous<WT). In contrast, no detectable change in ABCA1 protein expression was observed in macrophages (peritoneal or bone marrow derived) or liver, demonstrating selectivity for elimination of adipocyte ABCA1. Figure 1B confirms significant reduction in adipose tissue ABCA1 gene expression, but not liver. Although other ABCA1-expressing tissues were not examined, previous work has shown adipocyte specificity with the adiponectin Cre recombinase mouse [33] [19].

Adipocyte ABCA1 deletion reduces plasma total, and HDL cholesterol levels

The previous adipocyte ABCA1 knockout mouse model (made with an aP2 Cre recombinase mouse) had a slight, but significant decrease in total plasma

cholesterol and HDL cholesterol in two separate studies [17, 18] In our study, plasma total, LDL, and HDL cholesterol concentrations were measured in mice before (chow) and after 16 weeks of HFHC diet. ASKO mice had significantly reduced plasma cholesterol concentrations on both chow (53.5 ± 6.1 vs. 73.3 ± 4.5 mg/dl, P =0.0308) and HFHC (78.77 ± 8.215 vs. 6.1106.4±6.545 mg/dl, P=.0143) compared to control mice (Figure 2A); however, after 16 weeks of HFHC diet feeding, plasma cholesterol concentrations were elevated relative to control mice, but similar between HFHC fed control and ASKO mice. HDL cholesterol was significantly reduced in HFHC fed ASKO mice compared to HFHC fed WT mice (Figure 2B), but no differences were seen between chow fed groups. Both ASKO groups showed a trend toward reduced LDL cholesterol (Figure 2C) compared to their WT counterparts, however this did not reach significance. Plasma TG concentrations were similar for control and ASKO mice during chow and HFHC diet feeding (Figure 2 D).

ASKO Mice are Resistant to Diet-induced Weight Gain

To determine the systemic effect of adipocyte-specific ABCA1 knockout, mice were fed a HFHC diet for 16 weeks starting at 8 weeks of age or, in a smaller cohort, were maintained on chow from weaning until 24 weeks of age. All three possible genotypes of control mice (adiponectin Cre^{+} $Abca1^{+/+}$, $Abca1^{+/+}$, $Abca1^{flox/flox}$) were used and will be referred to hereafter as wild type (WT) mice as their phenotypes (body and fat pad weight, plasma cholesterol, adipocyte size, and adipose cholesterol content) were indistinguishable from $Abca1^{+/+}$ mice. Strikingly, ASKO mice had significantly less weight gain on the HFHC diet

compared to their WT counterparts (Figure 3A). In fact, weight gain for HFHC-fed ASKO mice was similar for both mouse genotypes maintained on chow for 24 weeks, resulting in terminal body weights that were significantly ($p<0.0017$) lower for WT (39.9 ± 1.1 g) vs. HFHC-fed ASKO (32.0 ± 1.4 g) mice (Figure 3B), demonstrating that ASKO mice are resistant to HFHC diet-induced weight gain.

Similar patterns were observed for fat pad weight. On a HFHC diet, WT mice had two-fold higher epididymal fat pad weights compared to ASKO mice (1.99 ± 0.140 vs. 1.12 ± 0.122 g, respectively, $p < 0.0001$), whereas weights were similar for chow-fed WT and ASKO (0.48 ± 0.10 vs. 0.55 ± 0.09 g, respectively; $p = 0.6$) (Figure 3C). Interestingly, HFHC diet-fed ASKO mice did have larger epididymal fat pads than chow-fed ASKO animals ($p = 0.02$) despite having no difference in body weight; however, the difference in fat pad weight between chow and HFHC diet-fed ASKO mice (0.568 g) was much smaller than that for WT mice (1.512 g). These differences were unaltered when EAT weight was normalized to body weight (data not shown). Liver to body weight ratio was similar for all mice. To measure body fat composition, the total amount of fat in epididymal (EAT), subcutaneous (SAT), brown (BAT), and retrorenal (RRAT) depots was measured and normalized to body weight. As seen in Figure 3D, HFHC diet-fed ASKO mice had a lower percentage of body weight as fat compared to WT mice (11.4 ± 0.9% vs. 8.8 ± 0.8%, $P = 0.04$). To make sure that a deletion of adipose ABCA1 did not result in general growth retardation, snout to tail base length was taken at time of necropsy. As seen in Figure 3E, no differences were observed between genotypes. Together, these data strongly suggest that, in the lean state, no

difference exists between WT and ASKO mouse body weight, but that ASKO mice are resistant to HFHC diet-induced weight gain.

ASKO Mice Have Significantly Smaller Adipocytes

Since adipocyte hypertrophy is believed to be the major mechanism for adipose mass increase during weight gain and is responsible for significant dysfunction, we examined adipocyte size in our study. Adipose tissue sections from ASKO and WT animals fed HFHC and chow diets were stained with H&E and examined microscopically. ASKO EAT and SAT adipocytes from HFHC fed mice appeared smaller than WT adipocytes (Figure 4A and C). Adipocyte cross-sectional area quantitative analysis confirmed that EAT and SAT ASKO adipocytes were smaller than WT adipocytes (Figure 4B and D). Interestingly, no differences in EAT adipocyte size were observed in chow-fed animals (Figure 4 E and F).

Metabolic Phenotyping of ASKO Mice

Since ASKO mice gained less weight on the HFHC diet compare with WT mice, we subjected both genotypes to metabolic phenotyping. Mice fed the HFHC diet for 16 weeks were placed in metabolic cages (CLAMS) for a total of four days, two days for acclimation and two days for measurements. No differences between genotypes were observed for respiratory exchange ratio (RER), heat, movement, or VCO_2 (Figure 5 A-D). Dark cycle VO_2 was higher in ASKO mice (Figure 5 E), likely due to the lower body weight for this group. Food intake, measured using the metabolic cages over a two day period or manually over a

week, was similar for both mouse genotypes (Figure 5F). Despite no difference in food intake, EAT leptin mRNA abundance was significantly decreased in ASKO mice compared to WT mice (fold change: 0.47 ± 0.20 vs. 1.03 ± 0.10, respectively; $p< 0.03$) (Figure 5G) as was plasma leptin measured by ELISA (8.0 ± 3.6 vs. 20.9 ± 2.4 ng/ml, $p = 0.01$; Figure 5H).

ASKO Mice Have Higher Adipose Tissue Cholesterol Content

We next examined the impact of adipocyte ABCA1 deletion on adipose tissue cholesterol content. Adipose tissue from ASKO mice had ~3-fold higher levels of cholesterol compared to WT mice, regardless of whether they were fed chow or the HFHC diet (Figure 6A). A significant increase in cholesterol was also observed in ASKO BAT, suggesting that the cholesterol enrichment was not isolated to the epididymal fat pad (Supplementary figure 1B). We examined expression of adipose tissue cholesterol metabolism genes in ASKO vs. WT mice and observed an upregulation of ABCG1, but not SR-BI, and a downregulation of cholesterol synthesis (HMGCoA reductase and synthase) and lipoprotein receptor genes (LDLr and VLDLr) (Figure 6B). Gene expression results for ABCG1 and LDLr were confirmed by Western blot analysis (Figure 6 C and D). Our previous work in macrophage-specific ABCA1 knockout mice demonstrated increased plasma membrane FC and lipid raft content that led to increases inflammatory signaling [34, 35] We isolated adipocyte plasma membranes and observed a two-fold increase in cholesterol content for ASKO mice compared to WT mice (Figure 6E). Lipid raft content was also estimated using fluorescent-labeled beta-cholera toxin on adipose tissue sections; ASKO

adipose tissue demonstrated increased lipid raft staining (Figure 6F). These data demonstrate a remarkable increase in adipocyte cholesterol in the absence of ABCA1 that cannot be overcome by the usual compensatory cellular responses to increased cholesterol load.

ASKO Mice Have Reduced Adipose Tissue TG

Previous studies have shown that the adipocyte cholesterol to TG ratio remains relatively constant regardless of adipocyte size [36]. Given the small adipocyte phenotype along with increased adipose cholesterol in ASKO mice, we next examined whether adipose TG metabolism was altered. We first measured TG content and determined that HFHC-diet fed ASKO adipose tissue contained less TG than WT mouse adipose tissue (Figure 7A). There are several reasons why TG may be lower in adipocytes. We first explored whether ASKO adipocytes were more lipolytic compared to WT adipocytes. At 16 weeks of age, after 8 weeks of HFHC diet, WT and ASKO mice were stimulated with a β3-specific adrenergic agonist to induce adipocyte TG lipolysis. Plasma NEFA levels were measured before and after stimulation. No differences between ASKO and WT mice were observed before or after stimulation, suggesting no difference in lipolysis (Figure 7B). Second, we examined uptake and incorporation of [^{3}H]-oleic acid into adipose tissue esterified lipids. Less [^{3}H]-TG was found in ASKO adipocytes, suggesting they are less able to take up and/or esterify oleic acid into TG compared to WT adipocytes (Figure 7C). Finally, we examined *de novo*

lipogenesis by incubating adipose tissue with [^{14}C]-acetic acid. The results suggest that ASKO adipose tissue is also less able to incorporate the [^{14}C]-acetate into TG, suggesting decreased *de novo* lipogenesis (Figure 7D). Taken together, these results suggest that ASKO adipocytes are smaller and contain less TG due to defective lipogenesis.

Adipocyte ABCA1 Deletion Does Not Affect Insulin Sensitivity

Insulin stimulates *de novo* lipogenesis via activation of SREBP-1 [37]. Since HFHC diet-fed ASKO mice had adipocytes that were smaller, contained less TG, and were defective in lipogenesis relative to WT adipocytes, we examined adipose tissue insulin signaling. Plasma insulin (Figure 8 A) and glucose (Figure 8 B) were similar in ASKO and WT mice. Insulin signaling-related gene expression in adipose tissue was also examined and only IRS-1 expression was decreased (Figure 8C). IRS-2 mRNA was not detectable (data not shown). Insulin signaling was examined in adipose tissue, liver and muscle five minutes after portal vein insulin injection. To our surprise, no difference in Akt phosphorylation was observed in ASKO and WT adipose tissue (Figure 8 D and E). These results suggest that diminished lipogenesis in ASKO adipose tissue was not due to defective insulin signaling.

Lipid Accretion Pathways are Down Regulated in ASKO Mice

Since reduced insulin signaling could not explain reduced adipocyte TG content and lipogenesis in ASKO mice, we examined lipid accretion pathways in more detail. Interestingly, PPARϒ and C/EBP1α and β gene expression were

significantly decreased in adipose tissue of ASKO mice (Figure 9A). These genes are the major transcription factors that regulate adipocyte TG accretion. For example, PPARγ expression is essential for TG accumulation during adipocyte differentiation [38]. Other genes involved in adipocyte TG synthesis, such as DGAT 1 and 2, or lipid uptake (i.e., CD36) were also decreased in ASKO adipose tissue. These data were confirmed by Western blot analysis of PPARγ, SCD-1, and CD36 (Figure 9B and C). One of the major regulatory pathways responsible for the promotion of lipid storage, including the transcription of PPARϒ, is SREBP1c. Accordingly, we examined activated SREBP1 levels in the nuclei of adipocytes isolated from 16 week HFHC fed adipose tissue and found decreased expression in ASKO tissue (Figure 9D). Based on these results, we hypothesize that adipocyte FC accumulation in ASKO mice results in enrichment the ER membrane with cholesterol, resulting in decreased activation of SREBP1 and PPARγ. This, in turn, will decrease lipid accretion, resulting in smaller adipoctyes with less TG.

Brown Adipose Tissue is Altered in ASKO Mice

BAT weight did not differ between WT and ASKO animals (Supplementary Figure 1 A) even though cholesterol content was elevated >2-fold in ASKO mice (Supplemental Figure 1 B). Expression of prototypical BAT genes was similar for both genotypes of mice (Supplemental Figure 1 C). Microscopic examination of ASKO BAT reveal markedly different histological appearance, including smaller cells, and an increased number of brown adipocytes, compared to WT BAT (Supplementary Figure 1D). In addition, several areas of small, multilocular

adipocytes were noted in ASKO WAT, which stained positive for UCP-1, a marker for adipose browning (Supplementary Figure 1E). While observational in nature, these results suggest that adipocyte ABCA1 expression and cholesterol content may play a significant role in adipocyte browning and the function of brown adipose tissue, and should be investigated in more detail.

Deletion of ABCA1 in Adipose Tissue Does Not Increase Adipose Tissue Inflammation

In previous studies, increased adipose tissue cholesterol content was associated with increased adipose tissue inflammation [5, 23]. Despite a 3-fold increase in adipose tissue cholesterol, ASKO mice did not exhibit such an increase in adipose tissue pro-inflammatory gene expression (Supplementary Figure 2A). ASKO EAT did, however, demonstrate lower expression of anti-inflammatory genes, including IL-10 and arginase 1, suggesting a decrease in anti-inflammatory macrophages rather than an increase in pro-inflammatory macrophages as described in previous studies. CD68 staining of adipose tissue agreed with the gene expression results (Supplementary Figure 2B), with no apparent increase in CD68 staining in ASKO adipose tissue compared to WT. Previous studies have shown a direct correlation between dietary cholesterol, adipose cholesterol, and inflammation [5, 23]; however, this is clearly not the case in our ASKO model.

Discussion

This study was designed to elucidate the role and contribution of adipocyte ABCA1, and to investigate the role of cholesterol in adipocyte dysfunction. Previous studies have shown that ABCA1 is abundantly expressed in adipose tissue; however, its exact contribution to adipocyte function is relatively unknown. The evidence presented herein is the first to describe the *in vivo* role of ABCA1 specifically in adipose tissue. Previous studies using the aP2 Cre recombinase mouse have had to contend with a concomitant and variable ABCA1 deletion in macrophages in addition to adipocytes [17, 18]. However, our model does not face such issues (Figure 1A).

We have shown here that adipocyte ABCA1 has minor effects on plasma cholesterol levels. ASKO mice fed chow had signficantly lower total plasma cholesterol than WT mice (Figure 2A). This is in agreement with previous studies on adipose ABCA1 *in vivo* [17, 18], but contradicts early *in vitro* evidence that reported little ABCA1-mediated cholesterol efflux from adipocytes [39]. Plasma cholesterol and HDL cholesterol trended lower in ASKO mice, but the results were not statically significant. Overall, this study confirms that in chow-fed animals, adipose ABCA1 contributes minimally (<15%) to total plasma cholesterol levels *in vivo*, which is in agreement with past studies showing that plasma HDL cholesterol is primarily determined by liver [40] and small intestine ABCA1 expression [41].

Despite having a small effect on plasma cholesterol concentration, adipocyte ABCA1 deletion resulted in significantly higher levels of adipose cholesterol as we observed a 3- to 5-fold increase in total cholesterol in both white and brown adipose (Figure 6 and supplementary figure 1). This dramatic increase in cholesterol was observed despite the fact that ASKO adipose upregulated ABCG1 and downregulated cholesterol synthesis and uptake genes (Figure 6). These results strongly suggest that adipocytes have no compensatory mechanism for the loss of ABCA1, and that ABCA1 is the major cholesterol efflux system in adipocytes. This is in contrast to early *in vitro* experiments which reported that ABCA1 only effluxes cholesterol under conditions of prolonged lipolysis [39], but agrees with other *in vivo* models that have attempted to examine adipose ABCA1 specifically [17, 18]. In addition to making conclusions about adipocyte ABCA1, we can also infer the importance of adipose cholesterol. Several examples in the literature have proposed that adipocyte cholesterol balance plays a significant role in determining adipocyte size and TG content in adipose tissue, particularly during obesity [16, 42, 43]. Specifically, several examples have proposed that cholesterol acts as a cell size signal in adipocytes, promoting lipid storage. This theory hinges on the idea that membrane cholesterol in hypertrophied adipocytes is “diluted”, and that adipocytes sense this dilution as true cholesterol depletion [16, 39, 42, 44], and our study provides additional evidence for this. Previous studies have shown that adipocyte cholesterol accumulation is associated with TG accretion during adipocyte hypertrophy [11]. In contrast, ASKO mice had a 3-fold increase in cholesterol, but

reduced adipocyte TG content and reduced adipocyte size (Figure 4 and 7). The reason for the difference in outcomes between our study and previous studies may lie in the high levels of membrane cholesterol observed in ASKO adipocytes (Figure 6). Several studies have shown that adipocyte cholesterol moves from the plasma membrane to the lipid droplet during hypertrophy, and the resulting enlarged adipocytes have plasma membranes that are relatively depleted of cholesterol [16, 45]. Supporting this hypothesis, reports have shown that hypertrophied adipocytes activate SREBP2 and upregulate cholesterol synthesis genes, despite having higher levels of total cholesterol [16]. By deleting adipocyte ABCA1, membrane cholesterol depletion is prevented during a HFHC diet. To determine the mechanism by which our ASKO mice had lower fat pad weight and smaller adipocytes, we looked more closely at TG storage pathways. We determined that no difference in lipolysis exists between genotypes (Figure 7B), but that ASKO adipose tissue is less able to incorporate both oleic acid and acetate into TG (Figure 7C and D). These results led us to examine lipogenic gene expression and pathways in ASKO mice. Both mRNA and protein levels of major lipogenic genes and transcription factors are downregulated in ASKO mice (Figure 9A and B). Surprisingly, despite no reduction in insulin signaling, nuclear SREBP1 was also decreased in ASKO adipose tissue (Figure 9D). A reduction in nuclear SREBP1 would collectively reduce expression of genes involved in FA uptake, *de novo* lipogenesis and FA esterification, resulting in reduced adipocyte TG accumulation, explaining reduced adipocyte size and TG content in ASKO mice. We hypothesize that by retaining high levels of membrane cholesterol

despite a HFHC diet, the SCAP-SREBP1 complex is retained in the ER, and TG accumulation is subsequently decreased.

While SREBP1 is highly responsive to insulin, previous work has shown that in extreme cholesterol-loaded conditions, adipocyte SREBP1c activation is decreased [46]. In fact, loading cholesterol in differentiating 3T3L1 cells prevents TG accumulation, downregulates lipogenic gene and transcription factor expression, and halts differentiation through prevention of SREBP1 activation [46]. In addition, early work on both SREBP1 and SREBP2 demonstrated that ER sterol content directly modulates SREBP1 movement to the nucleus [47, 48]. In adipocytes, cholesterol depletion induced by chromium piconate treatment increased nuclear SREBP1c content [49]. Additional research supports the hypothesis that 25-hydroxycholesterol can directly bind to Insig-1 and prevent the movement of SREBP1 to the nucleus [50]. Through these mechanisms, high levels of cholesterol in the ER can prevent the activation and translocation of SREBP1 to the nucleus. In ASKO adipose tissue, we observed a reduction in lipogenic gene and transcription factor expression, consistent with the reduction in nuclear SREBP1 (Figure 9). These data strongly suggest that membrane cholesterol (specifically ER cholesterol) content regulates adipocyte SREBP1 activation, and that this ultimately has significant effects on adipocyte function and TG storage. For example, high levels of membrane (including the ER) cholesterol, as found in lean adipocytes, are sensed in the ER and prevent the movement of SREBP1 (and likely SREBP2 [42]) to the nucleus, resulting in low levels of lipogenesis, FA esterification and reduced FA uptake (through CD36). In

contrast, theoretically, hypertrophied adipocytes with low levels of membrane cholesterol due to a redistribution of cholesterol to the lipid droplet would activate SREBP1 and ultimately increase TG and cholesterol content in adipocytes.

Despite having lower levels of adipose TG content, and fat pad and body weight, no striking metabolic differences were observed between HFHC-fed WT and ASKO mice. Given the lower levels of leptin in ASKO mice, we might have expected a difference in food intake; however, this was not observed (Figure 5 F-H). ASKO mice did have higher VO2 during the dark cycle (Figure 5E), suggesting higher energy utilization, but RER overall was not different. These data suggest that the metabolic measures we have used here are not sensitive enough to detect the metabolic differences between WT and ASKO mice during two days of monitoring. For instance, an 8 g difference in body weight between WT and ASKO mice over a 16 week period is an average body weight difference of 71 mg/day (8g/112 days), making it unlikely to detect the small difference in food consumption need to produce this small difference in body weight over a two day to one week observational period. However, the lower body weight of HFHC-fed ASKO mice must be the result of eating less and/or expending more energy. Due to the lower plasma leptin levels in the ASKO mice, we think it unlikely that ASKO mice are eating less; lower leptin levels are generally associated with higher food consumption, although this may be complicated by the existence of leptin resistance [51]. A more likely explanation is that ASKO animals expend more energy as heat through the uncoupling of oxidative phosphorylation in the adipose tissue. Supporting this notion, ASKO mice have

smaller, more numerous, BAT lipid droplets than WT mice. We also observed areas of UCP-1 staining in ASKO EAT (Supplementary figure 1E) that appear greater than their WT counterparts. These data suggest that ASKO animals may be expending energy as heat through the browning of WAT. Interestingly, others have proposed that adipose cholesterol metabolism and adipocyte browning are linked, namely that HDL/apoA1 act to reduce autophagy in adipocytes and prevent browning [44]. To investigate this further, ASKO animals should be directly monitored for elevations in body temperature, brown adipose should be further studied including additional gene expression and adipocyte size quantification, and white adipose should be evaluated fully for the expression of UCP-1, and beta oxidation tested *in vivo* or *ex vivo*. Cold tolerance studies that promote browning should also be performed to determine the reaction of ASKO mice non-shivering thermogenesis.

In addition to its significant effects on TG metabolism and adipocyte size, membrane cholesterol also plays a role in insulin signaling [52]. The insulin receptor localizes in cholesterol-rich plasma membrane caveolae and disruption of caveolae by cholesterol depletion with methly-β-cyclodextrin results in reduced insulin-induced glucose and FA uptake in cultured 3T3L1 adipocytes [16, 53, 54]. Given the reduced TG accumulation and SREBP1 activation in ASKO adipocytes, we originally considered that insulin signalling would be decreased in ASKO compared to WT adipocytes; however, this was not the case. In fact, *in vivo* insulin stimulation of adipose tissue showed no difference in insulin response between ASKO and WT adipose tissue (Figure 8D and E) and no

changes in plasma insulin or glucose were detected between genotypes on HFHC diet (Figure 8A and B). In addition, acute enrichment of membrane cholesterol with cholesterol-loaded methyl-β-cyclodextrin in 3T3L1 cells did not increase insulin-stimulated glucose or FA uptake [16]. Taken together, these results suggest that cholesterol depletion, but not overload, of plasma membrane blunts insulin signaling.

Interestingly, despite a 3-fold increase in EAT cholesterol content in ASKO mice, only minor effects on adipose tissue inflammation were found (Supplementary figure 2A and B). Previous studies have clearly shown that the addition of dietary cholesterol alone can lead to adipose inflammation [5, 23]. Several studies, including our own, have demonstrated that dietary cholesterol leads to increased adipose tissue cholesterol content, macrophage infiltration, and inflammation [5, 23, 55]. These studies show ~ 50% increase in adipose cholesterol content as a result of increased dietary cholesterol intake. In contrast, ASKO mice had ~ 3 fold increase in VAT cholesterol. This suggests that adipocyte cholesterol content alone is not the determining factor for adipose tissue inflammation and that macrophages, not adipocytes, are primarily responsible for dietary cholesterol-induced inflammation. In fact, macrophage infiltration into adipose tissue is likely the main contributor to adipose tissues inflammation that accompanies weight gain and obesity [5, 56-60]. We have also shown a small increase in macrophage membrane FC and lipid raft content induced by macrophage-specific deletion of ABCA1 results in an exaggerated proinflammatory response to Toll-like 4 receptor (TLR4) agonists (i.e. lipopolysaccharide, LPS) [35], supporting the idea

that an increase in adipose tissue macrophage cholesterol may result in increased adipose tissue inflammation. However, increased macrophage inflammation, *per se*, does not result in systemic insulin resistance that accompanies obesity [61], suggesting that the adipose tissue macrophages inflammation and adipocyte dysfunction are both necessary to display the full metabolic phenotype in obese states.

In all, the results from our mouse model provide additional mechanistic insights to what was previously known regarding adipose tissue cholesterol and raise several important mechanistic questions. Surprisingly, an adipose specific deletion of ABCA1 was mostly beneficial, and resulted in lower weight gain, reduced body fat, and no change in insulin sensitivity. We predict that the majority of these phenotypes are due to the high level of membrane cholesterol in ASKO adipocytes. By preventing cholesterol efflux, we artificially raised membrane cholesterol levels and prevented the adipocyte hypertrophy normally observed in HFHC-fed animals. This has significant implications, namely that preventing the loss of adipocyte membrane cholesterol can prevent adipocyte hypertrophy, reduce TG storage, maintain or even increase adipose insulin sensitivity, and perhaps promote adipocyte browning.

References

1. Finkelstein, E.A., et al., *Annual Medical Spending Attributable To Obesity: Payer-And Service-Specific Estimates.* Health Affairs, 2009. **28**(5): p. w822-w831.

2. Flegal Km, C.M.D.O.C.L.C.L.R., *PRevalence and trends in obesity among us adults, 1999-2008.* JAMA, 2010. **303**(3): p. 235-241.

3. Members, W.G., et al., *Heart Disease and Stroke Statistics—2012 Update: A Report From the American Heart Association.* Circulation, 2012. **125**(1): p. e2-e220.

4. Spiegelman, B.M. and J.S. Flier, *Adipogenesis and Obesity: Rounding Out the Big Picture.* Cell, 1996. **87**(3): p. 377-389.

5. Subramanian, S., et al., *Dietary Cholesterol Worsens Adipose Tissue Macrophage Accumulation and Atherosclerosis in Obese LDL Receptor–Deficient Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2008. **28**(4): p. 685-691.

6. Salans, L.B., J.L. Knittle, and J. Hirsch, *The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity.* The Journal of Clinical Investigation, 1968. **47**(1): p. 153-165.

7. Winkler, G., et al., *Expression of tumor necrosis factor (TNF)-alpha protein in the subcutaneous and visceral adipose tissue in correlation with adipocyte cell volume, serum TNF-alpha, soluble serum TNF-receptor-2 concentrations and C-peptide level.* European Journal of Endocrinology, 2003. **149**(2): p. 129-135.

8. Smith, U., *Effect of cell size on lipid synthesis by human adipose tissue in vitro.* J Lipid Res, 1971. **12**(1): p. 65-70.

9. Skurk, T., et al., *Relationship between Adipocyte Size and Adipokine Expression and Secretion.* Journal of Clinical Endocrinology & Metabolism, 2007. **92**(3): p. 1023-1033.

10. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* The Journal of Clinical Investigation, 2003. **112**(12): p. 1796-1808.

11. Krause, B.R. and A.D. Hartman, *Adipose tissue and cholesterol metabolism.* Journal of Lipid Research, 1984. **25**(2): p. 97-110.

12. R. H. Schreibman, R.B.D., *Human adipocyte Cholesterol. Concentration, localization, synthesis, and turnover.* Journal of clinical investigation, 1975. **55**(5): p. 986-993.

13. Kovanen, P.T., E.A. Nikkilä, and T.A. Miettinen, *Regulation of cholesterol synthesis and storage in fat cells.* Journal of Lipid Research, 1975. **16**(3): p. 211-23.

14. Tilvis, R.S., P.T. Kovanen, and T.A. Miettinen, *Release of newly synthesized squalene, methyl sterols and cholesterol from human adipocytes in the presence of lipoproteins.* Scandinavian Journal of Clinical & Laboratory Investigation, 1978. **38**(1): p. 83-87.

15. Umemoto, T., et al. (2013). "*Apolipoprotein A-I and HDL Have Anti-Inflammatory Effects on Adipocytes via Cholesterol Transporters: ATP-Binding Cassette (ABC) A-1, ABCG-1 and Scavenger Receptor B-1(SRB-1)*." Circulation Research **112**(10): 1345-1354.

16. Le Lay, S., et al., *Cholesterol, a Cell Size-dependent Signal That Regulates Glucose Metabolism and Gene Expression in Adipocytes.* Journal of Biological Chemistry, 2001. **276**(20): p. 16904-16910.

17. Chung, S., et al., *Adipose tissue ATP binding cassette transporter A1 contributes to high-density lipoprotein biogenesis in vivo.* Circulation, 2011. **124**(15): p. 1663-72.

18. de Haan, W., et al., *ABCA1 in adipocytes regulates adipose tissue lipid content, glucose tolerance, and insulin sensitivity.* J Lipid Res, 2014. **55**(3): p. 516-23.

19. Lee, K.Y., et al., *Lessons on Conditional Gene Targeting in Mouse Adipose Tissue.* Diabetes, 2013. **62**(3): p. 864-874.

20. Eguchi, J., et al., *Transcriptional control of adipose lipid handling by IRF4.* Cell metabolism, 2011. **13**(3): p. 249-259.

21. Brown, J.M., et al., *Targeted Depletion of Hepatic ACAT2-driven Cholesterol Esterification Reveals a Non-biliary Route for Fecal Neutral Sterol Loss.* Journal of Biological Chemistry, 2008. **283**(16): p. 10522-10534.

22. Berg, A.H. and P.E. Scherer, *Adipose Tissue, Inflammation, and Cardiovascular Disease.* Circulation Research, 2005. **96**(9): p. 939-949.

23. Chung, S., et al., *Dietary cholesterol promotes adipocyte hypertrophy and adipose tissue inflammation in visceral, but not in subcutaneous, fat in monkeys.* Arterioscler Thromb Vasc Biol, 2014. **34**(9): p. 1880-1887.

24. Lowry, O.H., et al., *PROTEIN MEASUREMENT WITH THE FOLIN PHENOL REAGENT.* Journal of Biological Chemistry, 1951. **193**(1): p. 265-275.

25. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative CT method.* Nat. Protocols, 2008. **3**(6): p. 1101-1108.

26. Galarraga, M., et al., *Adiposoft: automated software for the analysis of white adipose tissue cellularity in histological sections.* J Lipid Res, 2012. **53**(12): p. 2791-2796.

27. Ruan, H. and H.F. Lodish, *Regulation of insulin sensitivity by adipose tissue-derived hormones and inflammatory cytokines.* Current Opinion in Lipidology, 2004. **15**(3): p. 297-302.

28. Soukas, A., et al., *Leptin-specific patterns of gene expression in white adipose tissue.* Genes & Development, 2000. **14**(8): p. 963-980.

29. Liu, M., et al., *Uncleaved ApoM Signal Peptide Is Required for Formation of Large ApoM/Sphingosine 1-Phosphate (S1P)-enriched HDL Particles.* Journal of Biological Chemistry, 2015. **290**(12): p. 7861-7870.

30. Fischer, A.H., et al., *Hematoxylin and Eosin Staining of Tissue and Cell Sections.* Cold Spring Harbor Protocols, 2008. **2008**(5): p. pdb.prot4986.

31. Caviglia, J. M., et al. (2011). "Adipose-selective overexpression of ABHD5/CGI-58 does not increase lipolysis or protect against diet-induced obesity." Journal of Lipid Research **52**(11): 2032-2042.

32. Chung, S., et al., *A novel role for ABCA1-generated large pre-β migrating nascent HDL in the regulation of hepatic VLDL triglyceride secretion.* Journal of Lipid Research, 2010. **51**(4): p. 729-742.

33. Wang, Z.V., et al., *Identification and Characterization of a Promoter Cassette Conferring Adipocyte-Specific Gene Expression.* Endocrinology, 2010. **151**(6): p. 2933-2939.

34. Zhu, X., et al., *Macrophage ABCA1 reduces MyD88-dependent Toll-like receptor trafficking to lipid rafts by reduction of lipid raft cholesterol.* Journal of Lipid Research, 2010. **51**(11): p. 3196-3206.

35. Zhu, X., et al., *Increased Cellular Free Cholesterol in Macrophage-specific Abca1 Knock-out Mice Enhances Pro-inflammatory Response of Macrophages.* Journal of Biological Chemistry, 2008. **283**(34): p. 22930-22941.

36. Krause, B.R., et al., *Adipocyte Cholesterol Storage: Effect of Experimental Hypercholesterolemia in the Rat.* The Journal of Nutrition, 1979. **109**(12): p. 2213-2225.

37. Owen, J.L., et al., *Insulin stimulation of SREBP-1c processing in transgenic rat hepatocytes requires p70 S6-kinase.* Proceedings of the National Academy of Sciences, 2012. **109**(40): p. 16184-16189.

38. Tontonoz, P., E. Hu, and B.M. Spiegelman, *Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor.* Cell, 1994. **79**(7): p. 1147-1156.

39. Le Lay, S., et al., *Regulation of ABCA1 expression and cholesterol efflux during adipose differentiation of 3T3-L1 cells.* Journal of Lipid Research, 2003. **44**(8): p. 1499-1507.

40. Timmins, J.M., et al., *Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I.* The Journal of Clinical Investigation, 2005. **115**(5): p. 1333-1342.

41. Brunham, L.R., et al., *Intestinal ABCA1 directly contributes to HDL biogenesis in vivo.* The Journal of Clinical Investigation, 2006. **116**(4): p. 1052-1062.

42. Yu, B.L., S.P. Zhao, and J.R. Hu, *Cholesterol imbalance in adipocytes: a possible mechanism of adipocytes dysfunction in obesity.* Obesity Reviews, 2010. **11**(8): p. 560-567.

43. Turner, S.M., et al., *Dissociation between adipose tissue fluxes and lipogenic gene expression in ob/ob mice.* American Journal of Physiology - Endocrinology And Metabolism, 2007. **292**(4): p. E1101-E1109.

44. Wang, S. and D. Peng, *Regulation of adipocyte autophagy — The potential anti-obesity mechanism of high density lipoprotein and ApolipoproteinA-I.* Lipids in Health and Disease, 2012. **11**: p. 131-131.

45. Guerre-Millo, M., et al., *Alteration in membrane lipid order and composition in metabolically hyperactive fatty rat adipocytes.* Lipids, 1994. **29**(3): p. 205-209.

46. Li, H., et al., *Cholesterol suppresses adipocytic differentiation of mouse adipose-derived stromal cells via PPARγ2 signaling.* Steroids, 2013. **78**(5): p. 454-461.

47. Nohturfft, A., et al., *Regulated Step in Cholesterol Feedback Localized to Budding of SCAP from ER Membranes.* Cell. **102**(3): p. 315-323.

48. Wang, X., et al., *SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis.* Cell, 1994. **77**(1): p. 53-62.

49. Pattar, G.R., et al., *Chromium picolinate positively influences the glucose transporter system via affecting cholesterol homeostasis in adipocytes cultured under hyperglycemic diabetic conditions.* Mutat Res, 2006. **610**(1-2): p. 93-100.

50. Radhakrishnan, A., et al., *Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig.* Proc Natl Acad Sci U S A, 2007. **104**(16): p. 6511-6518.

51. Klok, M.D., S. Jakobsdottir, and M.L. Drent, *The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review.* Obesity Reviews, 2007. **8**(1): p. 21-34.

52. Le Lay, S., P. Ferre, and I. Dugail, *Adipocyte cholesterol balance in obesity.* Biochemical Society transactions, 2004. **32**(Pt 1): p. 103-106.

53. Parpal, S., et al., *Cholesterol Depletion Disrupts Caveolae and Insulin Receptor Signaling for Metabolic Control via Insulin Receptor Substrate-1, but Not for Mitogen-activated Protein Kinase Control.* Journal of Biological Chemistry, 2001. **276**(13): p. 9670-9678.

54. Covey, S.D., et al., *Cholesterol depletion inhibits fatty acid uptake without affecting CD36 or caveolin-1 distribution in adipocytes.* Biochemical and Biophysical Research Communications, 2007. **355**(1): p. 67-71.

55. Aguilar, D. and M.L. Fernandez, *Hypercholesterolemia induces adipose dysfunction in conditions of obesity and nonobesity.* Adv Nutr, 2014. **5**(5): p. 497-502.

56. Gregor, M.F. and G.S. Hotamisligil, *Inflammatory Mechanisms in Obesity.* Annual Review of Immunology, 2011. **29**(1): p. 415-445.

57. Hotamisligil, G.S., *Inflammation and metabolic disorders.* Nature, 2006. **444**(7121): p. 860-867.

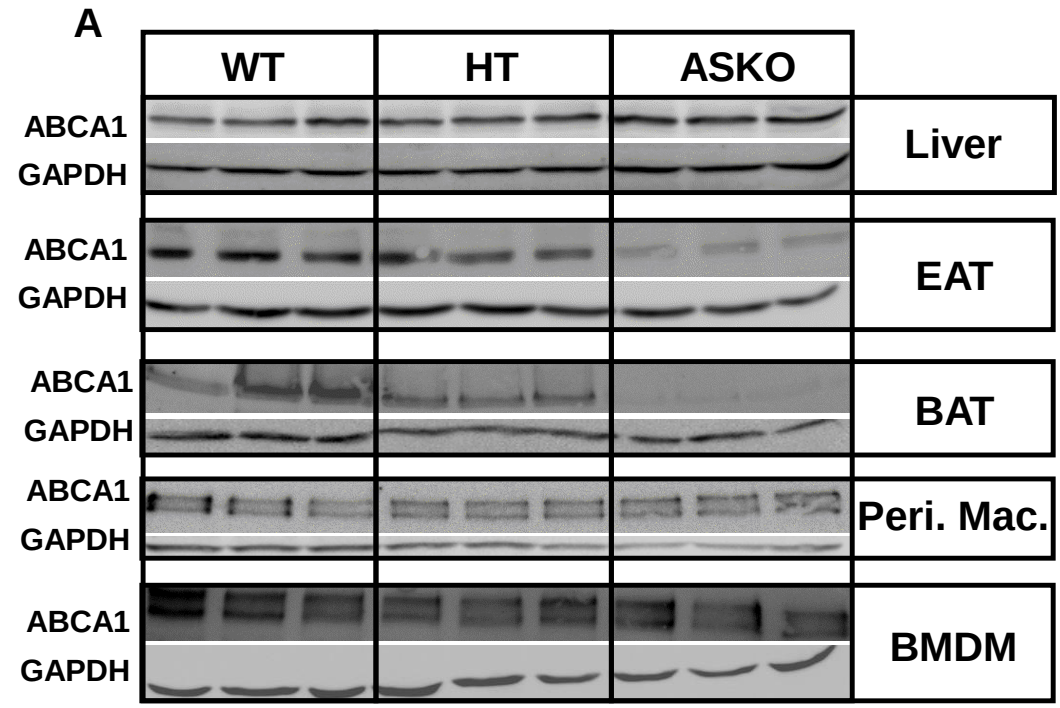
58. Ouchi, N., et al., *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol, 2011. **11**(2): p. 85-97.

59. Shoelson, S.E., L. Herrero, and A. Naaz, *Obesity, Inflammation, and Insulin Resistance.* Gastroenterology, 2007. **132**(6): p. 2169-2180.

60. Odegaard, J.I. and A. Chawla, *Pleiotropic actions of insulin resistance and inflammation in metabolic homeostasis.* Science, 2013. **339**(6116): p. 172-177.

61. Zhu, X., et al., *Myeloid cell-specific ABCA1 deletion does not worsen insulin resistance in HF diet-induced or genetically obese mouse models.* Journal of Lipid Research, 2013. **54**(10): p. 2708-2717.

Figure 1: ABCA1 is Specifically Deleted From Adipose Tissue



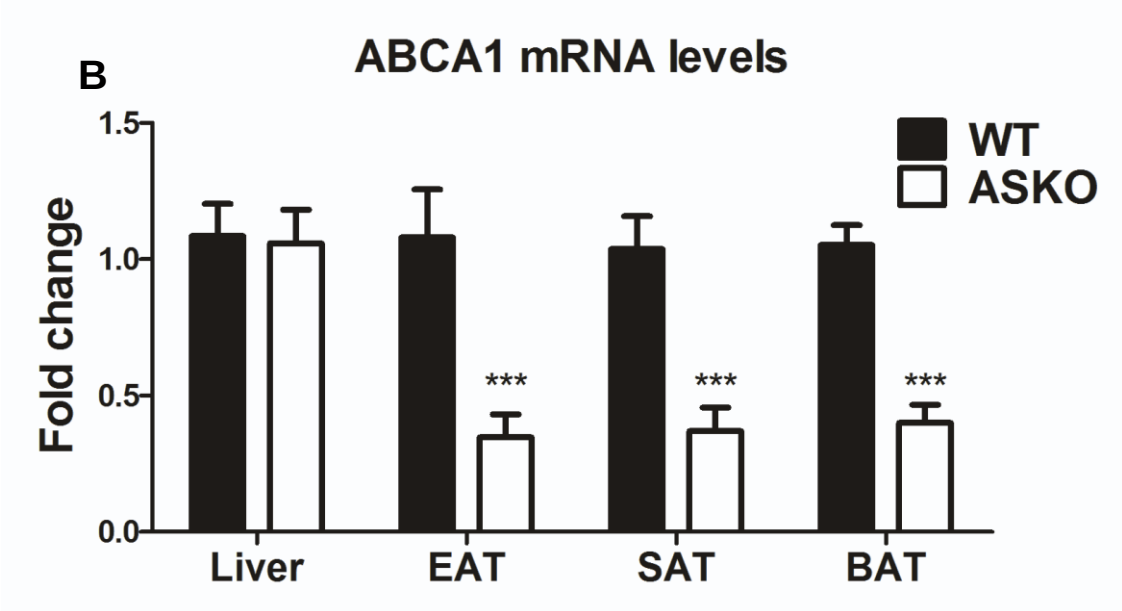


Figure 1: A) Western Blots fo ABCA1 and GAPDH in various tissues in WT, HT, and ASKO animals, B) ABCA1 gene expression from WT and ASKO, liver, EAT, SAT and BAT (n=6 per group), *** indicates P<.0001.

Figure 2: Adipocyte ABCA1 deletion reduces plasma total, and HDL cholesterol levels

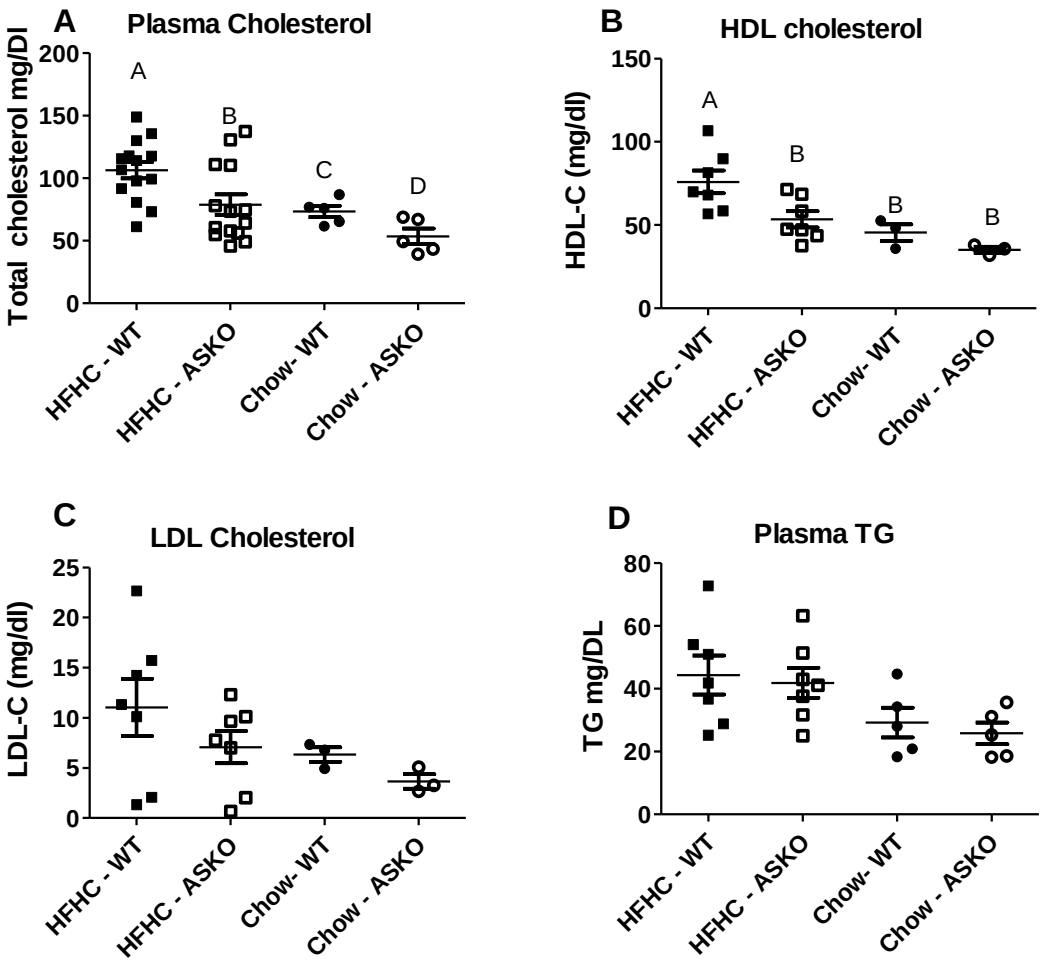


Figure 2: Plasma levels of A) total cholesterol, B) HDL-cholesterol, C) LDL-cholesterol, and D) triglycerides in WT and ASKO mice fed a HFHC or chow diet for 16 weeks. Total plasma cholesterol samples were determined from individual plasma samples, plasma cholesterol fractions were determined from samples pooled from 2 mice. Statistical significance was determined by ANOVA.

Figure 3: ASKO mice are resistant to diet-induced weight gain

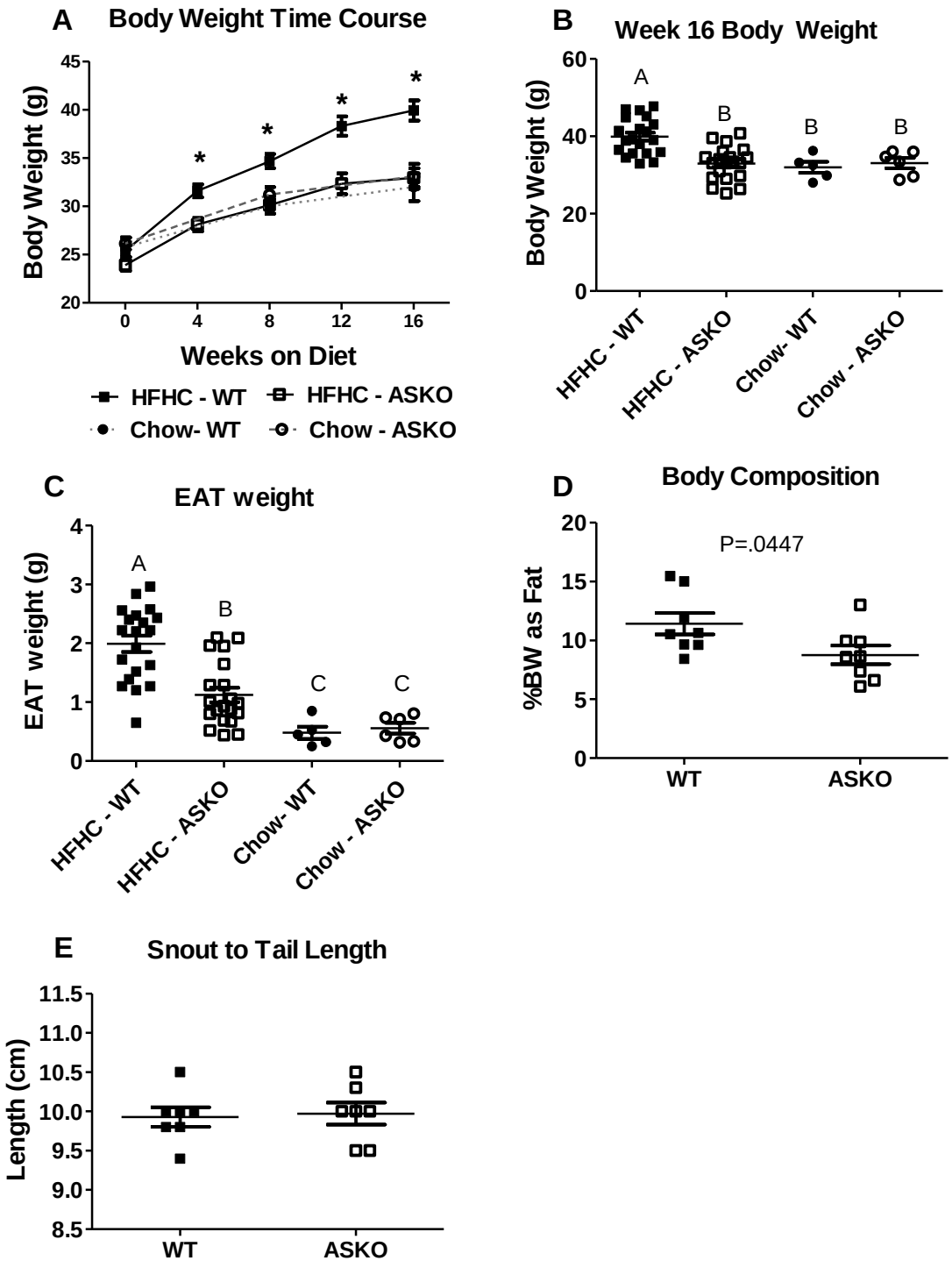
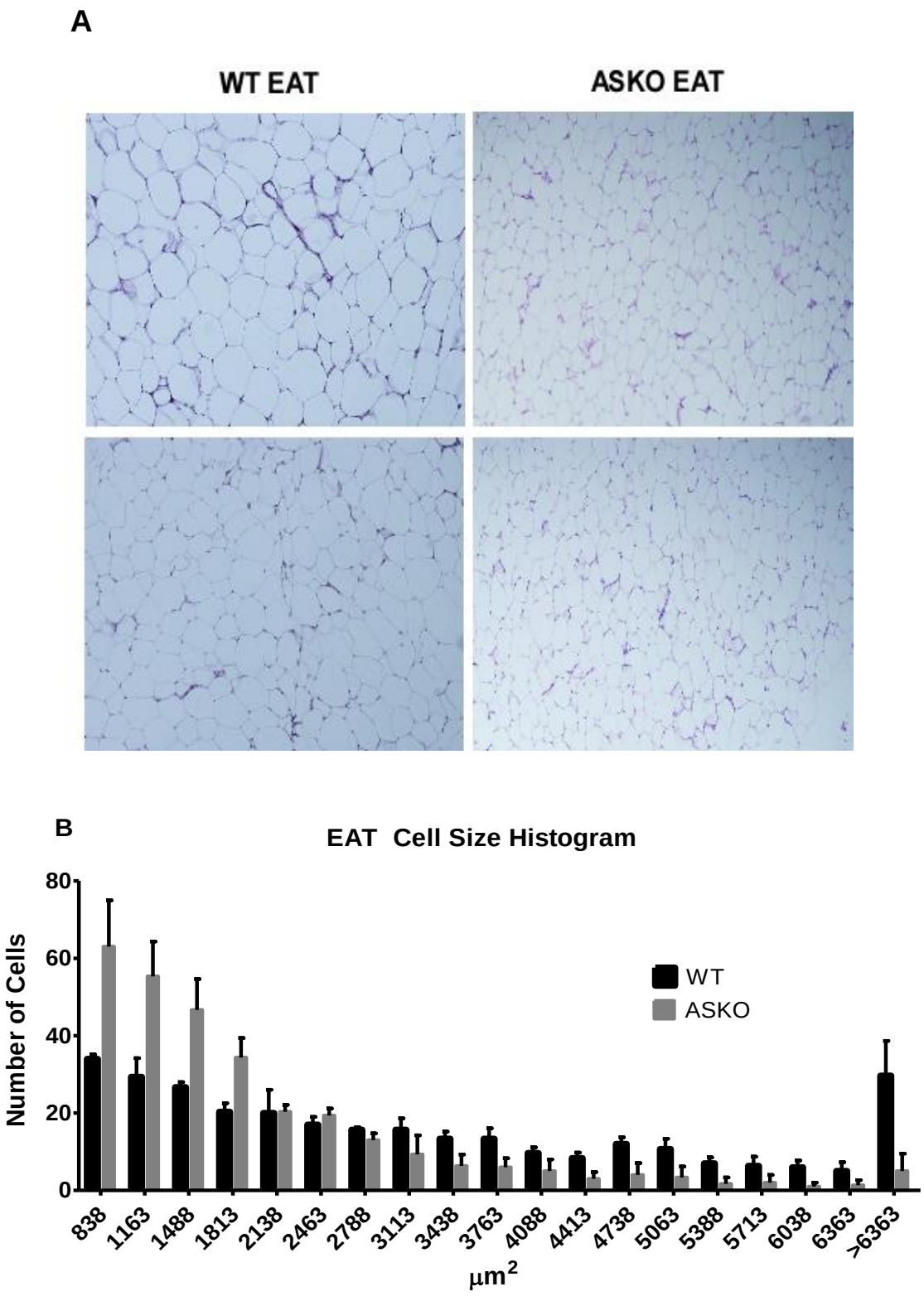


Figure 3: A) Timeline of body weight gain over the course of 16 weeks feeding either chow or HFHC diet (n=20 per genotype on HFHF, and 5 or 6 per genotype on chow). B) Body weights after 16 weeks chow and HFHC diet feeding. C) Epididymal Adipose Tissue weight expressed after 16 weeks diet feeding. D) Body composition, the total percentage of body weight occupied by epididymal, retrorenal, subcutaneous, and brown fat. E) Snout to tail length of 16 week HFHC fed mice. * indicates a signficant body weight difference between WT and ASKO mice on HFHC diet.

Figure 4: ASKO mice have significantly smaller adipocytes



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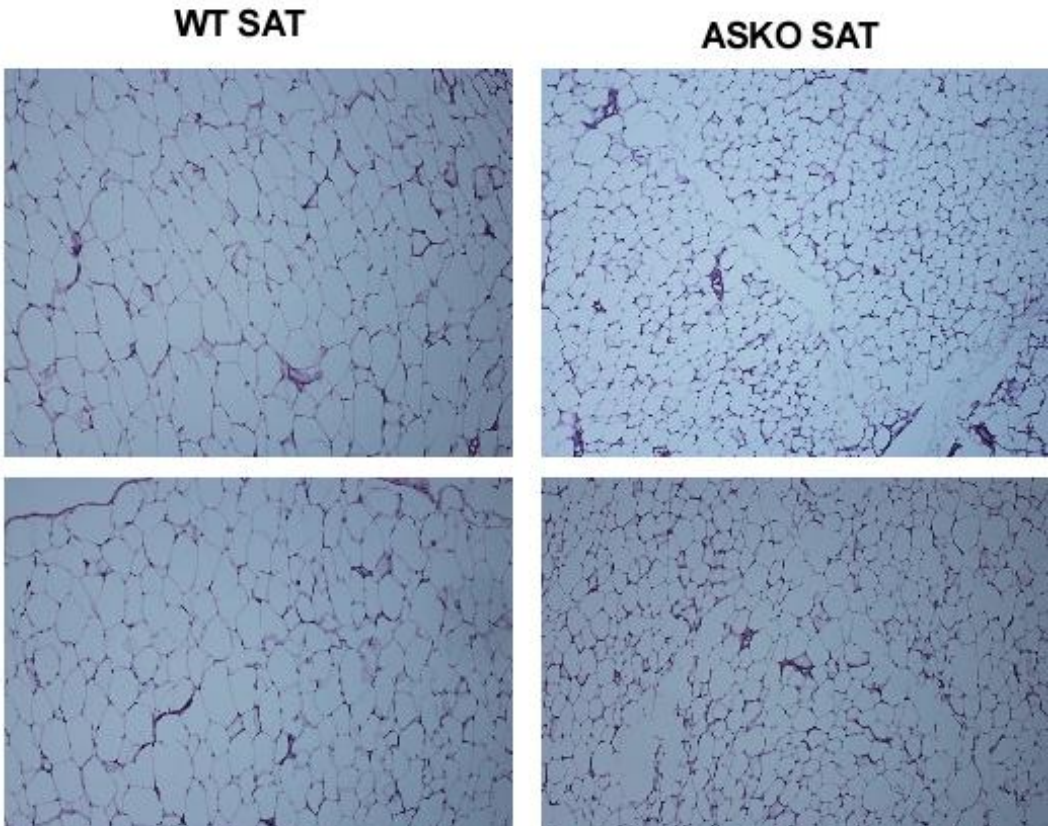
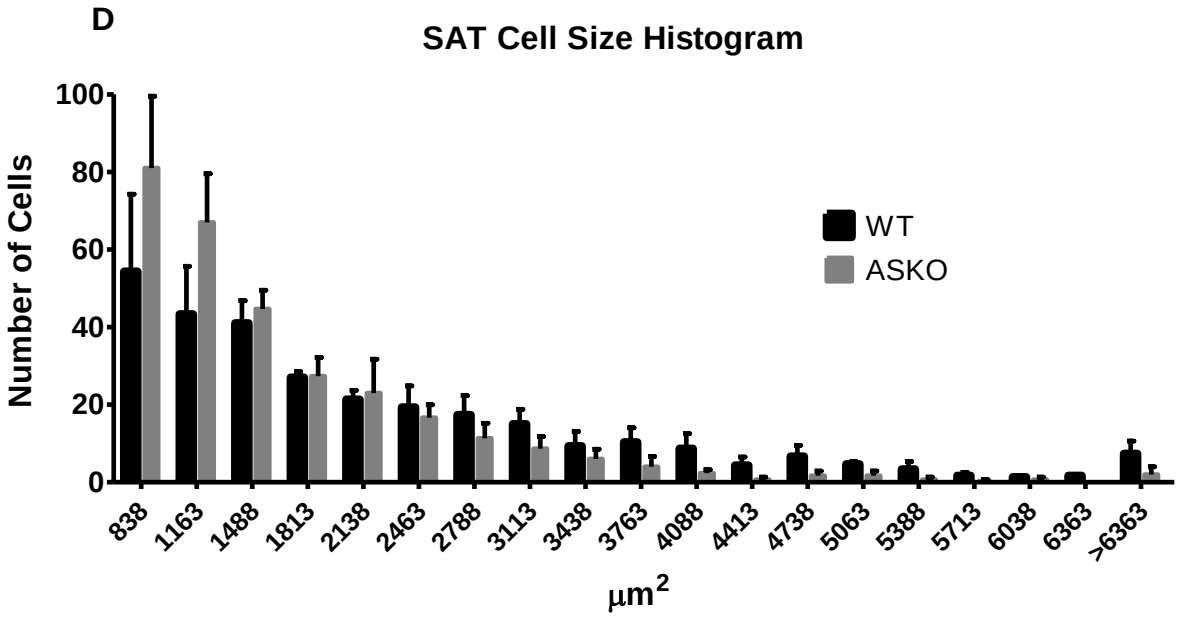
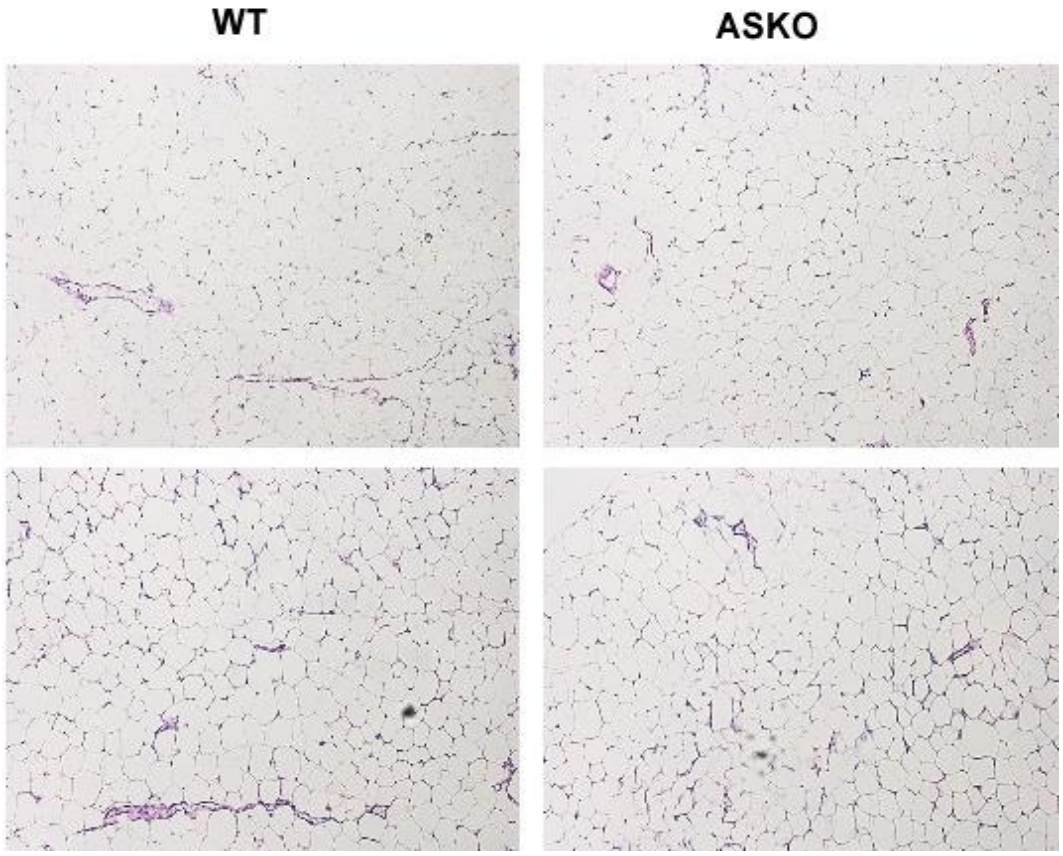

WT SAT
ASKO SAT

D


SAT Cell Size Histogram
Number of Cells
100
80
60
40
20
0
WT
ASKO
838
1163
1488
1813
2138
2463
2788
3113
3438
3763
4088
4413
4738
5063
5388
5713
6038
6363
>6363
μm2

E


WT
ASKO

F

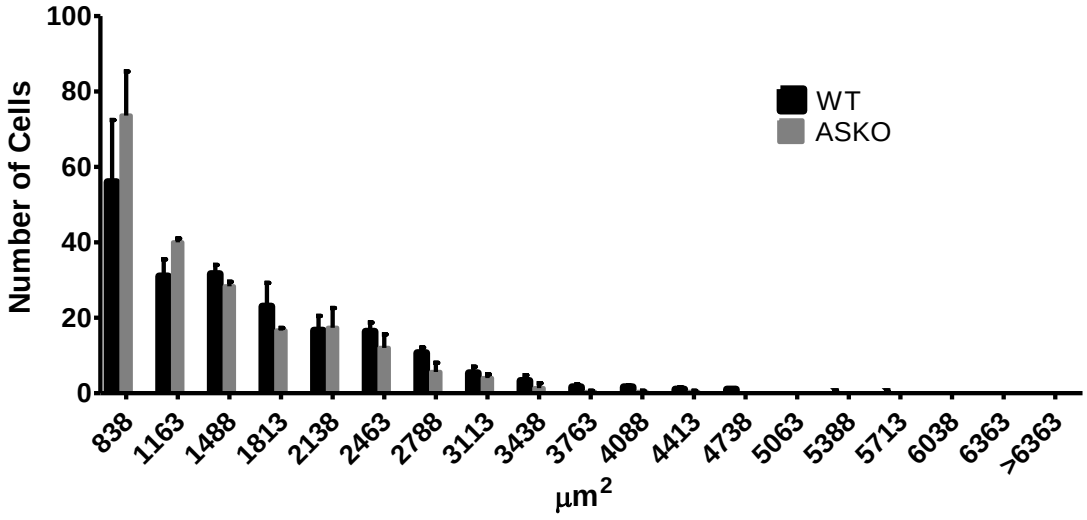
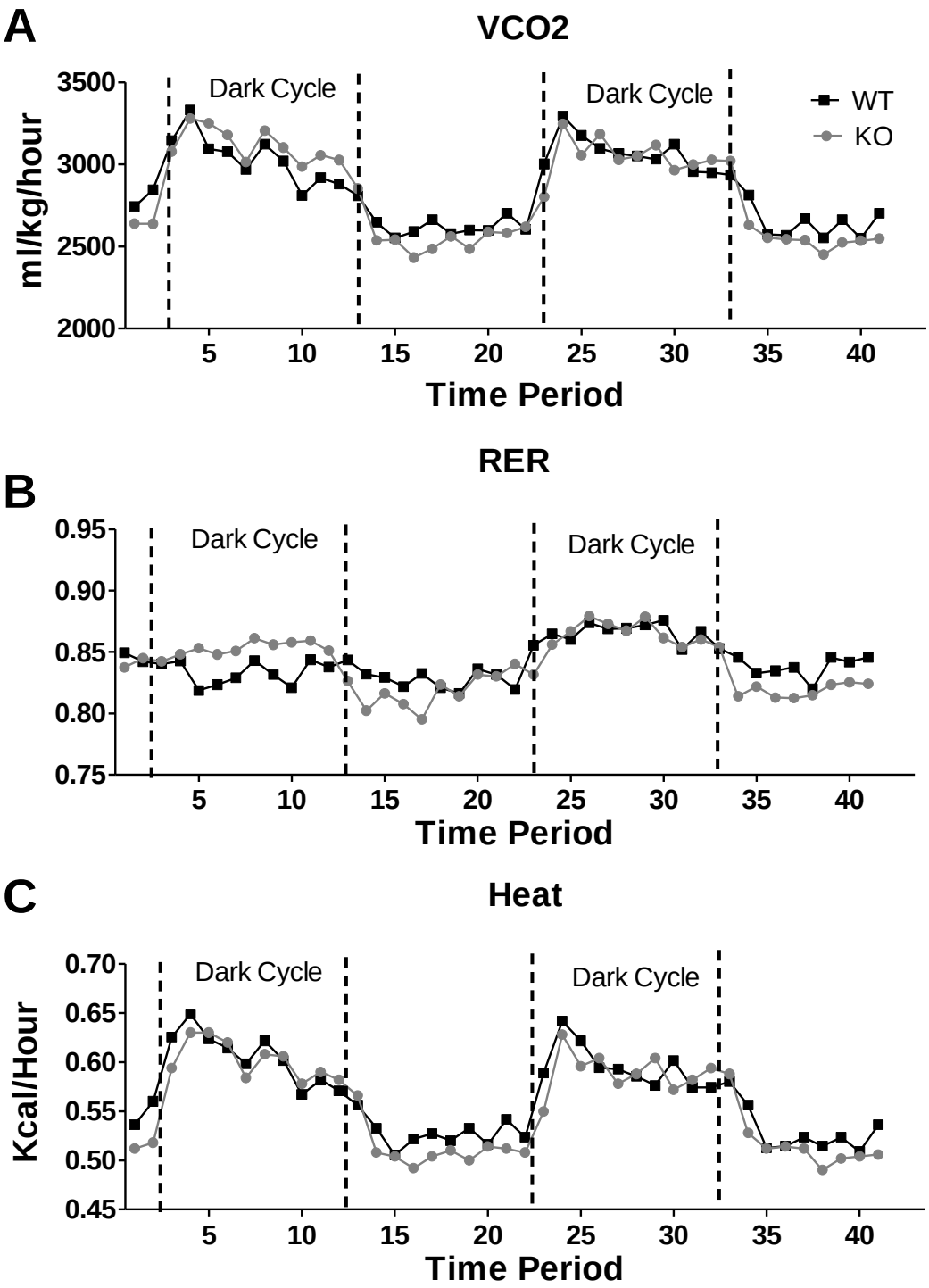
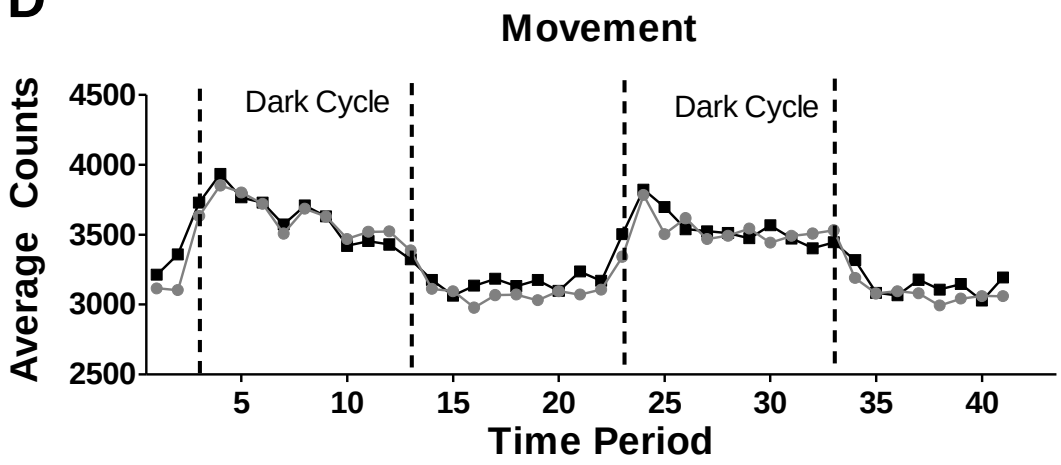

Chow EAT Cell Size Histogram
Number of Cells
100
80
60
40
20
0
WT
ASKO
838
1163
1488
1813
2138
2463
2788
3113
3438
3763
4088
4413
4738
5063
5388
5713
6038
6363
>6363
μm2

Figure 4: A) Representative photos of EAT from 16 week HFHC fed WT and ASKO mice, B) cell size histogram for EAT adipocytes, C) SAT photos from HFHC fed mice, D) SAT adipocyte size histogram, E) EAT photos from chow fed mice, F) Chow EAT adipocyte size histogram. For HFHC groups, n=5 mice per genotype, and 300 cell area measurements per mouse. For chow groups, n=3 mice per gentoype, and 300 cell area measurements per mouse.

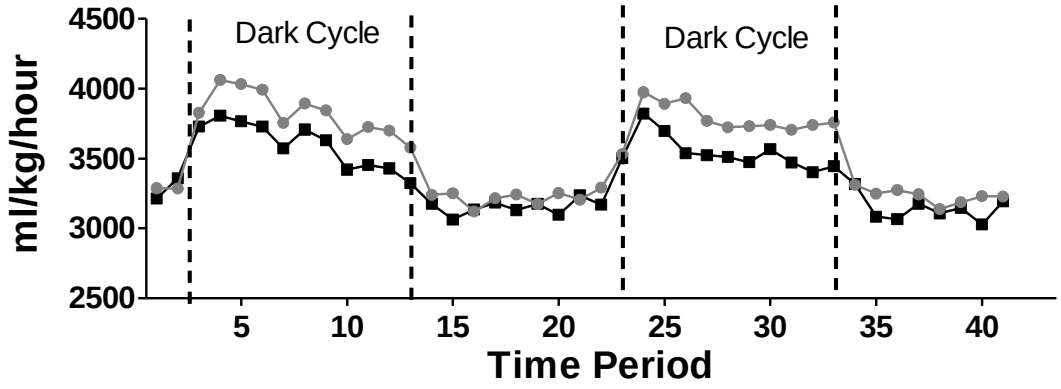
Figure 5: Metabolic phenotyping of ASKO mice



D


Movement
Average Counts
4500
4000
3500
3000
2500
Dark Cycle
Dark Cycle
5
10
15
20
25
30
35
40
Time Period

E


VO2
ml/kg/hour
4500
4000
3500
3000
2500
Dark Cycle
Dark Cycle
5
10
15
20
25
30
35
40
Time Period

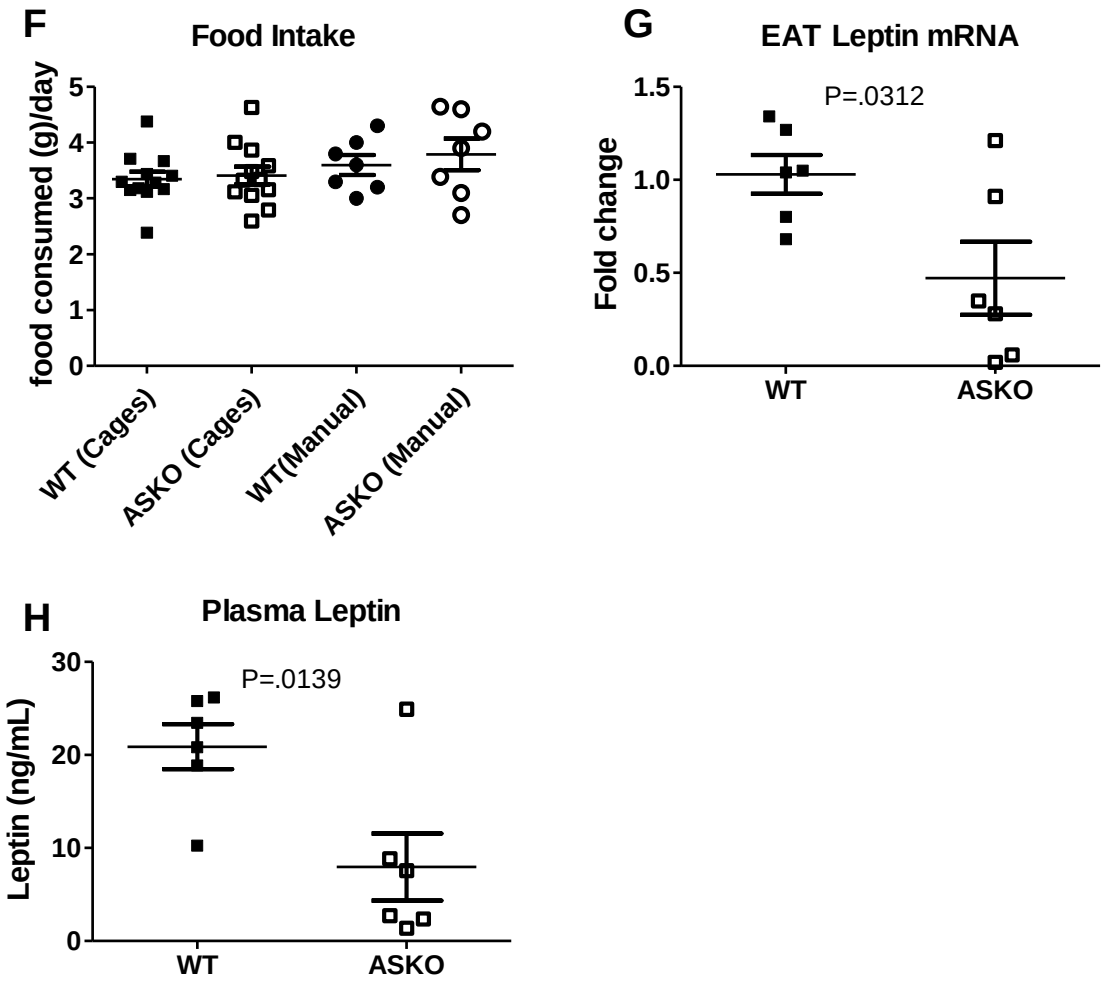
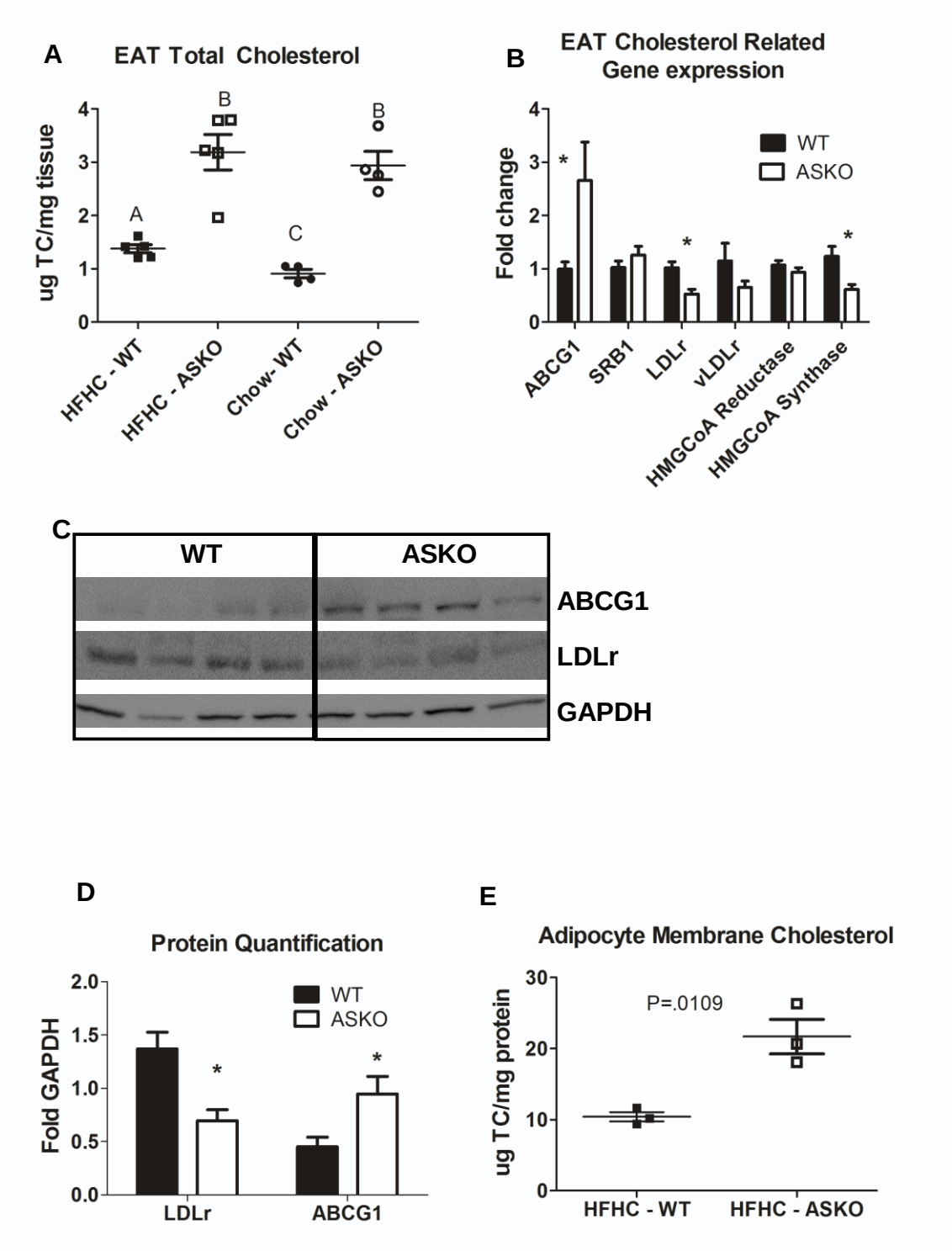

F
Food Intake
food consumed (g)/day
5
4
3
2
1
0
WT (Cages)
ASKO (Cages)
WT(Manual)
ASKO (Manual)
G
EAT Leptin mRNA
P=.0312
Fold change
1.5
1.0
0.5
0.0
WT
ASKO
H
Plasma Leptin
P=.0139
Leptin (ng/mL)
30
20
10
0
WT
ASKO

Figure 5: A-E) results from Clams metabolic Cages (n=11 per group). Values were averaged over 5 time points, and data is given for 48 hours from 3pm-3pm. Each time point roughly represents 1 hour. F) food intake measured by the CLAMS metabolic cages, or manually using wire bottomed cages. G) Gene expression of leptin in EAT, H) plasma leptin levels in male mice after 16 weeks HFHC diet.

Figure 6: ASKO mice have higher adipose tissue cholesterol content



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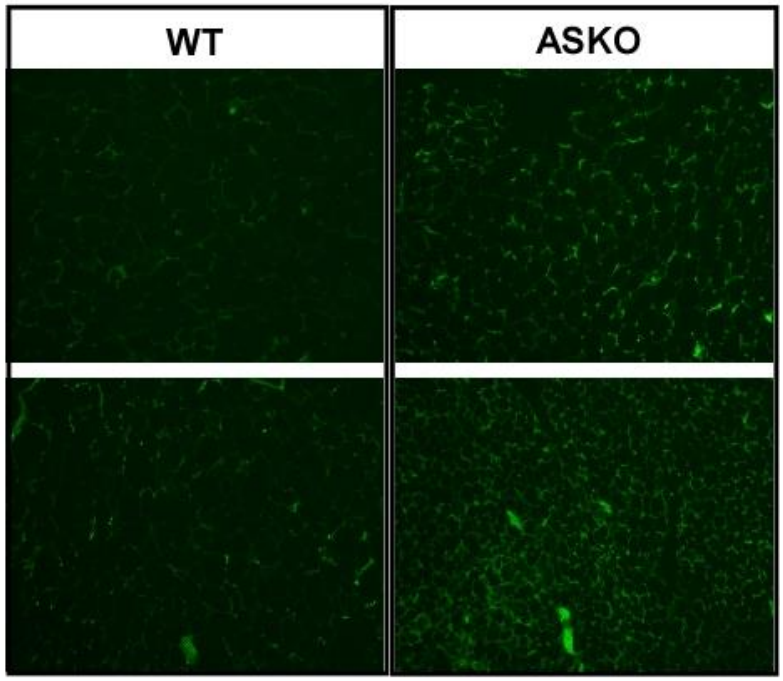
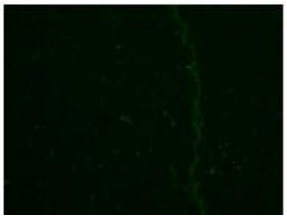




Negative Control

Figure 6: A) Total cholesterol content in EAT from male, 16 week HFHC or chow fed mice. B) Cholesterol related gene expression in EAT from HFHC fed mice (n= 6 per group), C) Western blot of ABCG1, LDLr and GAPDH from HFHC fed EAT D) western blot quantification, E) Membrane cholesterol content, F) Lipid raft staining

Figure 7: ASKO mice have reduced adipose tissue TG

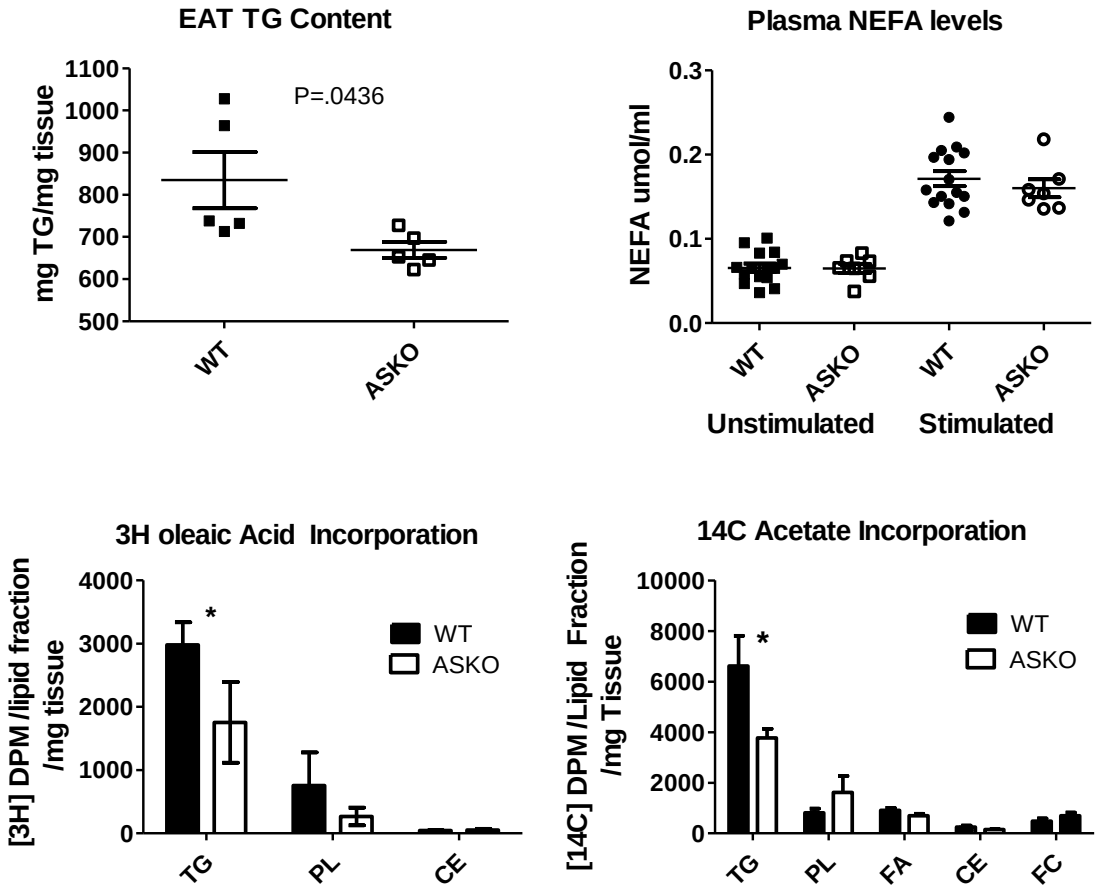
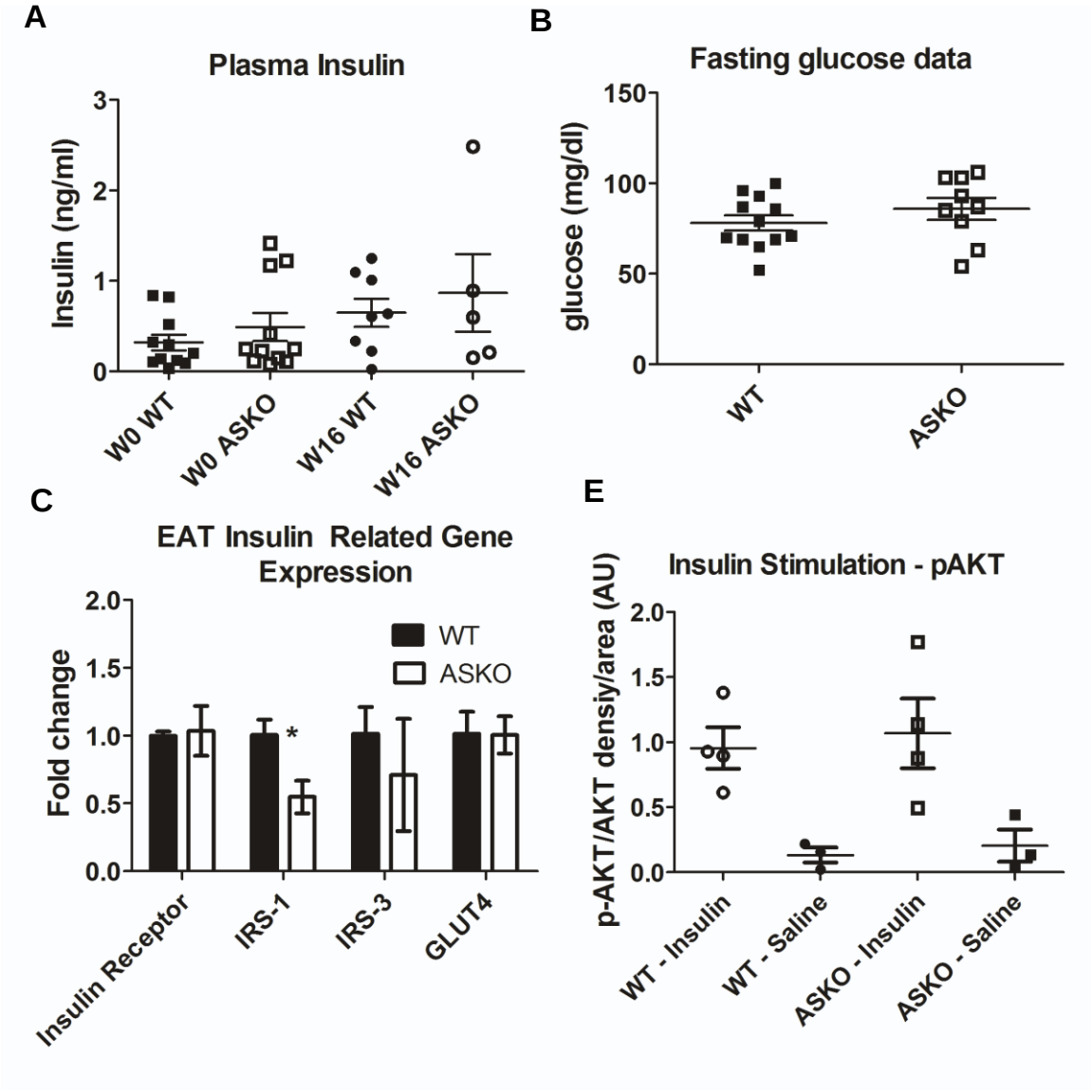


Figure 7: A) TG content of epididymal adipose tissue from male, HFHC (16 weeks) fed mice. B) Plasma NEFA levels before and after stimulation by a β-3 specific, adrenergic agonist in male mice after 8 weeks HFHC diet. C) Incorporation of [3H]-oleic acid into lipid fractions by adipose explants (n=6 per group). D) Incorporation of [14C]-acetate into lipid fractions by adipose explants (n=4 per group).

Figure 8: Adipocyte ABCA1 deletion does not affect insulin sensitivity



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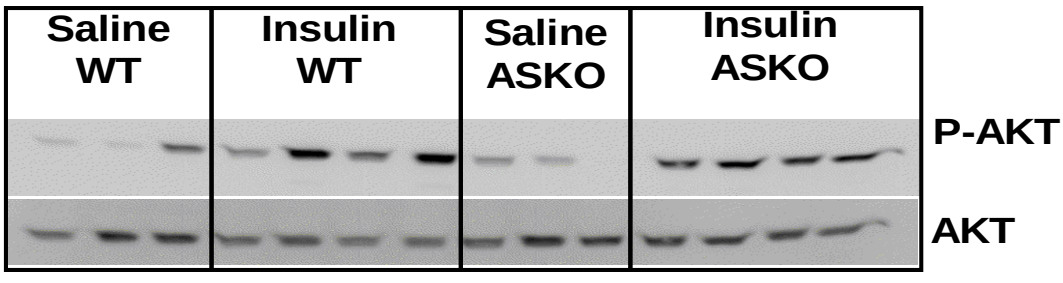


Figure 8: A) Fasting plasma insulin levels in male mice at 0 and 16 weeks of HFHC diet. B) Fasting glucose data in male mice after 8 weeks HFHC diet. C) Gene expression in epididymal adipose tissue of insulin related genes (n=6 per group). D) Western blot quantification of p-AKT/AKT from blots in F. * indicates significance by t-test, P<.05.

Figure 9: Lipid accretion pathways are down regulated in ASKO mice

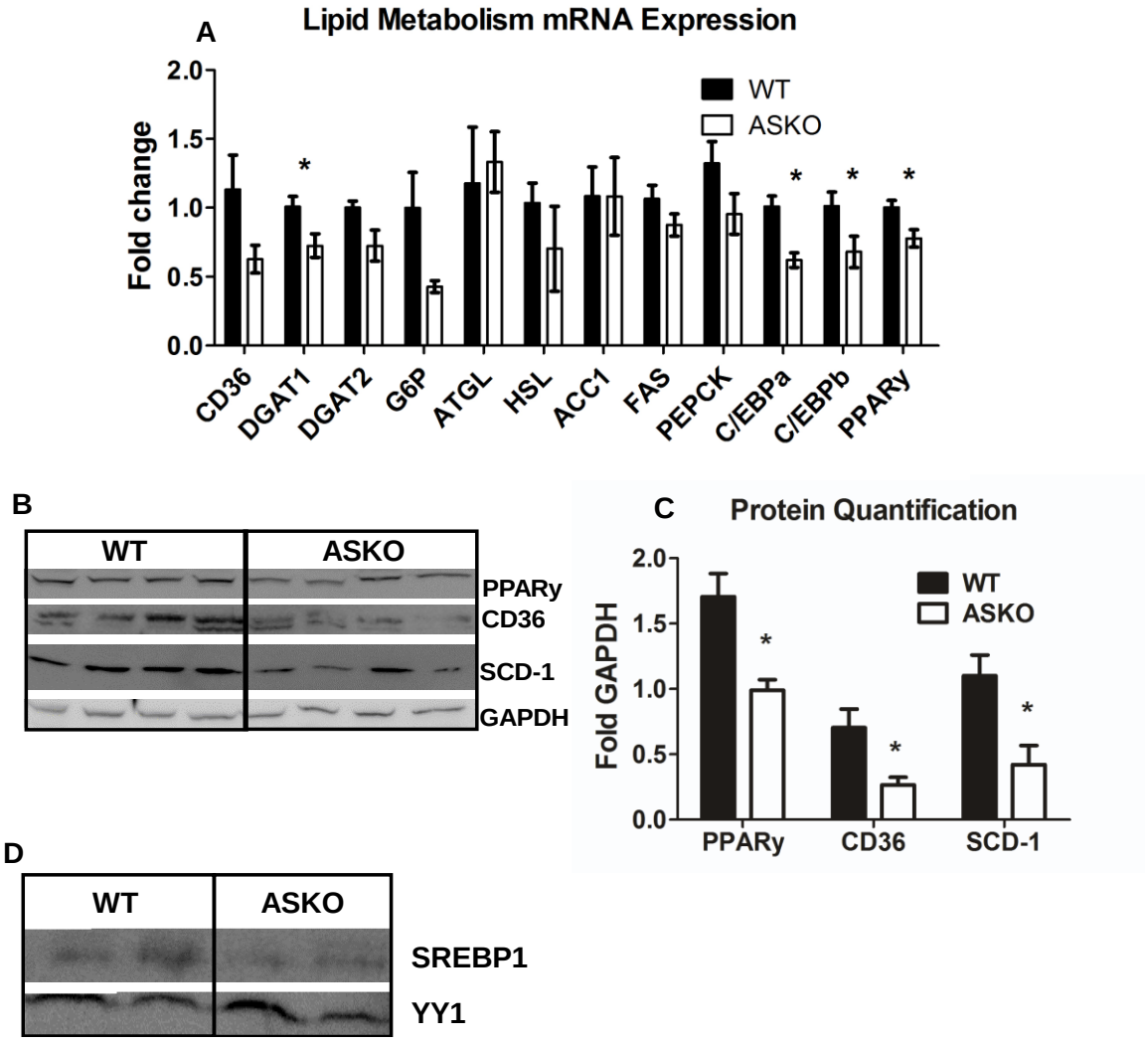


Figure 9: A) EAT gene expression of lipogenic genes and transcription factors (n=6 per group), B) Western blot of PPARγ, CD36, SCD-1 and GAPDH in EAT tissue, C) western blot quantification, D) western blot of SREBP1 in nuclear fractions from EAT.

Supplemental Table I: Primers used for rtpcr

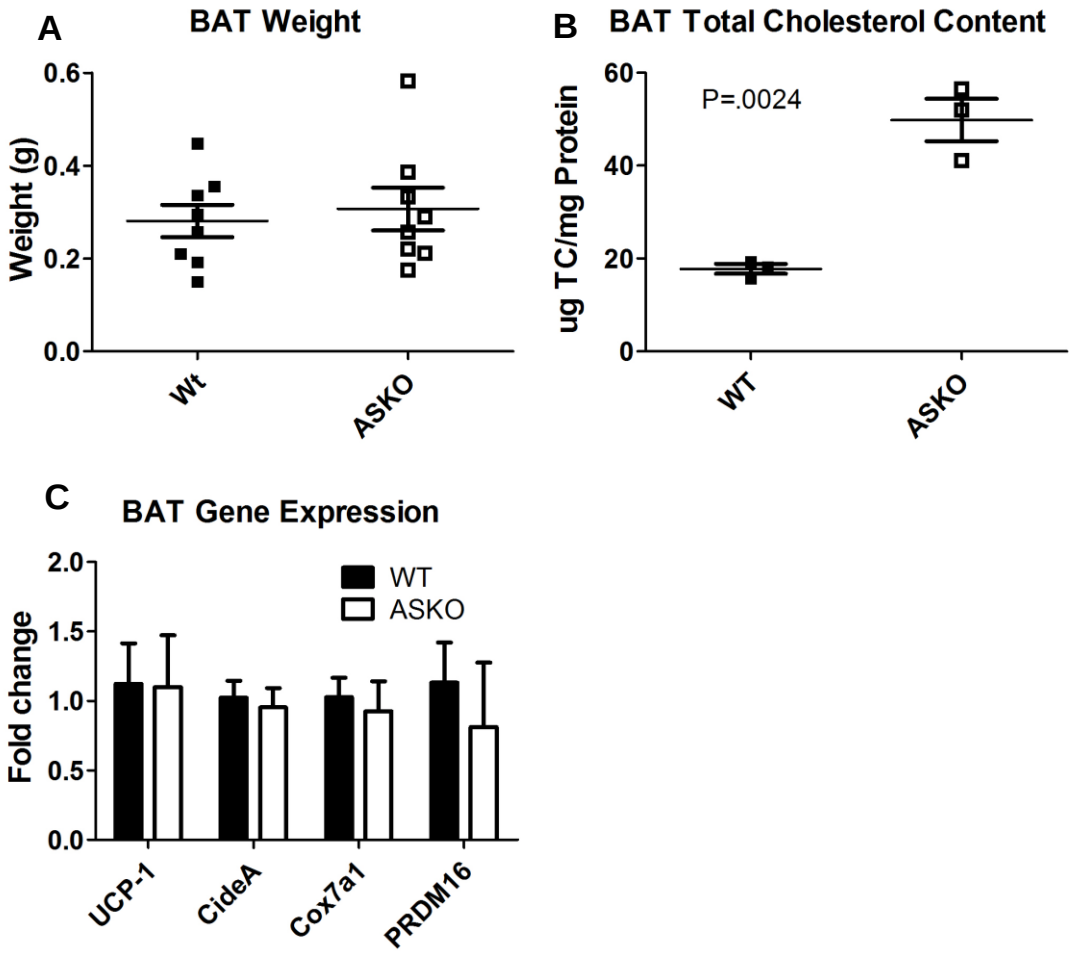
Gene		Primer Sequence
ABCA1	F	CGTTTCCGGGAAGTGTCCTA
	R	GCTAGAGATGACAAGGAGGATGGA
ABCG1	F	AGG TCT CAG CCT TCTAAA GTT CCT C
	R	TCT CTC GAA GTG AAT GAA ATT TAT CG
LDLr	F	AGGCTGTGGGCTCCATAGG
	R	TGCGGTCCAGGGTCATCT
Leptin	F	CAGCCTGCCTTCCCAAAAT
	R	AGATGGAGGAGGTCTCGGAGAT
SRB1	F	TCCCCATGAACTGTTCTGTGAA
	R	TGCCCGATGCCCTTGA
VLDLr	F	GCA GAT GAG TTC ACT GGC TCC
	R	AGG GCA GTT GAC CTC ATC GCT
HMGCoA Reductase	F	CGCTGGATAGCTGATCCTTCTC
	R	TTCGTCCAGACCCAAGGAAA
HMGCoA Synthase	F	GCCGTGAACTGGGTCGAA
	R	GCATATATAGCAATGTCTCCTGCAA
Insulin Receptor	F	CGAGTGCCCGTCTGGCTATA
	R	GGCAGGGTCCCAGACATG
IRS-1	F	CTATGCCAGCATCAGCTT
	R	TTGCTGAGGTCATTTAGGTCT
IRS-3	F	ACTAGATCTGGGCGGGAA
	R	CCCCATGTGGACATAGTCATT
GLUT4	F	ATGAGAAACGGAAGTTGGAGAGA
	R	GTGGGTGCGGCTGCC
CD36	F	GGAACTGTGGGCTCATTGC
	R	CATGAGAATGCCTCCAAACAC
DGAT 1	F	TTATCGTGGTATCCTGAATTG
	R	AAAAATAACCTTGCATTACTCAGG
DGAT2	F	CCGCAAAGGCTTTGTGAAG
	R	GGAATAAGTGGGAACCAGATCA
G6P	F	TGGGCAAAATGGCAAGGA
	R	TCTGCCCCAGGAATCAAAAAT
ATGL	F	GGACCCGGGAAGATTGGT
	R	AGCACAAAGTTCTGGGCAAGA

HSL	F	GGAGCACTACAAACGCAACGA
	R	TCGGCCACCGGTAAAGAG
ACC1	F	TGGACAGACTGATCGCAGAGAAAG
	R	TGGAGAGCCCCACACACA
FAS	F	GCTGCGGAAACTTCAGGAAAT
	R	AGAGACGTGTCACTCCTGGACTT
PEPCK	F	CCACAGCTGCTGCAGAACA
	R	GAAGGGTCGCATGGCAAA
C/EBPα	F	CTGGGGACCTAAACAGGAGC
	R	GAAGCCACCCTATAGCTCCC
C/EBPβ	F	GGCCTGGCTGGAACAGTA
	R	CGAAGGGAATTCAGGACAGT
PPARγ	F	CACAATGCCATCAGGTTTGG
	R	GCTGGTCGATATCACTGGAGATC
SREBP1c	F	GGAGCCATGGATTGCACATT
	R	GGCCCGGGAAGTCACTGT
GAPDH	F	TGTGTCCGTCGTGGATCTGA
	R	CCTGCTTCACCACCTTCTTGAT
arginase 1	F	GAATCTGCATGGGCAACC
	R	GAATCCTGGTACATCTGGGAAC
IL-10	F	CAGAGCCACATGCTCCTAGA
	R	TGTCCAGATGGTCCTTTGTT
TNFα	F	CTGTAGCCCACGTCGTAGC
	R	TTGAGATCCATGCCGTTG
MCP-1	F	GAGCCAGACGGGAGGAAG
	R	AGCTGGCTTCAGTGAGAGTTG
CD68	F	CCTCCACCCTCGCCTAGTC
	R	TTGGGTATAGGATTCGGATTTGA
IL-18	F	GACTCTTGCGTCAACTTCAAGG
	R	CAGGCTGTCTTTTGTCAACGA
UCP-1	F	GAGGTGTGGCAGTGTTCATTG
	R	GGCTTGCATTCTGACCTTCA

Supplemental Table II: Antibodies used for Western Blots and Immunostaining

Antibody	Dilution	Product info
ABCA1	1:1000	Home made
ABCG1	1:1000	Novus Biologicals, NB 400-132
PPARϒ	1:500	Santa Cruz, SC 7196
SCD-1	1:1000	Santa Cruz, SC 14719
CD36	1:500	Santa Cruz, SC 9154
GAPDH	1:2000	Santa Cruz, SC 32233
p-AKT	1:1000	Cell Signalling, 9271
AKT	1:1000	Cell Signalling, 9272
LDLr	1:500	Novus Biologicals, NB110-57162
CD68	1:200	AbDSerotec MCA1957
UCP-1	1:200	AbCam 10983

Supplemental Figure I: Brown adipose morphology is altered in ASKO mice



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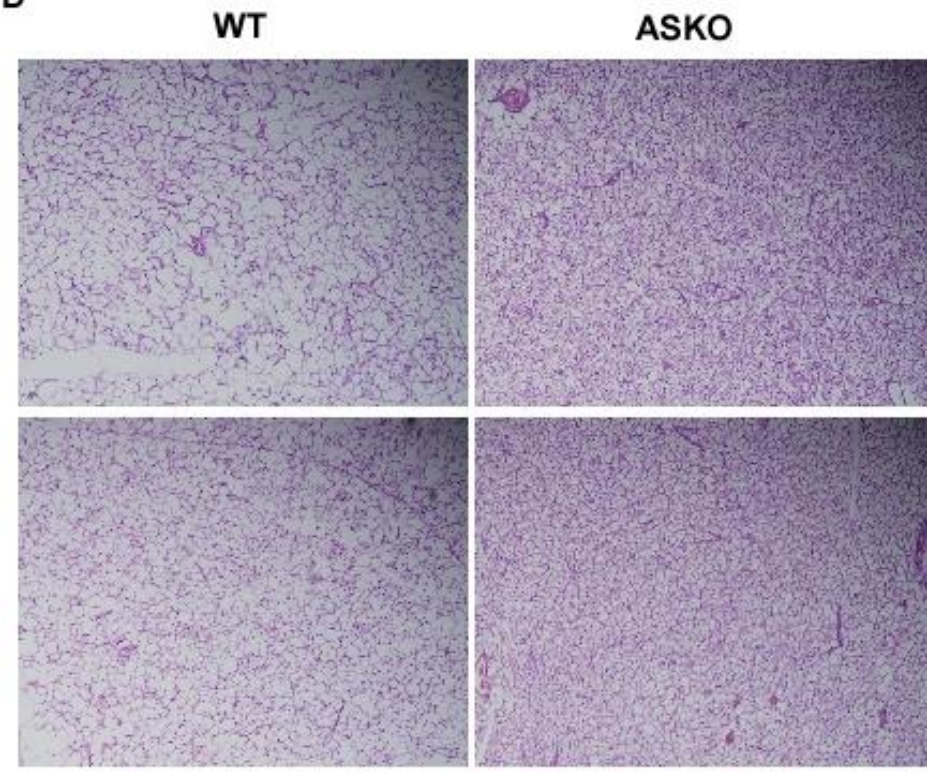

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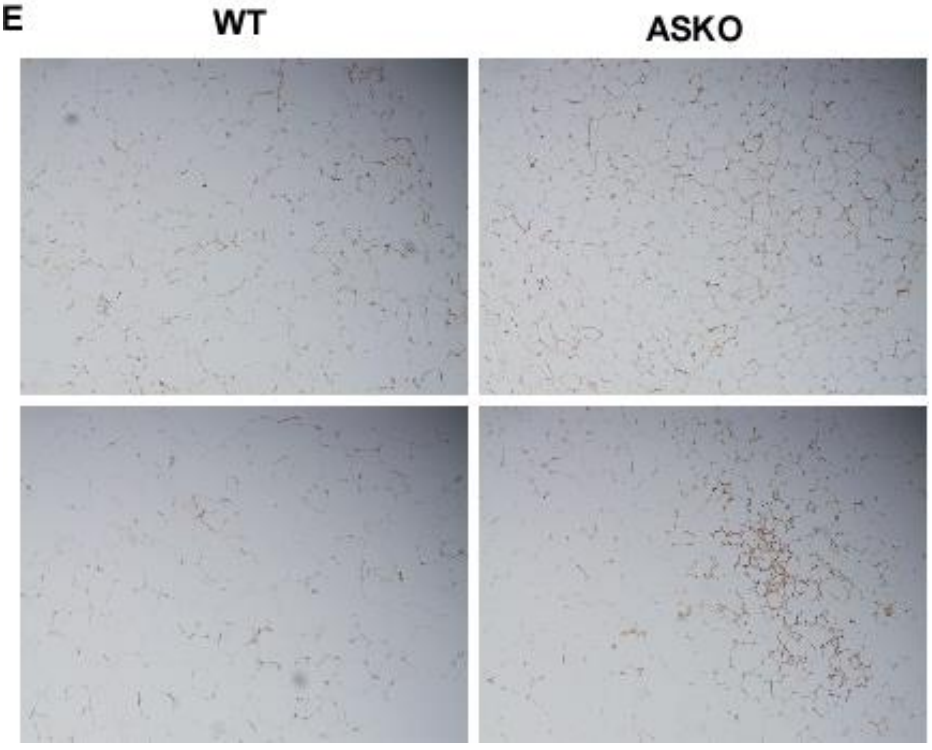
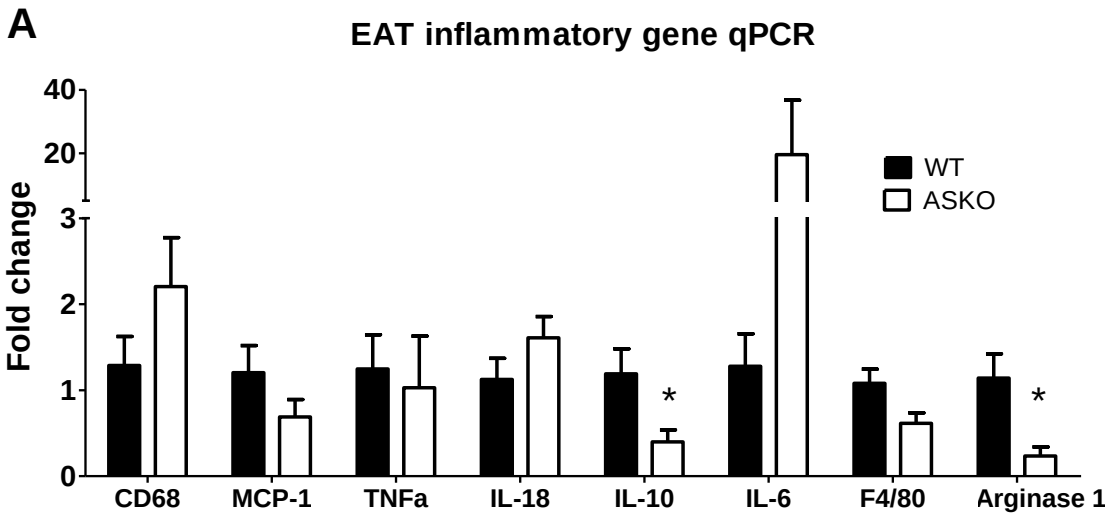
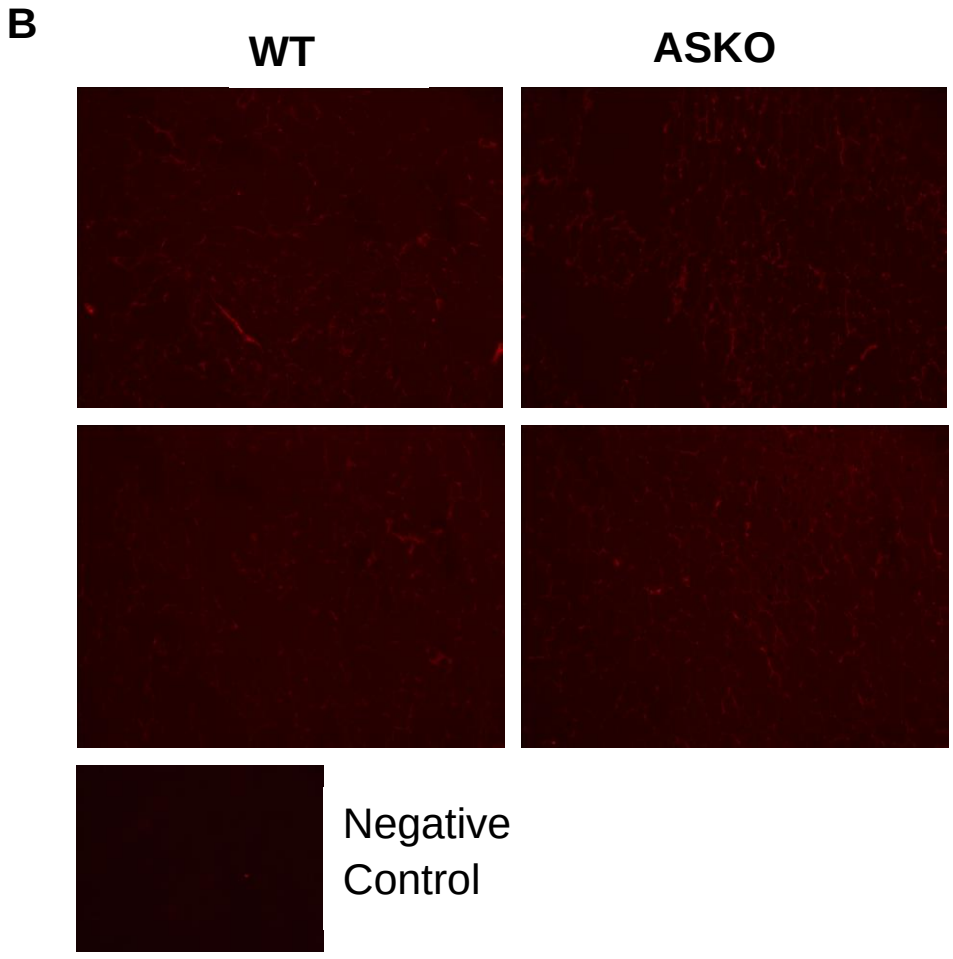


Supplementary Figure I: A) BAT weight, B) BAT cholesterol content, C) BAT gene expression, D) H and E stained BAT representative photos, E) UCP-1 staining in HFHC fed EAT.

Supplemental Figure II: Deletion of ABCA1 in Adipose Tissue Does Not Increase Adipose Tissue Inflammation





Supplementary Figure 2: A) inflammatory gene expression in EAT from HFHC fed mice, B) representative CD68 IF staining from EAT of HFHC fed mice.

Chapter IV

Discussion

Helen Cuffe prepared this chapter with John Parks acting in an advisory and editorial capacity

Adipose tissue is a key component of the obese state and its dysfunction during obesity plays a significant role in the development of comorbidities, such as diabetes, dyslipidemia, and the metabolic syndrome [1]. As the obesity epidemic increases, it is important to understand the role of specific dietary constituents in the development of obesity and associated comorbidities. While much research has focused on effects of adipose tissue TG accumulation, little is known about how cholesterol accumulation affects adipocyte function. In this thesis, I have presented data from two models, both of which address and examine the role of cholesterol in the development of adipocyte dysfunction. Cholesterol accumulates with TG in adipocytes, and regardless the extent of adipocyte hypertrophy, the TG to cholesterol ratio generally remains constant [2]. It was previously thought that TG, which is contained within the lipid droplet and makes up to 90% of adipocyte size, was primarily responsible for dictating both cholesterol content and adipocyte hypertrophy. The evidence in this thesis, along with pre-existing literature, paints a different picture, however, and suggests strongly that cholesterol has a significant effect on adipocyte hypertrophy, function, and inflammation, and may act as a signal in adipocytes to determine TG content.

This thesis presents two different models of adipose cholesterol accumulation. In the first, dietary cholesterol added to a HF diet resulted in roughly 1.5 fold increase in cholesterol in the visceral adipose tissue of African Green Monkeys. In the second, genetic deletion the ABCA1 cholesterol transporter specifically in adipocytes resulted in a ~ 3-fold increase in cholesterol in all examined depots.

Although both studies are models of cholesterol accumulation in adipose tissue, very different results were observed. In the first study (Chapter II), dietary cholesterol promoted adipocyte hypertrophy, dysfunction, and inflammation. In the second (Chapter III), increased adipose cholesterol actually protected mice from diet-induced obesity, adipocyte hypertrophy, and TG accumulation, did not alter insulin sensitivity in adipose tissue, and did not result in increased inflammation. This discussion attempts to explain the differences observed between the two models, give implications about adipocyte cholesterol in obesity, and suggest future directions for these studies.

The most striking difference between the two models is clearly the difference in adipocyte size. In our dietary cholesterol model, visceral adipocytes were significantly larger (1.5 fold), and contained more cholesterol (1.5 fold), in animals fed a high vs. low cholesterol diet (Chapter II). In this case, cholesterol content and adipocyte size were positively correlated, which is consistent with previous studies [2-4] that have shown larger adipocytes tend to contain more cholesterol. In stark contrast, compared to WT, adipocytes from HFHC fed ASKO mice had smaller adipocytes, despite having 3-fold more cholesterol content.

In ASKO mice, we show that the reason for smaller adipocytes is due to decreased activation (nuclear translocation) of SREBP1. SREBP1 is a master regulator of TG metabolism in adipocytes [5]. The reduction in SREBP1 activation is presumably responsible for the reduced protein and gene expression of PPARγ, CD36 and SCD-1 (along with additional genes) in ASKO adipose

tissue, the reduction in TG synthesis (both through FA re-esterification and *de novo* lipogenesis) and content, and ultimately, the reduction in adipocyte size and fat pad weight in ASKO animals (Chapter III). We hypothesize that the high levels of membrane cholesterol (including the ER) in ASKO adipocytes result in the retention of SREBP1 in the ER, and prevents TG accumulation. This hypothesis is consistent with previous studies in adipocytes that show membrane cholesterol overload reduces [6], and depletion increases [7], nuclear SREBP1, and that SREBP1 translocation can be modulated by ER cholesterol content [8, 9]. While we were not able to detect total cell SREBP1, SREBP1c mRNA expression was not decreased in ASKO adipose tissue, suggesting that the reduced nuclear SREBP1 in ASKO adipocytes is due to post-transcriptional regulation occurring in the ER.

In the case of our ASKO model, there is a clear mechanistic relationship between adipocyte cholesterol content and TG accumulation. This mechanism suggests that high levels of membrane (ER) cholesterol in adipocytes results in reduced nuclear SREBP1, reduced TG accumulation, and ultimately smaller adipocytes. This seems counterintuitive given that larger adipocytes contain more cholesterol [2-4]; however, several lines of evidence have shown that in adipocytes, TG accumulation is accompanied by a dilution in membrane cholesterol [10, 11]. It has also been reported that SREBP2 is paradoxically activated in larger adipocytes [12], again suggesting that adipocyte ER becomes depleted of cholesterol as adipocytes hypertrophy.

Given these results, we might predict that SREBP1 activation would be altered in our dietary cholesterol model (Chapter II); however this does not seem to be the case. Adipose from high cholesterol-fed monkeys did not exhibit any changes in lipogenic-related gene expression, including LPL, FAS, ACC1 or SREBP1c. In addition, we observed a decrease in adipose HMGCoA Reductase gene expression in response to the high cholesterol diet suggesting that SREBP2 is not activated in this tissue, and that ER cholesterol levels are high. These data does not necessarily contradict our ASKO model however. We did not observe any differences in adipocyte size in chow-fed ASKO mice, even though the tissue still had 3-fold the cholesterol content. These results suggest that a prolonged high fat diet is required to deplete cholesterol from the membrane, and that under these conditions, ER cholesterol content may regulate the activation of SREBP1. Taking this theory into the context of obesity, adipocytes accumulate both cholesterol and TG when a "western" diet is consumed by humans [2]. Also, as adipocytes hypertrophy, their membranes (likely including the ER) become relatively depleted of cholesterol [11, 13]. We predict, based on our ASKO study, that this results in an increase in nuclear SREBP1 and a subsequent increase in lipogenic gene expression, FA uptake and esterification, *de novo lipogenesis*, and overall TG storage.

Previous results regarding the regulation of adipocyte SREBP1 *in vivo* by cholesterol are mixed. Given our results, we would predict that a prolonged HFHC diet would result in increased adipocyte nuclear SREBP1; however, appropriate studies to confrim this seem to be lacking. Studies have shown that

high carbohydrate and high fat diets activate *de novo* lipogenesis (through SREBP1) in adipose and liver [14]; however, the contribution of added dietary cholesterol to this regulation is not well understood for adipocytes. Cholesterol (or oxysterols) may also be able to induce SREBP1 expression though activation of the LXR pathway. LXR agonists are well-characterized inducers of hepatic lipogenesis where both endogenous and synthetic LXR agonists induce lipid accumulation via stimulation of SREBP-1c and its response genes, including FAS and SCD1 [15, 16]. However, recent evidence suggests that this may not be the case for adipocytes [17]. Results comparing the effect of LXR KO on the liver and adipose show that, paradoxically, deleting LXR in adipose tissue results in increased SREBP1c expression and increased fat pad mass [17]. In our monkey study, a high cholesterol diet did not result in increased gene expression of LXR responsive genes (ABCA1, ABCG1 and ApoE), suggesting that the high cholesterol diet did not necessarily lead to increased oxysterol content in adipose tissue. This is consistent with previous studies showing that oxysterol accumulation in adipose tissue in response to a HFHC diet is relatively minor [18]. This throws into question the relevance of the LXR pathway in regulating adipose SREBP1 *in vivo*. In all, the regulation of SREBP1 in adipocytes is poorly understood, and comprehensive studies examining the consequences of dietary cholesterol and fat are needed.

Although we have shown that cholesterol can seemingly regulate SREBP1 activation *in vivo* in our ASKO mice, there are several limitations to these studies. First, we did not directly show that ER cholesterol was increased in ASKO

adipocytes. However, previous studies have shown that increases in total cell cholesterol rapidly result in increased ER cholesterol, that the magnitude of ER cholesterol increase is higher than the increase in the total cell [19], and that small changes in ER cholesterol content can modulate the SCAP-SREBP signaling complex [20]. In all, while ER cholesterol content was not directly measured, total membrane cholesterol is likely a reasonable indicator of ER cholesterol levels. Second, the regulation of SREBP1 by insulin signaling was not studied in these experiments. It is perhaps counter-intuitive that WT and ASKO adipocytes, with similar states of insulin sensitivity, have such differential regulation of SREBP1 (Chapter III); however, studies in adipocytes suggest that insulin signaling has dramatically different effects on INSIG and SREBP1 compared with the liver [17] and, that in adipose tissue, insulin may counterintuitively lead to less nuclear SREBP1. Due to the reported difficulties of studying these pathways in mouse tissue, additional models, perhaps using human or rat adipose tissue, may need to be employed to determine the relative contribution of ER cholesterol content and insulin signalling in the regulation of SREBP1 activation. Lastly, cholesterol modulation of SREBP1 was observed in a genetic model. ASKO mice had ~3-fold increases in adipose cholesterol regardless of diet. In contrast, WT mice fed a HFHC diet for 16 weeks only had around a 50% increase in adipose cholesterol (Chapter III), which is consistent with previous dietary cholesterol studies [21, 22] and our results in Chapter II. These data suggest that the increase in cholesterol observed in the ASKO mice is not physiological, and would not occur under normal, high cholesterol feeding

conditions. This highlights the need for additional studies of adipocyte cholesterol in WT cells/animals.

Since cholesterol can clearly regulate adipocyte hypertrophy, as demonstrated by both models in this thesis, close attention should be paid to factors that influence and control adipocyte cholesterol content and distribution. ABCA1, along with ABCG1 and SRB1, are major cholesterol transporters in adipocytes and other tissues and cell types. Using ASKO mice, we have shown that ABCA1 is the major efflux mechanism for adipocyte cholesterol, and that no other mechanism can compensate for its loss. We were also able to determine that in addition to total cholesterol levels, ABCA1 expression had significant effects on membrane cholesterol content (Chapter III). Interestingly, ABCA1 protein levels are highly regulated by diet in adipose tissue. High dietary cholesterol levels lead to increased adipose ABCA1 protein (Chapter II) and caloric restriction in db/db mice dramatically reduces ABCA1 protein levels [23]. Our own data (Discussion figure 1A) indicates that a HFHC diet dramatically upregulates adipose ABCA1 expression compared to a chow diet in WT mice, but that this is in stark contrast to what occurs in the livers from the same mice (Discussion Figure 1B). Increased dietary cholesterol in nonhuman primates (Chapter II) also resulted in increased ABCA1 protein levels in adipose tissue, but interestingly, did not alter hepatic ABCA1 gene expression, suggesting that adipose tissue is more sensitive to dietary cholesterol with regards to ABCA1 expression. These data suggest that ABCA1 is highly sensitive to dietary conditions in adipose tissue, but that factors regulating ABCA1 expression during weight gain or loss may be

different from the liver. Given the decrease of membrane cholesterol in hypertrophied adipocytes, the known contribution of ABCA1 to membrane cholesterol content (Chapter III), and the fact that ABCA1 is upregulated during weight gain, ABCA1 is likely responsible for the depletion of adipocyte membrane cholesterol that occurs during obesity [12, 13, 24, 25]. Given our data regarding the downregulation of SREBP1 by adipocyte cholesterol accumulation (Chapter III), the upregulation of ABCA1 during adipocyte hypertrophy may exacerbate and increase TG accumulation by decreasing adipocyte membrane cholesterol, with subsequent activation of SREBP1.

Interestingly, similar results to ours (chapter III) have been seen when studying adipocyte ABCG1 [26], suggesting that ABCA1 and ABCG1 act together to modulate cholesterol (specifically membrane cholesterol) in adipocytes. Silencing ABCG1 expression in adipocytes resulted in weight loss in mice, and reduced TG accumulation in 3T3L1 cells, but oddly resulted in lower levels of free cholesterol and increased expression of cholesterol synthesis genes. Silencing of ABCG1 also resulted in a decreased expression of PPARγ, CD36, and C/EBPα as observed in our ASKO model. Moreover, human adipose tissue ABCG1 expression is directly corollated with fat mass [26]. We predict that high levels of adipose ABCA1 protein in humans would correlate with increased adiposity and insulin resistance, and this research highlights the need for studies of human ABCA1 expression in the context of obesity to be performed.

Given the likelihood that ABCA1 upregulation in adipocytes will lead to adipocyte dysfunction, it is necessary to examine the factors that determine ABCA1 protein

expression and regulate cholesterol metabolism in adipocytes. Few studies have addressed this, and the literature that does exist is relatively confusing. For instance, the exact mechanisms by which cholesterol content is regulated in adipocytes are still unknown. In addition, it is not known how diet, specifically dietary cholesterol, regulates adipose cholesterol metabolism. In most cells, cholesterol content is modulated by a balance of uptake and synthesis through pathways regulated by two transcriptional factors (LXR and SREBP2) [5, 27]. SREBP2 and HMGCoA reductase levels are increased in hypertrophied adipocytes compared with lean adipocytes despite higher cholesterol content [12, 28]. These studies attributed this paradox to cholesterol location within the adipocyte, specifically hypothesizing that ER cholesterol is depleted in hypertrophied adipocytes. In our own studies, however, higher levels of adipose cholesterol resulted in decreased LDLr and HMGCoA reductase expression suggesting an activation of the SREBP2 pathway. Our studies seem to contradict the literature, however, it is important to remember that previous studies have been performed in lean vs. obese adipocytes [11, 12], whereas our study (chapter II) only compared different levels of dietary cholesterol and differences in adipocyte size were relatively small. Based on our studies, we predict that adipocyte hypertrophy does increase SREBP2 levels (as reported in the literature), but only under certain dietary conditions, namely, a prolonged HFHC diet. Overall, however, we still believe that a HFHC diet would result in decreased membrane and ER cholesterol, and an increase in SREBP2, compared with a chow diet, and future studies should examine this hypothesis.

Another key pathway in the regulation of ABCA1 levels is the LXR pathway. In conditions of high cholesterol (oxysterols), LXR activates the transcription of genes involved in cholesterol efflux, including ABCA1 and ABCG1 [27]. The role of LXR in adipocytes is not well understood. Studies have suggested that adipose LXR mRNA is increased in obese vs. lean adults [29, 30], but these data have yet to be replicated *in vitro* [29] and LXR co-regulators RIP-140 and CideA are known to be downregulated in obesity [31, 32]. LXRα/β knock out mice have increased fat pad size compared to WT mice [17], but other LXR-/- models show that these mice are resistant to diet-induced obesity [33], suggesting LXR modulates adiposity *in vivo*, but the exact mechanism is unknown. Our data (Chapter III) shows that cholesterol-enriched adipose tissue leads to activated LXR response genes (ABCA1, ABCG1, and APOE), suggesting that adipocyte total cholesterol content likely correlates with oxysterol content, and therefore LXR activation. However, we did not directly measure oxysterol content in this tissue, and previous studies have indicated relatively low levels of oxysterol accumulation in adipose in response to a HFHC diet [18]. In addition, ABCA1 expression is regulated by several post-transcriptional mechanisms that involve FAs [34, 35] and microRNAs (reviewed in [36]), but how these mechanisms affect adipocyte ABCA1 expression is unknown. More studies are needed to elucidate the pathways that regulate adipocyte ABCA1 expression and how obesity affects these pathways.

In addition to effects on TG accumulation, adipocyte cholesterol also has been shown to have significant effects on inflammation [22, 37]. Our dietary cholesterol

accumulation model (nonhuman primates, Chapter II) resulted in increased inflammatory gene expression, specifically IL-6, and IL-8, and increased inflammatory staining (CD68 and TNF-a) in high cholesterol vs. low cholesterol fed monkeys. This occurred with only a ~ 50% increase in adipose cholesterol. However, through co-localization of CD68 and TNF-α staining, we were able to determine that the majority of inflammation (Cytokine expression) came from tissue macrophages, not adipocytes. Our ASKO model showed very different results. Despite a 3 fold increase in ASKO adipose cholesterol compared to WT, no significant increase in inflammation was detected, either through macrophage staining or gene expression. This contradicts previous evidence showing that increased adipose tissue cholesterol leads to increased inflammation [22, 38]. The lack of inflammation in ASKO adipose tissue suggests that macrophages are primarily responsible for the dietary cholesterol induced inflammation seen in previous studies [22, 37]. Despite increased adipocyte cholesterol, the dietary cholesterol in our HFHC mouse study presumably had the same effect on macrophages in WT and ASKO adipose tissue, which is why no difference in inflammation was observed. In addition, we predict that added dietary cholesterol also results in increased macrophage cholesterol content in adipose tissue which has been associated with increased inflammatory response [39]. In either case, it is important to note that no matter the model, animals fed high levels of dietary cholesterol do have more inflamed adipose tissue when compared to lower levels of dietary cholesterol, confirming the idea that dietary cholesterol is detrimental to adipose tissue [40-44].

Another important concept for adipose tissue in obesity is insulin resistance. Adipose insulin resistance is associated with adipocyte hypertrophy and TG content, and more recently cholesterol content. In the study by Li et. al. [6], larger adipocytes had lower levels of membrane cholesterol and were more resistant to insulin. Depleting adipocyte cholesterol acutely dramatically reduced responsiveness to insulin [13, 45]; however, acute addition of cholesterol to the membranes did not affect insulin responsiveness [13]. We did not observe any difference in insulin resistance in our dietary cholesterol (monkey) model, but we did not examine this specifically in adipose tissue. We predict that adipose tissue from high cholesterol fed monkeys will be more insulin resistant compared to low cholesterol adipose. Our ASKO model showed a slight increase in response to insulin in the adipose tissue, but not in the liver or muscle, and did not show any signs of systemic insulin resistance (i.e., elevations in plasma glucose or insulin). These results suggest that increasing or maintaining cholesterol content in adipocyte membranes retains insulin sensitivity. Conversely, it also suggests membrane cholesterol depletion that occurs during adipocyte hypertrophy may be responsible for insulin resistance. ABCA1 may to be primarily responsible for the regulation of cholesterol content in adipocyte membranes (Chapter III); therefore, by upregulating ABCA1 during hypertrophy, adipocytes deplete their membranes of cholesterol, and in theory, become more insulin resistant. This theory should be tested in human samples by assessing the correlation of ABCA1 expression with states of insulin resistance, and we would predict that higher levels of ABCA1 would correlate with insulin resistance. It would also be

interesting to examine the results of adipose tissue overexpression of ABCA1. From our results, we would predict that adipocyte ABCA1 overexpression would result in adipocyte insulin resistance and increased diet-induced weight gain.

In general, results from this thesis have signficant implications for obesity and adipose function. Clearly, results from our ASKO model show that retaining membrane cholesterol has mostly beneficial effects on adipose tissue. Namely, a loss of ABCA1 results in decreased adipocyte size, a trend towards increased insulin sensitivity, no changes in inflamation, and reduced TG accretion. We attribute the majority of these observations to an increase in membrane cholesterol, rather than to the loss of ABCA1 itself. This is similar to many other tissue specific knockout models of ABCA1, including the pro-inflammatory effects observed in macrophages [39, 46], the defective insulin release from β-cells [47], and learning deficiencies in the absence of brain ABCA1 expression [48]. In essence, preventing the loss of membrane cholesterol by deleting ABCA1 in adipocytes prevents diet-induced weight gain and, seemingly, ameliorates many of the comorbidities found in adipose that are associated with a HFHC diet. In contrast to our ASKO model, feeding monkeys high levels of cholesterol had negative effects on adipose tissue including increased hypertrophy and inflammation. Together these results suggest that: 1) dietary cholesterol under normal (non-genetically altered) conditions is detrimental to adipose tissue and 2) paradoxically, adipocyte membrane cholesterol is beneficial. Overall, this suggests that preventing the depletion of membrane cholesterol during adipocyte

hypertrophy can ameliorate the negative effects of dietary cholesterol on adipose and prevent diet-induced obesity.

Future directions

It is clear from this work that ABCA1 function is of signficant importance in adipocytes. We have shown that deleting adipose ABCA1 protects mice from diet induced obesity, presumably due to high levels of membrane (ER) cholesterol resulting a the reduction of SREBP1 nuclear translocation. We have also shown that ABCA1 is highly regulated in adipocytes; specifically, that a high fat or high cholesterol diet upregulates protein expression in adipose tissue. Multiple future opportunities exist for this project regarding the study adipocyte cholesterol and ABCA1 function.

First, the regulation adipocyte ABCA1 protein needs to be evaluated under physiological conditions in both humans and mice. In humans, adipose tissue should be evaluated for ABCA1 protein expression and association with adiposity, TG content, adipocyte size and insulin resistance, similar to studies by Frisdale et al. [26]. In mice, the underlying mechanisms which result in the upregulation of ABCA1 should be determined; namely, what pathways lead to the upregulation of ABCA1 during HFHC diet feeding. This should also include time based studies that can determine how quickly ABCA1 upregulation occurs after initiation of a high fat diet. In addition, the regulation of nuclear SREBP1 in relation to cholesterol concentration in adipocytes should be determined to

identify whether physiological levels of membrane cholesterol result in a similar reduction as observed in our ASKO mice.

Secondly, our ASKO mice should be used to examine other adipose depots and cell types, in particular, BAT and WAT browning. These studies should involve more accurately measuring body temperature to determine whether ASKO mice expend energy as heat, as well as cold tolerance studies to determine whether ASKO white adipose is more susceptible to browning [49]. BAT adipocyte size and function should be examined more closely through protein and mRNA expression data. In all, these studies and the proposed future studies add significant evidence to the idea that adipose cholesterol can modulate adipocyte function. Dietary cholesterol accumulation in adipose tissue is clearly detrimental (chapter II), but the modulation of cholesterol location and content in adipocytes can prevent diet induced obesity (chapter III), suggesting that the influence of cholesterol on adipocytes is complicated and dynamic, and deserving of future studies.

References

1. Members, W.G., et al., *Heart Disease and Stroke Statistics—2012 Update: A Report From the American Heart Association.* Circulation, 2012. **125**(1): p. e2-e220.

2. Krause, B.R. and A.D. Hartman, *Adipose tissue and cholesterol metabolism.* Journal of Lipid Research, 1984. **25**(2): p. 97-110.

3. Angel, A. and J. Farkas, *Regulation of cholesterol storage in adipose tissue.* Journal of Lipid Research, 1974. **15**(5): p. 491-499.

4. Kovanen, P.T., E.A. Nikkilä, and T.A. Miettinen, *Regulation of cholesterol synthesis and storage in fat cells.* Journal of Lipid Research, 1975. **16**(3): p. 211-223.

5. Eberle, D., et al., *SREBP transcription factors: master regulators of lipid homeostasis.* Biochimie, 2004. **86**(11): p. 839-848.

6. Li, H., et al., *Cholesterol suppresses adipocytic differentiation of mouse adipose-derived stromal cells via PPARγ2 signaling.* Steroids, 2013. **78**(5): p. 454-461.

7. Pattar, G.R., et al., *Chromium picolinate positively influences the glucose transporter system via affecting cholesterol homeostasis in adipocytes cultured under hyperglycemic diabetic conditions.* Mutat Res, 2006. **610**(1-2): p. 93-100.

8. Nohturfft, A., M.S. Brown, and J.L. Goldstein, *Sterols regulate processing of carbohydrate chains of wild-type SREBP cleavage-activating protein (SCAP), but not sterol-resistant mutants Y298C or D443N.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 12848-12853.

9. Wang, X., et al., *SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis.* Cell, 1994. **77**(1): p. 53-62.

10. Storch, J., S.L. Shulman, and A.M. Kleinfeld, *Plasma membrane lipid order and composition during adipocyte differentiation of 3T3F442A cells. Studies in intact cells with 1-[4-(trimethylamino)phenyl]-6-phenylhexatriene.* Journal of Biological Chemistry, 1989. **264**(18): p. 10527-10533.

11. Guerre-Millo, M., et al., *Alteration in membrane lipid order and composition in metabolically hyperactive fatty rat adipocytes.* Lipids, 1994. **29**(3): p. 205-209.

12. Boizard, M., et al., *Obesity-related Overexpression of Fatty-acid Synthase Gene in Adipose Tissue Involves Sterol Regulatory Element-binding Protein Transcription Factors.* Journal of Biological Chemistry, 1998. **273**(44): p. 29164-29171.

13. Le Lay, S., et al., *Cholesterol, a Cell Size-dependent Signal That Regulates Glucose Metabolism and Gene Expression in Adipocytes.* Journal of Biological Chemistry, 2001. **276**(20): p. 16904-16910.

14. Strable, M.S. and J.M. Ntambi, *Genetic control of de novo lipogenesis: role in diet-induced obesity.* Critical reviews in biochemistry and molecular biology, 2010. **45**(3): p. 199-214.

15. Schultz, J.R., et al., *Role of LXRs in control of lipogenesis.* Genes & Development, 2000. **14**(22): p. 2831-2838.

16. Yoshikawa, T., et al., *Identification of Liver X Receptor-Retinoid X Receptor as an Activator of the Sterol Regulatory Element-Binding Protein 1c Gene Promoter.* Molecular and Cellular Biology, 2001. **21**(9): p. 2991-3000.

17. Beaven, S.W., et al., *Reciprocal regulation of hepatic and adipose lipogenesis by liver X receptors in obesity and insulin resistance.* Cell Metab, 2013. **18**(1): p. 106-17.

18. Wooten, J.S., et al., *The Influence of an Obesogenic Diet on Oxysterol Metabolism in C57BL/6J Mice.* Cholesterol, 2014. **2014**: p. 843468.

19. Lange, Y., et al., *Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol.* Journal of Lipid Research, 1999. **40**(12): p. 2264-2270.

20. Radhakrishnan, A., et al., *Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: a delicate balance.* Cell metabolism, 2008. **8**(6): p. 512-521.

21. Chung, S., et al. (2014). *Dietary cholesterol promotes adipocyte hypertrophy and adipose tissue inflammation in visceral, but not in subcutaneous, fat in monkeys.* Arterioscler Thromb Vasc Biol **34**(9): 1880-1887.

22. Aguilar, D. and M.L. Fernandez, *Hypercholesterolemia induces adipose dysfunction in conditions of obesity and nonobesity.* Adv Nutr, 2014. **5**(5): p. 497-502.

23. Edgel, K.A., et al., *Obesity and weight loss result in increased adipose tissue ABCG1 expression in db/db mice.* Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 2012. **1821**(3): p. 425-434.

24. Yu, B.L., S.P. Zhao, and J.R. Hu, *Cholesterol imbalance in adipocytes: a possible mechanism of adipocytes dysfunction in obesity.* Obesity Reviews, 2010. **11**(8): p. 560-567.

25. Dugail, I., et al., *New Insights into How Adipocytes Sense their Triglyceride Stores. Is Cholesterol a Signal?* Horm Metab Res, 2003. **35**(04): p. 204-210.

26. Frisdal, E., et al. (2015). "Adipocyte ATP-Binding Cassette G1 Promotes Triglyceride Storage, Fat Mass Growth, and Human Obesity." Diabetes **64**(3): 840-855.

27. Zelcer, N., et al., *LXR Regulates Cholesterol Uptake Through Idol-Dependent Ubiquitination of the LDL Receptor.* Science, 2009. **325**(5936): p. 100-104.

28. Le Lay, S., et al., *Regulation of ABCA1 expression and cholesterol efflux during adipose differentiation of 3T3-L1 cells.* Journal of Lipid Research, 2003. **44**(8): p. 1499-1507.

29. Dahlman, I., et al., *Liver X receptor gene polymorphisms and adipose tissue expression levels in obesity.* Pharmacogenet Genomics, 2006. **16**(12): p. 881-889.

30. Dahlman, I., et al., *Functional and genetic analysis in type 2 diabetes of liver X receptor alleles--a cohort study.* BMC Med Genet, 2009. **10**: p. 27.

31. Mejhert, N., et al., *Role of Receptor-Interacting Protein 140 in human fat cells.* BMC Endocr Disord, 2010. **10**: p. 1.

32. Nordstrom, E.A., et al., *A human-specific role of cell death-inducing DFFA (DNA fragmentation factor-alpha)-like effector A (CIDEA) in adipocyte lipolysis and obesity.* Diabetes, 2005. **54**(6): p. 1726-1734.

33. Kalaany, N.Y., et al., *LXRs regulate the balance between fat storage and oxidation.* Cell Metabolism, 2005. **1**(4): p. 231-244.

34. Tang, C., et al., *Diabetes reduces the cholesterol exporter ABCA1 in mouse macrophages and kidneys.* J Lipid Res, 2010. **51**(7): p. 1719-1728.

35. Wang, Y. and J.F. Oram, *Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a protein kinase C delta pathway.* J Lipid Res, 2007. **48**(5): p. 1062-1068.

36. Lv, Y.C., et al., *Posttranscriptional regulation of ATP-binding cassette transporter A1 in lipid metabolism.* DNA Cell Biol, 2013. **32**(7): p. 348-358.

37. Subramanian, S., et al., *Dietary Cholesterol Worsens Adipose Tissue Macrophage Accumulation and Atherosclerosis in Obese LDL Receptor–Deficient Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2008. **28**(4): p. 685-691.

38. Umemoto, T., et al. (2013). *"Apolipoprotein A-I and HDL Have Anti-Inflammatory Effects on Adipocytes via Cholesterol Transporters: ATP-Binding Cassette (ABC) A-1, ABCG-1 and Scavenger Receptor B-1(SRB-1)*." Circulation Research **112**(10): 1345-1354.

39. Zhu, X., et al., *Increased Cellular Free Cholesterol in Macrophage-specific Abca1 Knock-out Mice Enhances Pro-inflammatory Response of Macrophages.* Journal of Biological Chemistry, 2008. **283**(34): p. 22930-22941.

40. Gregor, M.F. and G.S. Hotamisligil, *Inflammatory Mechanisms in Obesity.* Annual Review of Immunology, 2011. **29**(1): p. 415-445.

41. Hotamisligil, G.S., *Inflammation and metabolic disorders.* Nature, 2006. **444**(7121): p. 860-867.

42. Ouchi, N., et al., *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol, 2011. **11**(2): p. 85-97.

43. Shoelson, S.E., L. Herrero, and A. Naaz, *Obesity, Inflammation, and Insulin Resistance.* Gastroenterology, 2007. **132**(6): p. 2169-2180.

44. Odegaard, J.I. and A. Chawla, *Pleiotropic actions of insulin resistance and inflammation in metabolic homeostasis.* Science, 2013. **339**(6116): p. 172-177.

45. Covey, S.D., et al., *Cholesterol depletion inhibits fatty acid uptake without affecting CD36 or caveolin-1 distribution in adipocytes.* Biochemical and Biophysical Research Communications, 2007. **355**(1): p. 67-71.

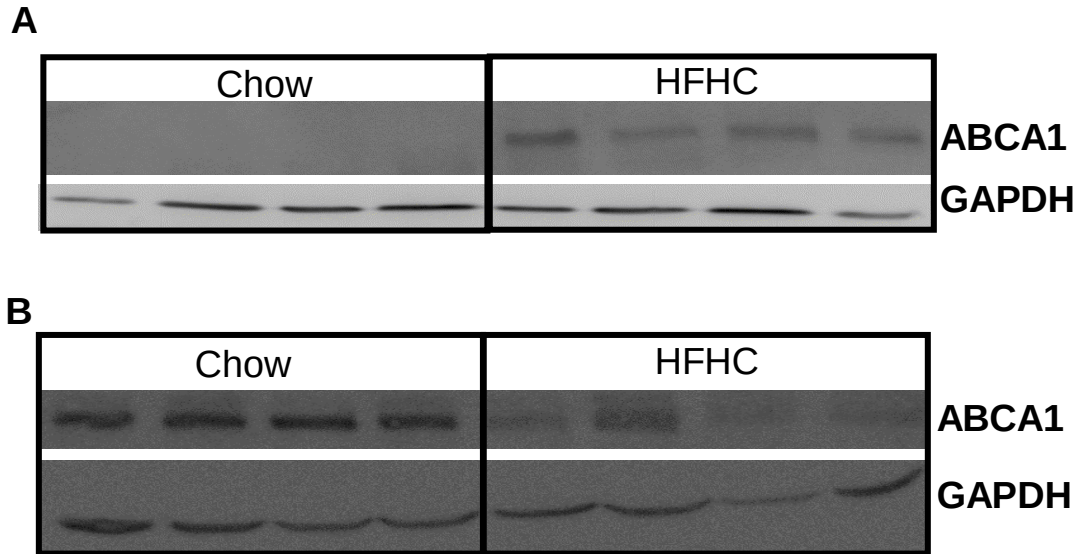
46. Zhu, X., et al., *Macrophage ABCA1 reduces MyD88-dependent Toll-like receptor trafficking to lipid rafts by reduction of lipid raft cholesterol.* Journal of Lipid Research, 2010. **51**(11): p. 3196-3206.

47. Brunham, L.R., et al., *[beta]-cell ABCA1 influences insulin secretion, glucose homeostasis and response to thiazolidinedione treatment.* Nat Med, 2007. **13**(3): p. 340-347.

48. Karasinska, J.M., et al., *Specific Loss of Brain ABCA1 Increases Brain Cholesterol Uptake and Influences Neuronal Structure and Function.* The Journal of Neuroscience, 2009. **29**(11): p. 3579-3589.

49. Fisher, f.M., et al., *FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis.* Genes & Development, 2012. **26**(3): p. 271-281.

Discussion Figure 1: ABCA1 protein is increased in adipose, and reduced in liver, in response to a HFHC diet



Discussion Figure 1: ABCA1 and GAPDH protein expression in WT mice fed 16 weeks of chow or HFHC diet in A) Adipose, and B) Liver.

Curriculum Vitae

Helen Ann Cuffe

Wake Forest University Health Sciences

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Education

PhD – Molecular Pathology	Wake Forest	05/15
MBA	Wake Forest	05/15
Master's degree - Biology	Purdue University	05/10
Bachelors of Science - Biochemistry	University of Evansville	05/08

Professional Experience

Graduate Research Assistant Wake Forest university Current

- Established mouse KO model of cholesterol transporter ABCA1 using adiponectin Cre tissue specific recombinase to determine the effects of cholesterol overload on adipose tissue.
- Established in vitro, and ex vivo adipose tissue metabolic assays for the lab to focus on the insulin resistance and inflammatory phenotypes in cholesterol loaded adipose tissue

Commercialization intern Wake Forest Innovations 08/2014 – Present

- Assisted licensing and commercialization professionals in the identification, development, protection, and commercialization of inventions
- Designed and created a functional database to cross reference Venture Capital funds with current research interests at Wake Forest University
- Performed prior art literature searches, market analysis, and regulatory analysis in preparation for the patent application process

Financial coordinator Wake Forest Case Competition 08/2014-03/2015

- Coordinated financing and budget for the 2015 Healthcare Case Competition at Wake Forest University including securing sponsorship

funds, developing a budget plan, negotiating costs with vendors and communicating finances to professors, sponsors and associates
- Documented all expenses and coordinated reimbursement and prizes through Wake Forest University

Biomedical Case Writer Boston Scientific 08/13 – 01/14

- Primary writer of biomedical case for the Wake Forest case competition in March 2014. Worked with Boston Scientific to analyze financial and market data and present as a written case for student competition.
- Coordinated inputs from Wake Forest professors, and Boston Scientific employees who contributed to the case.
- Performed extensive reasearch into the biomedical device field including: changing payer systems, pricing pressures, alternative buying practices, and the potential effects of the Affordable Care Act on medical devices and device companies.

Research Intern Ageno Lab – Stuttgart, Germany 05/11 – 09/11

- Developed German language skills to close to fluency for both business and science
- Improved proficiency for aseptic and sterile technique in a BSL3 lab environment
- Safely handled and analyzed unknown human tissue samples in an aseptic and sterile environment, and compared results with newly developed human stem cell derived skin model
- Developed an rtPCR assay for the detection of mycoplasma contamination in BSL3 lab environment, and was sucessfully able to implement a routine testing and inspection process for detection of common contaminant.
- Analyzed results and gave input for the improvement of human stem cell derived skin model for use in cosmetic testing

Reserach Intern BASF – Mannheim, Germany 05/10 – 08/10

- Transfected yeast with plant cytochrome P450 genes to determine effectiveness against pesticide and herbicide treatment with the ultimate goal of developing new genetically modified plants. Resulted in identification of multiple potential genes and initiated transfer of these genes into plant models.
- Developed an assay to determine the toxicity of herbicides and pesticides on yeast cultures as a first step in the development of new products
- Developed a high throughput culture and assay technique to determine the breakdown of over 100 pesticides and herbicides in 50 transfected yeast strains using LCMSMS and HPLC. Was sucessfully able to reduce process time by several days, and signficantly reduced use of lab materials and resources.

Awards

Beta Sigma Gamma Honor society March 2014

Description: invitation to join honor society for business students
Application Statistics: Top 20% of graduating students (GPA) from MBA program invited

CMCS Fellowship Recipient August 2014 – July 2015

Description: Fellowship award to cover research endeavours for the academic year 2014/2015
Application Statistics: Not released

Travel Award Kern Lipid Meeting July 2013

Description: Scholarship award to allow travel and accomodation at the Kern Lipid Sciences meeting in Vail Colorado, July 2013
Application Statistics: Not released

RISE pro DAAD scholarship recipient 2010, 2011

Description: Scholarship to cover living expenses while working in Germany during the summers of 2010 and 2011. Each year was awarded separately.
Application Statistics: roughly 15% acceptance rate each year

Division 1 Athletic Scholarship August 2004 – May 2008

Description: Full tuition scholarship to play division one golf at the University of Evansville.
Application Statistics: At time of high school graduation was ranked top 10 in the state among graduating seniors

Leadership/Community Experience

Graduate Student Association Secretary 2013 - 2014

Description: Carried out secretarial duties for the graduate student government association. Elected from student body numbering over 400.

Graduate Student Association Representative 2010 - 2013

Description: Represented students within department for GSA affairs, communcation with faculty, voicing of student concerns. Represented roughly 20 students over the course of 3 years.

Coach for The First Tee golf program 2011-2013

Description: Mentored and coached underprivileged children in the First Tee after school golf program. Was responsible for teaching golf skills and instilling honesty and integrity in action through the game of golf.

Varsity Golf captain 2007 – 2008

Description: Captain of University of Evansville womans golf team for senior season 07/08.

Publications and Presentations

Poster Presentation – ATVB conference May 2011
Poster Presentation – Kern Lipid Sciences Conference July 2013
Poster Presentation – Southeast Lipids Conference September 2013
Poster Presentation – Kern Lipids Conference August 2014

Helen Cuffe, Pam Clough, Alex Bashore, Mingxia Liu, Allison Weckerle, Elena Boudyguina and John S Parks. Adipocyte ABCA1 is essential for the regulation of cholesterol and TG content in adipose tissue during obesity (Manuscript in Preparation).

Chung, S., **H. Cuffe**, et al. (2014). "Dietary Cholesterol Promotes Adipocyte Hypertrophy and Adipose Tissue Inflammation in Visceral, but Not in Subcutaneous, Fat in Monkeys." Arteriosclerosis, Thrombosis, and Vascular Biology.

Zhu, X., S. Chung, et al. (2013). "Myeloid cell-specific ABCA1 deletion does not worsen insulin resistance in HF diet-induced or genetically obese mouse models." Journal of Lipid Research **54**(10): 2708-2717.