REGULATION OF MONOCYTE/MACROPHAGE ATP-BINDING CASSETTE TRANSPORTERS AND CHOLESTEROL EFFLUX BY ELEVATED GLUCOSE AND FREE FATTY ACID CONCENTRATIONS

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Abstract:

Individuals with diseases of poor glycemic control, including pre-diabetes and type 2 diabetes mellitus, are at increased risk of developing atherosclerosis, the major form of cardiovascular disease (CVD). Atherosclerotic lesion formation is promoted by monocyte/macrophage recruitment into the arterial wall and subsequent cholesterol accumulation in macrophages. However, the relationship between elevated circulating glucose and free fatty acids (FFA) concentrations, frequently associated with poor glycemic control, and macrophage cholesterol accumulation is yet to be fully elucidated. **We hypothesized that factors which regulate monocyte/macrophage cholesterol efflux, particularly ATP-binding cassette (ABC) transporter expression will be down-regulated in response to elevated glucose and FFA conditions *in vitro* and *in vivo*, mediated by liver-x-receptor (LXR)-α and sterol regulatory element binding protein (SREBP)-1c.**

The goal of this thesis was to determine the effect of elevated glucose and FFA concentrations on factors mediating macrophage cholesterol homeostasis using two different systems: human monocytes and cultured murine bone marrow-derived macrophage cells (BMDM). Human monocytes were isolated from individuals in the fasting state and after an oral glucose challenge to determine the relationship between elevated blood glucose concentration and changes in monocyte ABC-transporter (ABCA1 and ABCG1) mRNA expression. Additionally, murine BMDM were used as a model system to determine the effect of elevated glucose and FFA concentrations on factors governing macrophage intracellular cholesterol accumulation and cellular cholesterol efflux through ABC-transporters and scavenger receptor (SR)-B1. LXR-α and SREBP-1a, -1c and -2 were also targeted for their potential role as transcriptional mediators of transporters involved in macrophage cholesterol efflux, specifically with respect to glucose and FFA concentrations.

In humans a glucose challenge increased leukocyte ABCA1 and ABCG1 mRNA expression but had no significant effect on monocyte ABCA1 or ABCG1 mRNA expression. We also determined that leukocyte ABC-transporter mRNA expression was significantly higher than PBMC or monocytes *in vivo*. However, in an *in vitro* macrophage model, BMDM, cholesterol efflux was suppressed after exposure to elevated glucose concentrations, a FFA mixture (linoleic acid [18:2], palmitic acid [16:0], and oleic acid [18:1]) as well as 18:2 alone, but not 16:0 alone. Elevated glucose concentrations did not have a significant effect on ABC-transporter mRNA or protein expression in oxidized low density lipoprotein (oxLDL)-stimulated BMDM. In contrast, ABC-transporter and SREBP-1c mRNA expression and ABCA1 protein expression was suppressed by 18:2. Neither LXR-α or SREBP-1 protein expression was affected by 18:2, indicating that changes in expression of these transcription factors are not mediating the effect.

The results of this research will add to our understanding of the relationship between glucose and FFA concentrations, and monocyte/macrophage cholesterol homeostasis, *in vivo* and *in vitro*. These data will advance efforts to gain perspective on novel mechanisms by which diseases of poor glycemic control accelerate the development of CVD.

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CHAPTER 1

INTRODUCTION

1. Statement of Significance

Cardiovascular disease (CVD) is the leading cause of death in industrialized societies [1]. Individuals with poor glycemic control, such as type 2 diabetes mellitus (T2DM) and impaired glucose tolerance (IGT), are at increased risk to develop CVD [2-4]. There are several mechanisms which may contribute to accelerated atherosclerosis progression in these patients. Common factors include hyperglycemia and elevated free fatty acid (FFA) concentrations.

Hyperglycemia has an important role in pathogenicity of macrovascular complications of diabetes [5]. Observational studies have identified a positive association between hyperglycemia and CVD risk [6-9]. These data are stronger for non-fasting than fasting plasma glucose concentrations [10-12].

Individuals with T2DM also frequently present with elevated FFA concentrations [13], and are reported to have impaired macrophage cholesterol efflux [14, 15], which would be predicted to favor atherosclerotic lesion progression. *In vitro* work supports these observations, demonstrating, mainly in immortalized macrophage cell line, that elevated glucose and FFA concentrations impair cholesterol efflux [16-21].

Data provided by *in vitro* studies and animal models are critical to elucidate mechanisms for observations generated in humans but do not mimic the *in vivo* state. Immortalized macrophage cell lines do not always respond similarly to stimuli as macrophages, *in vivo* [22, 23]. Therefore, it is important to adjudicate data generated in *in vitro* studies and animal models with well design studies in humans, *in vivo*, and with primary cell models.

Due to the formidable increase in prevalence of T2DM in the recent past [24], there has been an increased focus in the research community on therapeutic measures to prevent complications associated with this disease. It is well documented that individuals with T2DM are at increased risk for CVD [2-4], but the mechanisms remain to be fully elucidated. A more thorough understanding of the mechanisms is critical before more targeted approaches can be developed for the prevention of complications of T2DM. Results of this study will aid in the understanding of mechanisms underlying complications of T2DM and suggestion of potential targets for CVD risk reduction.

2. Specific Aims

Individuals with poor glycemic control (including pre-diabetes, IGT and T2DM) are at increased risk of developing atherosclerosis, the major form of CVD [2-4]. Hallmarks of poor glycemic control include elevated blood glucose and FFA concentrations [25, 26]. Atherosclerotic lesion formation is promoted by monocyte/macrophage recruitment into the arterial wall and subsequent macrophage cholesterol accumulation [27].

The objective of this thesis was to use two different experimental models (human monocytes and murine bone marrow-derived macrophages [BMDM]) to characterize the effect of elevated glucose and FFA concentrations on ATP-binding cassette (ABC) transporters, ABCA1 and ABCG1, and scavenger receptor (SR)-B1 and their influence in cellular cholesterol homeostasis; as well as mediation of these factors by liver-x-receptor (LXR)-α and sterol regulatory element binding protein (SREBP)-1c.

Specific Aim 1: To determine the relationship between blood glucose concentrations and changes in monocyte ATP-binding cassette transporters (ABC)A1 and ABCG1 mRNA expression during an oral glucose challenge in human subjects.

Hypothesis 1: Monocyte, but not leukocyte, ABCA1 and ABCG1 mRNA expression will be suppressed following administration of an oral glucose challenge in human subjects.

Specific Aim 2: To determine the effect of elevated glucose and FFA concentrations on intracellular lipid accumulation (triglycerides, total cholesterol, and cholesteryl esters), HDL- and apoA1-mediated cholesterol efflux, expression of genes and proteins involved in cellular cholesterol efflux (scavenger receptor (SR)-B1, ABCA1, ABCG1) and their potential transcriptional regulators (LXR-alpha and SREBP-1c) in murine BMDM.

Hypothesis 2: Elevated glucose and FFA concentrations will increase lipid accumulation and suppress cellular cholesterol efflux and expression of SRB1 and ABC-transporters corresponding to suppression of LXR and SREBP-1c.

Results from these studies will aid in the understanding of the relationship between glucose and FFA concentrations and macrophage cholesterol homeostasis, *in vivo* and *in vitro*, and also to gain new perspectives on the mechanisms by which poor glycemic control is a risk factor for developing atherosclerosis and CVD.

3. References

1. Lloyd-Jones, D.M., *Cardiovascular risk prediction: basic concepts, current status, and future directions.* Circulation, 2010. **121**(15): p. 1768-1777.
2. Ryden, L., et al., *Guidelines on diabetes, pre-diabetes, and cardiovascular diseases: executive summary. The Task Force on Diabetes and Cardiovascular Diseases of the European Society of Cardiology (ESC) and of the European Association for the Study of Diabetes (EASD).* Eur Heart J, 2007. **28**(1): p. 88-136.
3. Beckman, J.A., M.A. Creager, and P. Libby, *Diabetes and atherosclerosis: epidemiology, pathophysiology, and management.* JAMA, 2002. **287**(19): p. 2570-81.
4. Park, L. and D. Wexler, *Update in diabetes and cardiovascular disease: synthesizing the evidence from recent trials of glycemic control to prevent cardiovascular disease.* Curr Opin Lipidol, 2010. **21**(1): p. 8-14.
5. Brownlee, M., *The pathobiology of diabetic complications: a unifying mechanism*. 2005: Diabetes. 2005 Jun;54(6):1615-25.
6. Balkau, B., et al., *Prediction of the risk of cardiovascular mortality using a score that includes glucose as a risk factor. The DECODE Study.* Diabetologia, 2004. **47**(12): p. 2118-28.
7. *Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnostic criteria. The DECODE study group. European Diabetes Epidemiology Group. Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria in Europe.* Lancet, 1999. **354**(9179): p. 617-621.
8. Khaw, K.T., et al., *Glycated haemoglobin, diabetes, and mortality in men in Norfolk cohort of european prospective investigation of cancer and nutrition (EPIC-Norfolk).* Bmj, 2001. **322**(7277): p. 15-8.
9. Turner, R.C., et al., *Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23).* Bmj, 1998. **316**(7134): p. 823-8.
10. Temelkova-Kurktschiev, T.S., et al., *Postchallenge plasma glucose and glycemic spikes are more strongly associated with atherosclerosis than fasting glucose or HbA1c level.* Diabetes Care, 2000. **23**(12): p. 1830-1834.
11. Hanefeld, M., et al., *Post-challenge hyperglycaemia relates more strongly than fasting hyperglycaemia with carotid intima-media thickness: the RIAD Study. Risk Factors in Impaired Glucose Tolerance for Atherosclerosis and Diabetes.* Diabetic medicine : a journal of the British Diabetic Association, 2000. **17**(12): p. 835-840.
12. Heine, R.J., et al., *What does postprandial hyperglycaemia mean?* Diabet Med, 2004. **21**(3): p. 208-13.
13. Garvey, W.T., et al., *Effects of insulin resistance and type 2 diabetes on lipoprotein subclass particle size and concentration determined by nuclear magnetic resonance.* Diabetes, 2003. **52**(2): p. 453-462.
14. Mauldin, J.P., et al., *Reduced expression of ATP-binding cassette transporter G1 increases cholesterol accumulation in macrophages of patients with type 2 diabetes mellitus.* Circulation, 2008. **117**(21): p. 2785-92.
15. Zhou, H., et al., *Determinants of leukocyte adenosine triphosphate-binding cassette transporter G1 gene expression in type 2 diabetes mellitus.* Metabolism, 2008. **57**(8): p. 1135-40.
16. Uehara, Y., et al., *Polyunsaturated fatty acids and acetoacetate downregulate the expression of the ATP-binding cassette transporter A1.* Diabetes, 2002. **51**(10): p. 2922-2928.

17. Uehara, Y., et al., *Unsaturated fatty acids suppress the expression of the ATP-binding cassette transporter G1 (ABCG1) and ABCA1 genes via an LXR/RXR responsive element.* Atherosclerosis, 2007. **191**(1): p. 11-21.
18. Wang, Y. and J.F. Oram, *Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a phospholipase D2 pathway.* J Biol Chem, 2005. **280**(43): p. 35896-903.
19. 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. 1062-8.
20. Passarelli, M., et al., *Advanced glycation end product precursors impair ABCA1-dependent cholesterol removal from cells.* Diabetes, 2005. **54**(7): p. 2198-2205.
21. Mauerer, R., S. Ebert, and T. Langmann, *High glucose, unsaturated and saturated fatty acids differentially regulate expression of ATP-binding cassette transporters ABCA1 and ABCG1 in human macrophages.* Experimental and Molecular Medicine, 2009. **41**(2): p. 126.
22. Kohro, T., et al., *A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage.* J Atheroscler Thromb, 2004. **11**(2): p. 88-97.
23. Qin, Z., *The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature.* Atherosclerosis, 2012. **221**(1): p. 2-11.
24. CDC, *Increasing prevalence of diagnosed diabetes - United States and puerto rico, 1995-2010.* MMWR Morb Mortal Wkly Rep, 2012. **61**(45): p. 918-21.
25. Reaven, G.M., et al., *Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM.* Diabetes, 1988. **37**(8): p. 1020-1024.
26. Seigneur, M., et al., *Serum fatty acid profiles in type I and type II diabetes: metabolic alterations of fatty acids of the main serum lipids.* Diabetes research and clinical practice, 1994. **23**(3): p. 169-177.
27. Glass, C.K. and J.L. Witztum, *Atherosclerosis. the road ahead.* Cell, 2001. **104**(4): p. 503-516.

CHAPTER 2

LITERATURE REVIEW

1. Diabetes and cardiovascular disease

Individuals with type 2 diabetes mellitus (T2DM) and other conditions of poor glycemic control, characterized by elevated glucose and free fatty acid (FFA) concentrations, are at increased risk of developing cardiovascular disease (CVD) [1-3]. There are several mechanisms by which poor glycemic control may increase CVD risk including hyperglycemia, advanced glycation end products (AGE), insulin resistance, inflammation, and diabetic dyslipidemia (including increased circulating FFA). The role that these factors play in atherosclerosis progression, the leading cause of CVD, is still poorly defined.

Diabetes mellitus is diagnosed if an individual has one of the following criteria: fasting plasma glucose ≥126 mg/dL, two-hour plasma glucose ≥ 200 mg/dL during an oral glucose tolerance test, hemoglobin A1c (HbA1c) ≥ 6.5%, or a random plasma glucose ≥ 200 mg/dL [4]. Ninety eight percent of patients with diabetes mellitus have T2DM (or non-insulin dependent diabetes mellitus), while 2% have type 1 diabetes mellitus (T1DM) [4]. In addition to a diagnosis of diabetes, there are other conditions of poor glycemic control such as pre-diabetes and metabolic syndrome. Pre-diabetes can be diagnosed by fasting plasma glucose between 100 -125 mg/dL (impaired fasting glucose), two-hour plasma glucose between 140-199 mg/dL during an oral glucose tolerance test (impaired glucose tolerance [IGT]), or HbA1c between 5.7-6.4% [4]. Metabolic syndrome is a clustering of cardiovascular risk factors: hyperglycemia/insulin resistance, dyslipidemia, obesity and hypertension [5].

While there are many pathogenic outcomes which characterize diseases of poor glycemic control, this review will focus on the potential mechanisms which explain the association between poor glycemic control and elevated CVD/atherosclerosis risk.

Many of the biomarkers associated with diabetes are also associated with metabolic syndrome and pre-diabetes, which typically precede T2DM [6, 7]. Individuals with pre-diabetes, T2DM and metabolic syndrome also present with insulin resistance, a condition during which disruption of insulin signaling prevents glucose from entering peripheral tissues. Insulin resistance often leads to hyperglycemia and elevated FFA due to reduced uptake of glucose and reduced utilization of FFA by muscle tissues and increased adipose FFA release [8].

Two of the factors which are often elevated in individuals with T2DM are glucose and FFA concentrations. Elevated glucose and FFA concentrations may have some similar effects on peripheral tissues. Hyperglycemia can increase oxidative stress, activate NF-kB transduction pathways and form advanced glycation end products (AGE), all of which signal cells to produce inflammatory cytokines and growth factors [9-12]. These factors are associated with elevations in modified (glycosylated or oxidized) lipoprotein particles or cellular proteins [13]. Additionally, exposure to elevated glucose or FFA concentrations can lead to protein kinase-C (PK)C activation, which has a variety of effects on gene expression, reducing antiatherogenic factors such as vasodilators, while increasing expression of pro-inflammatory genes such as NF-kB and oxidative stress pathways [14, 15].

All of the proatherogenic effects of hyperglycemia seem to have one mechanism in common: oxidative stress. Oxidative stress caused by high glucose concentrations leads

to generation of intracellular superoxides and hydroxyl radicals which damage tissues in the local microenvironment of the arterial wall [16-18]. These free radicals may lead to disruption of many important protective mechanisms, such as macrophage involvement in reverse cholesterol transport, which will be discussed in length in this review. However, yet to be determined is whether hyperglycemia itself alters atherosclerotic risk independent of atherogenic factors commonly associated with T2DM [19-22].

In addition to loss of glycemic control, T2DM is characterized by dyslipidemia, characterized by low high density lipoprotein (HDL)-cholesterol, high triglyceride (TG)-rich lipoprotein concentrations, increased postprandial TG concentrations, increased proportion of small dense low density lipoprotein (LDL) particles, and elevated FFA concentrations [23, 24]. There is an abundance of *in vitro* evidence to support the proatherogenic effect of TG-rich lipoproteins, mainly through increased cholesterol accumulation, inflammation and apoptosis of macrophages and endothelial cells [25, 26]. We chose to study the effects of elevated FFA, which can increase inflammation and oxidative stress in peripheral tissue exposure [27] and may also disrupt macrophage cholesterol efflux [28-30].

In a healthy individual, circulating glucose and FFA concentrations vary throughout the day in response to ingestion of food and hormonal levels (**Figure 1**) [31]. In individuals with IGT, hormonal levels outside the normal range result in elevated glucose, insulin, FFA and TG concentrations (**Figure 1**). Regardless of glycemic status the three most abundant FFA in circulation are oleic acid (18:1), linoleic acid (18:2) and palmitic acid (16:0) [32]. Diet, lifestyle, and specific disease states can modify the

relative proportions of circulating FFA, but these three FFA remain the most abundant [33] and were the focus of this thesis research.

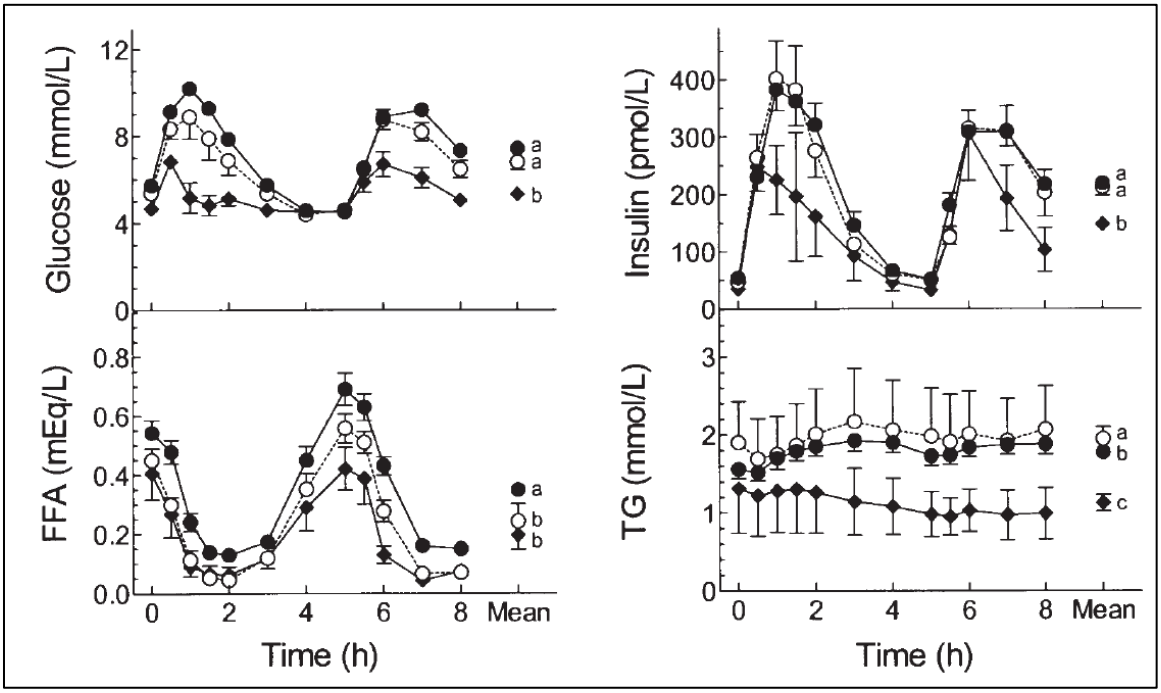

Figure 1. Glucose, insulin, free fatty acid (FFA), and triacylglycerol (TG) concentrations during mealtime in lean, obese, and impaired glucose tolerant (IGT) individuals. 8-h metabolic profile in 34 subjects with IGT (●), 7 obese control subjects (O), and 8 lean control subjects (♦). Breakfast was consumed at 0 h and lunch at 5 h. Error bars are not shown if they are smaller than the symbol or overlap other error bars. Means with different letters are significantly different, $p < 0.05$ [31].

2. Macrophages and atherosclerosis development

Atherosclerotic lesions, the leading cause of CVD, often begin in regions of arterial branching or curvature where blood flow tends to be disturbed and the intimal endothelial cells are exposed to high fluid sheer stress. This endothelial layer of cells is vulnerable to infiltration by LDL particles [34, 35], which activate an inflammatory response. Activated endothelial cells secrete cytokines and adhesion molecules which attract circulating monocytes [36, 37]. These monocytes transmigrate into the subendothelial space [38] (**Figure 2, Part I**). The presence of macrophages in atherosclerotic plaques

was first reported in 1960 [39]. This observation advanced our understanding of atherosclerotic lesion progression, with specific emphasis on the involvement of immune cells.

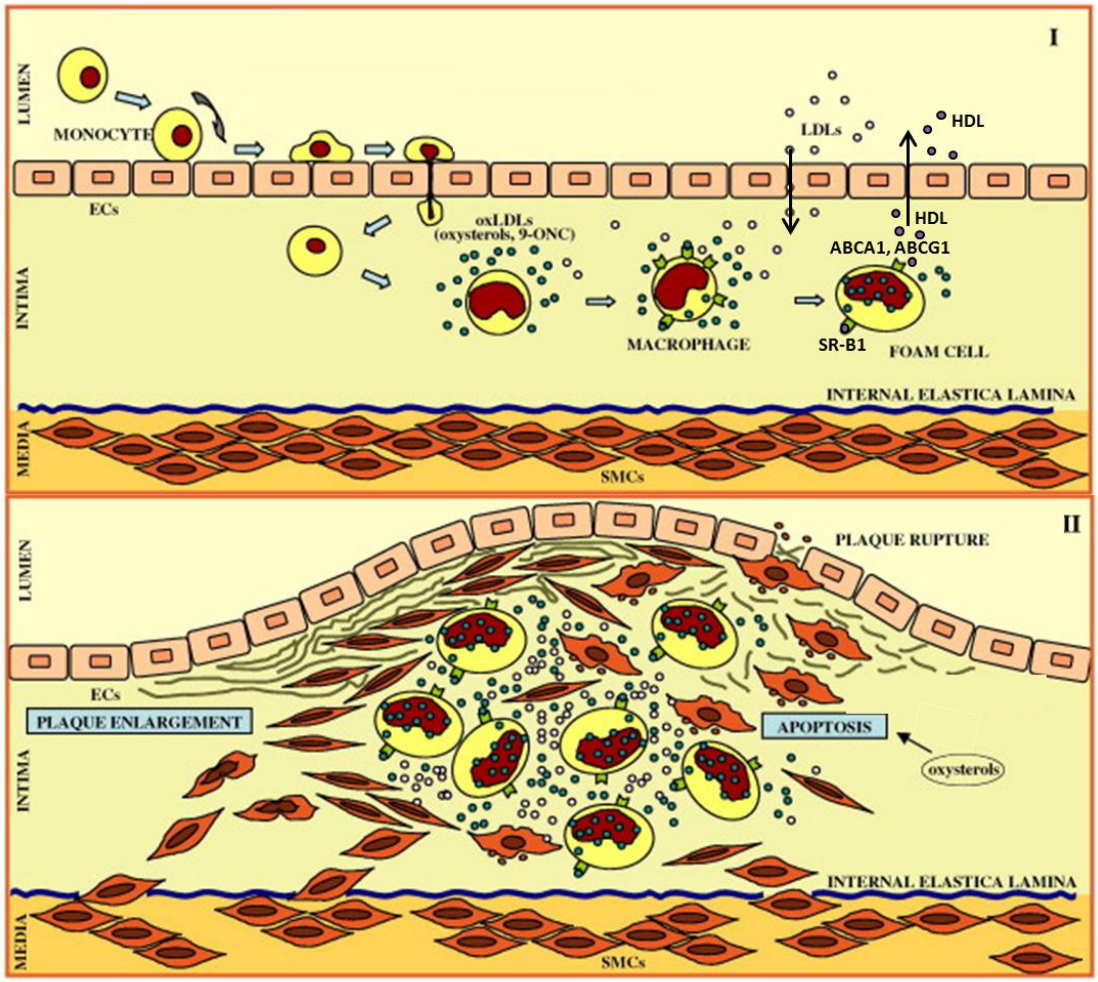

Figure 2. Macrophages in atherosclerosis development. Part I. Monocytes enter the intimal wall where they engulf modified low density lipoproteins (LDL), such as oxidized (ox)LDL, and become macrophage foam cells. Proteins on the surface (ATP-binding cassette transporters (ABC) A1 and G1 and scavenger receptor (SR)-B1) associate with high density lipoprotein (HDL) particles, effluxing cholesterol to HDL, which can exit the intimal space. Part II. Intracellular accumulation of cholesterol is not a feedback inhibited process, and thus eventually leads to apoptosis and cell death. An atherosclerotic plaque continues to enlarge until there is a rupture and plaque contents are released into the lumen as a thrombus. Endothelial cells (ECs), smooth muscle cells (SMCs). Adapted from Poli G. et al, 2009 [40].

Once monocytes enter the subendothelial matrix of the vessel wall, macrophage-colony stimulating factor (M-CSF) and modified-LDL promote differentiation of the monocytes into macrophages [41, 42]. These macrophages engulf modified LDL

particles through phagocytosis promoted by scavenger receptors (SR)-A, LDL-receptor (LDLR) and CD36 [36, 43]. The resulting cholesterol-loaded macrophages are frequently termed foam cells due to their "foamy" appearance. In advanced lesions, foam cells apoptose, release cholesteryl esters, and form a necrotic core under a fibrous cap (**Figure 2, Part II**). This cap can be unstable and burst, triggering the formation of a thrombus, increasing the risk of a heart attack or stroke. Minimizing macrophage cholesterol accumulation is important in the prevention of foam cell formation and atherosclerotic lesion progression [44].

3. Macrophage lipid homeostasis

Macrophage lipid homeostasis is an important process in the evolution of atherosclerotic plaque [41]. Cholesterol accumulates in arterial wall macrophages, as described in the previous section (**Figure 2**). Cholesterol in the macrophage's cytoplasm is esterified by acetyl-CoA acetyltransferase (ACAT)-1 and packaged into lipid droplets by a variety of proteins, including adipocyte differentiation-related protein (ADFP) [42]. Macrophages are unable to compensate for this influx of cholesterol, even though there is a down-regulation of 3-hydroxy-3-methyl-glutaryl (HMG)CoA reductase, the rate-limiting enzyme for cholesterol synthesis [43]. The uptake of LDL in macrophages is not a feedback inhibited process. Although, cholesterol is vital to all cells, excess free cholesterol is toxic and must be removed by cholesterol efflux [44]. Macrophages regulate cellular free cholesterol levels through esterification of cholesterol and by accelerating cholesterol efflux to HDL, mediated primarily by ATP-binding cassette (ABC) transporters A1 and G1, and scavenger receptor (SR)-B1 [45, 46] (**Figure 2, Part**

I). Subsequently, HDL particles transport cholesterol from these peripheral tissues back to the liver, through reverse cholesterol transport (RCT) [36].

The first step of RCT occurs in peripheral tissues such as the macrophage, where intracellular neutral cholesteryl ester hydrolase (NCEH)-1 hydrolyzes stored cholesteryl esters, releasing free cholesterol into the cytoplasm [47]. Cholesterol transporter, ABCA1, transfers free cholesterol to extracellular apolipoprotein (apo) A1, the major protein of HDL. ABCG1 and SR-B1 transfer free cholesterol directly to HDL particles [48-50]. When macrophage LDL uptake exceeds efflux, the cells accumulate cholesterol, promoting atherosclerotic lesion formation [41].

Although elevated HDL cholesterol concentrations are associated with decreased CVD risk [51, 52], the mechanisms by which many elements of the RCT system are regulated have yet to be fully elucidated. Both macrophage ABC-transporter activity and NECH1 minimize lesion development by facilitating macrophage cholesterol efflux [47] [41]. In contrast, ADFP has been show to facilitate lesion development by promoting macrophage cholesterol accumulation [42].

Studies using selective inhibition/activation of ABC-transporters in mouse macrophages, *in vivo*, have found that in hyperlipidemic mice (such as apo E-deficient and LDLR-deficient mice) increased macrophage ABCA1 expression resulted in decreased lesion formation [53-55]. The significance of this observation in individuals with glucose intolerance is unresolved [56]. Mitigating factors may be the effect of elevated glucose and FFA concentrations, frequently associated with poor glycemic control.

4. Regulation of macrophage cholesterol efflux

ABC-transporters are a super-family of trans-membrane proteins that couple ATP hydrolysis with trans-membrane substrate transport [57]. Two of these transporters, ABCA1 and ABCG1, are integral to the efflux of cholesterol to plasma HDL particles. There are two additional proteins located on the cell membrane which have also been demonstrated to facilitate cholesterol efflux, caveolins and SR-B1 [58]. Additionally, cholesterol efflux can also occur via passive diffusion by differences in concentration gradient.

In individuals with Tangier Disease, there are mutations in the ABCA1 gene [59-62]. Individuals with Tangier Disease have low plasma HDL-C concentrations, associated with cholesterol accumulation in a variety of peripheral tissues including macrophages, tonsils, liver, and spleen as well as potentially resulting in peripheral neuropathy [63]. Fibroblasts collected from Tangier Disease patients have substandard/impaired apoA1 binding, low rates of cholesterol efflux, and individuals with this disease have increased incidence of early atherosclerosis development [59-62, 64]. In addition to Tangier Disease, mutations in other ABC-transporter genes have been demonstrated to contribute to other diseases such as cystic fibrosis [65] and retinal degeneration [66, 67].

ABCG1 is a “half transporter” which must homodimerize with itself or heterodimerize with other ABCG family proteins in order to function [68]. ABCA1 preferentially associates with lipid-poor apoA1 to promote cholesterol efflux [69, 70], whereas ABCG1 preferentially associates with HDL2 and HDL3 [50, 71]. There is some evidence that the activity of ABC transporters are coordinated; when one is inhibited the other increases activity [72].

4.1. Transcriptional regulation of ABC-transporters

ABC-transporters are regulated at the transcriptional level by liver X receptor (LXR)-α, a ligand-activated transcription factor which is a member of the nuclear hormone receptor superfamily [73]. In order to act as a promoter, LXR-α forms an obligate heterodimer with retinoid X receptor (RXR) and binds to the promoter region of a specific gene. When a ligand binds to LXR-α, this induces a conformational change that results in binding to the promoter region of target genes such as ABCA1 and ABCG1 [74]. Ligands of LXR-α are derivatives of cholesterol, termed oxysterols, which are in abundance in cholesterol-loaded cells. RXR is activated by its ligand, the vitamin A derivative 9-cis-retinoic acid (9CRA) [75]. Binding of either of these ligands to the respective transcription factors can promote transcription of the target gene, but binding of both oxysterols and 9CRA can lead to a synergistic induction in promoter activity [74]. Therefore, upon loading a cell with cholesterol, production of oxysterols up-regulate genes such as ABCA1 and ABCG1.

Peroxisome proliferator-activated receptors (PPAR) are another family of transcription factors that bind to RXR to promote transcription of genes. PPAR-α and γ activators (fibrate and glitasone pharmaceutical therapies) have been shown to induce macrophage ABCA1 expression through induction of LXR-α [76]. This may be an important pathway to examine in the context of T2DM and elevated FFA concentrations because unsaturated fatty acids have been reported to induce PPAR [77] and impair LXR-α activity [78].

There are many physiological stimuli suggested to play a role in regulation of ABC-transporters: inflammatory cytokines (tumor necrosis factor-α, interferon-γ, interleukin-

1β), oxysterols, retinoids, hormones, glucose and unsaturated fatty acids [79, 80]. Many, but not all, of the mechanisms proposed for the transcriptional regulation of ABC-transporter expression involve LXR-α.

LXR-α is indirectly involved in regulating de novo lipogenesis by modifying levels of sterol regulatory element binding protein (SREBP)-1c [81]. SREBPs act as transcriptional activators for genes involved in biosynthesis of cholesterol, phospholipids, fatty acids and TG. Three isoforms, SREBP-1a, -1c and 2 play different roles in this family of lipid biosynthesis regulators. SREBP-1c may be directly involved in promoting transcription of ABCG1 [82], while there is contrasting evidence reporting that SREBP-2 is either involved in promoting or repressing ABCA1 transcription [83, 84]. Overall, the role of SREBP in ABC-transporter expression has not been widely studied so this was a mechanism that was approached in this thesis work.

4.2. Post-translational regulation of ABC-transporters

ABCA1 has an estimated half-life of 1-3 hours [85-87]. Due to such a short half-life, post translational regulation may play an important role in determining the activity of these transporters. Protein kinases, PKA and PKC, have been implicated in the regulation of ABCA1 [88-90]. Phosphorylation of ABCA1 by PKA appears to help facilitate apoA-1 mediated phospholipid efflux [88, 89]; while phosphorylation by PKC increases ABCA1 turnover [90]. PKA and PKC appear to affect ABCA1 efflux activity without affecting the ability of ABCA1 to interact with apoA1; however, another kinase, janus kinase 2 phosphorylates ABCA1 to enhance binding of apoA1 [89]. In contrast, casein kinase 2 appears to constitutively phosphorylate ABCA1 in such a way in which absence of this phosphorylation leads to an increase in apoA1 binding and efflux [91].

The pathway of ABCA1 phosphorylation by PKC has been demonstrated to be activated by the unsaturated fatty acids [28, 29, 86], as will be discussed further. It is unclear whether there is similar post-translational control over ABCG1 expression.

4.3. Regulation of SR-B1

SR-B1 associates not only with HDL, but also with modified LDL, facilitating the bidirectional movement of lipids between cells and lipoprotein particles [92]. Although the human form of SR-B1 has not been studied as extensively as the mouse SR-B1 homolog, their physiological roles are assumed to be similar [93]. Data suggest that PPAR-γ influences SR-B1 expression but it is unclear whether this effect is mediated by LXR-α [94], as has been suggested for the influence of PPAR-γ on ABC-transporter expression [95]. Lack of consistent data regarding the regulation of SR-B1 and prominence in cholesterol efflux among different macrophage models makes its inclusion in our study important.

5. T2DM and elevated glucose and FFA concentrations influence cholesterol efflux

Macrophage cholesterol efflux is impaired in individuals with T2DM [96, 97]. Human monocyte-derived macrophages isolated and differentiated from individuals with T2DM compared to nomoglycemic individuals have lower ABCG1 mRNA and protein expression and cholesterol efflux rates [96, 97]. Additionally, hyperglycemia has been associated with suppression of leukocyte ABCA1 mRNA expression [98].

Cholesterol efflux and ABC-transporter expression is also impaired in animal models of diabetes (**Table 1**), such as a streptozotocin-induced diabetic hyperlipidemic swine model [99] and diabetic mouse models, including T1DM models (streptozotocin-induced

diabetic mice, cyclophosphamide-induced non-obese diabetic [NOD] mice and LDLR-null mice crossed with mice transgenic for lymphocytic choriomenigitis virus glycoprotein [LDLR/GP mice] [78, 100]) and T2DM models (mice transgenic for the leptin receptor [*db/db*] and KK mice crossed with Ay/a mice [*KKay*] [101]). Diabetic, hyperlipidemic swine and LDLR/GP mice have been found to develop extensive atherosclerotic lesions and have lower macrophage ABCA1 protein expression compared to non-diabetic animals [99, 100]. Corresponding results have been reported in other diabetic mouse models [78, 100, 101]. Streptozotocin-induced diabetic mice have lower peritoneal macrophage ABCA1 mRNA [78] and NOD mice have lower ABCA1 protein expression and apoA1-mediated cholesterol efflux than control mice [100]; whereas *Db/db, KKay* and NOD mice have lower ABCG1 mRNA and protein expression and impaired HDL-mediated cholesterol efflux in peritoneal macrophages than control mice [78, 100, 101]. These results are consistent with other data reporting that ABCA1 is the key transporter of cholesterol to apoA1, whereas ABCG1 is the key transporter to HDL [50]. These human and animal data suggest that diabetes mellitus is associated with a decrease in macrophage ABC-transporter expression and cholesterol efflux.

In order to understand the mechanisms responsible for the findings of macrophage cholesterol efflux impairment in individuals with T2DM, there have been several *in vitro* studies to test metabolic factors that are often elevated in individuals with T2DM: glucose and FFA. Elevated glucose and FFA concentrations have been found to decrease either apoA1- or HDL-mediated cholesterol efflux. However, the effect on ABC-transporters has been variable among different macrophage model systems [28, 29, 79, 86, 101].

	Model	Description of model	Athero. Lesion	Chol. Efflux	ABCA1 mRNA	ABCA1 protein	ABCG1 mRNA	ABCG1 protein	Ref.
T1DM models	Diabetic swine	Streptozotocin-induced	↑			↓			[99]
	LDLR/GP mice	Transgenic	↑		↔	↓			[100]
	NOD mice	Cyclophophamide-induced		↓A	↔	↓	↓	↓	[100]
	Diabetic mice	Streptozotocin-induced			↓				[78]
T2DM models	Db/db mice	Transgenic		↓H	↔		↓	↓	[101]
	KKay mice	Transgenic		↓h	↔		↓	↓	[101]

A = apoA1-mediated cholesterol efflux was impaired (but not HDL-mediated efflux)

H = HDL-mediated cholesterol efflux was impaired (but not apoA1-mediated efflux)

h = HDL-mediated cholesterol efflux was impaired (apoA1-mediated efflux not tested)

Table 1. Atherosclerosis, ATP-binding cassette (ABC)-transporter expression and cholesterol efflux in diabetic animal models. Outcome measures used for comparison include: atherosclerosis lesion (athero. lesion), macrophage cholesterol efflux (chol. efflux), and macrophage ABCA1 and ABCG1 mRNA and protein. Up or down arrows indicate significant differences ($p<0.05$) in outcomes for each model compared to control (non-diabetic) animals, as described in each reference. Shaded area indicates outcomes that were not measured in specific models.

5.1. Elevated glucose and cholesterol efflux, in vitro

Human monocyte-derived macrophages (HMDM) exposed to 25 mM glucose *in vitro* down-regulate both ABCA1 and ABCG1 mRNA and protein expression [79]. But these results have not been consistently reported in other macrophage models. In peritoneal macrophages elicited from C57BL/6J mice, 25 mM glucose concentrations suppressed ABCG1 mRNA and protein expression [101]; but, unlike that observed in human macrophages [79], elevated glucose concentrations did not affect ABCA1 expression [101].

In studies conducted in immortalized macrophage cell lines ABC-transporter expression is often induced artificially with LXR-ligands (such as 8-Br-cAMP, 22-(R)-hydroxycholesterol/9-cis-retinoic acid (22R-HC), or TO901317), which reduces the translational application of these data.

RAW 264.7 macrophages (pre-stimulated with 8-Br-cAMP) were not found to alter ABCA1 mRNA expression in response to elevated glucose concentrations [78, 86].

However, in a separate study J774 macrophages (also pre-stimulated with 8-Br-cAMP) were found to respond to glycolaldehyde or glyoxal concentrations by destabilizing ABCA1 protein and suppressing cholesterol efflux [99]. Glycolaldehyde and glyoxal are AGE precursors, and their concentrations are relatively high in hyperglycemic conditions such as T2DM [102]. Therefore, it is possible that there is an effect of AGE or AGE precursors on ABCA1 protein turnover, but not an effect of elevated glucose concentrations on ABCA1 at the mRNA level in these immortalized macrophage cell lines. These results differ from the primary human macrophage (HMDM) study explained previously [79] potentially because of differences in response to metabolic stimulus or threshold for a response in immortalized cell lines compared to primary macrophages. To explore the regulation of ABC-transporter expression, previous studies have utilized immortalized cell lines, which are often relatively easy to transfect with small interfering RNA (siRNA) [103]. However, given the pervasive use of immortalized cell lines to understand the role of macrophages in atherosclerosis mechanisms it is important to understand the complexity in translating such results [103, 104].

5.2. Elevated FFA concentrations and cholesterol efflux, in vitro

Similar to the effect of elevated glucose concentrations on ABC-transporter expression, the effect of FFA has been somewhat inconsistent across macrophage models. In HMDM, 100 uM 18:2 exposure decreased ABCA1, but not ABCG1 mRNA expression [79]. In contrast, in J774 cells (pre-stimulated with Br-8-cAMP) ABCA1 mRNA expression was unaffected by 125 uM 18:2 exposure [86]. However, there are data to suggest that there is a suppressive effect of unsaturated fatty acids (18:1, 18:2, and

20:5), but not saturated fatty acids, on ABCA1 and ABCG1 gene promoters in human ABCA1 and ABCG1 promoter-transfected RAW 264.7 macrophages (stimulated with 22R-HC) due to disruption of LXR-α activity on these promoters [78, 105, 106]. The picture is further complicated by the PPAR-stimulating properties of unsaturated fatty acids, which have been reported to increase transcription of LXR-α [77]. This effect seems to be overridden by the direct suppression by unsaturated fatty acids of LXR-α activity and thus ABC-transporter transcription, but it is clear that the suppressive effect of unsaturated fatty acids on ABC-transporter mRNA expression is not consistent in all macrophage models.

In contrast to the inconsistent data on the effect of unsaturated fatty acids on macrophage ABC-transporter mRNA expression, there is a clear suppressive effect of unsaturated fatty acids on cholesterol efflux in immortalized macrophage cell lines [28, 29, 78, 106]. Whether this effect is due to changes in ABCA1 or ABCG1 protein expression, or both, has not been resolved. In HMDM, 100 uM 18:2 markedly suppressed both ABCA1 and ABCG1 protein expression [79]. Exposure of RAW 264.7, J744 and THP-1 macrophages (as well as non-macrophage cell lines such as HepG2 liver cells and baby hamster kidney cells) to unsaturated fatty acids has also resulted in reduced ABC-transporter protein expression and cholesterol efflux [28, 29, 78, 86, 105, 106].

A promising mechanism has been identified that may account for increased ABCA1 protein turnover in response to elevated unsaturated fatty acid concentrations and possibly elevated glucose concentrations. Fatty acids and glucose increase intracellular diacyl glycerol (DAG) concentrations, which activate PKC –α, -β and –δ [14]. PKC-δ, in

turn, phosphorylates ABCA1, leading to degradation of the transporter [28, 29]. Mechanisms linked to ABCG1 protein disruption have not been thoroughly studied.

6. Research Objectives:

The primary objective of this thesis research was to study the effects of elevated glucose and FFA concentrations on ABC-transporter expression and cholesterol efflux in human primary immune cells (monocytes and leukocytes) and primary murine bone marrow-derived macrophage (BMDM) cells. Human monocytes and leukocytes and murine BMDM were chosen as the models for these studies rather than immortalized cell lines because the data derived are more similar to normal physiology. Multiple immortalized macrophage cell lines have been used to study the effects of elevated glucose and FFA concentrations on ABC-transporter expression, but few studies have used primary macrophage cells.

While chronic hyperglycemia and elevated FFA concentrations associated with T2DM have been demonstrated to suppress macrophage ABC-transporter expression in humans and mice, the acute effect of an oral glucose challenge has not, to our knowledge, been examined *in vivo*. Since the suppressive effect of elevated glucose concentrations on ABC-transporter expression is less consistent and the effect is often smaller than that observed after exposure to unsaturated fatty acids, there have been fewer mechanistic studies to explore the causal pathways.

Cells stimulated with oxLDL are known to increase ABC-transporter expression through oxysterol (OS)-mediated LXR-α activation (**Figure 3**). Therefore, the model of oxLDL-stimulated BMDM was chosen as a model for the environment of the arterial wall. Our objective was to determine the effect of elevated glucose and FFA

concentrations on macrophage cholesterol efflux and ABC-transporter and SR-B1 expression, in addition to mechanisms potentially responsible for these effects (**Figure 3**).

Data suggest that there is a transcriptional effect of elevated glucose concentrations on ABC-transporter expression independent of LXR-α transcription [101], possibly due to changes in LXR-α activity. One hypothesis explaining this effect is that elevated glucose concentrations lead to a reactive oxygen species (ROS)-associated down-regulation of sirtuin-1 (SIRT1) [107], a deacetylase known to promote removal of acetyl groups from LXR-α, affecting LXR-α activity but not expression [108] (**Figure 3**). Also, as noted previously, the effect of elevated glucose concentrations on ABC-transporter expression may be attributed to AGE products and/or glycation of cholesterol transporters or lipoproteins [99] (**Figure 3**).

We hypothesize that a sterol regulatory element (SRE) in the ABCG1 promoter [83] may facilitate suppression of ABC-transporter expression by unsaturated fatty acids (**Figure 3**). Since unsaturated fatty acids have been reported to down regulate transcription of sterol regulatory element binding protein (SREBP)-1c through reduced LXR activity [109], it will be important to determine whether reduced SREBP-1c expression in response to unsaturated fatty acids may contribute to the suppression of cholesterol efflux.

In addition to transcriptional effects of elevated glucose and FFA concentrations on ABC-transporter activity, unsaturated fatty acids have been demonstrated to suppress cholesterol efflux through DAG-mediated increase in PKCδ, which leads to degradation of ABCA1 [28, 29] (**Figure 3**). It is unclear whether elevated glucose concentrations in

macrophages, which increase intracellular DAG concentrations [14], act through the same posttranslational modifications as unsaturated fatty acids to suppress ABCA1 expression. Also unclear is whether elevated glucose or FFA concentrations alter SR-B1 expression and the mechanism thereof. The elucidation of these mechanisms will expand our understanding of how factors associated with T2DM increase CVD risk.

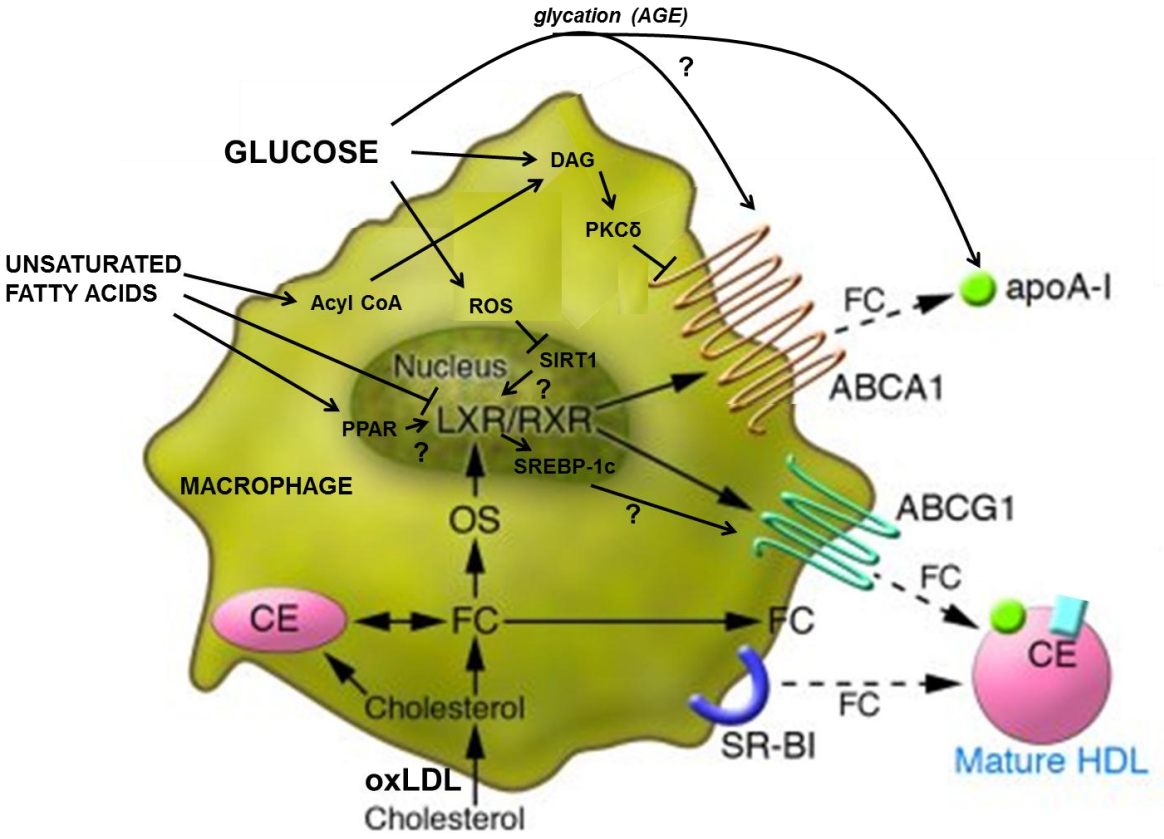

Figure 3. Potential effects of elevated glucose and unsaturated fatty acid concentrations on regulation of macrophage cholesterol efflux. Question marks (?) indicate that many of the pathways have not been confirmed in macrophage models. Advanced glycation end products (AGE), reactive oxygen species (ROS), diacyl glycerol (DAG), phosphokinase-C-δ (PKCδ), sirtuin-1 (SIRT1), peroxisome proliferator-activated receptor (PPAR), liver-x-receptor (LXR), retinoid-x-receptor (RXR), sterol regulatory element-binding protein-1c (SREBP-1c), oxidized low density lipoprotein (oxLDL), high density lipoprotein (HDL), cholesteryl ester (CE), free cholesterol (FC), scavenger receptor-B1 (SR-B1), ATP-binding cassette transporters (ABC) A1 and G1, apolipoprotein (apo) A1 and E. Adapted from Rader D.J., 2006 [110].

7. References

1. Ryden, L., et al., *Guidelines on diabetes, pre-diabetes, and cardiovascular diseases: executive summary. The Task Force on Diabetes and Cardiovascular Diseases of the European Society of Cardiology (ESC) and of the European Association for the Study of Diabetes (EASD).* Eur Heart J, 2007. **28**(1): p. 88-136.
2. Beckman, J.A., M.A. Creager, and P. Libby, *Diabetes and atherosclerosis: epidemiology, pathophysiology, and management.* JAMA, 2002. **287**(19): p. 2570-81.
3. Park, L. and D. Wexler, *Update in diabetes and cardiovascular disease: synthesizing the evidence from recent trials of glycemic control to prevent cardiovascular disease.* Curr Opin Lipidol, 2010. **21**(1): p. 8-14.
4. ADA, *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2012. **35**(1): p. S64-71.
5. Huang, P.L., *A comprehensive definition for metabolic syndrome.* Dis Model Mech, 2009. **2**(5-6): p. 231-7.
6. Haffner, S.M., et al., *Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction.* The New England journal of medicine, 1998. **339**(4): p. 229-234.
7. Cefalu, W.T., *Insulin resistance: cellular and clinical concepts.* Exp Biol Med, 2001. **226**(1): p. 13-26.
8. Kelley, D.E. and J.A. Simoneau, *Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus.* J Clin Invest, 1994. **94**(6): p. 2349-56.
9. Abordo, E.A. and P.J. Thornalley, *Synthesis and secretion of tumour necrosis factor-alpha by human monocytic THP-1 cells and chemotaxis induced by human serum albumin derivatives modified with methylglyoxal and glucose-derived advanced glycation endproducts.* Immunology letters, 1997. **58**(3): p. 139-147.
10. Doi, T., et al., *Receptor-specific increase in extracellular matrix production in mouse mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor.* Proc Natl Acad Sci U S A, 1992. **89**(7): p. 2873-2877.
11. Kirstein, M., et al., *Receptor-specific induction of insulin-like growth factor I in human monocytes by advanced glycosylation end product-modified proteins.* J Clin Invest, 1992. **90**(2): p. 439-446.
12. Schmidt, A.M., et al., *Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes.* J Clin Invest, 1995. **96**(3): p. 1395-1403.
13. Quintao, E.C., W.L. Medina, and M. Passarelli, *Reverse cholesterol transport in diabetes mellitus.* Diabetes Metab Res Rev, 2000. **16**(4): p. 237-250.
14. Koya, D. and G.L. King, *Protein kinase C activation and the development of diabetic complications.* Diabetes, 1998. **47**(6): p. 859-866.

15. Brownlee, M., *The pathobiology of diabetic complications: a unifying mechanism*. 2005: Diabetes. 2005 Jun;54(6):1615-25.
16. Pennathur, S., et al., *A hydroxyl radical-like species oxidizes cynomolgus monkey artery wall proteins in early diabetic vascular disease.* J Clin Invest, 2001. **107**(7): p. 853-860.
17. Pennathur, S. and J.W. Heinecke, *Mechanisms for oxidative stress in diabetic cardiovascular disease.* Antioxidants & redox signaling, 2007. **9**(7): p. 955-969.
18. Davi, G., et al., *In vivo formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation.* Circulation, 1999. **99**(2): p. 224-229.
19. Balkau, B., et al., *Prediction of the risk of cardiovascular mortality using a score that includes glucose as a risk factor. The DECODE Study.* Diabetologia, 2004. **47**(12): p. 2118-28.
20. *Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnostic criteria. The DECODE study group. European Diabetes Epidemiology Group. Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria in Europe.* Lancet, 1999. **354**(9179): p. 617-621.
21. Khaw, K.T., et al., *Glycated haemoglobin, diabetes, and mortality in men in Norfolk cohort of european prospective investigation of cancer and nutrition (EPIC-Norfolk).* Bmj, 2001. **322**(7277): p. 15-8.
22. Turner, R.C., et al., *Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23).* Bmj, 1998. **316**(7134): p. 823-8.
23. Garvey, W.T., et al., *Effects of insulin resistance and type 2 diabetes on lipoprotein subclass particle size and concentration determined by nuclear magnetic resonance.* Diabetes, 2003. **52**(2): p. 453-462.
24. Deeg, M.A., et al., *Pioglitazone and rosiglitazone have different effects on serum lipoprotein particle concentrations and sizes in patients with type 2 diabetes and dyslipidemia.* Diabetes Care, 2007. **30**(10): p. 2458-2464.
25. Shin, H.K., et al., *Remnant lipoprotein particles induce apoptosis in endothelial cells by NAD(P)H oxidase-mediated production of superoxide and cytokines via lectin-like oxidized low-density lipoprotein receptor-1 activation: prevention by cilostazol.* Circulation, 2004. **109**(8): p. 1022-1028.
26. De Pascale, C., et al., *Fatty acid composition of chylomicron remnant-like particles influences their uptake and induction of lipid accumulation in macrophages.* The FEBS journal, 2006. **273**(24): p. 5632-5640.
27. Shi, H., et al., *TLR4 links innate immunity and fatty acid-induced insulin resistance.* J Clin Invest, 2006. **116**(11): p. 3015-3025.
28. Wang, Y. and J.F. Oram, *Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a phospholipase D2 pathway.* J Biol Chem, 2005. **280**(43): p. 35896-903.
29. 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. 1062-8.

30. Wang, Y. and J.F. Oram, *Unsaturated fatty acids inhibit cholesterol efflux from macrophages by increasing degradation of ATP-binding cassette transporter A1.* J Biol Chem, 2002. **277**(7): p. 5692-5697.
31. Wolever, T.M. and C. Mehling, *Long-term effect of varying the source or amount of dietary carbohydrate on postprandial plasma glucose, insulin, triacylglycerol, and free fatty acid concentrations in subjects with impaired glucose tolerance.* Am J Clin Nutr, 2003. **77**(3): p. 612-21.
32. Liu, L., et al., *Free fatty acid metabolic profile and biomarkers of isolated post-challenge diabetes and type 2 diabetes mellitus based on GC-MS and multivariate statistical analysis.* J Chromatogr B Analyt Technol Biomed Life Sci, 2010. **878**(28): p. 2817-25.
33. Mitropoulos, K.A., et al., *Dietary fat induces changes in factor VII coagulant activity through effects on plasma free stearic acid concentration.* Arteriosclerosis and Thrombosis : A Journal of Vascular Biology / American Heart Association, 1994. **14**(2): p. 214-222.
34. Gimbrone, M.A., Jr., *Vascular endothelium, hemodynamic forces, and atherogenesis.* The American journal of pathology, 1999. **155**(1): p. 1-5.
35. Williams, K.J. and I. Tabas, *The response-to-retention hypothesis of early atherogenesis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 1995. **15**(5): p. 551-561.
36. Glass, C.K. and J.L. Witztum, *Atherosclerosis. the road ahead.* Cell, 2001. **104**(4): p. 503-516.
37. Mestas, J. and K. Ley, *Monocyte-endothelial cell interactions in the development of atherosclerosis.* Trends in cardiovascular medicine, 2008. **18**(6): p. 228-232.
38. Kamei, M. and C.V. Carman, *New observations on the trafficking and diapedesis of monocytes.* Current opinion in hematology, 2010. **17**(1): p. 43-52.
39. Anitschkow, N., *On the morphodynamics of coronary sclerosis of the heart.* Acta Pathologica et Microbiologica Scandinavica, 1960. **49**(Journal Article): p. 426-432.
40. Poli, G., et al., *Cholesterol oxidation products in the vascular remodeling due to atherosclerosis.* Mol Aspects Med, 2009. **30**(3): p. 180-9.
41. Tall, A.R., *Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins.* Journal of internal medicine, 2008. **263**(3): p. 256-273.
42. Paul, A., et al., *Deficiency of adipose differentiation-related protein impairs foam cell formation and protects against atherosclerosis.* Circ Res, 2008. **102**(12): p. 1492-1501.
43. Brown, M.S. and J.L. Goldstein, *The LDL receptor and HMG-CoA reductase--two membrane molecules that regulate cholesterol homeostasis.* Current topics in cellular regulation, 1985. **26**(Journal Article): p. 3-15.
44. Tabas, I., *Consequences of cellular cholesterol accumulation: basic concepts and physiological implications.* J Clin Invest, 2002. **110**(7): p. 905-911.
45. Jessup, W., et al., *Roles of ATP binding cassette transporters A1 and G1, scavenger receptor BI and membrane lipid domains in cholesterol export from macrophages.* Curr Opin Lipidol, 2006. **17**(3): p. 247-257.

46. Van Eck, M., et al., *Scavenger receptor BI and ATP-binding cassette transporter A1 in reverse cholesterol transport and atherosclerosis.* Curr Opin Lipidol, 2005. **16**(3): p. 307-315.
47. Sekiya, M., et al., *Ablation of neutral cholesterol ester hydrolase 1 accelerates atherosclerosis.* Cell metabolism, 2009. **10**(3): p. 219-228.
48. Yvan-Charvet, L., et al., *ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation.* Science (New York, N.Y.), 2010. **328**(5986): p. 1689-1693.
49. Kennedy, M.A., et al., *ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation.* Cell metabolism, 2005. **1**(2): p. 121-131.
50. Wang, N., et al., *ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins.* Proc Natl Acad Sci U S A, 2004. **101**(26): p. 9774-9.
51. Assmann, G., et al., *High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM experience and pathophysiological implications for reverse cholesterol transport.* Atherosclerosis, 1996. **124**(20): p. S11-20.
52. National Cholesterol Education Program (NCEP) Expert Panel on Detection, E., and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), *Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report.* Circulation, 2002. **106**(25): p. 3143-421.
53. van Eck, M., et al., *Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues.* Proc Natl Acad Sci U S A, 2002. **99**(9): p. 6298-303.
54. Van Eck, M., et al., *Macrophage ATP-binding cassette transporter A1 overexpression inhibits atherosclerotic lesion progression in low-density lipoprotein receptor knockout mice.* Arterioscler Thromb Vasc Biol, 2006. **26**(4): p. 929-34.
55. Aiello, R.J., et al., *Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2002. **22**(4): p. 630-637.
56. Vergeer, M., et al., *The HDL hypothesis: does high-density lipoprotein protect from atherosclerosis?* J Lipid Res, 2010. **51**(8): p. 2058-2073.
57. Bishop, L., et al., *Reconstitution of a bacterial periplasmic permease in proteoliposomes and demonstration of ATP hydrolysis concomitant with transport.* Proc Natl Acad Sci U S A, 1989. **86**(18): p. 6953-6957.
58. Ohashi, R., et al., *Reverse cholesterol transport and cholesterol efflux in atherosclerosis.* QJM : monthly journal of the Association of Physicians, 2005. **98**(12): p. 845-856.
59. Rust, S., et al., *Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1.* Nature genetics, 1999. **22**(4): p. 352-355.
60. Bodzioch, M., et al., *The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease.* Nature genetics, 1999. **22**(4): p. 347-351.

61. Brooks-Wilson, A., et al., *Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency.* Nature genetics, 1999. **22**(4): p. 336-345.
62. Lawn, R.M., et al., *The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway.* J Clin Invest, 1999. **104**(8): p. R25-31.
63. Fredrickson, D.S., et al., *Tangier DiseaseCombined Clinical Staff Conference at the National Institutes of Health.* Annals of Internal Medicine, 1961. **55**(6): p. 1016-1031.
64. Francis, G.A., R.H. Knopp, and J.F. Oram, *Defective removal of cellular cholesterol and phospholipids by apolipoprotein A-I in Tangier Disease.* 1995. **96**(1): p. 78-87.
65. Riordan, J.R., et al., *Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA.* Science (New York, N.Y.), 1989. **245**(4922): p. 1066-1073.
66. Sun, H., P.M. Smallwood, and J. Nathans, *Biochemical defects in ABCR protein variants associated with human retinopathies.* Nature genetics, 2000. **26**(2): p. 242-246.
67. Allikmets, R., et al., *A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy.* Nature genetics, 1997. **15**(3): p. 236-246.
68. Cserepes, J., et al., *Functional expression and characterization of the human ABCG1 and ABCG4 proteins: indications for heterodimerization.* Biochem Biophys Res Commun, 2004. **320**(3): p. 860-867.
69. Wang, N., et al., *Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1.* J Biol Chem, 2000. **275**(42): p. 33053-33058.
70. Wang, N., et al., *ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein.* J Biol Chem, 2001. **276**(26): p. 23742-23747.
71. Gelissen, I.C., et al., *ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2006. **26**(3): p. 534-540.
72. Tall, A.R., et al., *HDL, ABC transporters, and cholesterol efflux: implications for the treatment of atherosclerosis.* Cell metabolism, 2008. **7**(5): p. 365-375.
73. Repa, J.J. and D.J. Mangelsdorf, *The liver X receptor gene team: potential new players in atherosclerosis.* Nature medicine, 2002. **8**(11): p. 1243-1248.
74. Costet, P., et al., *Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor.* J Biol Chem, 2000. **275**(36): p. 28240-28245.
75. Levin, A.A., et al., *9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha.* Nature, 1992. **355**(6358): p. 359-361.
76. Chinetti, G., et al., *PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway.* Nature medicine, 2001. **7**(1): p. 53-58.
77. Tobin, K.A., et al., *Cross-talk between fatty acid and cholesterol metabolism mediated by liver X receptor-alpha.* Mol Endocrinol, 2000. **14**(5): p. 741-52.

78. Uehara, Y., et al., *Polyunsaturated fatty acids and acetoacetate downregulate the expression of the ATP-binding cassette transporter A1*. Diabetes, 2002. **51**(10): p. 2922-2928.
79. Mauerer, R., S. Ebert, and T. Langmann, *High glucose, unsaturated and saturated fatty acids differentially regulate expression of ATP-binding cassette transporters ABCA1 and ABCG1 in human macrophages.* Experimental and Molecular Medicine, 2009. **41**(2): p. 126.
80. Schmitz, G. and T. Langmann, *Transcriptional regulatory networks in lipid metabolism control ABCA1 expression.* Biochim Biophys Acta, 2005. **15**(1): p. 1-19.
81. Repa, J.J., et al., *Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta.* Genes Dev, 2000. **14**(22): p. 2819-2830.
82. Ecker, J., et al., *Isomer specific effects of Conjugated Linoleic Acid on macrophage ABCG1 transcription by a SREBP-1c dependent mechanism. Biochem Biophys Res Commun*, 2007. 352(3): p. 805-11.
83. Zeng, L., et al., *Sterol-responsive element-binding protein (SREBP) 2 down-regulates ATP-binding cassette transporter A1 in vascular endothelial cells: a novel role of SREBP in regulating cholesterol metabolism.* J Biol Chem, 2004. 279(47): p. 48801-7.
84. Wong, J., C.M. Quinn, and A.J. Brown, *SREBP-2 positively regulates transcription of the cholesterol efflux gene, ABCA1, by generating oxysterol ligands for LXR.* Biochem J, 2006. 400(3): p. 485-91.
85. Vedhachalam, C., et al., *ABCA1-induced cell surface binding sites for ApoA-I.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2007. **27**(7): p. 1603-1609.
86. Wang, Y. and J.F. Oram, *Unsaturated fatty acids inhibit cholesterol efflux from macrophages by increasing degradation of ATP-binding cassette transporter A1*. J Biol Chem, 2002. **277**(7): p. 5692-7.
87. Rechsteiner, M. and S.W. Rogers, *PEST sequences and regulation by proteolysis.* Trends in biochemical sciences, 1996. **21**(7): p. 267-271.
88. Haidar, B., et al., *Apolipoprotein A-I activates cellular cAMP signaling through the ABCA1 transporter.* J Biol Chem, 2004. **279**(11): p. 9963-9969.
89. Tang, C., A.M. Vaughan, and J.F. Oram, *Janus kinase 2 modulates the apolipoprotein interactions with ABCA1 required for removing cellular cholesterol.* J Biol Chem, 2004. **279**(9): p. 7622-7628.
90. Yamauchi, Y., et al., *Apolipoprotein A-I activates protein kinase C alpha signaling to phosphorylate and stabilize ATP binding cassette transporter A1 for the high density lipoprotein assembly.* J Biol Chem, 2003. **278**(48): p. 47890-47897.
91. Roosbeek, S., et al., *Phosphorylation by protein kinase CK2 modulates the activity of the ATP binding cassette A1 transporter.* J Biol Chem, 2004. **279**(36): p. 37779-37788.
92. Valacchi, G., et al., *Scavenger receptor class B type I: a multifunctional receptor.* Ann N Y Acad Sci, 2011. **7**(10): p. 1749-6632.
93. Murao, K., et al., *Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes.* J Biol Chem, 1997. **272**(28): p. 17551-7.

94. Chinetti, G., et al., *CLA-1/SR-BI is expressed in atherosclerotic lesion macrophages and regulated by activators of peroxisome proliferator-activated receptors.* Circulation, 2000. **101**(20): p. 2411-2417.
95. Chawla, A., et al., *A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis.* Molecular cell, 2001. **7**(1): p. 161-171.
96. Zhou, H., et al., *Determinants of leukocyte adenosine triphosphate-binding cassette transporter G1 gene expression in type 2 diabetes mellitus.* Metabolism, 2008. **57**(8): p. 1135-40.
97. Mauldin, J.P., et al., *Reduced expression of ATP-binding cassette transporter G1 increases cholesterol accumulation in macrophages of patients with type 2 diabetes mellitus.* Circulation, 2008. **117**(21): p. 2785-92.
98. Albrecht, C., et al., *Leukocyte ABCA1 gene expression is associated with fasting glucose concentration in normoglycemic men.* Metabolism, 2004. **53**(1): p. 17-21.
99. Passarelli, M., et al., *Advanced glycation end product precursors impair ABCA1-dependent cholesterol removal from cells.* Diabetes, 2005. **54**(7): p. 2198-2205.
100. Tang, C., et al., *Diabetes reduces the cholesterol exporter ABCA1 in mouse macrophages and kidneys.* J Lipid Res, 2010. **51**(7): p. 1719-1728.
101. Mauldin, J.P., et al., *Reduction in ABCG1 in Type 2 diabetic mice increases macrophage foam cell formation.* J Biol Chem, 2006. **281**(30): p. 21216-24.
102. Brownlee, M., A. Cerami, and H. Vlassara, *Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications.* N Engl J Med, 1988. **318**(20): p. 1315-21.
103. Qin, Z., *The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature.* Atherosclerosis, 2012. **221**(1): p. 2-11.
104. Kohro, T., et al., *A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage.* J Atheroscler Thromb, 2004. **11**(2): p. 88-97.
105. Ku, C.S., et al., *Unsaturated fatty acids repress expression of ATP binding cassette transporter A1 and G1 in RAW 264.7 macrophages.* J Nutr Biochem, 2012. **23**(10): p. 1271-6.
106. Uehara, Y., et al., *Unsaturated fatty acids suppress the expression of the ATP-binding cassette transporter G1 (ABCG1) and ABCA1 genes via an LXR/RXR responsive element.* Atherosclerosis, 2007. **191**(1): p. 11-21.
107. Hayek, T., et al., *Macrophage NADPH oxidase activation, impaired cholesterol fluxes, and increased cholesterol biosynthesis in diabetic mice: a stimulatory role for D-glucose.* Atherosclerosis, 2007. **195**(2): p. 277-86.
108. Li, X., et al., *SIRT1 deacetylates and positively regulates the nuclear receptor LXR.* Molecular cell, 2007. **28**(1): p. 91-106.
109. Yoshikawa, T., et al., *Cross-talk between peroxisome proliferator-activated receptor (PPAR) alpha and liver X receptor (LXR) in nutritional regulation of fatty acid metabolism.* Mol Endocrinol, 2003. **17**(7): p. 1240-54.
110. Rader, D.J., *Molecular regulation of HDL metabolism and function: implications for novel therapies.* J Clin Invest, 2006. **116**(12): p. 3090-100.

CHAPTER 3

MANUSCRIPT A:

Regulation of ATP-binding cassette transporters and cholesterol efflux by glucose in primary human monocytes and murine bone marrow-derived macrophages

Regulation of ATP-binding cassette transporters and cholesterol efflux by glucose in primary human monocytes and murine bone marrow-derived macrophages

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Abstract

Background: Individuals with type 2 diabetes mellitus are at increased risk of developing atherosclerosis. This may be partially attributable to suppression of macrophage ATP-binding cassette (ABC) transporter mediated cholesterol efflux by sustained elevated blood glucose concentrations. Two models were used to assess this potential relationship: human monocytes/leukocytes and murine bone marrow-derived macrophages (BMDM).

Methods: Ten subjects (4F/6M, 50-85yrs, BMI 25-35kg/m^2) underwent an oral glucose challenge. Baseline and 1- and 2-hour post-challenge ABC-transporter mRNA expression was determined in monocytes, leukocytes and peripheral blood mononuclear cells (PBMC). In a separate study, murine-BMDM were exposed to 5mmol/L D-glucose (control) or additional 20mmol/L D- or L-glucose and 25ug/mL oxidized low density lipoprotein (oxLDL). High density lipoprotein (HDL)-mediated cholesterol efflux and ABC-transporter (ABCA1 and ABCG1) expression were determined.

Results: Baseline ABCA1and ABCG1 expression was lower (>50%) in human monocytes and PBMC than leukocytes ($p<0.05$). One hour post-challenge leukocyte ABCA1 and ABCG1 expression increased by 37% and 30%, respectively ($p<0.05$), and began to return to baseline thereafter. There was no significant change in monocyte ABC-transporter expression. In murine BMDM, higher glucose concentrations suppressed HDL-mediated cholesterol efflux (10%; $p<0.01$) without significantly affecting ABCA1 and ABCG1 expression.

Conclusion: Data demonstrate that leukocytes are not a reliable indicator of monocyte ABC-transporter expression. Human monocyte ABC-transporter gene

expression was unresponsive to a glucose challenge. Correspondingly, in BMDM, hyperglycemia attenuated macrophage cholesterol efflux in the absence of altered ABC-transporter expression, suggesting that hyperglycemia, *per se*, suppresses cholesterol transporter activity. This glucose-related impairment in cholesterol efflux may potentially contribute to diabetes-associated CVD.

1. Introduction

Individuals with poor glycemic control, such as type 2 diabetes mellitus, are at increased risk of developing atherosclerosis [1-3]. In the arterial wall, monocyte-derived macrophages engulf modified low density lipoprotein (LDL), playing a key role in the progression of atherosclerosis [4, 5]. High density lipoprotein (HDL) particles can modify arterial cholesterol accumulation by facilitating reverse cholesterol transport (RCT), the process by which excess cholesterol is removed from macrophages in the arterial wall [5].

Critical to the RCT process are the ATP binding cassette (ABC) transporters ABCA1 and ABCG1, and the scavenger receptor (SR)-B1, located on the macrophage membrane [5-7]. Transcription of ABC-transporters is tightly regulated by liver-x-receptor (LXR)-alpha (α) [6, 7]. The importance of these transporters in atherosclerosis progression has been observed both in animal models [8-10] and patients with Tangier Disease, the latter carrying mutations in the ABCA1 gene [11-13].

Data from human and animal studies suggest that hyperglycemia may suppress ABC-transporters in monocytes/macrophages [14-21]. Due to the relative difficulty of isolating human monocytes without activating their differentiation into macrophages, either leukocytes, peripheral blood mononuclear cells (PBMC) or lymphocytes are often used as a surrogate for monocytes in studies designed to evaluate gene and protein expression [20-22]. Leukocytes, otherwise referred to as white blood cells, include a mixture of granulocytes (neutrophils, eosinophils, and basophils) and PBMC (primarily monocytes/macrophages and lymphocytes). Some data suggest that PBMC may not be a good indicator of monocyte ABC-transporter expression [22]. Data are lacking as to

whether leukocyte ABC-transporter expression is a good indicator of monocyte expression.

The aims of this work were to assess the reliability of leukocyte ABC transporter expression to serve as an indicator of monocyte expression and to determine the effect of elevated glucose concentrations on cholesterol efflux in primary monocyte/macrophage models. To address these aims (1) leukocyte and monocyte ABC-transporter expression was characterized, in vivo, in response to an acute change in blood glucose concentrations, and (2) ABC-transporter and SR-B1 expression, and macrophage cholesterol efflux in murine bone marrow-derived macrophages (BMDM) was determined, in vitro, in response to elevated glucose concentrations.

2. Methods

2.1. Subjects

Ten subjects (four postmenopausal women and six men; age 50-85 years; BMI 25-35 kg/m^2) were recruited to participate in this study. Our sample size varied from 4-9 for some outcome measures due to limited sample volumes (see figure legends for details). Subjects were excluded from participation if they took medications known to affect glucose metabolism, gastrointestinal motility or lipid metabolism, and if they reported consuming more than 7 alcoholic drinks per week. Subjects were asked to refrain from consuming alcohol or engaging in strenuous physical activity 72 hours and fast overnight (12 hours) prior to the test day. This protocol was approved by the Human Investigation Review Committee of Tufts University/Tufts Medical Center. All subjects gave written informed consent.

2.2. Oral Glucose Challenge

A baseline blood sample (time=0) was collected after an overnight fast from the medial antecubital vein. Thereafter, subjects were given an oral glucose challenge, 50 g glucose/50 mL water, which is the standard amount of carbohydrate employed during a glycemic index test. Additional blood samples were drawn 30, 60 and 120 minutes post challenge. Blood was collected in EDTA containing tubes and plasma was immediately separated by centrifugation at 1100 x g at 4oC for 15 minutes. During the 2 hour time period, subjects were restricted to sedentary activities. Plasma glucose concentrations were measured using an automated chemistry analyzer (Olympus AU400) with reagents from Beckman-Coulter (Brea, CA). Plasma insulin concentrations were measured using a radioimmunoassay (Human Insulin Specific RIA Kit; Millipore, St Charles, MO). Additionally, blood was collected in BD vacutainer cell preparation tubes (CPT) containing sodium citrate (Becton Dickinson GmbH, Heidelberg, Germany) and PAXgene blood RNA tubes (Qiagen, Valencia, CA) for analysis of gene expression, described below. Preliminary experiments were conducted at additional time points (**Appendix A, Section 1**).

2.3. Magnetic-activated cell sorting (MACS)

PBMC were separated from other blood cells using the BD vacutainer CPT. Monocytes were isolated from PBMC using Monocyte Isolation Kit II with a magnetic cell sorter (autoMACS pro, Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, PBMC were incubated with biotin-conjugated monoclonal antibodies to CD3, CD7, CD16, CD19, CD56, CD123 and glycophrin A, followed by incubation with anti-biotin monoclonal antibodies conjugated to magnetic beads. The autoMACS pro was used to

deplete PBMC of magnetically labeled cells, leaving a monocyte-rich fraction. To determine the potential effect of the cell sorter procedure on PBMC gene expression, PBMC from fasting blood samples (*n=4*) were incubated with the Monocyte Isolation Kit II antibodies conjugated to magnetic beads but were not subjected to cell sorting.

2.4. Murine-BMDM Cell Culture

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were maintained 2 per cage, 12:12, light:dark cycle and fed a standard mouse chow diet (Harlan Teklad 7012) in accordance with institutional guidelines. The protocol was approved by the institutional review board of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University. Mice were killed at the age of 10-12 weeks by CO2 followed by cervical dislocation. Bone marrow cells were isolated from the femurs and tibias by flushing the bone cavity with RPMI medium (Gibco, Life Technologies, Grand Island, NY) as previously described [23]. The harvested cells were washed, plated and differentiated into BMDM with 100 ng/mL macrophage-colony stimulating factor (eBioscience, San Diego, CA) and grown in medium containing 20% low endotoxin fetal bovine serum (FBS) (Gibco, Life Technologies) and 1% streptomycin/penicillin (Sigma-Aldrich, St. Louis, MO) in humidified air at 37°C in 5% CO2. After 4-5 days, macrophages adhered to the culture dishes, allowing non-adherent cells to be discarded. BMDM differentiation was confirmed by monitoring the expression of the cell surface marker F4/80, preferentially expressed by mature macrophages [23]. Passage of BMDM was achieved by incubation (37°C, 10 minutes) with 8 mg/mL lidocaine solution (Sigma-Aldrich). After passage, BMDM were pre-treated for 16 hours with 5 to 25 mmol/L D- or L-glucose (Sigma-Aldrich) in the presence of 20% lipoprotein deficient (LD)-FBS.

Preliminary experiments were conducted to support the development of the BMDM model for this study (**Appendix A, Section 2-6**). L-glucose was used to control for osmolality. LD-FBS was obtained by ultracentrifugation of FBS at a density of 1.215 g/ml. Cellular lipid accumulation and mRNA and protein expression were assessed after exposure to 25 ug/mL oxidized LDL (oxLDL) (Intracel, Frederick, MD) for 24 hours.

2.5. Quantitative real-time PCR

Leukocyte RNA was extracted from whole blood using the PAXgene blood RNA tubes and the PAXgene blood RNA kit (Qiagen). RNA was extracted from human PBMC, monocytes, PBMC depleted of monocytes, and murine BMDM using TRIzol reagent (Ambion, Life Technologies) and Rneasy mini kit (Qiagen). Reverse transcription was performed using the Reverse Transcription Kit (Promega, Madison, WI). Real Time PCR was performed using Qiagen's Quantitect primer assays for human ABCA1, ABCG1, and beta (β)-actin (QT00064869, QT00021035, QT01680476) and murine ABCA1, ABCG1, SR-B1, LXR-α and β-actin (QT00165690, QT00113519, QT00166495, QT00113729, QT01136772). Relative quantification (ΔΔCt) was used to assess expression of target genes in the BMDM samples, using β-actin as the reference gene.

2.6. Western blot analysis

BMDM were lysed in RIPA buffer (Sigma-Aldrich) containing protease inhibitors (Bio-Rad, Hercules, CA). Protein concentrations were determined by MicroBCA protein assay (Bio-Rad). Proteins were separated by SDS-PAGE (Bio-Rad) under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad). Quantification by Western blotting was performed using the following primary antibodies: ABCA1

(Abcam, Cambridge, MA), ABCG1 (Santa Cruz Biotechnology, Santa Cruz, CA), SR-B1 (Santa Cruz), LXR-α (Abcam), and β-actin (Sigma-Aldrich). Secondary antibodies were purchased from Santa Cruz Biotechnology. Signals were visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified using a GS-800 calibrated densitometer (Bio-Rad).

2.7. Assessment of BMDM lipid composition

After lipid extraction [24], cellular triglyceride (TG) content was determined using a colorimetric kit (Sigma-Aldrich). Cellular total cholesterol (TC) and free cholesterol (FC) content was determined by gas chromatography using 5-α cholestane as an internal standard [25]. Cholesteryl esters (CE) were calculated as the difference between TC and FC. Cellular lipids (FC, TC, CE and TG) were expressed as μg/mg cellular protein.

2.8. Cholesterol efflux assay

After 16 hr incubation with different glucose concentrations BMDM were incubated with 1 uCi/ml [^{3}H] cholesterol (Perkin Elmer, Waltham, MA) and 25 ug/mL oxLDL for 24 hours [26, 27]. Immediately thereafter the cells were equilibrated in RPMI containing 2 mg/mL fatty acid-free bovine serum albumin for 6 hours. BMDM were then incubated with either 50 ug/mL HDL (Intracel) or 10 ug/mL apoA1 (Sigma-Aldrich) in serum-free media for 4 hours [27]. D- or L-glucose concentrations in the media were maintained constant throughout the experiments. Fraction cholesterol efflux was calculated as the ratio of the radiotracer in the medium to the total (medium + cells), normalized for cellular protein.

2.9. Statistical analysis

All data are reported as mean ± standard deviation (SD). Student's t-test or analysis of variance (ANOVA) was used to test for significant differences between or among groups, respectively. Post-hoc comparisons were performed using Tukey's test (SAS version 9.3, SAS Institute Inc, Cary, NC). A p-value < 0.05 was considered significant.

3. Results

3.1. In vivo, fasting PBMC and monocyte ABC-transporter mRNA is lower than in leukocytes

To assess the reliability of leukocyte and PBMC expression of ABC transporters as surrogate indicators of monocyte ABCA1 and ABCG1 expression, comparisons were made across these cell types isolated from subjects at time=0. The expression of ABCA1 and ABCG1 mRNA was significantly lower in monocytes (51% [$p<0.05$] and 85% [$p<0.0001$], respectively) and PBMC (57% and 53% [both $p< 0.01$]) than in total leukocytes (**Figure 1A and B**), suggesting that leukocyte expression is not a good surrogate for monocyte expression. To rule out the possibility that the antibodies used during the cell sorting protocol changed ABC-transporter mRNA expression, PBMC were incubated with and without the sorting antibodies conjugated to magnetic beads. There was no significant effect of treatment with antibodies on ABC-transporter mRNA expression.

While subsequent cell sorting had no significant effect on ABCA1 mRNA expression in monocytes or PBMC depleted of monocytes (**Figure 1A**), a significantly lower expression of ABCG1 mRNA was observed in monocytes ($p< 0.01$) than in PBMC depleted of monocytes (**Figure 1B**). These results indicate that the antibodies conjugated to magnetic beads had no significant effect on ABC-transporter expression in

PBMC, but there is a differential expression of ABC-transporters in various blood cell types.

3.2. Leukocyte ABC-transporter expression increases during an oral glucose challenge

Having established that the isolation procedure did not affect the measures of interest, leukocyte and monocyte ABC-transporter expression was evaluated during a 2-hour oral glucose (50 g) challenge. Mean plasma glucose and insulin concentrations peaked at 60 minutes and then began to return to baseline (**Figure 2**). Average leukocyte ABCA1 and ABCG1 mRNA expression increased significantly by 37% ($p< 0.01$) and 30% ($p<0.05$), respectively, at 60 minutes post-glucose challenge but did not remain significantly elevated through 120 minutes (**Figure 3A and B**). There was no significant effect of the glucose challenge on monocyte ABCA1 or ABCG1 expression (**Figure 3C and D**). These results suggest that monocyte ABC-transporter mRNA expression is not sensitive to an acute increase in plasma glucose concentrations, in vivo. On the basis of these data we assessed the effect of high glucose concentrations on cholesterol efflux and ABC-transporter expression *in vitro*.

3.3. Elevated glucose concentrations suppress HDL-mediated cholesterol efflux in BMDM

Treatment of BMDM with high D- and L-glucose concentrations resulted in lower HDL-mediated cholesterol efflux (10% [$p< 0.01$] and 5% [$p < 0.05$], respectively) compared to the low D-glucose concentration (**Figure 4A**). There was no significant effect of either D- or L-glucose concentration on apoA1-mediated cholesterol efflux. Since L-glucose is not metabolized by the cells, these results suggest that the effect of

high glucose on HDL-mediated cholesterol efflux is not solely dependent on glucose metabolism.

3.4. OxLDL exposure, but not glucose, stimulates BMDM CE accumulation

To assess the effect of high glucose on lipid accumulation, BMDM were cultured in 5 mmol/L or 25 mmol/L glucose, followed by exposure to oxLDL. Treatment of BMDM with oxLDL for 24 hours resulted in an increase in CE content from undetectable levels to approximately 30 ug/mg protein ($p< 0.0001$) in the absence of significant changes in FC or TG content (**Figure 4C**). The increase in CE content was similar across the different glucose concentrations **(Figure 4B)**. Moreover, glucose concentration in the media did not affect the FC or TG cellular content.

3.5. Elevated glucose concentrations do not affect BMDM ABC-transporter expression

Incubation of BMDM with oxLDL resulted in increased ABCA1, ABCG1 and LXR-α mRNA expression and a corresponding increase in ABCA1 protein levels ($p< 0.0001$, **Figure 5A, B and C**). There was no significant effect of oxLDL on SR-B1 mRNA expression or protein levels. These data indicate that exposure to oxLDL in BMDM up-regulates some of the transporters involved in cholesterol efflux. Although high D- and L-glucose decreased HDL-mediated cholesterol efflux (**Figure 4A**), there was no significant effect on cholesterol transporter or LXR- α mRNA or protein expression (**Figure 5A, B and C**). In absence of an effect of high glucose concentration on ABC-transporter and SR-B1 transcripts or protein expression, our data suggest that high glucose concentration may impair HDL-mediated cholesterol efflux by attenuating transporter activity.

4. Discussion

ABC-transporters play an important role in monocyte/macrophage cellular cholesterol balance [5]. Monocyte/macrophage ABC-transporters are involved in the first step of RCT and forestall atherosclerotic lesion progression [5]. Hyperglycemia has been implicated as a potential factor in the disruption of RCT by down-regulating monocyte/macrophage ABC-transporter expression and subsequent cholesterol efflux [14-21, 28]. Using BMDM as an experimental model, our results demonstrate that elevated glucose concentrations attenuate cholesterol efflux. In contrast, elevated glucose concentrations in the media had no significant effect on ABC-transporter expression in BMDM or in human monocytes following an oral glucose challenge.

Due to technical challenges in isolating primary monocytes/macrophages, human leukocytes and other blood cell fractions (such as PBMC or lymphocytes), as well as immortalized cell lines, have often been used as surrogate cells in studies intended to assess the mechanisms related to cellular cholesterol balance [14, 19, 21, 28]. One aim of this study was to determine the effect of an oral glucose challenge on monocyte ABC-transporter expression and to determine whether leukocyte ABC-transporter expression correlated with this effect. Since there was no significant change in monocyte ABC-transporter expression following the glucose challenge, the increase observed in leukocyte ABC-transporter expression may have been coincident with, rather than directly related to, monocyte/macrophage cellular cholesterol balance. Alternatively, the increase in leukocyte ABC-transporter expression may have been associated with the decrease in plasma free fatty acid concentration which has previously been demonstrated to occur during a glucose challenge [19, 29, 30]. It is important to note that changes in

leukocyte ABC-transporter mRNA expression represent an average of multiple cell types and therefore may not be used as evidence of changes in monocyte gene expression.

Monocyte ABCA1 and ABCG1 mRNA expression in a commercial preparation of human monocytes has been reported to be higher than in PBMC [22]. In contrast, our results indicate that in non-activated human monocytes ABC-transporter expression is similar to PBMC and lower than in leukocytes. The discrepancy between prior work and our work is likely due to an artifact of the isolation procedures which alters ABC-transporter activation. For example, isolation of monocytes from PBMC by plate adherence has been reported to accelerate conversion to human monocyte-derived macrophages (HMDM) and increases ABC-transporter expression [19]. By design, in the present study, monocytes were isolated by magnetic-activated cell sorting to minimize conversion to macrophages. This method was chosen because it has a shorter sample processing time prior to RNA extraction, hence, maximizes the ability to detect an effect of the glucose challenge on ABC-transporter expression were one present.

Monocyte ABC-transporter expression has not previously been examined in response to a glucose challenge in vivo. However, in vitro studies using a range of 5-25 mmol/L glucose have documented a suppressive effect on HMDM ABC-transporter expression [19]. In our study, the mean change in plasma glucose response was modest, only 5.6 to 8.3 mmol/L (100 to 150 mg/dL), after an oral glucose challenge in normoglycemic subjects. This may explain the lack of effect on ABC-transporter expression.

In light of this null effect of the oral glucose challenge on human monocyte ABC-transporter expression, it was of interest to determine the impact of elevated glucose concentrations on ABC-transporter expression in a primary macrophage model, BMDM,

when exposed to oxLDL. Consistent with prior observations, exposing BMDM to oxLDL resulted in intracellular CE accumulation [27], and an increase in ABCA1 and ABCG1 mRNA transcripts and ABCA1 protein expression, possibly mediated by changes in LXR-α activity. However, the suppressive effect of high glucose concentrations on BMDM cholesterol efflux, in the absence of changes in ABC-transporter or SR-B1 protein expression, was unexpected. We hypothesize that the contrast between our results and prior work [19] may be due to stimulation of BMDM with oxLDL. This stimulation may have overridden potential effects of elevated glucose on cholesterol transporter expression. In the current study, elevated D- and L-glucose concentrations may have acted directly to inhibit ABC-transporter activity. We were unable to determine whether this effect was mediated by glycation or glycoxidation of transporters or lipoproteins, which has the potential to suppress cholesterol efflux [31], or an osmotic effect of elevated glucose concentrations.

5. Conclusion

This is the first study, to our knowledge, to demonstrate that there is a difference between ABC-transporter expression in human leukocytes compared to monocytes and PBMC. Additionally, our data suggest that elevated glucose concentrations impair HDL-mediated cholesterol efflux and that this effect is not mediated by transcript or protein expression but may be mediated by an attenuated transporter activity. Future studies should focus on primary monocytes and macrophages to address issues related to elevated glucose concentrations and cholesterol efflux and ABC-transporter expression, rather than using other blood cells or immortalized cell lines as surrogates.

Conflict of Interest

None declared.

Sources of funding

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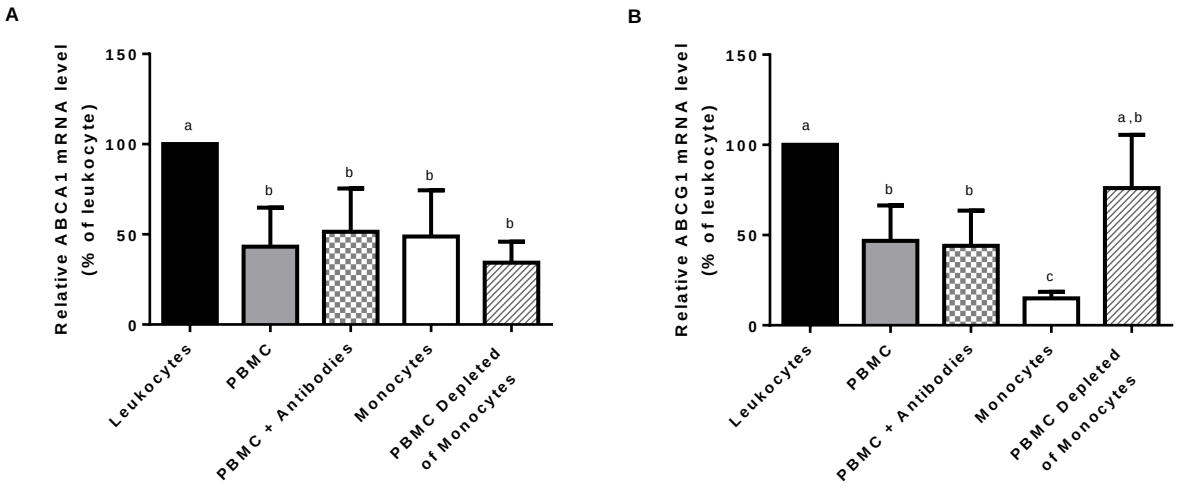

Figure 1. Percent differences of mRNA expression in cells isolated from subjects in the fasting state. (A) ABCA1 and (B) ABCG1 mRNA expression was evaluated by real-time PCR. β-actin was used as the standard housekeeping gene. The abundance of mRNA in leukocytes was defined as 100%, and the amounts of ABCA1 and ABCG1 mRNA from each cell fraction were then expressed as percentages of this control value. Values represent mean ± SD (n=4). Bars without common letters are significantly different, p<0.05.

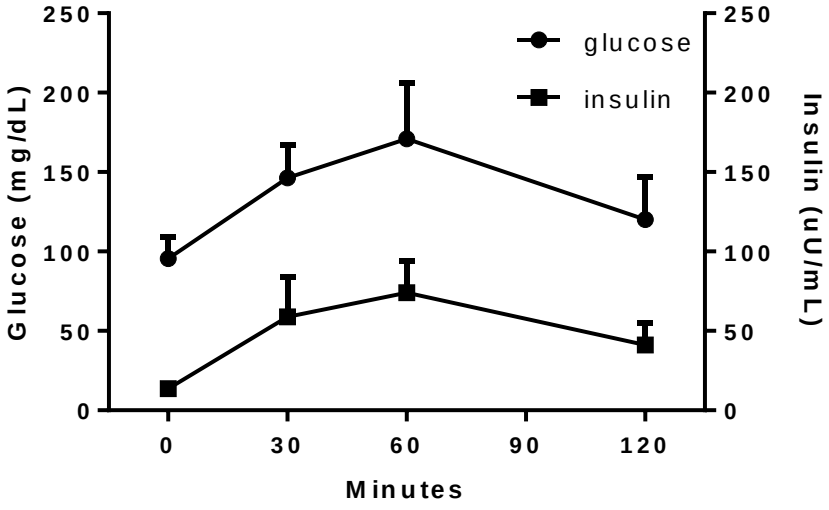

Figure 2. **Plasma glucose (mg/dL) and insulin (uU/mL) concentrations during an oral glucose challenge**. Values represent mean ± SD (*n*=9).

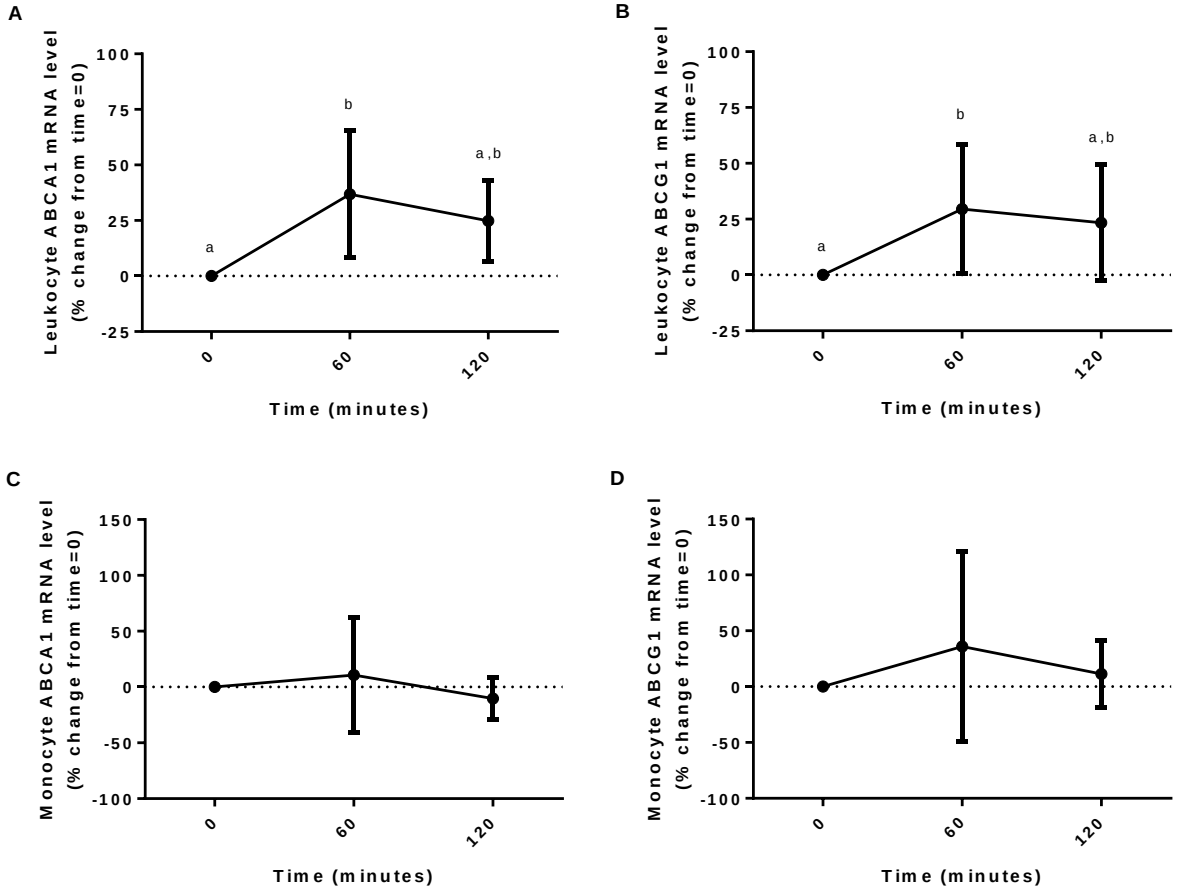

Figure 3. Effect of an oral glucose challenge on ABC-transporter expression in leukocytes and monocytes. (A) Leukocyte ABCA1 and (B) ABCG1 and (C) monocyte ABCA1 and (D) ABCG1 mRNA expression was determined by real-time PCR. β-actin was used as a standard housekeeping gene. Values represent mean ± SD; leukocytes (n=9) or monocytes (n=7). Outcome values without common letters are significantly different, $p<0.05$.

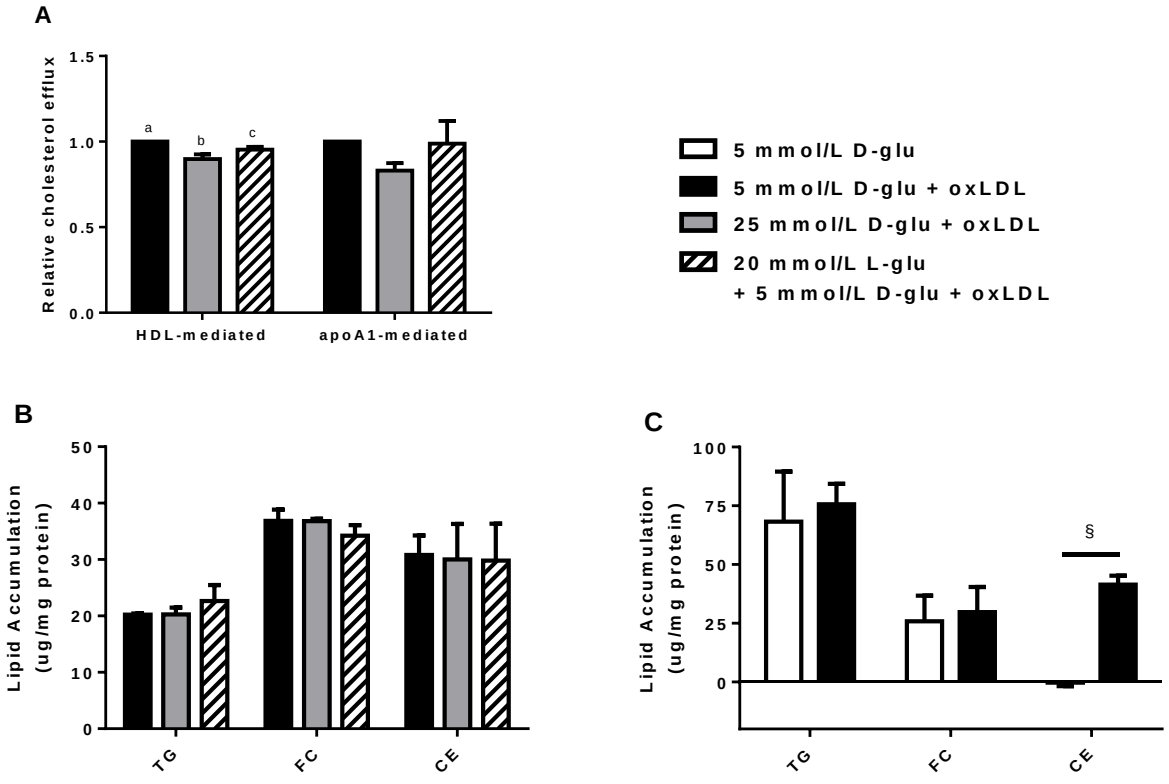

Figure 4. Effect of elevated glucose (glu) on lipid accumulation and cholesterol efflux in BMDM. (A) OxLDL- and [^{3}H] cholesterol-loaded BMDM were stimulated with 50 ug/mL HDL or 10 ug/mL apoA1 for 4 hours. Fractional cholesterol efflux was estimated from the ratio of the radiotracer in the medium to the total (medium + cells) and normalized for cellular protein. (B and C) Lipid was first extracted from cells. Triglyceride (TG) was determined by colorimetric TG Accumulation Kit to analyze the effect of glucose concentrations (B) and oxLDL (C) on lipid accumulation. Free cholesterol (FC) and cholesteryl esters (CE) were determined using gas chromatography. Values are representative of 3 independent experiments in triplicate. Within each comparison, bars without common letters are significantly different, $p<0.05$. §$p <$ 0.0001 for the comparison of 5 mmol/L D-glu with or without oxLDL.

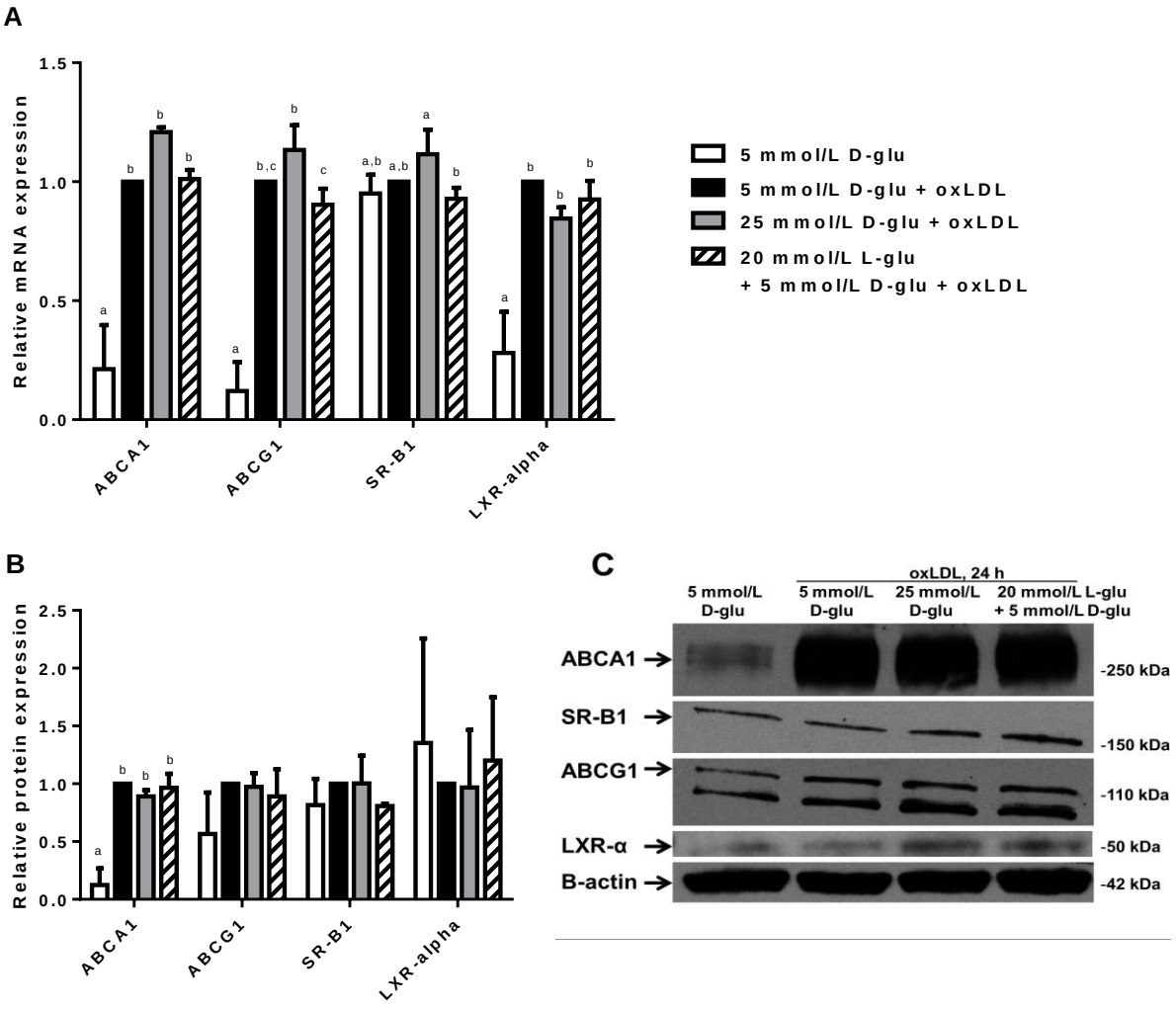

Figure 5. Effect of elevated glucose (glu) and oxLDL on ABCA1, ABCG1, SR-B1 and LXR-α mRNA and protein expression in BMDM. (A) ABCA1, ABCG1, SR-B1 and LXR-α mRNA expression was determined by real-time PCR. β-actin was used as a standard housekeeping gene. (B) ABCA1, ABCG1, SR-B1, LXR-α and β-actin protein expression was determined by Western blot analysis of BMDM cell lysates. β-actin was used to control equal loading. (C) A representative Western blot used for evaluation of protein expression (B). Values are representative of 3 independent experiments in triplicate. Within each gene or protein, bars without common letters are significantly different, $p<0.05$.

6. References

1. Ryden, L., et al., *Guidelines on diabetes, pre-diabetes, and cardiovascular diseases: executive summary. The Task Force on Diabetes and Cardiovascular Diseases of the European Society of Cardiology (ESC) and of the European Association for the Study of Diabetes (EASD).* Eur Heart J, 2007. **28**(1): p. 88-136.
2. Beckman, J.A., M.A. Creager, and P. Libby, *Diabetes and atherosclerosis: epidemiology, pathophysiology, and management.* JAMA, 2002. **287**(19): p. 2570-81.
3. Park, L. and D. Wexler, *Update in diabetes and cardiovascular disease: synthesizing the evidence from recent trials of glycemic control to prevent cardiovascular disease.* Curr Opin Lipidol, 2010. **21**(1): p. 8-14.
4. Williams, K.J. and I. Tabas, *The response-to-retention hypothesis of early atherogenesis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 1995. **15**(5): p. 551-561.
5. Glass, C.K. and J.L. Witztum, *Atherosclerosis. the road ahead.* Cell, 2001. **104**(4): p. 503-516.
6. Jessup, W., et al., *Roles of ATP binding cassette transporters A1 and G1, scavenger receptor BI and membrane lipid domains in cholesterol export from macrophages.* Curr Opin Lipidol, 2006. **17**(3): p. 247-257.
7. Wang, X. and D.J. Rader, *Molecular regulation of macrophage reverse cholesterol transport.* Current opinion in cardiology, 2007. **22**(4): p. 368-372.
8. Aiello, R.J., et al., *Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2002. **22**(4): p. 630-637.
9. van Eck, M., et al., *Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues.* Proc Natl Acad Sci U S A, 2002. **99**(9): p. 6298-303.
10. Van Eck, M., et al., *Macrophage ATP-binding cassette transporter A1 overexpression inhibits atherosclerotic lesion progression in low-density lipoprotein receptor knockout mice.* Arterioscler Thromb Vasc Biol, 2006. **26**(4): p. 929-34.
11. Bodzioch, M., et al., *The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease.* Nature genetics, 1999. **22**(4): p. 347-351.
12. Brooks-Wilson, A., et al., *Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency.* Nature genetics, 1999. **22**(4): p. 336-345.
13. Rust, S., et al., *Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1.* Nature genetics, 1999. **22**(4): p. 352-355.
14. Passarelli, M., et al., *Advanced glycation end product precursors impair ABCA1-dependent cholesterol removal from cells.* Diabetes, 2005. **54**(7): p. 2198-2205.
15. Tang, C., et al., *Diabetes reduces the cholesterol exporter ABCA1 in mouse macrophages and kidneys.* J Lipid Res, 2010. **51**(7): p. 1719-1728.
16. Mauldin, J.P., et al., *Reduction in ABCG1 in Type 2 diabetic mice increases macrophage foam cell formation.* J Biol Chem, 2006. **281**(30): p. 21216-24.

17. Mauldin, J.P., et al., *Reduced expression of ATP-binding cassette transporter G1 increases cholesterol accumulation in macrophages of patients with type 2 diabetes mellitus.* Circulation, 2008. **117**(21): p. 2785-92.
18. Zhou, H., et al., *Determinants of leukocyte adenosine triphosphate-binding cassette transporter G1 gene expression in type 2 diabetes mellitus.* Metabolism, 2008. **57**(8): p. 1135-40.
19. Mauerer, R., S. Ebert, and T. Langmann, *High glucose, unsaturated and saturated fatty acids differentially regulate expression of ATP-binding cassette transporters ABCA1 and ABCG1 in human macrophages.* Experimental and Molecular Medicine, 2009. **41**(2): p. 126.
20. Albrecht, C., et al., *Leukocyte ABCA1 gene expression is associated with fasting glucose concentration in normoglycemic men.* Metabolism, 2004. **53**(1): p. 17-21.
21. Ghanbari-Niaki, A., M. Saghebjoo, and M. Hedayati, *A single session of circuit-resistance exercise effects on human peripheral blood lymphocyte ABCA1 expression and plasma HDL-C level.* Regul Pept, 2011. **166**(1-3): p. 42-7.
22. DiBlasio-Smith, E.A., et al., *Discovery and implementation of transcriptional biomarkers of synthetic LXR agonists in peripheral blood cells.* J Transl Med, 2008. **6**: p. 59.
23. Nguyen, M.T., et al., *A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways.* J Biol Chem, 2007. **282**(48): p. 35279-92.
24. Schwartz, D.M. and N.E. Wolins, *A simple and rapid method to assay triacylglycerol in cells and tissues.* J Lipid Res, 2007. **48**(11): p. 2514-20.
25. Matthan, N.R., et al., *Impact of simvastatin, niacin, and/or antioxidants on cholesterol metabolism in CAD patients with low HDL.* J Lipid Res, 2003. **44**(4): p. 800-806.
26. 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. 1062-8.
27. Meyer, J.M., et al., *High-capacity selective uptake of cholesteryl ester from native LDL during macrophage foam cell formation.* J Lipid Res, 2012. **53**(10): p. 2081-91.
28. Zhou, H., et al., *Cellular cholesterol efflux to serum is impaired in diabetic nephropathy.* Diabetes Metab Res Rev, 2008. **24**(8): p. 617-23.
29. Uehara, Y., et al., *Unsaturated fatty acids suppress the expression of the ATP-binding cassette transporter G1 (ABCG1) and ABCA1 genes via an LXR/RXR responsive element.* Atherosclerosis, 2007. **191**(1): p. 11-21.
30. Punyadeera, C., et al., *Weight-related differences in glucose metabolism and free fatty acid production in two South African population groups.* Int J Obes Relat Metab Disord, 2001. **25**(8): p. 1196-205.
31. Machado-Lima A., et al. *Advanced glycated albumin isolated from poorly controlled type 1 diabetes mellitus patients alters macrophage gene expression impairing ABCA-1-mediated reverse cholesterol transport.* Diabetes/metabolism research and reviews. 2013;29(1):66-76.

CHAPTER 4

MANUSCRIPT B:

Linoleic acid suppresses cholesterol efflux and ATP-binding cassette transporters in murine bone marrow-derived macrophages

Linoleic acid suppresses cholesterol efflux and ATP-binding cassette transporters in murine bone marrow-derived macrophages

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Abstract

Individuals with type 2 diabetes mellitus (T2DM) are at increased risk of developing cardiovascular disease (CVD), possibly associated with elevated plasma free fatty acid concentrations. Paradoxically, evidence suggests that unsaturated fatty acids, rather than saturated fatty acids, suppress macrophage cholesterol efflux, favoring cholesterol accumulation in the artery wall. Murine bone marrow-derived macrophages (BMDM) were used to further explore the relationship between elevated saturated and unsaturated fatty acids, and cholesterol efflux mediated by ATP-binding cassette transporters (ABCA1 and ABCG1) and transcription factors liver-x-receptor-alpha (LXR-α) and sterol receptor element binding protein (SREBP)-1. BMDM isolated from C57BL/6 mice were exposed to 100 uM linoleic acid (18:2) or palmitic acid (16:0) for 16 hr, and 25 ug/mL oxidized low density lipoprotein for an additional 24 hr. Relative to control, 18:2 suppressed ABCA1 mRNA expression to a greater extent than 16:0 (60% and 30%, respectively) and ABCG1 mRNA expression (54% and 29%, respectively) (all $p<0.01$). 18:2 decreased high density lipoprotein (HDL) mediated cholesterol efflux by 53% and ABCA1 protein levels by 94% (both $p<0.05$), and had no significant effect on ABCG1, LXR-α or SREBP-1 protein levels. 16:0 had no effect of HDL-mediated cholesterol efflux or ABC-transporter, LXR-α or SREBP-1 protein expression. These results suggest that 18:2, relative to 16:0, attenuated macrophage HDL-mediated cholesterol efflux through down regulation of ABCA1 mRNA and protein levels but not through changes in LXR-α or SREBP-1 expression. The effect of 18:2 relative to 16:0 on macrophage cholesterol homeostasis may exacerbate the predisposition of individuals with T2DM to increased CVD risk.

1. Introduction

High density lipoprotein (HDL) particles promote reverse cholesterol transport via their interaction with macrophage membrane proteins, ATP-binding cassette (ABC) transporters and scavenger receptor (SR)-B1 [1]. Two components of this system, HDL concentrations [2, 3] and macrophage ABC-transporter expression [4, 5] are low in individuals with type 2 diabetes mellitus (T2DM) [6]. These individuals are at increased risk of developing atherosclerosis.

Among the metabolic disturbances associated with T2DM are chronically elevated free fatty acids (FFA) concentrations [7]. One class of FFA, unsaturated fatty acids, has been reported to suppress expression of the macrophage ABC-transporters ABCA1 and ABCG1, resulting in reduced cholesterol efflux [8-11]. In healthy individuals as well as those with T2DM, linoleic (18:2) and palmitic acid (16:0) are among the highest circulating FFA and therefore were the focus of this study [12]. Both ABCA1 and ABCG1 protein expression have been reported to be suppressed by 18:2 in human monocytes-derived macrophages [11]. ABC-transporter expression and cholesterol efflux are regulated at multiple levels. With few exceptions [11] most of the work done on the relationship between FFA and ABC-transporter expression has been conducted using immortalized macrophage cell lines.

At the post-translational level, 18:2 has been reported to increase ABCA1 protein turnover via protein kinase C (PKC)-δ activation in RAW 264.7 macrophages [8, 9]. At the transcriptional level, ABC-transporters are regulated by liver-x-receptor (LXR)-α [13, 14]. Polyunsaturated fatty acids (PUFA) such as 18:2 and eicosapentaenoic acid (20:5), and to a lesser extent monounsaturated fatty acids (MUFA) such as oleic acid (18:1), have

been reported to disrupt LXR-α activity on ABC-transporter expression in transfected RAW 264.7 macrophages [10]. PUFA have also been reported to down regulate transcription of sterol regulatory element binding protein (SREBP)-1c through reduced LXR-α activity [15]. There is a sterol regulatory element (SRE) in the promoter for the ABCG1 gene [16], suggesting an additional mechanism by which unsaturated fatty acids may regulate SREBP-1c activity and subsequent cholesterol efflux. The role of SREBP-2 in ABC-transporter expression is more controversial, with contrasting evidence suggesting that SREBP-2 is either a promoter or repressor of ABCA1 transcription [17, 18].

While there are several mechanisms by which unsaturated fatty acids may cause a suppression of ABC-transporter expression, the entire regulatory process of cholesterol transporters has not been fully elucidated. The aim of this study was to assess the mechanism by which 18:2 disrupts transcriptional regulation of ABC-transporters and cholesterol efflux using bone marrow-derived macrophages (BMDM), a primary macrophage cell model, compared to 16:0, focusing on the role of SREBP-1c in mediating the relationship between fatty acids and macrophage ABC-transporter expression.

2. Methods

2.1. Murine-BMDM Cell Culture

Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were maintained 2 per cage, 12:12, light:dark cycle and fed a standard mouse chow diet (Harlan Teklad 7012) in accordance with institutional guidelines. Mice were killed at the age of 10-12 weeks by CO2 followed by cervical dislocation. Bone marrow cells were isolated from the femurs

and tibias by flushing the bone cavity with RPMI medium (Gibco, Life Technologies, Grand Island, NY) as previously described [19]. The harvested cells were washed, plated and differentiated into BMDM with 100 ng/mL macrophage-colony stimulating factor (eBioscience, San Diego, CA) and grown in medium containing 20% low endotoxin fetal bovine serum (FBS) (Gibco, Life Technologies) and 1% streptomycin/penicillin (Sigma-Aldrich, St. Louis, MO) in humidified air at 37°C in 5% CO2. After 4-5 days, macrophages had adhered to the culture dishes allowing non-adherent cells to be discarded. BMDM differentiation was confirmed by monitoring the expression of the cell surface marker F4/80, preferentially expressed in mature macrophages [19]. Passage of BMDM was achieved by incubation (37°C, 10 minutes) with 8 mg lidocaine /mL phosphate buffered saline (Sigma-Aldrich). After passage, cells were pre-treated for 16 hours with 0 or 100 uM 18:2 or 16:0 (Nu-Chek Prep, Elysian, MN) in the presence of 50 uM low endotoxin, fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich) and 20% lipoprotein deficient (LD)-FBS. Free fatty acid concentrations in the media were maintained throughout the experiment. Fatty acids:BSA stock solutions (1 mM:0.5 mM) were made up in RPMI [20]. Fatty acid treatment concentrations were maintained throughout the experiment. LD-FBS was obtained by ultracentrifugation of FBS at a density of 1.215 g/ml. Lipid accumulation, mRNA and protein expression were assessed, as described below, after exposure to 25 ug/mL oxidized low density lipoprotein (oxLDL) (Intracel, Frederick, MD) for 24 hours. Preliminary experiments were conducted with a 200 uM mixture of FFA (66.7 uM 18:2, 66.7 uM 16:0, and 66.7 uM oleic acid [18:1]) (**Appendix A, Section 7**).

2.2. Quantitative real-time PCR

RNA was extracted from BMDM using TRIzol reagent (Ambion, Life Technologies) and Rneasy mini kit (Qiagen, Valencia, CA). Reverse transcription was performed using the Reverse Transcription Kit (Promega, Madison, WI). Real Time PCR was performed using Qiagen's Quantitect primer assays for murine ABCA1, ABCG1, SR-B1, LXR-α and beta (β)-actin (QT00165690, QT00113519, QT00166495, QT00113729, QT01136772). Primers were designed for the following genes (**Table 1**): SREBP-1a, 1c, 2, acetyl CoA carboxylase (ACC), and stearoyl CoA desaturase 1 (SCD1). All templates were initially denatured for 5 minutes at 95˚C, followed by a standard thermal cycle (repeated 40 times): denaturation at 94˚C for 1 min, annealing at 55˚C for 30 sec, and extension at 72˚C for 34 sec. Relative quantification (ΔΔCt) was used to assess expression of target genes and standardized to internal reference gene, β-actin.

2.3. Western blot analysis

BMDM were lysed in RIPA buffer (Sigma-Aldrich) containing protease inhibitors (Bio-Rad, Hercules, CA) for whole cell protein extraction. Nuclear protein extraction was performed as previously described [21]. Briefly, cells were resuspended in a fractionation buffer containing protease inhibitors (Bio-Rad), passed through a 22.5 gauge needle 30 times, and centrifuged at 1000 x g for 7 min at 4°C to remove membrane protein. The pellet was resuspended in another fractionation buffer containing protease inhibitors (Bio-Rad), rotated at 4°C for 1 hr, and centrifuged at 100,000 x g for 30 min at 4°C in a Beckman TLA 100.2 rotor. The supernatant from this spin was designated the nuclear extract.

Whole cell and nuclear protein concentrations were determined by MicroBCA protein assay (Bio-Rad). Proteins were separated by SDS-PAGE (Bio-Rad) under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad). Quantification by Western blotting was performed using the following primary antibodies: ABCA1 (Abcam, Cambridge, MA), ABCG1 (Santa Cruz Biotechnology, Santa Cruz, CA), SR-B1 (Santa Cruz), LXR-α (Abcam), SREBP-1 (Abcam), and β-actin (Sigma-Aldrich). Secondary antibodies were purchased from Santa Cruz Biotechnology. Signals were visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantified using a GS-800 calibrated densitometer (Bio-Rad).

2.4. Assessment of BMDM lipid composition

After lipid extraction [22] cellular triglyceride (TG) content was determined using a colorimetric enzyme-linked kit (Sigma-Aldrich). Cellular total cholesterol (TC) and free cholesterol (FC) content was determined by gas chromatography using 5-α cholestane as an internal standard as previously described [23]. Cholesteryl ester (CE) was calculated as the difference between TC and FC. Cellular lipids (FC, TC, CE and TG) were expressed as ug/mg cellular protein.

2.5. Cholesterol efflux assay

BMDM were pre-incubated with 1 uCi/ml [^{3}H] cholesterol (Perkin Elmer, Waltham, MA) and 25 ug/mL oxLDL for 24 hours [9, 24]. Immediately thereafter the cells were equilibrated in RPMI containing 2 mg/mL fatty acid-free BSA for 6 hours. BMDM were then incubated with 50 ug/mL HDL (Intracel) in serum-free media for 4 hours [24]. FFA concentrations in the media were maintained constant throughout the incubation period.

Fraction cholesterol efflux was estimated from the ratio of the radiotracer in the medium to the total (medium + cells) and normalized for cellular protein.

2.6. Statistical analysis

All data are reported as mean ± standard deviation (SD). Student's t-test or analysis of variance (ANOVA) was used to test for significant differences among groups. Post-hoc comparisons were performed using Tukey's test (SAS version 9.3, SAS Institute Inc, Cary, NC). A p-value < 0.05 was considered significant.

3. Results

3.1. OxLDL, but not 18:2 or 16:0, increases CE accumulation

To characterize the model of oxLDL-stimulated BMDM, the effects of oxLDL on lipid accumulation was examined. After 24 hours of exposure to oxLDL CE concentrations increased from undetectable levels to approximately 30 ug CE/mg protein (**Figure 1A**, $p<0.0001$) whereas there was no significant effect on FC and TG concentrations. In a separate set of experiments pretreatment of BMDM with 100 uM 18:2 or 16:0, followed by exposure to oxLDL, had no additional effects on lipid accumulation (**Figure 1B**). From these data we concluded that, in the presence of oxLDL, exposure to 18:2 or 16:0 did not alter BMDM lipid accumulation.

3.2. 18:2 suppresses HDL-mediated cholesterol efflux

The effect of pre-treating BMDM with 18:2 and 16:0 on cholesterol efflux was next assessed. Exposure of BMDM to 100 uM 18:2, but not 16:0, resulted in 53% lower cholesterol efflux (**Figure 2**, $p<0.05$). On the basis of these data cholesterol transporter expression was next assessed.

3.3. 18:2 decreases ABCA1 mRNA and protein expression

Pre-treating BMDM with 18:2 and 16:0 and then exposing the cells to oxLDL decreased ABCA1 mRNA expression by 60% ($p<0.001$) and 30% ($p<0.01$), respectively, and ABCG1 mRNA expression by 54% ($p<0.0001$) and 29% ($p<0.01$), respectively (**Figure 3A**) compared to BSA + oxLDL treated cells. In contrast, 18:2, but not 16:0, suppressed SR-B1 mRNA expression by 21% (**Figure 3A**, $p<0.05$) and ABCA1 protein expression by 93% (**Figure 3B**, $p<0.05$). There was no significant effect of either fatty acid on LXR-α mRNA or protein expression, or ABCG1 and SR-B1 protein expression. These data suggest that the effect of 18:2 and 16:0 on ABC-transporter mRNA expression is not mediated by regulation of LXR-α expression. On the basis of these data, we next examined the relationship between 16:0 and 18:2, and the transcription factor SREBP-1c. SREBP-1c has been suggested to be involved in regulation of ABC-transporters at the transcriptional level [16, 25].

3.4. 18:2 decreases SREBP-1 mRNA, but not SREBP-1 nuclear protein expression

In oxLDL-stimulated BMDM, treatment with 18:2, but not 16:0, suppressed SREBP-1c mRNA expression by 97% ($p<0.0001$), consistent with suppression of mRNA expression of its target genes, SCD1 by 97% ($p<0.001$) and ACC by 48% ($p<0.05$), (**Figure 4A**). Expression of SREBP-1a mRNA was likewise suppressed by 18:2 (28% [$p<0.05$]), but not 16:0, whereas SREBP-1 nuclear protein expression was unaffected by exposure to16:0 or 18:2 (**Figure 4B and C**). These data suggest that SREBP-1 did not play a major role in the effect of 18:2 or 16:0 on ABC-transporter expression.

4. Discussion

Elevated circulating FFA concentrations, in particular unsaturated fatty acids, may contribute to the accelerated development of CVD in individuals with T2DM. Diet, lifestyle, and certain disease states alter total circulating FFA concentrations [12]. For example, in individuals with T2DM tend to have twice the FFA concentration of healthy subjects, resulting in two-fold higher 18:2 concentrations [12].

In the current study, we found that 18:2, but not 16:0, caused a sharp suppression of cholesterol efflux from BMDM after exposure to an acceptor, HDL. This suppression may occur at multiple levels of cellular regulation. Exposure of BMDM to high18:2 concentrations led to a suppression in ABC-transporter gene expression as well as ABCA1 protein expression. These changes likely contributed to the corresponding suppression of cholesterol efflux.

Studies implicating activation of PKC-δ and impairment of LXR-α activity as the mechanisms responsible for ABC-transporter suppression in response to unsaturated fatty acids have been reported previously in immortalized cell lines [8-10]. However, the translation of results from immortalized cell lines to the *in vivo* state must be done cautiously. For example, THP-1 macrophages differ from primary human macrophages in LXR-α activation in response to stimuli [26]. Mechanistic work to identify the role transcriptional regulation has on ABC-transporters has not previously been reported in primary macrophage models. Therefore, the use of an *ex vivo* primary macrophage model, BMDM, was used to further explore this issue.

While LXR-α directly affects ABC-transporter expression, it also influences SREBP-1c mRNA expression [15]. Previous research suggests that PUFA disrupts LXR-α

activity on the SREBP-1c promoter, leading to a strong suppression of SREBP-1c mRNA expression [15]. Data from the current study are consistent with this observation. While neither 18:2 or 16:0 had a significant effect on LXR-α expression, we could not rule of the possibility that these fatty acids affected LXR-α activity, as indicated in previous studies [10]. SREBP-1c is predicted to have a binding site in the ABCG1 promoter region [16]. On this basis we hypothesized that SREBP-1c suppression would mediate the effect of 18:2 on ABC-transporter expression. However, we found that although SREBP-1c and 1a mRNA expression were reduced by 18:2, SREBP-1 nuclear protein expression was unaffected. Our results differ from previous studies which reported that nuclear maturation of SREBP-1 is lower in response to unsaturated than saturated fatty acids [15]. We cannot rule out the possibility that the inconsistency in the data is due to the presence of oxLDL in our study which may have overridden the effect of 18:2 on SREBP-1 nuclear protein. Evidence from previous studies suggest that LXR-α and PKC-δ mediate the suppressive effect of 18:2 on cholesterol efflux [8, 9].

Results from our study suggest that 18:2 suppresses macrophage HDL-mediated cholesterol efflux through down regulation of ABC-transporter expression and that this suppression is not mediated by effects on SREBP-1c expression. The effect of 18:2 on macrophage cholesterol efflux may be partially responsible for the increased risk of CVD in individuals with T2DM, associated with elevated circulating FFA concentrations.

Conflict of Interest

None declared.

Sources of funding

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Gene	Primer sequence	Reference
SREBP-1a	F 5'-ACACAGCGGTTTTGAACGACATC-3' R 5'-ACGGACGGGTACATCTTTACAG-3'	[27]
SREBP-1c	F 5'-GGAGCCATGGATTGCACATT-3' R 5'-ACGGACGGGTACATCTTTAC-3'	Adapted from [27]
SREBP-2	F 5'-CACAATATCATTGAAAAGCGCTACC-3' R 5'-TTTTTCTGATTGGCCAGCTTCAGCA-3'	[27]
SCD1	F 5'-CTGACCTGAAAGCCGAGAAG-3' R 5'-GCGTTGAGCACCAGAGTGTA-3'	[28]
ACC	F 5'-ACAGTGAAGGCTTACGTCTG-3' R 5'-AGGATCCTTACAACCTCTGC-3'	[27]

* Other primers were purchased from Qiagen as indicated in *Methods 2.2*

Table 1. Real Time PCR primer sequences for SREBP-1a, 1c, 2, SCD1 and ACC

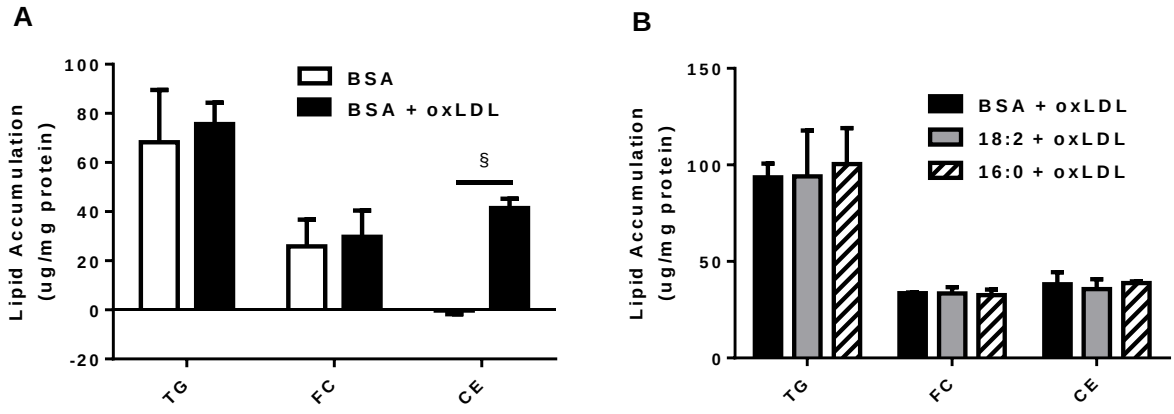


Figure 1. Effect of (A) oxLDL (B) oxLDL +/- FFA on lipid accumulation in BMDM. Lipids were first extracted from cells. Triglyceride (TG) was determined using a colorimetric TG Accumulation Kit to analyze the effect of oxLDL (A) and FFA (B) on lipid accumulation. total and free cholesterol (TC and FC) were determined by gas chromatography, cholesteryl esters (CE) was determined by the difference between TC and FC. Values are representative of 3 independent experiments performed in triplicate. §$p < 0.0001$ for the comparison of BSA *versus* BSA + oxLDL.

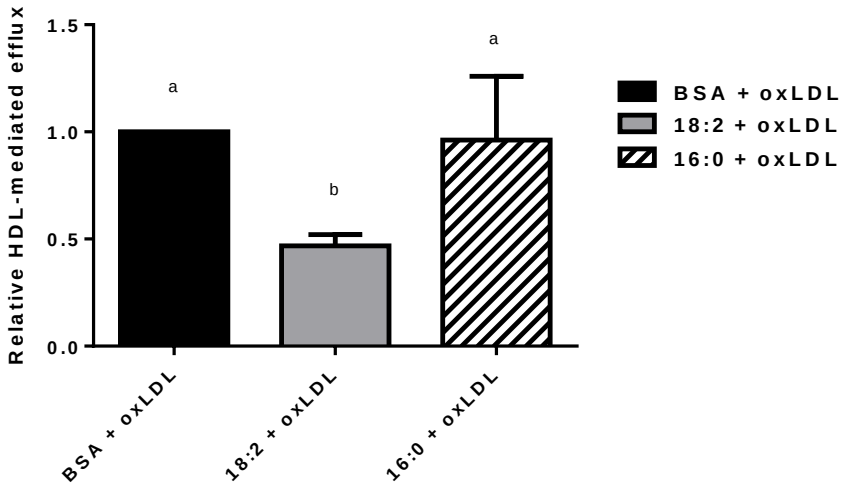


Figure 2. Effect of 18:2 and 16:0 on cholesterol efflux in BMDM. OxLDL- and [^{3}H] cholesterol-loaded BMDM were stimulated with 50 ug/mL HDL for 4 hours. Fraction cholesterol efflux was estimated from the ratio of the radiotracer in the medium to the total (medium + cells) and normalized for cellular protein. Values are representative of 3 independent experiments in triplicate. Bars without a common letter are significantly different (p<0.05).

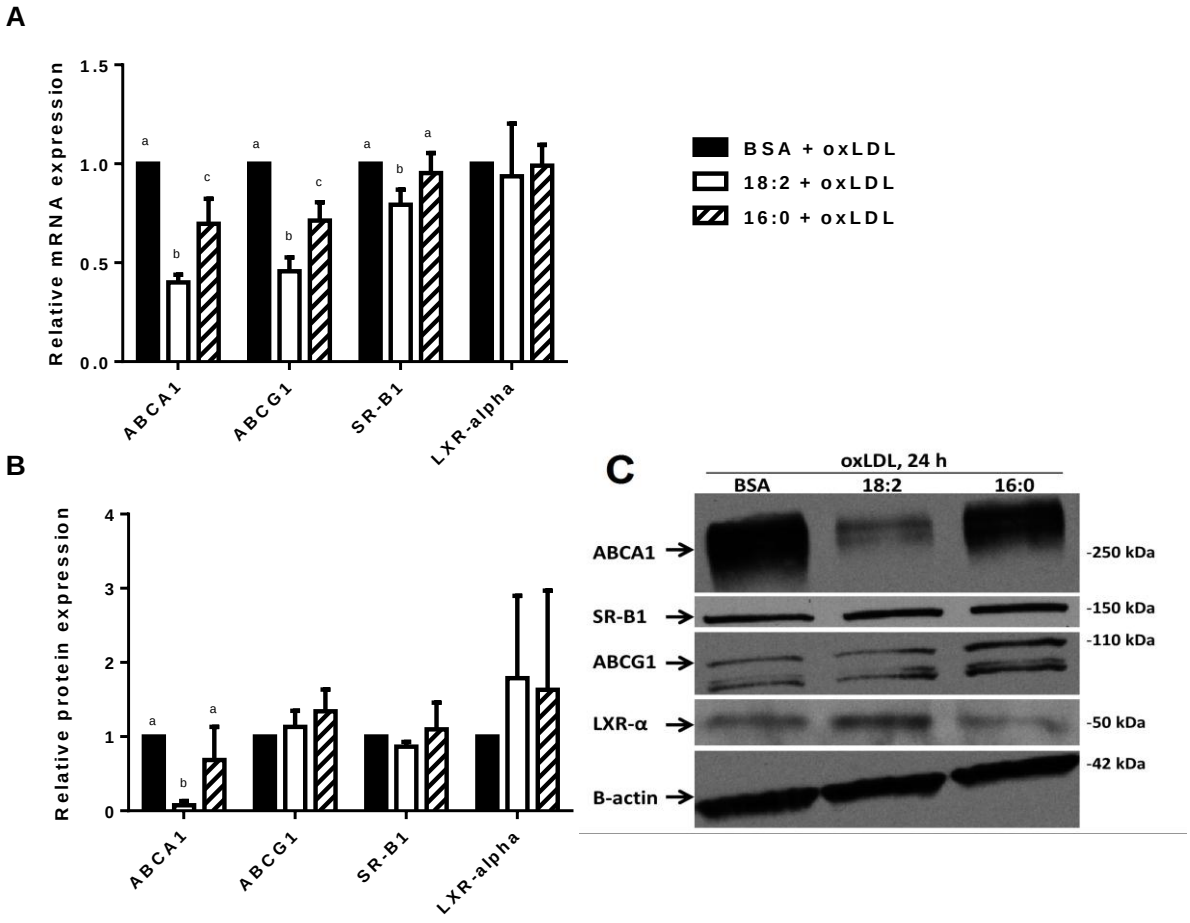


Figure 3. Effect of 18:2 and 16:0 on ABCA1, ABCG1, SR-B1 and LXR-α mRNA and protein expression in BMDM. (A) ABCA1, ABCG1, SR-B1 and LXR-α mRNA expression was determined by real-time PCR. β-actin was used as a standard housekeeping gene. (B) ABCA1, ABCG1, SR-B1, LXR-α and β-actin protein expression was determined by Western blotting of BMDM cell lysates. β-actin was used to control equal loading. (C) Representative Western blot. Values are the mean +/- SD 3 independent experiments performed in triplicate. Bars without a common letter are significantly different (p<0.05).

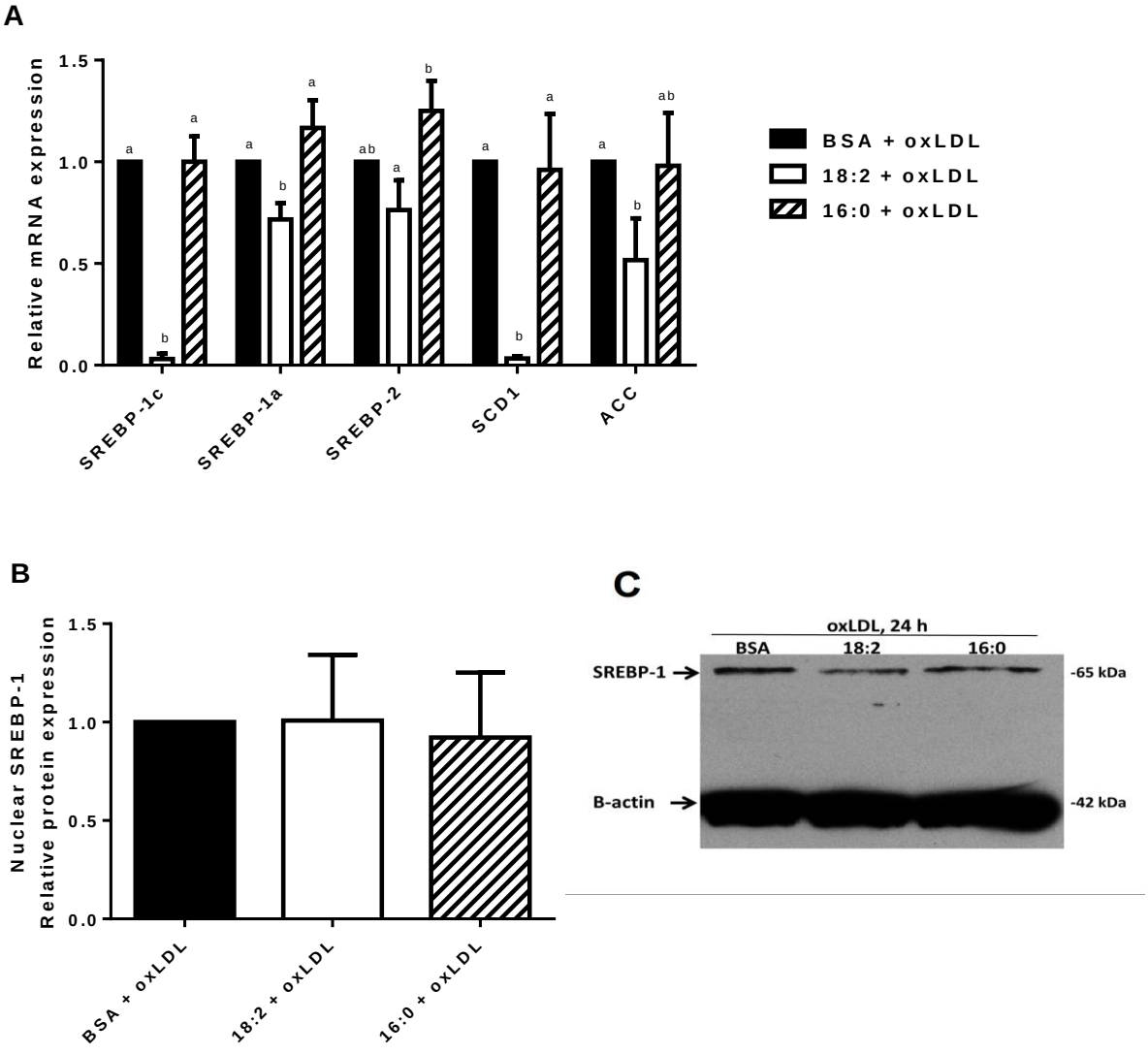


Figure 4. Effect of 18:2 and 16:0 on SREBP-1a, 1c, 2, SCD1, and ACC mRNA and SREBP-1 nuclear protein expression in BMDM. (A) SREBP-1a, 1c, 2, SCD1, and ACC mRNA expression was determined by real-time PCR. β-actin was used as a standard housekeeping gene. (B) SREBP-1 nuclear protein expression was determined by Western blotting of BMDM nuclear extracts. β-actin was used to control equal loading. (C) Representative Western blot. Values represent the mean +/-SD of 3 independent experiments performed in triplicate. Bars without a common letter are significantly different (p<0.05).

6. References

1. Ohashi, R., et al., Reverse cholesterol transport and cholesterol efflux in atherosclerosis. QJM : monthly journal of the Association of Physicians, 2005. 98(12): p. 845-856.
2. National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation, 2002. 106(25): p. 3143-421.
3. Assmann, G., et al., High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM experience and pathophysiological implications for reverse cholesterol transport. Atherosclerosis, 1996. 124(20): p. S11-20.
4. Mauldin, J.P., et al., Reduced expression of ATP-binding cassette transporter G1 increases cholesterol accumulation in macrophages of patients with type 2 diabetes mellitus. Circulation, 2008. 117(21): p. 2785-92.
5. Zhou, H., et al., Determinants of leukocyte adenosine triphosphate-binding cassette transporter G1 gene expression in type 2 diabetes mellitus. Metabolism, 2008. 57(8): p. 1135-40.
6. Ryden, L., et al., Guidelines on diabetes, pre-diabetes, and cardiovascular diseases: executive summary. The Task Force on Diabetes and Cardiovascular Diseases of the European Society of Cardiology (ESC) and of the European Association for the Study of Diabetes (EASD). Eur Heart J, 2007. 28(1): p. 88-136.
7. Garvey, W.T., et al., Effects of insulin resistance and type 2 diabetes on lipoprotein subclass particle size and concentration determined by nuclear magnetic resonance. Diabetes, 2003. 52(2): p. 453-462.
8. Wang, Y. and J.F. Oram, Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a phospholipase D2 pathway. J Biol Chem, 2005. 280(43): p. 35896-903.
9. 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. 1062-8.
10. Uehara, Y., et al., Unsaturated fatty acids suppress the expression of the ATP-binding cassette transporter G1 (ABCG1) and ABCA1 genes via an LXR/RXR responsive element. Atherosclerosis, 2007. 191(1): p. 11-21.
11. Mauerer, R., S. Ebert, and T. Langmann, High glucose, unsaturated and saturated fatty acids differentially regulate expression of ATP-binding cassette transporters ABCA1 and ABCG1 in human macrophages. Experimental and Molecular Medicine, 2009. 41(2): p. 126.
12. Liu, L., et al., Free fatty acid metabolic profile and biomarkers of isolated post-challenge diabetes and type 2 diabetes mellitus based on GC-MS and multivariate statistical analysis. J Chromatogr B Analyt Technol Biomed Life Sci, 2010. 878(28): p. 2817-25.

13. Jessup, W., et al., Roles of ATP binding cassette transporters A1 and G1, scavenger receptor BI and membrane lipid domains in cholesterol export from macrophages. Curr Opin Lipidol, 2006. 17(3): p. 247-257.
14. Wang, X. and D.J. Rader, Molecular regulation of macrophage reverse cholesterol transport. Current opinion in cardiology, 2007. 22(4): p. 368-372.
15. Yoshikawa, T., et al., Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. J Biol Chem, 2002. 277(3): p.1705-11.
16. Langmann, T., et al., Genomic organization and characterization of the promoter of the human ATP-binding cassette transporter-G1 (ABCG1) gene. Biochim Biophys Acta, 2000. 15: p. 1-2.
17. Wong, J., C.M. Quinn, and A.J. Brown, SREBP-2 positively regulates transcription of the cholesterol efflux gene, ABCA1, by generating oxysterol ligands for LXR. Biochem J, 2006. 400(3): p. 485-91.
18. Zeng, L., et al., Sterol-responsive element-binding protein (SREBP) 2 down-regulates ATP-binding cassette transporter A1 in vascular endothelial cells: a novel role of SREBP in regulating cholesterol metabolism. J Biol Chem, 2004. 279(47): p. 48801-7.
19. Nguyen, M.T., et al., A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. J Biol Chem, 2007. 282(48): p. 35279-92.
20. Dillard, A., N.R. Matthan, and A.H. Lichtenstein, Tamm-Horsfall protein 1 macrophage lipid accumulation unaffected by fatty acid double-bond geometric or positional configuration. Nutr Res, 2011. 31(8): p. 625-30.
21. DeBose-Boyd, R.A., et al., Transport-dependent proteolysis of SREBP: relocation of site-1 protease from Golgi to ER obviates the need for SREBP transport to Golgi. Cell, 1999. 99(7): p. 703-12.
22. Schwartz, D.M. and N.E. Wolins, A simple and rapid method to assay triacylglycerol in cells and tissues. J Lipid Res, 2007. 48(11): p. 2514-20.
23. Matthan, N.R., et al., Impact of simvastatin, niacin, and/or antioxidants on cholesterol metabolism in CAD patients with low HDL. J Lipid Res, 2003. 44(4): p. 800-806.
24. Meyer, J.M., et al., High-capacity selective uptake of cholesteryl ester from native LDL during macrophage foam cell frmation. J Lipid Res,2012. 53(10):p.2081-91.
25. Ecker, J., et al., Isomer specific effects of Conjugated Linoleic Acid on macrophage ABCG1 transcription by a SREBP-1c dependent mechanism. Biochem Biophys Res Commun, 2007. 352(3): p. 805-11.
26. Kohro, T., et al., A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage. J Atheroscler Thromb, 2004. 11(2): p. 88-97.
27. Kakuma, T., et al., Leptin, troglitazone, and the expression of sterol regulatory element binding proteins in liver and pancreatic islets. Proc Natl Acad Sci U S A, 2000. 97(15): p. 8536-41.
28. Yecies, J.L., et al., Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. Cell Metab, 2011. 14(1): p. 21-32.

CHAPTER 5

SUMMARY AND DISCUSSION

The risk for developing cardiovascular disease (CVD), the leading cause of mortality in the U.S. [1], is elevated in individuals with type 2 diabetes mellitus (T2DM) [2-4]. Although this association is well established, the underlying mechanisms linking T2DM to increased risk of developing atherosclerosis, the leading cause of CVD, are not well characterized.

The overall aim of this thesis project was to determine the influence of elevated glucose and free fatty acid (FFA) concentrations, as in individuals with T2DM, on monocyte/macrophage ABC-transporter expression and cholesterol efflux capacity. Previous studies have examined these relationships in immortalized macrophage cell lines [5-7]. However, the characteristics of these cell lines limits the translational nature of the resulting data to *in vivo* conditions that mimic the biological state of individuals with T2DM [8, 9]. Therefore, we used primary monocyte and macrophage models to complete this work, which may be more suitable to explore mechanisms relevant to T2DM and atherosclerosis.

1. Discussion of Specific Aim 1:

Specific Aim 1 was addressed in manuscript A (**Chapter 3**), to determine the relationship between blood glucose concentrations and monocyte ATP-binding cassette transporters (ABC)A1 and ABCG1 mRNA expression during an oral glucose challenge in human subjects. Additionally, we assessed the reliability of leukocyte ABC-transporter expression as an indicator of monocyte expression.

Results from studies summarized in **Chapter 3** suggest that human leukocyte ABC-transporter expression is not a good indicator of expression in monocytes. A cross-sectional comparison of blood cells from subjects in the fasting state demonstrated that

monocyte ABC-transporter expression was similar to peripheral blood mononuclear cells (PBMC) and lower than leukocytes (**Chapter 3, Figure 1**). One hour after an oral glucose challenge in these subjects, leukocyte ABCA1 and ABCG1 mRNA expression was elevated (**Chapter 3, Figure 3**). In contrast, human monocyte ABC-transporter expression was not significantly affected by the glucose challenge. These results do not support the use of leukocyte ABC-transporter expression as an appropriate surrogate measure for monocyte ABC-transporter expression.

The mechanism responsible for causing an elevation in leukocyte ABC-transporter expression during the oral glucose challenge in normoglycemic individuals is unclear. We hypothesize that this observation is related to the decrease in circulating FFA concentrations as has previously been reported in response to an oral glucose challenge [10]. One class of FFA, unsaturated fatty acids, has a suppressive effect on ABC-transporter expression [5, 11]. Therefore, decreased FFA concentrations during an oral glucose challenge may be responsible for the increased leukocyte ABC-transporter expression previously observed. The lack of an effect of FFA on monocyte ABC-transporter expression *in vivo* may be due to the length of sample processing time before RNA extraction from monocytes. Alternatively, changes may be undetectable in monocytes due to comparatively low ABC-transporter expression in these undifferentiated cells [11] (**Chapter 3, Figure 1**).

In light of the null effect of the oral glucose challenge on human monocyte ABC-transporter expression, it was of interest to determine the impact of elevated glucose and FFA concentrations on ABC-transporter expression in a primary murine macrophage model, bone marrow-derived macrophages (BMDM).

2. Discussion of Specific Aim 2:

The next aim of this thesis was to determine the mechanisms by which individuals with T2DM exhibit lower macrophage ABC-transporter expression and suppressed cholesterol efflux [12, 13]. T2DM is often associated with chronically elevated glucose and FFA concentrations, among other metabolic abnormalities, compared to individuals without T2DM [14, 15]. Therefore, in addition to studying the effects of an oral glucose challenge on ABC-transporter expression in primary human monocytes *in vivo*, we examined the effects of elevated glucose and FFA concentrations *in vitro* using a macrophage model, murine-BMDM. We also examined the role of potential transcriptional regulators: liver-x-receptor (LXR)-α and sterol regulatory element binding protein (SREBP)-1c.

A hallmark of atherosclerosis is the presence of cholesteryl ester (CE)-loaded macrophages in the artery wall [16]. Therefore, it was important that the macrophage model used for these experiments was consistent with these characteristics in order to best mimic macrophage foam cell development *in vivo*. Exposure of BMDM to oxidized low density lipoprotein (oxLDL) resulted in an increased intracellular CE content (**Chapter 3, Figure 1; Appendix A, Figures 3 and 4**), consistent with prior observations [17], and increased ABCA1 and ABCG1 mRNA transcripts and ABCA1 protein expression (**Chapter 3, Figure 5**).

Much of the background data, indicating that elevated glucose and FFA concentrations have a suppressive effect on ABC-transporter expression has been conducted in immortalized cell lines, often using transformed genes and after stimulation

with pharmaceutical compounds [5, 18-20]. Therefore, the use of a primary macrophage model stimulated with oxLDL is an advantage of this study, mimicking the *in vivo* state.

Data from this study indicated that oxLDL-loaded BMDM exposed to elevated D- and L-glucose and FFA concentrations (in a mixture [18:1, 18:2 and 16:0], or 18:2, but not 16:0 alone) suppressed high density lipoprotein (HDL)-mediated cholesterol efflux (**Chapter 3, Figure 4A; Chapter 4, Figure 2; Appendix A, Figure 7**). However, neither D- nor L- glucose concentrations had an effect on ABC-transporter or SR-B1 expression (**Chapter 3, Figure 5**). In contrast, 18:2 suppressed ABCA1 and ABCG1 mRNA expression and ABCA1 protein expression (**Chapter 4, Figure 3**). It was further established that this effect was not mediated by changes in LXR-α or SREBP-1c protein expression (**Chapter 3, Figures 3 and 4**). We were not able to exclude the possibility that effects are mediated by changes in activity of these transcription factors.

Although no effect of elevated glucose concentrations on ABC-transporter expression was observed in BMDM (**Chapter 3, Figure 5**), previously it has been reported that elevated glucose concentrations suppress human monocyte-derived macrophage ABC-transporter expression [11]. The contrast between our results and the prior study is likely due to exposure of the BMDM to oxLDL in concert with exposure to elevated glucose. We hypothesize that the resulting CE accumulation may have overridden any effects of glucose concentration on cholesterol transporter expression. Instead, our study demonstrated that elevated D- and L-glucose concentrations may act directly to inhibit ABC-transporter activity. Whether this effect is mediated by glycation or glycoxidation of transporters or lipoproteins or an osmotic effect of elevated D- and L-glucose concentrations on BMDM remains to be determined.

The effect of 18:2 on ABC-transporter expression is likely driven by suppression of LXR-α activity and increased ABC-transporter protein turnover, mediated by protein kinase C (PKC)-δ [5, 6]. In contrast, unsaturated fatty acids have been reported to induce peroxisome proliferator-activated receptor (PPAR), which stimulates LXR-α [21]. LXR-α expression was unaffected after BMDM was exposed to 18:2 (**Chapter 4, Figures 3 and 4**). These data suggested that the possible effect of PPAR stimulated by 18:2 on LXR-α was overridden in this model. Similarly, there were no effects of elevated glucose concentrations on LXR-α expression (**Chapter 3, Figure 5**).

Results of these experiments strongly suggest that elevated glucose and FFA concentrations, as observed in individuals with T2DM, suppress cholesterol efflux in primary macrophages *in vitro* (**Chapter 3, Figure 4A; Chapter 4, Figure 2; Appendix A, Figure 7**) . A number of mechanisms may be responsible for these effects, possibly acting on transcription rates, protein expression or directly on ABC-transporter activity. Yet to be determined are the mechanisms that are conserved *in vivo* and relevant to the pathological consequence of T2DM on atherosclerosis risk.

3. Limitations:

We chose to focus on blood monocyte mRNA expression in **Specific Aim 1** because these cells are most closely related to atherosclerotic lesion development. However, expression of cholesterol transporters in these blood cells may not directly reflect the characteristics of differentiated macrophages in the arterial wall. Additionally, isolation of human monocytes by cell sorting, the method used for this thesis, took approximately 3 hours and required that the cells be exposed to a variety of buffers and antibodies. In an attempt to determine whether this method modified the cells we conducted a control

experiment to assess the impact of these buffers on expression of ABC-transporters. The results of that work indicated that the mononuclear cells were unaffected by the cell sorting process (**Chapter 3, Figure 1**). However, although it is unlikely that methodological issues contributed to the results observed it is impossible to rule out the possibility that glucose concentrations on ABC-transporter gene expression was lost during the sorting process.

Limitations in the volume of blood available for the *in vivo* work limited our ability to adequately compare different primary blood cell isolation techniques. We were also limited in the number of subjects we were able to study, which affected our ability to test other methods of isolating monocytes, such as by plate adherence to generate human monocyte-derived macrophages.

Another potential limitation to our human study was that the participant population was normoglycemic (set by inclusion criteria for the ongoing Glycemic Index Study). Changes in gene expression may have been larger in individuals with abnormal glucose homeostasis. However, based on *in vivo* studies describing the regulatory impact that habitual blood glucose concentrations in normoglycemic subjects have on ABC-transporter expression [22, 23], it was an appropriate study population to determine the effect of an oral glucose challenge on ABC-transporter expression.

The primary murine BMDM cells that were chosen to carry out **Specific Aim 2** was a new model for our laboratory, so there were inherent limitations in working through this new model. As a primary cell model, BMDM have many advantageous properties. The use of immortalized cell lines to examine the role of macrophages in atherosclerosis progression is pervasive and the comprehensive translation of such results to the *in vivo*

state is questionable due to potential differences from primary macrophages. Unfortunately, the use of primary cells is also complicated, due to difficulties in isolating adequate quantities from humans or animals and subsequent inability of these cells to thrive in culture in absence of growth hormones. Compared to many other primary cells, BMDM have the advantages of being homogenous, relatively easy to isolate in abundance, having proliferative capacity, and can survive in culture for longer than one week [24]. They are also more malleable to transfection and work well as a model to study gene function *in vitro*. For example, bone marrow cells can be isolated from transgenic mice, which may be useful for future experiments exploring these mechanisms [25, 26].

Initial efforts were focused on optimizing BMDM culture conditions so that the work proposed in **Specific Aim 2** could proceed. This work built on the observations made when addressing **Specific Aim 1** and from preliminary experiments summarized in **Appendix A**.

It was also necessary to establish optimal controls for the *in vitro* studies to determine the effect of elevated glucose concentrations on BMDM. L-glucose was used as a control rather than mannitol because L-glucose is a substrate for glycation and glycoxidation of proteins and lipids [27]. Both L-glucose and mannitol contribute similar osmotic effects as D-glucose on cells but neither can be metabolized by cells. The use of mannitol as a control would have precluded our ability to distinguish between its effects from that of D-glucose. Since we hypothesized that glycation may play a role in mediating effects of D-glucose, L-glucose was used as the control.

The controls for the FFA treatments were also carefully considered. The multiple unsaturated fatty acid bonds of 18:2 make this fatty acid susceptible to potential oxidation. To minimize *in vitro* oxidation, FFA were conjugated to bovine serum albumin prior to introduction into the cell culture media (**Appendix B, Section 5**). We were not able to confirm that no oxidation of 18:2 occurred. The use of antioxidants was precluded due to potential independent effects of these compounds on the BMDM. We cannot rule of the possibility that the oxidation of 18:2 *in vivo* may have contributed to the effects of unsaturated fatty acids on ABC-transporter expression and cholesterol efflux.

4. Conclusions and Future Directions

The research which has been discussed in this thesis addressed the mechanisms by which elevated glucose and FFA concentrations influence macrophage cholesterol efflux. This is the first study, to our knowledge, to demonstrate the difference between ABC-transporter expression in human leukocytes compared to monocytes and PBMC. Additionally, our data suggest that high glucose concentrations impair HDL-mediated cholesterol efflux, perhaps by attenuating transporter activity rather than transcript or protein expression. In the case of elevated FFA concentrations, data indicate that the suppressive effect on HDL-mediated cholesterol efflux and ABC-transporter expression is driven by 18:2 (**Chapter 4, Figures 2 and 3; Appendix A, Figure 7**). However, it seems that this observation may be dependent on the monocyte/macrophage model used to test this hypothesis [7, 11, 18, 19].

A complete understanding of the mechanisms responsible for the suppression of cholesterol efflux by elevated glucose and FFA concentrations would be an important

contribution, which could be exploited for T2DM treatment strategies and other obesity-related metabolic diseases associated with increased CVD risk. Due to limitations of these experiments performed for this thesis, further work is needed to investigate the effects of acute and chronically elevated glucose and FFA on monocyte/macrophage cholesterol homeostasis *in vivo*. It may be necessary to use a different method of monocyte isolation for future studies due to the comparatively low levels of ABC-transporter expression that was observed in the non-activated monocytes (**Chapter 3, Figure 1**) [11].

Additionally, more work is needed to elucidate the mechanisms by which elevated glucose and FFA concentrations directly affect macrophage cell membrane ABC-transporter expression and activity. Whole cell ABC-transporter expression, measured by Western blot in this study (**Chapter 3, Figure 5B and C; Chapter 4, Figure 3B and C**), may not predict cell membrane ABC-transporter expression. Future studies may focus on whether there is a difference in ABC-transporter protein expression on the cell membrane by performing flow cytometry.

As mentioned previously, future studies should focus on primary monocytes and macrophages to address issues related to elevated glucose concentrations and cholesterol efflux and ABC-transporter expression, rather than relying on other blood cells or immortalized cell lines as surrogates models. Potential targets for treatment strategies are LXR-α and PKC-δ, identified previously to be involved in suppression of cholesterol efflux by unsaturated fatty acids [5, 6, 18, 19]. Pharmaceutical LXR-α agonists have been shown to reduce atherosclerotic lesion progression in mouse models [28] but use in humans has not been useful due to concomitant liver lipid accumulation [29].

While many groundbreaking drugs, such as the statin drug-group, are currently used to improve lipoprotein profiles and retard CVD progression, CVD remains the largest cause of mortality in the U.S. [1]. Therefore, new mechanisms need to be targeted in order to further decrease the incidence of CVD. The mechanism that was focused on in this thesis work, macrophage cholesterol efflux, provides an important potential target for new therapies to retard atherosclerosis progression or to actually induce its regression [30]. Therapeutically targeting this mechanism could be particularly successful if aimed at certain individuals who are known to have defective macrophage cholesterol efflux rates such as individuals with T2DM or low HDL cholesterol concentrations [12, 13]. Macrophage cholesterol transporters are potentially modifiable through lifestyle changes [31] and are not currently targeted clinically. Interestingly, statin treatment decreases ABCA1expression [32] in contrast to pioglitazone, used to treat T2DM, which increases ABC-transporter expression *in vitro* [33]. These data indicate the importance of fully understanding regulation of macrophage cholesterol efflux and factors influencing this athero-protective mechanism before supplemental approaches to prevention and treatment CVD can be developed.

5. References

1. Lloyd-Jones, D.M., *Cardiovascular risk prediction: basic concepts, current status, and future directions.* Circulation, 2010. **121**(15): p. 1768-1777.
2. Ryden, L., et al., *Guidelines on diabetes, pre-diabetes, and cardiovascular diseases: executive summary. The Task Force on Diabetes and Cardiovascular Diseases of the European Society of Cardiology (ESC) and of the European Association for the Study of Diabetes (EASD).* Eur Heart J, 2007. **28**(1): p. 88-136.
3. Beckman, J.A., M.A. Creager, and P. Libby, *Diabetes and atherosclerosis: epidemiology, pathophysiology, and management.* JAMA, 2002. **287**(19): p. 2570-81.
4. Park, L. and D. Wexler, *Update in diabetes and cardiovascular disease: synthesizing the evidence from recent trials of glycemic control to prevent cardiovascular disease.* Curr Opin Lipidol, 2010. **21**(1): p. 8-14.
5. Brownlee, M., *The pathobiology of diabetic complications: a unifying mechanism*. 2005: Diabetes. 2005 Jun;54(6):1615-25.
6. Balkau, B., et al., *Prediction of the risk of cardiovascular mortality using a score that includes glucose as a risk factor. The DECODE Study.* Diabetologia, 2004. **47**(12): p. 2118-28.
7. *Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnostic criteria. The DECODE study group. European Diabetes Epidemiology Group. Diabetes Epidemiology: Collaborative analysis Of Diagnostic criteria in Europe.* Lancet, 1999. **354**(9179): p. 617-621.
8. Khaw, K.T., et al., *Glycated haemoglobin, diabetes, and mortality in men in Norfolk cohort of european prospective investigation of cancer and nutrition (EPIC-Norfolk).* Bmj, 2001. **322**(7277): p. 15-8.
9. Turner, R.C., et al., *Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23).* Bmj, 1998. **316**(7134): p. 823-8.
10. Temelkova-Kurktschiev, T.S., et al., *Postchallenge plasma glucose and glycemic spikes are more strongly associated with atherosclerosis than fasting glucose or HbA1c level.* Diabetes Care, 2000. **23**(12): p. 1830-1834.
11. Hanefeld, M., et al., *Post-challenge hyperglycaemia relates more strongly than fasting hyperglycaemia with carotid intima-media thickness: the RIAD Study. Risk Factors in Impaired Glucose Tolerance for Atherosclerosis and Diabetes.* Diabetic medicine : a journal of the British Diabetic Association, 2000. **17**(12): p. 835-840.
12. Heine, R.J., et al., *What does postprandial hyperglycaemia mean?* Diabet Med, 2004. **21**(3): p. 208-13.
13. Garvey, W.T., et al., *Effects of insulin resistance and type 2 diabetes on lipoprotein subclass particle size and concentration determined by nuclear magnetic resonance.* Diabetes, 2003. **52**(2): p. 453-462.
14. Mauldin, J.P., et al., *Reduced expression of ATP-binding cassette transporter G1 increases cholesterol accumulation in macrophages of patients with type 2 diabetes mellitus.* Circulation, 2008. **117**(21): p. 2785-92.
15. Zhou, H., et al., *Determinants of leukocyte adenosine triphosphate-binding cassette transporter G1 gene expression in type 2 diabetes mellitus.* Metabolism, 2008. **57**(8): p. 1135-40.
16. Uehara, Y., et al., *Polyunsaturated fatty acids and acetoacetate downregulate the expression of the ATP-binding cassette transporter A1.* Diabetes, 2002. **51**(10): p. 2922-2928.

17. Uehara, Y., et al., *Unsaturated fatty acids suppress the expression of the ATP-binding cassette transporter G1 (ABCG1) and ABCA1 genes via an LXR/RXR responsive element.* Atherosclerosis, 2007. **191**(1): p. 11-21.
18. Wang, Y. and J.F. Oram, *Unsaturated fatty acids phosphorylate and destabilize ABCA1 through a phospholipase D2 pathway.* J Biol Chem, 2005. **280**(43): p. 35896-903.
19. 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. 1062-8.
20. Passarelli, M., et al., *Advanced glycation end product precursors impair ABCA1-dependent cholesterol removal from cells.* Diabetes, 2005. **54**(7): p. 2198-2205.
21. Mauerer, R., S. Ebert, and T. Langmann, *High glucose, unsaturated and saturated fatty acids differentially regulate expression of ATP-binding cassette transporters ABCA1 and ABCG1 in human macrophages.* Experimental and Molecular Medicine, 2009. **41**(2): p. 126.
22. Kohro, T., et al., *A comparison of differences in the gene expression profiles of phorbol 12-myristate 13-acetate differentiated THP-1 cells and human monocyte-derived macrophage.* J Atheroscler Thromb, 2004. **11**(2): p. 88-97.
23. Qin, Z., *The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature.* Atherosclerosis, 2012. **221**(1): p. 2-11.
24. CDC, *Increasing prevalence of diagnosed diabetes - United States and puerto rico, 1995-2010.* MMWR Morb Mortal Wkly Rep, 2012. **61**(45): p. 918-21.
25. Reaven, G.M., et al., *Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM.* Diabetes, 1988. **37**(8): p. 1020-1024.
26. Seigneur, M., et al., *Serum fatty acid profiles in type I and type II diabetes: metabolic alterations of fatty acids of the main serum lipids.* Diabetes research and clinical practice, 1994. **23**(3): p. 169-177.
27. Glass, C.K. and J.L. Witztum, *Atherosclerosis. the road ahead.* Cell, 2001. **104**(4): p. 503-516.

APPENDIX A

PRELIMINARY STUDIES

1. Five hour oral glucose challenge

Ten subjects were recruited for the human pilot study (**Chapter 3, Methods 2.1**). Blood samples were collected at baseline and 1, 2, 3, 4 and 5 hours after an oral glucose challenge. But due to limited sample volumes, results for leukocyte and monocyte mRNA expression for **Chapter 3** analysis were only available from time points at baseline, 1 and 2 hours following the glucose challenge. Below, are results from leukocyte and monocyte mRNA expression at baseline and 1-5 hours following the glucose challenge **(Figures 1 and 2).** Missing data points were due to inadequate sample volume. Some of the missing samples at time points 0-2 hours were able to be reanalyzed using duplicate samples when possible. However, duplicates were not available for each subject at each time point. Since not all samples from a given subject were reanalyzed, only the initial results are displayed in **Figures 1 and 2**.

2. BMDM model development:

The bone marrow-derived macrophage (BMDM) cell model was used to address **Specific Aim 2** which was new to our laboratory. Preliminary incubation times and concentrations were based on literature data and/or personal communication. Initial efforts were focused on a pilot study to optimize culture conditions. Prior to starting the subsequent work, presented in **Chapters 3 and 4**, the following experiments were performed to establish feasibility and define appropriate treatment parameters and laboratory methods to carry out the aforementioned aims of this thesis.

3. BMDM response to LD-FBS *versus* LR-FBS

Despite similar free fatty acid (FFA) profiles of lipid reduced-fetal bovine serum (LR-FBS, HyClone SH30855.03) and lipoprotein deficient (LD)-FBS **(Table 1)**, BMDM were

not able to grow in LR-FBS but grew well in LD-FBS, data not shown. LD-FBS was made through an ultracentrifugation procedure to remove the lipoprotein fraction of FBS (Gibco, Life Technologies) (**Appendix B, Section 6**). Although LR-FBS contained a lower glucose concentration than LD-FBS, increasing the glucose concentration to match the concentration in LD-FBS did not restore the proliferative ability to LR-FBS. One hypothesis to explain the lower rate of growth observed using LR-FBS may be the absence of growth factors necessary for differentiation and maintenance of these cells. One the basis of the preliminary data, LD-FBS was used for all subsequent experiments.

4. BMDM oxLDL treatment parameters

Oil Red O (ORO) lipid staining demonstrates the increase in intracellular lipid accumulation following exposure of BMDM to 25 ug/mL oxidized low density lipoprotein (oxLDL) for 24 hours **(Figures 3A and 3B)**. Cellular cholesteryl ester (CE) content was undetectable in absence of oxLDL **(Figure 4A)**. Exposure to oxLDL resulted in approximately 50 ug CE accumulation per mg cellular protein (**Figures 4A and 4B**). Elevated glucose or FFA concentrations do not significantly affect CE accumulation **(Figures 4A and 4B)**.

Saturating the extent to which BMDM accumulated CE would not allow us to test the hypothesis regarding the effects of glucose and FFA concentrations on this outcome. On the basis of preliminary data CE accumulation was similar regardless of whether BMDM were exposed to 50 or 100 ug/mL oxLDL (**Figure 4**). Therefore, in order to determine the effect of FFA and/or glucose alter cholesterol accumulation, we repeated the work using a lower concentration of oxLDL, 25 ug/mL.

5. Effect of elevated glucose concentration on ABCA1, ABCG1 and SIRT1 expression in BMDM

In a preliminary experiment we examined the effect of elevated glucose concentrations (25 mmol/L) on ATP-binding cassette (ABC) transporters (ABCA1 and ABCG1) and sirtuin (SIRT)-1 mRNA expression in BMDM in the absence of oxLDL-stimulation. Although this experiment was not powered to determine statistical significance it allowed us to determine whether SIRT1 played a role in mediating suppression of ABC-transporter expression by elevated glucose concentrations. Results indicate that SIRT1 mRNA expression, a deacetylase enzyme, tended to decrease in response to elevated glucose concentrations, comparable to that reported in previous studies [1]. But ABCA1 and ABCG1 mRNA expression increased in this model, which is the opposite of what has been reported in the human monocyte-derived macrophage model in response to elevated glucose concentrations [2].

Originally, we hypothesized that glucose-mediated SIRT1 suppression would lead to increased acyetyl-liver-x-receptor (LXR)-α expression and decreased activity, as previously reported [3], leading to a suppression of ABC-transporter expression. Given the increase in ABC-transporter expression in response to elevated glucose concentration in this preliminary experiment (**Figure 5**), the aim to explore SIRT1 and acetyl-LXR-α was abandoned. To continue exploring other effects of glucose on ABC-transporter expression and cholesterol efflux we stimulated cholesterol accumulation and ABC-transporter expression, mimicking foam cell behavior by exposing BMDM to oxLDL. In oxLDL-stimulated BMDM, elevated glucose did not have an effect on ABC-transporter expression (**Chapter 3, Figure 5**) but did suppress HDL-mediated cholesterol efflux (**Chapter 3, Figure 4A**).

6. Effect of elevated glucose on cholesterol efflux time-course

We examined cholesterol efflux in the oxLDL-stimulated BMDM model because there was no previous data in this model to provide suggestion as to the optimal time required for adequate efflux to occur. Based on the results in **Figure 6**, we determined that the amount of cholesterol efflux that occurred within the first 2 hours was comparatively low and that there may be a lower rate of cholesterol efflux after 8 hours. In order to adequately assess differences in cholesterol efflux among treatments, it was important that efflux rate had not plateaued. Therefore, we chose to measure efflux after 4 hours, during the highest rate of efflux, for all subsequent experiments.

7. Effect of FFA mixture (18:1, 18:2, 16:0) on cholesterol efflux in BMDM

We next addressed the question of why individuals with type 2 diabetes mellitus (T2DM) have lower macrophage ABC-transporter expression and suppressed cholesterol efflux [4, 5]. T2DM is often associated with elevated glucose and FFA concentrations, among other metabolic abnormalities, compared to individuals without T2DM [6, 7]. Therefore, in addition to studying the effects of elevated glucose on ABC-transporter expression, we examined the effects of elevated FFA concentrations as well.

Previous studies have suggested that unsaturated fatty acids suppress ABC-transporter expression and cholesterol efflux [8, 9]. But in circulation, FFA are present in a mixture of saturated and unsaturated FFA with varying lengths and location of double bonds. Predominant in concentrations in this mixture are linoleic acid (18:1), oleic acid (18:2), and palmitic acid (16:0) [10]. In a preliminary experiment, we tested the effect of a 200 uM mixture of FFA (66.6 uM 18:1, 66.6 uM 18:2, 66.6 uM 16:0) on HDL- and apoA1-mediated cholesterol efflux (**Figure 7**).

The FFA mixture was found to suppress both HDL- and apoA1-mediated cholesterol efflux in this model. However, given the reports of different effects of FFA based on degree of saturation [8, 9], we chose to explore effects of two of these most predominant FFA in circulation for the remainder of the studies in this thesis: 18:1 and 16:0.

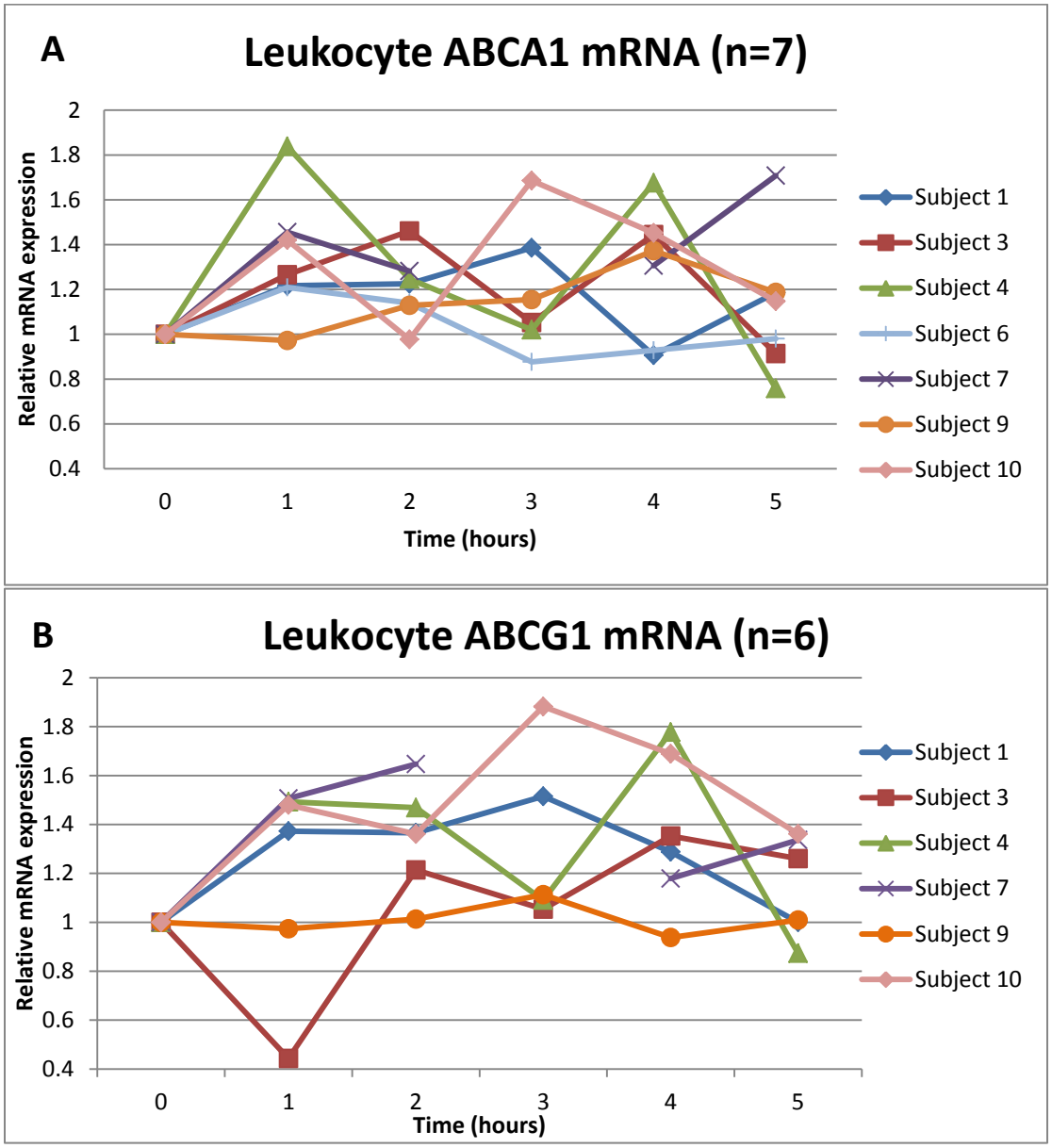


Figure 1. Human leukocyte ATP-binding cassette (ABC) transporter, ABCA1 and ABCG1 mRNA expression during a 5-hour oral glucose challenge. Whole blood (leukocyte) RNA was isolated from fasted subjects at time=0. Immediately thereafter, subjects consumed 50 g/50 mL glucose drink. Leukocyte RNA was isolated at 1, 2, 3, 4, and 5 hours post-challenge. Data are missing from subjects due to low sample volumes. (A) Leukocyte ABCA1 and (B) ABCG1mRNA expression was determined by real-time PCR. β-actin was used as a standard housekeeping gene.

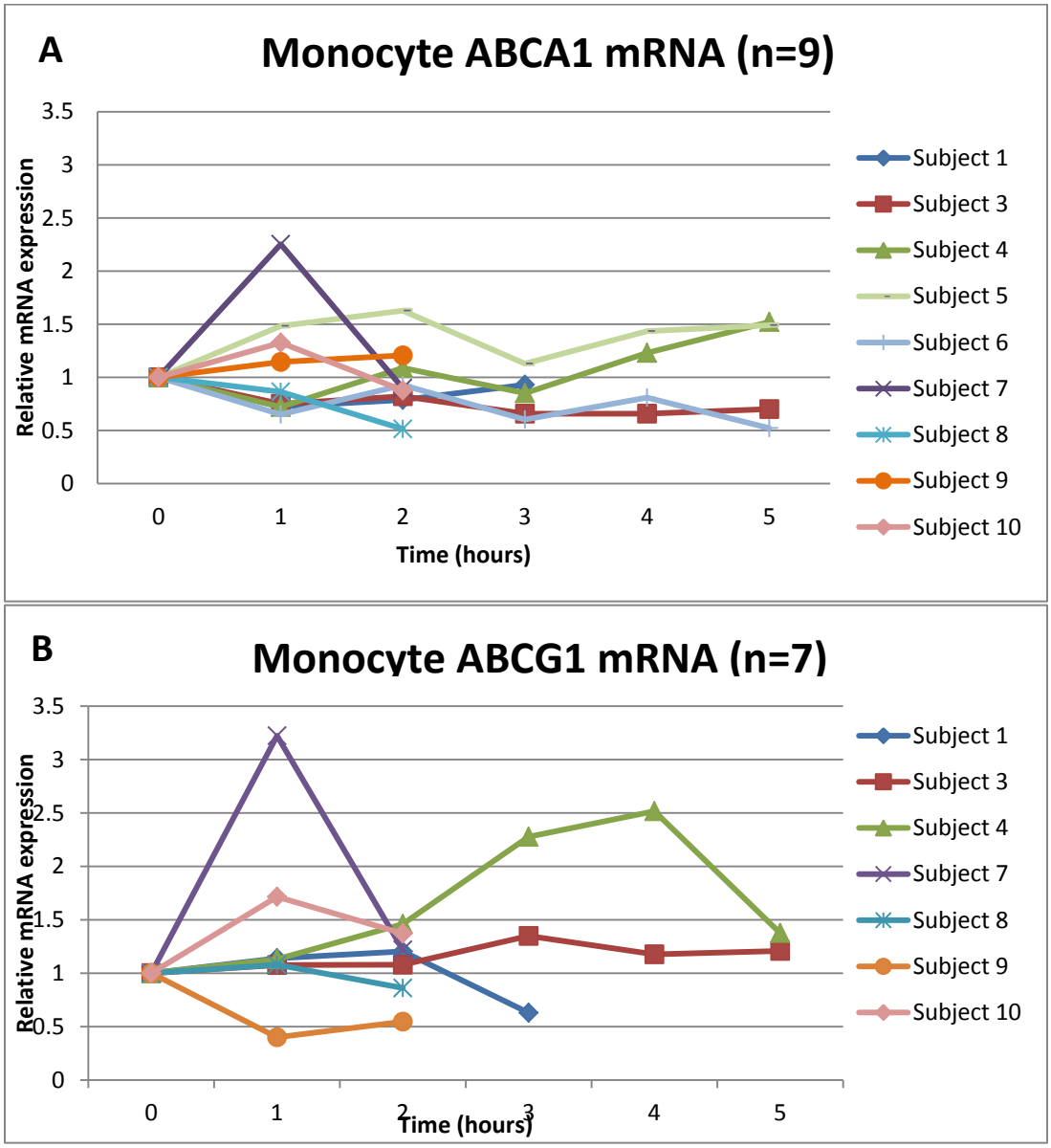


Figure 2. Human monocyte ATP-binding cassette (ABC) transporter, ABCA1 and ABCG1, mRNA expression during a 5-hour oral glucose challenge. Monocytes were isolated by an autoMACS method (described in **Chapter 3, Section 2.3**) from fasted subjects at time=0. Immediately thereafter, subjects consumed 50 g/50 mL glucose drink. Monocytes were isolated at 1, 2, 3, 4, and 5 hours post-challenge. Data are missing from subjects due to low sample volumes. (A) Monocyte ABCA1and (B) ABCG1 mRNA expression was determined by real-time polymerase chain reaction. β-actin was used as a standard housekeeping gene.

FBS type	SFA	MUFA	PUFA	PUFA n-3	PUFA n-6
FBS (Gibco)	0.151	0.106	0.077	0.024	0.052
LD-FBS (Gibco, altered)	0.032	0.014	0.014	0.005	0.006
LR-FBS (HyClone)	0.033	0.013	0.010	0.003	0.006

Table 1. Fatty acid analysis for various fetal bovine serum (FBS) samples (ug fatty acid/ ul FBS). Lipid from 200 ul FBS samples were extracted by standard methods and determined by gas chromatograph for analysis[11] . Lipoprotein deficient (LD), lipid reduced (LR), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA).

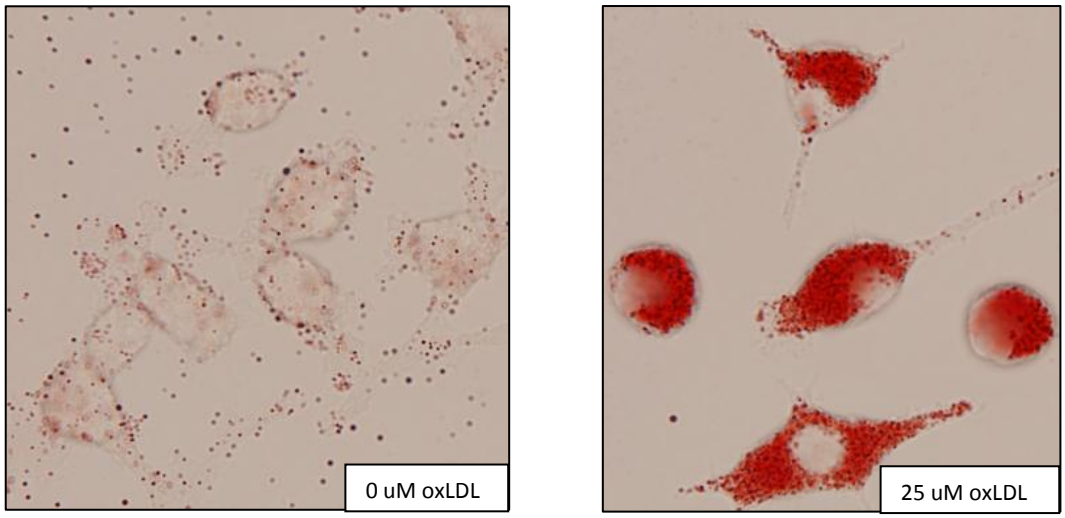


Figure 3. Oil-Red-O (ORO) staining in bone marrow-derived macrophages (BMDM). BMDM were treated with or without 25 ug/mL oxidized low density lipoprotein (oxLDL) for 24 hours. Cells washed with cold 1X phosphate buffered saline, fixed with formalin, and stained with ORO for 5 minutes (adapted from [12]).

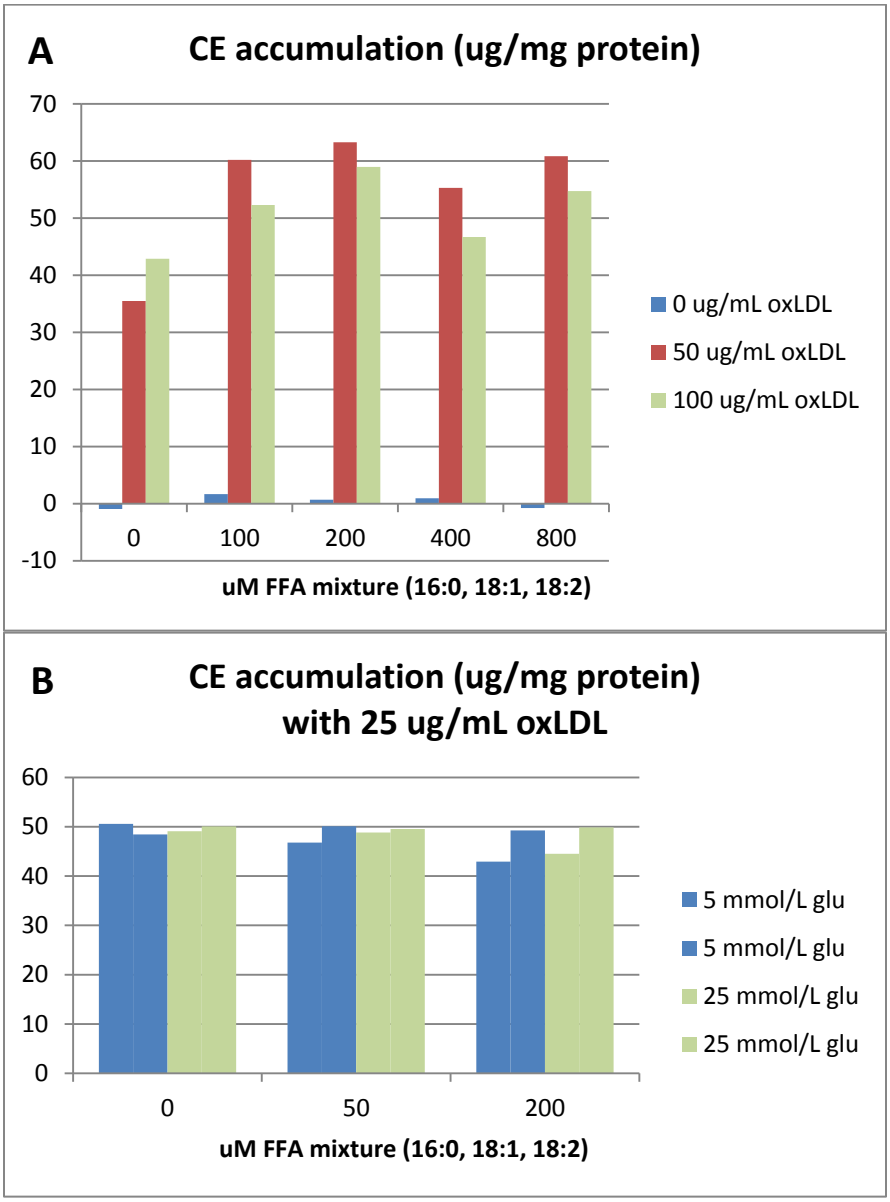


Figure 4. Bone marrow-derived macrophage (BMDM) cholesteryl ester (CE) accumulation in response to elevated glucose (glu), free fatty acid (FFA) and oxidized low density lipoprotein (oxLDL) treatments. BMDM were incubated with 5-25 mmol/L glucose and 0-800 uM FFA mixture (18:1, 18:2, and 16:0) for 16 hours, followed by exposure to 0, 25, 50, or 100 ug/mL oxLDL for 24 hours. Lipid was extracted from cells, followed by running samples of the gas chromatograph to determine free and total cholesterol concentrations. CE was determined by calculating the difference between free and total cholesterol.

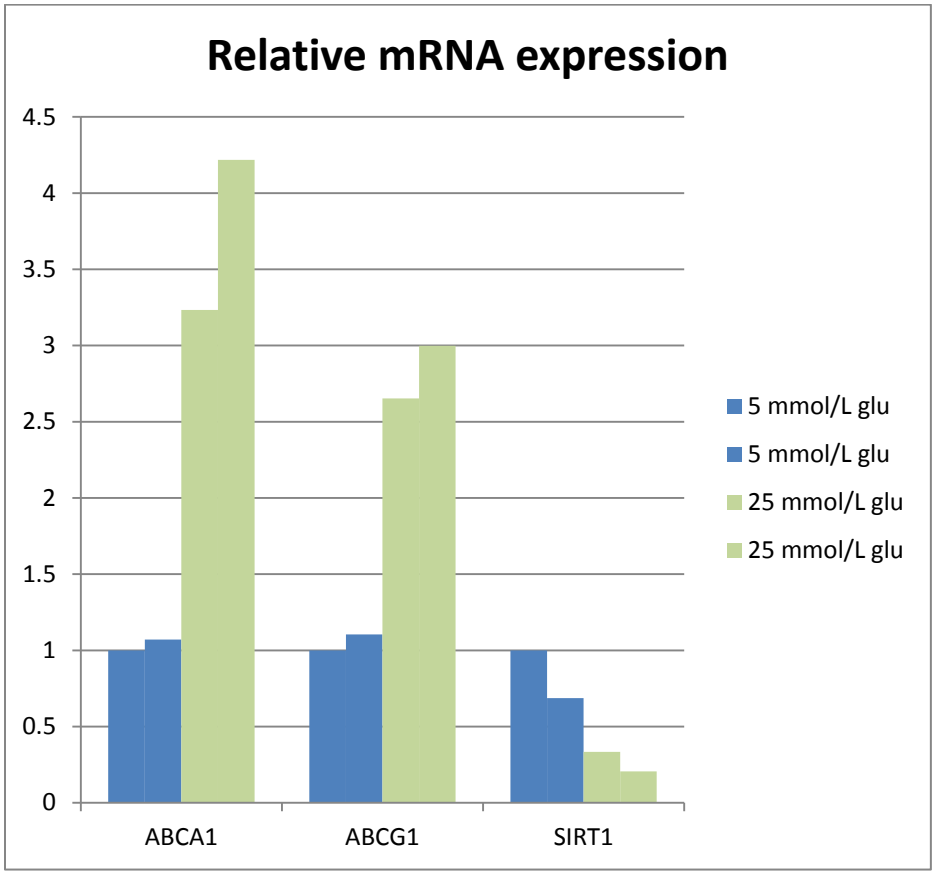
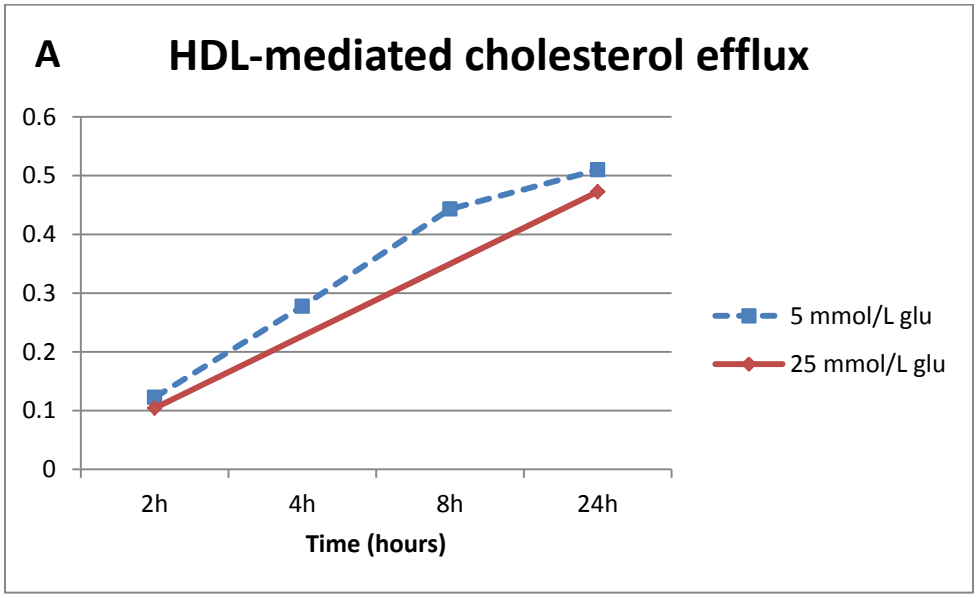


Figure 5. Relative ATP-binding cassette transporter (ABC)-transporter and sirtuin (SIRT)-1 mRNA expression in bone marrow-derived macrophages (BMDM). BMDM were treated with 5 or 25 mmol/L glucose (glu) for 16 hours. Cells were immediately collected and RNA was extracted. ABCA1, ABCG1, and SIRT1 mRNA expression was determined by real-time PCR. β-actin was used as a standard housekeeping gene.



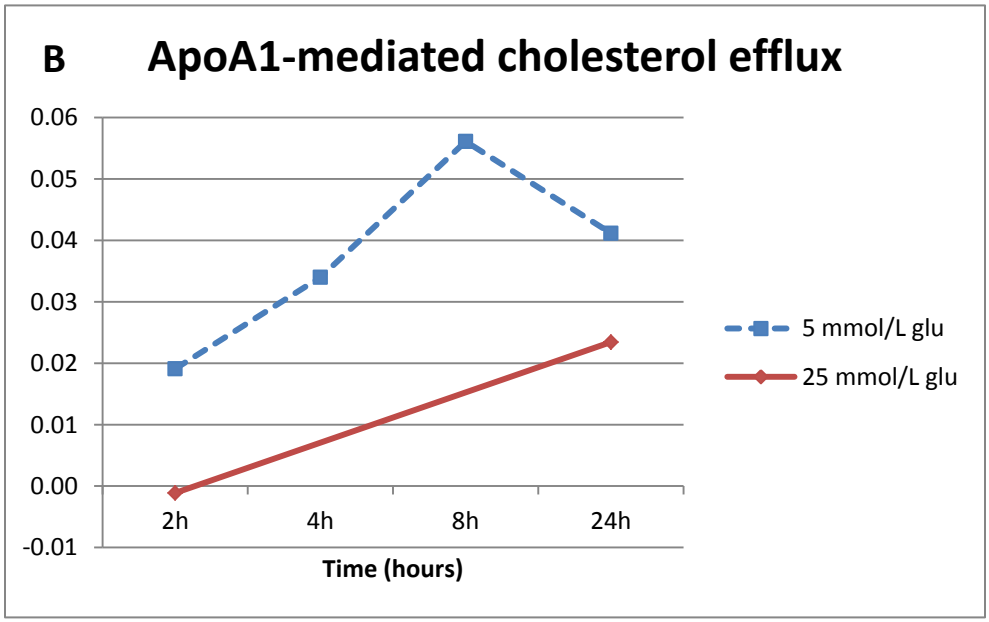


Figure 6. Effect of elevated glucose (glu) on cholesterol efflux time-course. Oxidized low density lipoprotein and [^{3}H] cholesterol-loaded BMDM were stimulated with (A) 50 ug/mL high density lipoprotein (HDL) or (B) 10 ug/mL apolipoprotein (apo)-A1 for 2, 4, 8, or 24 hours. Fractional cholesterol efflux was estimated from the ratio of the radiotracer in the medium to the total (medium + cells) and normalized for cellular protein.

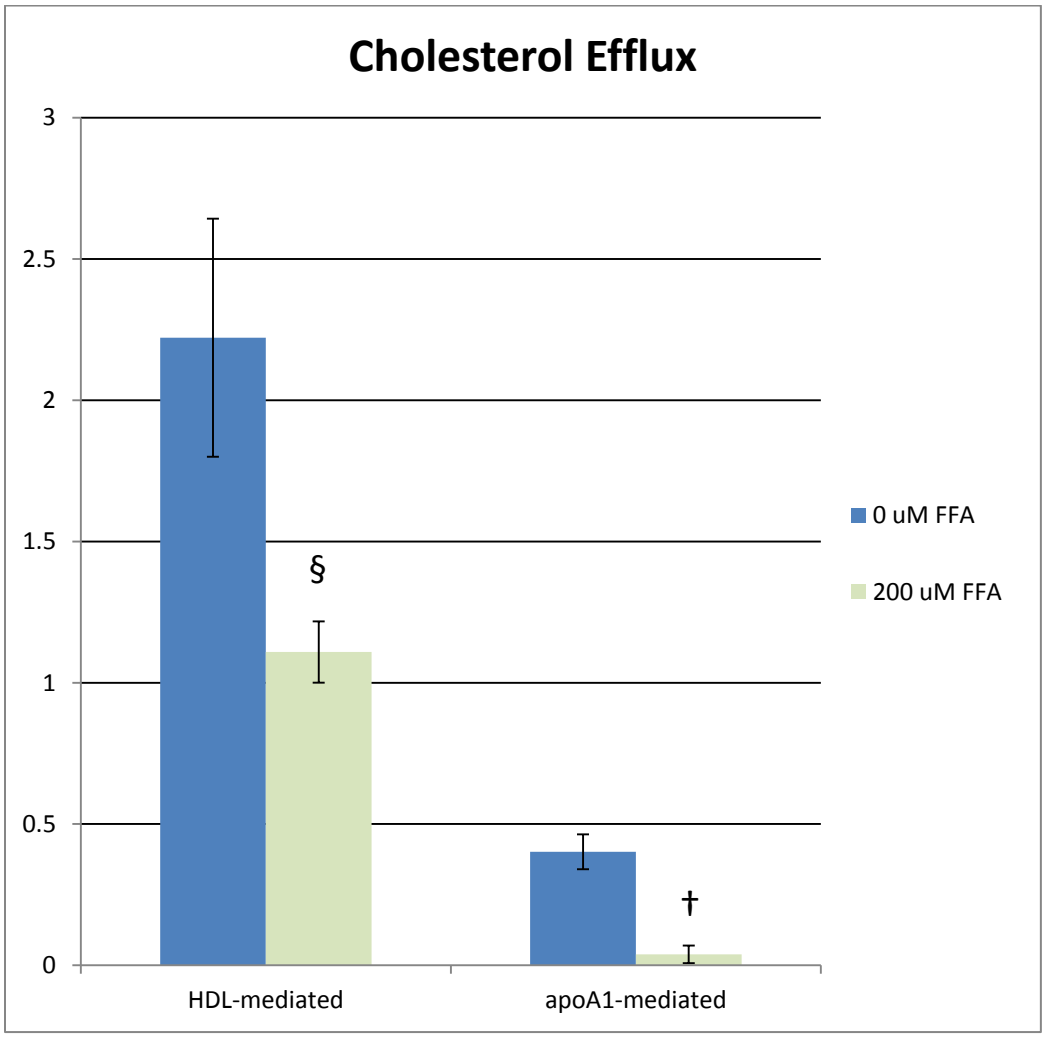


Figure 7. Effect of free fatty acid (FFA) mixture (18:1, 18:2, 16:0) on cholesterol efflux in bone marrow-derived macrophages (BMDM). Oxidized low density lipoprotein and [^{3}H] cholesterol-loaded BMDM were stimulated with 50 ug/mL high density lipoprotein (HDL) or 10 ug/mL apolipoprotein (apo)-A1 for 4 hours. Fractional cholesterol efflux was estimated from the ratio of the radiotracer in the medium to the total (medium + cells) and normalized for cellular protein. Values are representative of 3 independent experiments in triplicate. †$p < 0.01$, §$p < 0.0001$ compared to 0 uM FFA treatment.

8. References

1. Hayek, T., et al., *Macrophage NADPH oxidase activation, impaired cholesterol fluxes, and increased cholesterol biosynthesis in diabetic mice: a stimulatory role for D-glucose.* Atherosclerosis, 2007. **195**(2): p. 277-86.
2. Mauerer, R., S. Ebert, and T. Langmann, *High glucose, unsaturated and saturated fatty acids differentially regulate expression of ATP-binding cassette transporters ABCA1 and ABCG1 in human macrophages.* Experimental and Molecular Medicine, 2009. **41**(2): p. 126.
3. Li, X., et al., *SIRT1 deacetylates and positively regulates the nuclear receptor LXR.* Molecular cell, 2007. **28**(1): p. 91-106.
4. Mauldin, J.P., et al., *Reduced expression of ATP-binding cassette transporter G1 increases cholesterol accumulation in macrophages of patients with type 2 diabetes mellitus.* Circulation, 2008. **117**(21): p. 2785-92.
5. Zhou, H., et al., *Determinants of leukocyte adenosine triphosphate-binding cassette transporter G1 gene expression in type 2 diabetes mellitus.* Metabolism, 2008. **57**(8): p. 1135-40.
6. Reaven, G.M., et al., *Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM.* Diabetes, 1988. **37**(8): p. 1020-1024.
7. Seigneur, M., et al., *Serum fatty acid profiles in type I and type II diabetes: metabolic alterations of fatty acids of the main serum lipids.* Diabetes research and clinical practice, 1994. **23**(3): p. 169-177.
8. Uehara, Y., et al., *Unsaturated fatty acids suppress the expression of the ATP-binding cassette transporter G1 (ABCG1) and ABCA1 genes via an LXR/RXR responsive element.* Atherosclerosis, 2007. **191**(1): p. 11-21.
9. 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. 1062-8.
10. Liu, L., et al., *Free fatty acid metabolic profile and biomarkers of isolated post-challenge diabetes and type 2 diabetes mellitus based on GC-MS and multivariate statistical analysis.* J Chromatogr B Analyt Technol Biomed Life Sci, 2010. **878**(28): p. 2817-25.
11. Dillard, A., N.R. Matthan, and A.H. Lichtenstein, *Tamm-Horsfall protein 1 macrophage lipid accumulation unaffected by fatty acid double-bond geometric or positional configuration.* Nutr Res, 2011. **31**(8): p. 625-30.
12. Jun, H.J., et al., *Induction of ABCA1 and ABCG1 expression by the liver X receptor modulator cineole in macrophages.* Bioorg Med Chem Lett, 2012. **15**(12): p. 01455-2.

APPENDIX B

RESEARCH METHODS

1. **Supplemental Recruitment Protocol for Study 2343: Oral glucose challenge in human subjects**

Subjects for this pilot study will be recruited primarily from the Tufts University Medical Campus. No potential study subject will be supervised by the PI or anticipated to be supervised by the PI in the future, in the capacity of student, trainee or employee. Subjects will include men and postmenopausal women, 50-85 years, and with a BMI of 25 to 35 kg/m^2.

The pilot study will be advertised in buildings at the Tufts University Medical Campus using informative IRB approved flyers posted on bulletin boards. If these advertisements fail to draw an inadequate number of participants we will widen our recruitment strategy to include advertisements in community and citywide newsletters and newspapers. Prior to participation all subjects will sign a Tufts University IRB approved consent form.

Employees of the HNRCA (employee-subjects) who voluntarily want to participate in the study will also be enrolled. In order to qualify for the study, employee-subjects must respond to IRB-approval advertisement of their own accord and will not be directly approached by any person seeking to recruit them for participation in the study. Members of the research team, as direct-report subordinates of the PI and anyone who is direct-report subordinate to any of the research team members in any other capacity will not be eligible to participate in the study. If employee-subjects qualify to participate in the study, they will use personal vacation time for participating in any research activity that takes place during their work hours.

During the screening visit blood pressure, body weight and height, waist and hip circumferences, a 40ml blood sample to run various tests, electrocardiogram, and

pregnancy test are the procedures that may be performed to identify potential health problems and ensure inclusion into the pilot study.

If we get positive results with the pilot study and decide to continue with the GI sub-study, we will recruit subjects that are already participants in the GI Study at the HNRCA. The GI Study used similar strategies as described above in the Greater Boston Area to recruit 100 subjects for Phase 2 of the study. From this pool of GI Study participants, we will ask participants if they would like to take part in this sub-study until we have the number of volunteers that are deemed necessary for the study based on sample size calculations from the pilot study. These volunteers will sign separate approved informed consent form that indicates knowledge of the additional volume of blood that will be drawn for this sub-study.

Participants that have been recruited through the GI Study will have undergone a series of screenings including a telephone interview and screening visit to the MRU. The exclusion criteria for the GI study are the same as for the pilot study. Should any abnormal test results be identified, volunteers will be referred to their personal physician for follow up.

Exclusion Criteria:

1. Age < 50 or > 85 years
2. BMI < 25 or > 35 kg/m^2
3. Smoking within the past 6 months
4. Use of medications known to affect glucose metabolism (insulin, sulfonylureas, metformin, glucosidase inhibitors, thiazolidinedione insulin sensitizers) or medications known to affect gastrointestinal motility (such as prokinetic agents)
5. Use of medication known to affect lipid metabolism (bile acid sequestrants, cholesterol absorption inhibitors, fibric acids, HMG-Coa reductase [statin] inhibitors, nicotinic acids, anabolic steroids, diphenyl hydantions, dilantin, hydrocortisone, oral contraceptives, fish oil, red yeast rice).
6. Alcohol consumption > 7 drinks/week
7. Unwillingness to adhere to study protocol

2. Study 2343 Laboratory Methods: Oral glucose challenge in human subjects

Supplies:

- Cell Preparation Tubes (CPT) (BD 362761)
- PAXgene Tubes (Qiagen, 761115)
- Dulbecco's 10X Phosphate Buffered Saline (PBS) (Fisher Scientific, 17515-Q)
- RIPA (Thermo Scientific, 89901 – 250mL)
- Protease and Phosphatase Inhibitor (Thermo Scientific, 78440)
- autoMACs Running Buffer (Miltenyi Biotech Inc., 130-091-221)
- autoMACs Wash Solution (Miltenyi Biotech Inc., 130-092-987)
- MACs Bleach Solution (Miltenyi Biotec Inc., 130-093-663)
- Monocyte Isolation Kit II (Miltenyi Biotech Inc., 130-091-153)
- Ethanol (Sigma, 270741-2L)
- Trizol (Invitrogen, 15596-026)
- Borosilicate glass disposable pasteur pipettes (5 ¾") – (VWR, 14673-010)
- Cell Preparation Tubes (CPT) (BD, 362761)
- autoMACs columns (Miltenyi Biotech Inc., 130-021-101)
- Refrigerated centrifuge (Sorvall RT6000B, Dupont) with H-1000B Rotor
- Microcentrifuge
- autoMACs Pro cell sorter + rack for 15 mL tubes (Miltenyi Biotech Inc.,)

Preparation Protocol

1. Refrigerate autoMACs tube rack the night before and sign up to use machine.
2. In the morning, set the centrifuge in Room 440 to 4°C, set the centrifuge in Room 443 to 25°C, set microcentrifuge in Room 439 to 4°C, thaw autoMACs running buffer.
3. On lab bench, set up two 15 mL conical tubes (label #1 and #2) for 6 times points (12 tubes total)
4. Set up two more 15 mL conical tubes for 6 time points for autoMACs output
5. Prepare ice buckets, cold 1X PBS, make up RIPA/PI mix
6. Install autoMACs columns:
    - Fill autoMACs bottles with running and wash buffers, turn on machine
    - Select: Option, Col_ex, Run (wait 5 min)
    - May be prompted to select: Install
    - Wait until prompted to exchange columns
    - Open door, pull column out of magnetic slot, place container under column
    - Unscrew top of column, tip into container, unscrew bottom
    - Screw in new column (bottom first, then top, push into slot)
    - Repeat for second column (blank?), press done (wait 2 min)
    - When green, run pre-sort wash (under separation, select: wash now then rinse)

BLOOD DRAW PROTOCOL

During the visit conducted in the Medical Research Unit (MRU) of the Human Nutrition Research Center on Aging, subjects were provided with a glucose drink and will have blood drawn 7 times over a five hour period (timepoints: 0, 30, 60, 120, 180, 240, and 300 minutes). One ml of blood was discarded at each time point before subsequent blood is drawn. Details regarding blood volume and tubes are listed below:

Time points: 0, 60, 120, 180, 240 & 300 minutes

Vacutainer	Size (ml)	No.	Test Required	Aliquot Volume	Special instructions
Red Top	3 ml	1	Glucose Insulin Save	500 ul 500 ul Remainder	Bring to NEL Room Temp (RT)
Blue/Black (CPT)	8 ml	3	Gene/prot expr	N/A	Draw below arm Invert 8-10 times Bring to CNL, RT
PAXgene	2.5 ml	2	Gene expr	N/A	Draw below arm Invert 8-10 times Bring to CNL, RT

Time point: 30 minutes

Vacutainer	Size (ml)	No.	Test Required	Aliquot Volume	Special instructions
Red Top	3 ml	1	Glucose Insulin Save	500 ul 500 ul Remainder	Bring to NEL Room Temp (RT)

PBMC ISOLATION (from CPT)

Retrieve 3 CPT tubes from the MRU immediately after each blood draw (except the 30 min time point). Ensure that the CPT tubes were inverted 8-10 times prior to bringing them down to lab.

1. Remix blood sample immediately prior to centrifugation by gently inverting 8-10X
2. Centrifuge at 1800 x g for 15 min at room temperature
3. Aspirate approx half of the plasma without disturbing cell layer (whitish, just above gel)
4. Collect cell layer with a Pasteur pipette from the first CPT, transfer to tube #1
5. Collect cell layers from the next two CPT and transfer both to tube #2
6. Bring conical tube volumes to 15 ml with 1X PBS. Cap tubes. Mix cells by inverting 5X
7. Centrifuge at 300 x g for 15 min at 4C. Pipet out as much supernatant as possible without disturbing the pellet. Leave about 1 cm of PBS. Store on ice.
8. Resuspend cell pellet by gently vortexing or tapping tube with finger (scrape on bench)
9. Add PBS to bring volume to 10 ml. Cap tube. Mix cells by inverting 5 times

10. Centrifuge at 300 x g for 10 min at 4C. Pipet out as much supernatant without disturbing the pellet.
11. Tube #1 (PBMC) will be stored on ice for protein collection (once other cells are sorted). Add 1 ml 1x PBS to PBMC sample, transfer to eppi
12. Tube #2 (containing 2x PBMC) will be sorted for monocytes

Collection tube set up for autoMACS output

- Sample (A)
- Unlabeled (B) - these cells go through column (depleted)
- Labeled (C) - cells bind to column and released later (positive selection [pos-sel])

CELL SORTING (autoMACS Pro)

Negative (Deplete) Selection (Monocyte Isolation Kit II)

1. Resuspend pellet in 30 ul running buffer (mix well), 10 ul FcR Block Reagent, 10 ul Biotin Ab-Cocktail per 10 million cells (mix again)
2. Incubate for 10 min in the refrigerator
3. Add 30 ul running buffer and 20 ul Anti-Biotin Microbeads
4. Mix well and incubate for 15 min in refrigerator
5. Wash cells with buffer by adding 1-2 ml buffer, centrifuge 300 x g, 10 min (4˚C)
6. Pipette off supernatant, resuspend up to 100 million cells in 500 ul buffer (mix)
7. Under separation, select "Deplete" (touch 1 well) wait for green, select run, 5 min

PROTEIN/RNA COLLECTION (on ice)

1. Split sorted monocyte sample (from 2x PBMC) – 1 ml for RNA, 1 ml protein
2. Spin monocytes and PBMC for 5 min at 2500 (2.6x1000 RPM) at 4C. Remove PBS leaving only pellet
3. Add 1 ml trizol to the monocytes designated for RNA, vortex, set on ice 5 min (store in -80˚C)
4. Add 30-100ul Prot. I Combo to monocyte/PBMC samples for protein. Mix well by scraping across rack
    a. Set on ice for 20 min
    b. Spin at full speed for 10 min (12,000 RPM)
    c. Transfer supernatant into newly labeled 1.5 ml tube
    d. Store in -80C (save 15 ul for protein quant, aliquot for 40 ug protein)

Bleach/Biohazard Wash of AutoMACS

1. Options, special, col_ex, run (put in empty columns), done
2. Options, special, safe, run (disconnect all tubing except from waste + reconnect to bottles with 15 ml 10% bleach, put 10% bleach in 50 ml conical tube in rack)
3. Press ok, (asks for the 50 ml), ok (15-20 min)
4. When done, reconnect bottles, ok (system will rinse for 5 min)
5. Tells you to replace columns – insert the ones that were left in the machine
6. Then it prompts you to clean the needle (but it never lifts)
7. When green, press Φ in top right corner of screen, yes
8. After it rinses it will tell you to shut off (rinse for 5-10 min)
9. Refrigerate running buffer, empty waste

3. RNA Isolation, Reverse Transcription and Real Time PCR

RNA Isolation using RNeasy Kit (from cell pellet stored in trizol)
Materials/Equipment
Microcentrifuge at 4˚C
Qiagen RNeasy Kit: Label (2)1.5 ml tubes, (1)column per sample
2 mL chloroform in beaker
70% EtOH
Prepare DNase 1 solution (70 ul RDD + 10 ul DNase – per sample) in refrigerator (DO NOT VORTEX) or 910 ul RDD, 130 ul DNase

1. Thaw on ice (takes 30 min) – the rest of this protocol takes 2 hours after thawed
2. Add 200 ul chloroform. Vortex. Set on ice for 5 min. Spin 15 min at 12,000rpm, 4˚C
3. Remove upper (aqueous) layer into new 1.5 ml tube.
4. Add ~500 ul (or one volume) 70% ethanol, mix well by pipetting
5. Transfer 600 ul to RNeasy spin column – may need to repeat with remaining sample
6. Centrifuge 15s at 10,000 rpm (8000xg). Discard flow-through (re-use collection tube each time)
7. Add 350 ul Buffer RW1 to RNeasy spin column. Centrifuge 15s at 12,000rpm. Discard flow-through
8. Add 80 ul DNase 1 solution. Incubate on bench 15 min.
9. Add 350 ul Buffer RW1 to RNeasy spin column. Centrifuge 15s at 12,000rpm. Discard flow-through
10. Add 500 ul Buffer RPE to RNeasy spin column. Centrifuge 15s at 12,000rpm. Discard flow-through
11. Add 500 ul Buffer RPE to RNeasy spin column. Centrifuge 2 min at 12,000rpm. Discard flow-through
12. Optional: place spin column in new collection tube. Centrifuge for 1 min
13. Place spin column in new 1.5 ml tube, add 20 ul (30-50 ul) RNase-free water directly onto membrane. Centrifuge 1 min at 10,000rpm to elute RNA
14. Can repeat if expect high yield (>30 ug). This will increase the total RNA yield by 15-30%, but the final RNA concentration will be lower.
15. Quantify by nano-drop. Store in -20C

RNA Isolation using PAXgene Blood RNA Kit
Materials/Equipment:
Qiagen PAXgene Blood RNA Kit: buffers 1-5, PK, RNase free water, DNase and RDD
New cap(s), shredder+spin column, (3) 1.5ml tubes, (6) 2ml processing tubes
Pipetmen, tips (sterile-filter), pipetaid, 5ml pipet, glass tips, waste, ice, 100% EtOH
Centrifuge at RT (for blood tubes), Microcentrifuge at RT (for 1.5ml tubes)
Small bench-top microcentrifuge, vortex

Preparation:
Set PAXgene Blood RNA Tubes at room temp for at least 2 hours
Set shaker-incubator to 55°C
Make up BR4 and DNase I stock solution (if using kit for the first time)

Protocol:

1. Centrifuge PAXgene Blood RNA Tube 10 min at 4000 x g (at RT)
2. Remove the supernatant by decanting. Dry rim with kimwipe. Add 4 ml RNase-free water to the pellet, and close the tube using a new BD Hemogard closure
3. Vortex until the pellet is visibly dissolved, and centrifuge for 10 min at 4000 x g. Remove and discard the entire supernatant (glass pipette).
4. Add 450 µl BR1 (changed from original), vortex + scrape against surface until the pellet is dissolved
5. Pipet (glass) sample into a 1.5 ml tube. Add 300 µl BR2 and 40 µl proteinase K. Mix by vortexing for 5 sec, and incubate for 10 min at 55°C using a shaker–incubator at 400–1400 rpm. After incubation, set the temp of the shaker–incubator to 65°C (for later)
6. Pipet 600 ul lysate into a Shredder spin column (lilac), centrifuge for 3 min at 13,000 x g
7. Transfer supernatant of the flow-through fraction to a fresh 1.5 ml tube without disturbing the pellet in the processing tube (use pipet tips)
8. Repeat step 6 + 7 with remaining sample. (Discard Shedder spin column)
9. Add 350 µl ethanol (100%). Mix by vortexing, and microcentrifuge briefly (1–2 sec)
10. Pipet 700 µl sample into Pink spin column, and centrifuge for 1 min at 13,000 x g. Place spin column in a new processing tube, and discard old. Repeat with remaining sample
11. Pipet 350 µl BR3 into the spin column. Centrifuge for 1 min at 13,000 x g. Place the spin column in a new processing tube, and discard old
12. Add 10 µl DNase I stock solution to 70 µl Buffer RDD in a 1.5 ml tube. Mix by gently flicking the tube, and microcentrifuge briefly (2sec). For 12 samples, add 130 µl DNase I stock solution to 910 µl Buffer RDD
13. Pipet the DNase I incubation mix (80 µl) directly onto the spin column membrane (not on ring), and incubate on the benchtop (20–30°C) for 15 minutes
14. Pipet 350 µl BR3 into the spin column, and centrifuge for 1 min at 13,000 x g. Place the spin column in a new processing tube, and discard old
15. Pipet 500 µl BR4 to the spin column, and centrifuge for 1 min at 13,000 x g. Place the spin column in a new processing tube, and discard old
16. Add another 500 µl BR4 to the spin column. Centrifuge for 3 min at 13,000 x g. Place the spin column in a new processing tube, and discard old
17. Centrifuge for 1 min at 13,000 x g. Place the spin column in a 1.5 ml microcentrifuge tube, and discard old
18. Pipet ~~40~~ 20 µl BR5 onto the spin column membrane. Centrifuge for 1 min at 13,000 x g to elute the RNA
19. Repeat the elution step (step 18) as described, using ~~40~~ 20 µl Buffer BR5 and the same microcentrifuge tube

20. Incubate the eluate for 5 minutes at 65°C in the shaker–incubator (from step 5) without shaking. After incubation, chill immediately on ice
21. If the RNA samples will not be used immediately, store at –20°C or –70°C. Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the incubation at 65°C. If using the RNA samples in a diagnostic assay, follow the instructions supplied by the manufacturer.

Quantification of RNA

bring 1-10ul pipetter, tips + samples, RNase free water, and BR5 on ice
Double Click "ND-1000"
Click "Nucleic Acid" (make sure on "RNA" setting)
Apply 2ul RNase free water to reader, Click "OK"
Apply 2ul BR5, Click "Blank"
Apply 2ul sample, Click "Measure"
Wipe down reader with water

Reverse Transcription using Promega Kit (A3500)

- Do not vortex AMVRT (enzyme) – it is very sensitive, was told not even to leave it on ice (it does not freeze so just take from freezer when ready to add to master mix)
- Usually use 1ug RNA samples and bring them up to 10ul with water. If your sample is too dilute to do this, you should be able to bring up to 15ul (you have to bring all your samples up to the same volume and make up the x1.25 master mix per sample)
- Heat water baths to 42˚C, 70˚C, 95˚C
- Thaw master mix buffers on ice

Master Mix Preparation:

	per 10ul sample	per 15ul (x1.25)	x #samples (+extra)
MgCl2	4ul	5ul	
10xRT	2ul	2.5ul	
dNTPs	2ul	2.5ul	
RNasIN	0.5ul	0.625ul	
AMVRT	0.6ul	0.75ul	
Rand. Prim	1ul	1.25ul	

Protocol

1. For 10ul RNA samples, add 10.1ul master mix per sample (on ice) (For 15ul RNA samples, add 12.6ul master mix per sample, but all samples must be made up to 15ul)
2. Incubate at 70C for 10 min, then ice for at least 5 min
3. Add 10.1ul (or 12.6ul) master mix, mix well
4. RT 10 min, 42˚C 15 min, 95˚C 5 min, ice 5 min (+)
5. Aliquot cDNA samples

- I used 5ul cDNA for each gene, then added 10ul water for PCR → 4ul per well x 3 wells
- I was testing 3 genes (including B-actin) so I aliquoted 3 samples, 5ul each (and saved what was left)
- cDNA can be stored in -20C

Real Time PCR

Materials/Equipment:
Applied Biosystems 7300 machine
QuantiTect SYBR Green PCR Master Mix (Qiagen, 204145)
QuantiTect Primer Assays described in methods for **Chapters 3 and 4**
Primers described in **Chapter 4, Table 1**
Tris EDTA (Fluka, 93283)

Reconstitute 10x QuantiTect Primer Assays to make 5 uM solution:

- 1 ml of 1 M Tris·Cl, pH 8.0 (autoclaved)
- 0.2 ml of 0.5 M EDTA, pH 8.0 (autoclaved)
- 98.8 ml of distilled water
- Alternatively, ready-made TE can be purchased from chemicals suppliers (Tris EDTA, Fluka 93283)

Reconstitute primers made by Tufts Core to make 5 uM solution:

- Determine amount of TE to add using optical density (OD) and molecular weight (MW):
  - ODx330,000/MW= amount of TE which should be added to each primer
  - 5 uL forward primer + 5 uL reverse primer + 90 uL diH2O → 5 uM solution

Important points before starting:

- PCR must start with an initial incubation step of 15 minutes at 95°C to activate HotStarTaq DNA Polymerase (included in 2x QuantiTect SYBR Green Master Mix)
- Always readjust the threshold value for analysis of every run
- Do not use final reaction volumes of less than 25 µl when using this instrument
- Must run primer efficiency assays to determine appropriate cDNA dilutions: see Nicole Spartano's lab notebooks for details
- The volume of the cDNA added (from the undiluted RT reaction) should not exceed 10% of the final PCR volume
- Due to the hot start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler
- No optimization of the Mg2+ concentration is required. The final Mg2+ concentration of 2.5 mM provided by 2x QuantiTect SYBR Green PCR Master Mix gives optimal results

Procedure:

1. Thaw 2x QuantiTect SYBR Green PCR Master Mix (if stored at –20°C), 10x QuantiTect Primer Assay, template cDNA, and RNase-free water. Mix the individual solutions
2. Prepare a reaction mix:

    - 2x QuantiTect SYBR Green PCR Master Mix* -- 25 µl
    - 10x QuantiTect Primer Assay -- 5 µl
    - Template cDNA (added at step 4) -- Variable (≤100 ng/reaction)
    - RNase-free water -- Variable
    - Total volume 50 µl

3. Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or plates
4. Add template cDNA (≤100 ng/reaction) to individual PCR tubes or wells containing the reaction mix
5. Program the real-time cycler (described below)
6. Place the PCR tubes or plates in the real-time cycler, and start the cycling program

Cycling conditions for two-step RT-PCR

- PCR initial activation step -- 15 min at 95°C
- 3-step cycling (35-40 cycles):
    - Denaturation* 15 s 94°C
    - Annealing 30 s 55°C
    - Extension† 30 s 72°C (fluorescence data collection step)
- Dissociation

† Due to software requirements, the fluorescence detection step must be at least 30 s with the ABI PRISM 7000, or 34 s with the Applied Biosystems® 7300 and 7500.
†† If using the Applied Biosystems 7500, we recommend adjusting the default "Manual Ct" threshold value of 0.2 to a lower value (e.g., 0.02) in order to analyze the data properly.
Optional: Melting curve analysis of the PCR product(s) may be performed to verify their specificity and identity. Melting curve analysis is an analysis step built into the software of real-time cyclers. Please follow the instructions provided by the supplier.
Note: The *T*m of a PCR product depends on buffer composition and salt concentration. *T*m values obtained when using QuantiTect SYBR Green PCR reagents may differ from those obtained using other reagents.

4. Isolation and Culture of Murine Bone Marrow Cells

Materials:

1. RPMI 1640 medium — *Gibco # 11875-093*
   - Glucose (2g/L),Phenol red, L-glutamine, No sodium pyruvate, no HEPES
2. RPMI 1640 medium – (glucose-free) — *Gibco # 11879-020*
3. Fetal Bovine Serum (FBS) (storage -20) — *Gibco, 160000-44*
   - Remove lipoprotein (LD-FBS protocol)
4. D-Glucose, L-Glucose — *Sigma G-7021 , G-5500*
5. Penicillin-Streptomycin — *Sigma P4333*
6. Lidocaine HCL — *Sigma L5647*
7. EDTA (storage: room temperature) — *Sigma E7889-100ml*
8. recombinant M-CSF (Human) — *eBioscience ,16-7336-85*
   - Aliquot into 100ug/ml PBS, final treatment concentration is 100ng/ml
9. Culture dishes 100 X 15 mm (untreated) — *Fisher 08-757-13*
10. 6-well plates (tissue culture treated) — *Falcon 353046*
11. 10 ml syringes — *BD 301029*
12. 25 or 27 gauge needle — *BD305109, BD305122*
13. Milipore Filter — *SLGP033RS*
14. PBS (sterilize to use in hood) — *Sigma P5493*
15. Oleate, Palmitate, Linoleate — *Nu-chek S-1120, S-1109, S-1127*
16. BSA (for conjugation) — *Sigma A8806*
17. oxLDL, HDL — *Intracel RP-047, RP-037*
18. apoA1 — *Sigma A0722*
19. H3 cholesterol — *Perkin Elmer NET139250UC*
20. NH4-HCO3
    (1M = 79.056g/L or 0.79056g/10mL, so 20 mM = 15.8112 mg/10 mL)

Preparation of Media for differentiating Macrophages from Bone Marrow Cell

Macrophage Growth Media

1. 80% RPMI (2 g/L glucose)
2. 20% FBS
3. 5 mg/ml Penn/Strep (5 ml in 1000ml media)
4. 100 ng/ml M-CSF (1ul/ml of the stock solution) in -80, add day of use

LD-Media

1. 80% RPMI (glucose-free)
2. 20% LD-FBS
3. 5 mg/ml Penn/Strep
4. 100 ng/ml M-CSF
5. Glucose (1g/L)

Glucose Stocks: 160 mg D- or L-glucose + 10 mL PBS, filter sterilized

FFA/BSA Stocks: see preceding protocol

Treatment Media (glucose or FFA) – make day of use

- 500 uL treatment media is added to 1 mL LD-media present in wells

1. RPMI (glucose-free)
2. 20% LD-FBS
3. 100 ng/ml M-CSF
4. Stock Treatments solutions:

    a. FFA/BSA stock solution calculated for100 uM FFA +

    a. 50 uM BSA final concentrations (in 1.5 mL)

        - Final D-glucose concentration: 1 g/L

    b. D- or L- glucose stock calculated for final concentrations:
        - Low D-glucose: 1 g/L D-glucose
        - High D-glucose: 4.5 g/L D-glucose
        - High L-glucose: 3.5 g/L D-glucose + 1 g/L D-glucose

<u>OxLDL/Treatment Media (glucose or FFA)</u> – make day of use

- 1 mL oxLDL/treatment media is added directly to cells

2. RPMI (glucose-free)
3. 20% LD-FBS
4. 100 ng/ml M-CSF
5. 25 ug/mL oxLDL (final concentration)
6. (1 uCi/mL H cholesterol for cholesterol efflux experiment)
7. Stock Treatments solutions:
    a. FFA/BSA stock solution calculated for100 uM FFA + 50 uM BSA final concentrations (in 1 mL)
        - Final D-glucose concentration: 1 g/L
    b. D- or L- glucose stock calculated for final concentrations:
        - Low D-glucose: 1 g/L D-glucose
        - High D-glucose: 4.5 g/L D-glucose
        - High L-glucose: 3.5 g/L D-glucose + 1 g/L D-glucose

The following protocol was adapted for macrophage differentiation by Dr. Yun Lee from a procedure published for dendritic cells [1].

Day 1. (Friday)

Isolation and culture of bone marrow derived macrophages

1. Prepare a working station, sign up for necropsy room
    a. 50 mL conical tube with 30 mL of just RPMI (glucose containing)
    b. conical w/ 40 mL growth media (take out 40ul M-CSF, add during spin)
    c. Sterilized facet, scissors, 70% EtOH bottle, syringes and needles
    d. (4) 100 mm dishes/animal + 2 dishes (trash, sterile PBS) + 50 mL tube
2. Euthanize the mouse by CO2 narcosis followed by cervical dislocation
3. On a hind leg cut the skin around the ankle and pull the skin back towards the body revealing the entire leg
4. Hold the leg toward you, remove muscle/fat, cut off ankle
5. Cut the leg off as high up as possible, remove additional muscle and fat
6. Cut the joint of the leg, and snip both ends of the bone (not too much because cells are concentrated near the joints)
7. Insert the syringe into the bone and push ~ 6 ml of RPMI through the bone into a 50 ml conical tube
8. Repeat for each section of bone (2 per leg)
9. Resuspend the cells well by pipetting up and down
10. Centrifuge 5min at 1500 rpm
11. Aspirate supernatant being careful not to disturb pellet (may be a little red, OK)
12. Add 40 ml of growth media (+ 40 ul M-CSF) to the pellet and resuspend
    a. Add 10 ml first, pipet up and down, then add the rest
13. Plate cells evenly across the (4) 100 mm dishes

Day 2. (Sat) - Observe the cells (majority of the cells floating and not attached)
Day 3. (Sun) - Macrophages begin attaching to the bottom of the plate (still a lot floating)
Day 4. (Mon morning) - Observe cells and decide to change the media or harvest them
Must at least change growth media even if cells are not fully stuck

1. Harvest by making removal solution - primary macrophages are very adhesive
    a. 400 mg Lidocaine HCL in 50 mL tube, add 500 uL 0.5 M EDTA (pH 8.0)
    b. Bring final volume to 50 mL with PBS in the conical tube, mix well
    c. Filter sterilize it using filter and syringe, warm up the solution
    d. Filter sterilize 100 mL PBS
    e. Warm 40-80 mL LD-media per animal
2. Suck out old media, wash w/ 10 ml PBS, suck out
3. Add 10 mL lidocaine per each 100 mm dish
4. Incubate them in the incubator for 5-10 minutes
5. Pipet up and down in plate, collect the cells in a new conical tube
6. Centrifuge them for 5 min at 1500 rpm
7. Remove the supernatant and wash with PBS
8. Centrifuge them for 5 min at 1500 rpm
    a. Take out the M-CSF at this point to add to LD-media
9. Resuspend them in LD-media (**10 mL at first, then add 10mL**)
10. Count the number of cells (1-1.5 million cells per well in 6-well plate)
    a. Take out 10ul, may want to dilute with 10ul water
    b. _#_ x 2000 x 2(dilution factor) = # cells/mL (in 20 mL)
    c. Or _#_ x 2000 x 1(no dilution) = # cells/mL (in 20 mL)
    d. 1.5 million cells/mL (6-well plates), dilute accordingly
    e. Or 5 million cells/4.5 mL (100 mm dishes)
11. Remember to put in coverslips if preparing for ORO
12. Plate 1.5 million cells in 1 mL media per well (6-well plates)
13. Check on them tonight (3-6 hours later) whether they are ready to be treated. You may need to wait until Tuesday evening (push all treatments back 1 day).

Treatment (Mon night)

1. Add 500 uL glucose or L-glucose or FFA/BSA treatment solution to necessary wells in 6-well plate (which already contains 1 mL media)
2. Tuesday morning (16 hours later) remove media and add 1 mL glucose or FFA/BSA oxLDL treatment solution (see oxLDL treatment solution below)

<u>Collecting BMDM (for outcome measures other than cholesterol efflux):</u>

- Cells will be collected 24 hours after treatment with oxLDL
- First, rinse 2x with 5 ml PBS

For Cholesterol/TG Analysis:

- Bring up in 1 ml PBS, scrape, transfer to eppi
- Transfer 100 ul to new eppi for protein, 800 ul to new eppi for chol/TG
- Spin at 2,600 rpm (mini-centrifuge) 5 min, 4C, remove supernatant
    - Chol: bring up in 250 ul water
    - TG: bring up in 100 ul water
    - Vortex and freeze -20C
- Prot: bring up in 30 ul RIPA/PI mix, scrape, 20 min ice, spin 10 min 12,000 rpm, transfer supernatant to new eppi, store -80C

For RNA Analysis:

- Bring up in 1 ml trizol, scrape, transfer to eppi, vortex, 5 min ice, store -80C

For Protein Analysis:

- Bring up in 500 ul RIPA/PI mix, scrape, transfer to eppi, scrape on rack, 20 min ice, spin 10 min 12,000 rpm, transfer supernatant to new eppi, store -80C

Solutions to make up for Cholesterol Efflux Experiment:

2% BSA stock solution: 15 ml RPMI (glucose-free) + 300 mg BSA → sterilize
RPMI (1 g/L glucose): 250 mL RPMI (2 g/L glucose) + 250 mL RPMI (glucose-free)

Equilibration Media (glucose or FFA) – make day of use

*This media contains no LD-FBS!!

- 1 mL equilibration media is added directly to cells

1. RPMI (glucose-free)
2. 100 ng/ml M-CSF
3. 0.2% BSA final concentration (using 2% BSA stock solution)
4. Stock Treatments solutions:
    a. FFA/BSA stock solution calculated for100 uM FFA + 50 uM BSA final concentrations (in 1 mL)
        - Final D-glucose concentration: 1 g/L
    b. D- or L- glucose stock calculated for final concentrations:
        - Low D-glucose: 1 g/L D-glucose
        - High D-glucose: 4.5 g/L D-glucose
        - High L-glucose: 3.5 g/L D-glucose + 1 g/L D-glucose

Efflux Media: - prepare as in the box form (previous page) – use final concentrations or percents, not actual volumes to be added!!!!

*This media contains no LD-FBS!!

For example, if you need to make 10 mL media (3 treatments in triplicate) for each HDL, apoA1 and baseline then you will need to add enough BSA, glucose, M-CSF and p/s for 11.11 mL because you will be adding 10 % FFA after the HDL/apoA1.

*same principles apply for glucose treatments but below you will find FFA example:

In 33.3 mL efflux media (HDL, apoA1, baseline):

- 28.94 mL RPMI (877 uL/mL)
- 3.663 mL BSA (111 uL/mL)
- 229 uL glucose stock (6.94 uL/mL)
- 183.15 uL p/s (5.55 uL/mL)
- 36.63 uL M-CSF (1.11 uL/mL)

Baseline

10 mL efflux media + 29.52 ul NH4-HCO3 (stock =20 mM)

*apoA1 contains 10 mM ammonium bicarb so we have to add ½ the volume of apoA1 we added in 20 mM NH4-HCO3 as control to the HDL and baseline solutions

apoA1

10 mL efflux media + 59.04 uL apoA1 (stock = 1.88 mg/uL)

final concentration=10 ug/mL or 0.01 mg/mL

0.01mg/ml x 11.11 mL = 0.111 mg 0.111mg/1.88mg = 59.04 uL

HDL

10 mL efflux media + 27.78 uL HDL (stock 20 mg/uL) + 29.52 ul NH4-HCO3 (20 mM)

final concentration=50 ug/mL or 0.05 mg/mL

0.05 mg/mL x 11.11 mL = 0.556 mg 0.556 mg/20 mg = 27.78 uL

Baseline (10 mL)	**apoA1 (10 mL)**	**HDL (10 mL)**
3.15 mL + 0.35 mL FFA	3.15 mL + 0.35 mL FFA	3.15 mL + 0.35 mL FFA
3.15 mL + 0.35 mL FFA	3.15 mL + 0.35 mL FFA	3.15 mL + 0.35 mL FFA
3.15 mL + 0.35 mL FFA	3.15 mL + 0.35 mL FFA	3.15 mL + 0.35 mL FFA

Cholesterol Efflux Experiment (24 hours following treatment with oxLDL/H3 cholesterol):

1. Remove media, rinse 2x with 2 mL RPMI (1 g/L glucose)
    a. Waste must go in radioactive waste bins
2. Add equilibration media (containing glucose or FFA treatments), incubate 6-8 hours
3. Remove media, rinse 2x with RPMI (1 g/L glucose)
4. Add HDL, apoA1 or baseline efflux media, incubate 4 hours
5. Collect as described on the following page

Collecting for efflux study:

- Set up 6 vials per well (4ml scintillation fluid per vial)
- Collect 750 ul media from each well into eppi
- Rinse cells 2x with 5 ml cold PBS (radioactive waste)
- Add 500 ul RIPA (no PI) to each well, scrape, transfer to eppi
- Spin (10,000 x g, 10 min, RT) transfer supernatant (cell lysate) to new tube
    - (can store in -20C overnight if needed)
    - Add 100 uL cell lysate or 200 uL media to scintillation vials, cap, shake
    - Keep the rest of cell lysate for protein quantitation (I usually do this first)
- Fraction effluxed = medium dpm/(medium dpm + cell dpm)
  Dpm = disintegrations per minute
- At the end, adjust fraction efflux to cellular protein:
    - Final value = fraction effluxed/mg protein
- 8th floor (vitamin bioavailability lab)
- Place racks into scintillation counter (back right) with flag #3 (or other program with the right parameters: isotope H3 at 1 min count)
    - Lever on flag should be pulled out
    - Let samples settle for 10-20 min before starting

5. FA/BSA Conjugation

Materials

Sodium Stearate C18:0 (Nu-Chek Prep S-1111) MW 306.48
Sodium Elaidate C18:1t (Nu-Chek Prep S-1121) MW 304.48
Sodium Palmitate C16:0 (Nu-Chek Prep S-1109) MW 278.41
Sodium Oleate C18:1 (Nu-Chek Prep S-1120) MW 304.48
Sodium Linoleate C18:2 (Nu-Chek Prep S-1127) MW 302.48
Sodium Linolenate C18:3 (Nu-Chek Prep S-1129) MW 300.48
Low endotoxin, fatty acid-free bovine serum albumin (Sigma: A8806)
RPMI1640 mediun (ATCC: 30-2001),
1N NaOH (1M) MW 40.00, 1N HCl MW 36.46 (we have 1N HCl in acid cabinet)
Scale, Weighing paper, Weighing boat, Water bath, pH meter, Biosafety cabinet
Pipet and tips (10µL, 200µL and 1000µL), 10mL pipet, 10mL syringe (Fisher: 14-829-22A)
Microcentrifuge tubes (Fisher: 02-681-320), 15mL-sterile tubes (Fisher:05-539-5)
Millipore* Millex* sterile syringe filters (0.22um, Low protein binding) (Fisher: SLGV 033 RS)

Preparation

1. Turn on water bath and set temperature to 37°C and 70°C (for SFA or TransFA)
2. ONLY FOR THP-1 CELLS: Add 2µL of 2-mercaptoethanol into 500mL of RPMI1640 medium, and put it into water bath – remove amount needed into 50mL conical tubes while still in hood to keep rest of media sterile (I removed 75mL – 37.5mL into 2 50mL conical tubes)

Procedures

1. Make 0.5mM albumin: 0.033g/mL (0.99g into 30mL or 1.32g into 40mL)
2. Invert conical tubes slowly and put onto rocker at RT to slowly dissolve the BSA into media, rock until albumin is completely dissolved (changes from pink to slightly yellow)
3. Put the albumin solution into water bath (37°C)
4. Weigh fatty acid and dissolve it into diH_2O in a microcentrifuge tube and make concentration to 10mg/mL. For example: weight is 8.5mg, then add 850µL of diH_2O

* Must flush Na-FA powder with nitrogen and make air-tight for storage in -20°C

5. Vortex and put the tube in water bath
    a. UnsatFA need to be put into 37°C to dissolve
    b. SFA and Trans FA need to be put into 70°C (or greater) to dissolve **(after in solution you must do next step IMMEDIATELY or they will fall out of solution)**
6. **Make 1mM fatty acids:**
    a. Add 10mL (minus the volume of FA you want to add) of media+BSA into a 15mL tube
    b. Make 1mM FA solution by adding calculated amount of 10mg/mL stock solution to the above media+BSA, drop by drop and vortex every 10 drops

For example: stearic acid sodium salt MW 306.48mg/mL (so 0.306 mg/mL = 1 mM)
Since you have a 10 mL solution, need to add 3.06 mg total
So add 306µL of 10mg/mL (= 3.06mg) to 9.694 mL media(+ BSA)
**** For 2mM FA solution**: add 612 uL of 10 mg/mL to 9.388 mL media(+BSA)
*****Must add 18:0 and 18:1t immediately after dissolving (do it next to the water bath or it will fall out of solution!) After the 306uL is added drop wise to 10mL media it stays in solution, but must do it quickly.**

7. Make (control) 0 mM FA solution of media + BSA by adding same amount of water as your treatment solution (so above example: would add 0.306 mL water to 9.694 mL media+BSA)
8. Incubate at 37°C for 30min with occasion mixing/shaking (to help the FA, BSA mix)
9. Adjust pH to 7.6 by adding either NaOH or HCl (I had to add 1-2 drops of 1N NaCl)
10. In the sterile hood, pass the above 1mM fatty acid through 0.22µm Millipore filters by using 10mL syringe in the biosafety cabinet, and the sterile fatty acid is collected into a new 15mL-sterile tube
11. Aliquot the fatty acid into several autoclaved microcentrifuge tubes, **flush them with nitrogen** and store at −80°C

Examples:
If you want a final treatment concentration of 100 uM FA (50 uM BSA):
use 100 uL of **1mM FA (0.5 mM BSA)** treatment solution + 900 uL normal media

200 uM FA (50 uM BSA):
100 uL of **2mM FA (0.5 mM BSA)** + 900 uL normal media

50 uM FA (50 uM BSA):
100 uL of **0.5mM FA (0.5 mM BSA)** + 900 uL normal media

0uM FA (50 uM BSA):
100 uL of **0mM FA (0.5 mM BSA)** + 900 uL normal media

** Make sure you make separate treatment solutions for each concentration of treatment because you do not want BSA concentration to vary. Must make sure you add the same volume of treatment to each well of cells (same BSA and everything else, only different FA)

6. Lipoprotein deficient FBS (LD-FBS) preparation

Materials

Fetal bovine serum (FBS) (Invitrogen:16000-036)
Potassium bromide (KBr) MW 119.00 (Sigma: P0838)
Sodium chloride (NaCl) MW 58.44 (Sigma: S7653)
Dialysis tubing (Fisher: 21-152-10)
50mL sterile tube (Fisher: 14-432-22)
15mL sterile tube (Fisher: 14-959-49B)
Beckman QS PA bell tube (Beckman: 344619)
Tube spacers (Beckman, discontinued?? – borrowed from rm 524)
Millipore* Millex* sterile syringe filters (0.22um, Low protein binding) (Fisher: SLGV 033 RS)
Ultracentrifuge (Beckman, Optima XL-80K, 5th floor common room), Biosafety cabinet
Rotor (Beckman, Type 50.3Ti, 4th floor common room) – kept in refrigerator
Tube sealer (Beckman: 342420), Tube slicer (Beckman: 347960)
10cc Syringe with 21-gauge needle (Fisher, catalog No. 14-823-12)
Stirring bar, Stirring plate, Beaker, Scale, Weighing paper
Glass pipette, 100ml Graduated cylinder, 5L Graduated cylinders, 5L Flat-bottom container

Preparation

1. Autoclave 2-100 mL graduated cylinders, 200 mL beaker, stirbar
2. Equation for KBr calculation [2]:

--For 100 mL of FBS, the amount of solid KBr to be added is,

$$g\ KBr = \frac{V\ (d_F - d_I)}{1 - K \times d_F}$$

V = Initial volume of FBS at 4°C (mL)
d_F = Final density (1.215 is chosen b/c HDL has a density < 1.21 g/ml)
d_I = Initial density
K = ~~constant to calculate~~ partial specific volume of KBr at final density
The K for final density of 1.1215 is 0.30639

$$g\ KBr = \frac{100\ (1.215 - 1.006)}{1 - 0.30639 \times 1.215} = 33.30$$

Procedures

1. Turn on ultracentrifuge and set temperature to 4°C, push vacuum to start decreasing temperature (will hear hissing sound as vacuum accumulates pressure)
2. Add 33.30g KBr to 100mL FBS
    a. use sterilized beaker and stir bar when dissolving KBr in the FBS

3. Use glass pipette to fill the tubes and get rid of bubbles
4. Seal the tubes, put them in the rotors and put the spacers on the tubes
5. Grease O-rings
6. Ultracentrifuge in 6mL Beckman bell tubes at 39,000rpm for 48 hrs at 4°C, use max acceleration and slow deceleration (option 6)
    a. Push vacuum to be able to open (vacuum was on to decrease temp to 4 C)
    b. Put in rotor, close
    c. Push vacuum and wait for microns < 300 (5-10min)
    d. Push ENTER and then Start (and always check about 8-10min after pushing start to make sure it gets up to correct speed)

After ~40 hours:

7. Prepare 22L of 150mM NaCl (8.766 g/L)
8. Cut 3 pieces of dialysis tubing (25 cm each), put in water 30 min, then 150mM NaCl
9. Slice tubes – set slicer at ~2.8-2.9cm and you should remove ~2.0mL of liquid from the top fraction
    a. Top is clear/whitish (where lipoproteins are located), bottom is yellow/orangish (everything else is located here) – cut in the middle of the clear/whitish fraction
10. Use needle (less than or equal to 21g needle) to suck out the upper fraction – this is the lipoprotein fraction, discard
11. Rinse blade and slicer with DiH_2O before removing the blade
12. Collect bottom fraction by glass pipette to 50mL sterile tubes
13. Rinse the bottom of the 6 mL tubes with small amount of 150mM NaCl and add to conical tube
14. Clip bottom of dialysis tubing (2-4 cm from bottom)
15. Open top with 1000uL plastic pipette tip, hold plastic tip in place as you use glass pipette to transfer FBS into dialysis tubing (avoid bubbles), leave 10% additional length for expansion/contraction of sample volume, clip 2-4 cm from top
16. Dialyze extensively 5 times at 4°C in 4L of 150 mM NaCl with stirring in cold room.
    a. 1hr for the first 3 dialysis
    b. overnight for the 4^{th}
    c. 1 hr for the 5^{th}
17. After dialysis, transfer FBS into 100 ml graduated cylinder and bring to original volume (100mL) with 150 mM NaCl.
18. Sterilize by passing through 0.22µm filter and collect into ~10 12mL-sterile tubes in the biosafety cabinet
19. Store LPDF at -20°C

7. **Triacylglycerol assay:** adapted from Schwartz D.M., et al. [3]

Reagents:

Isopropanol-hexane-water (80:20:2 v/v/v)(2 mL per sample)
PBS-10mM EDTA, pH 7.4 (>200 uL per sample) – I used the liquid EDTA in fridge (1ml:49ml 1xPBS), then pH (ObMet lab uses E-5391)
Hexane-diethyl Ether (1:1) (500 uL per sample)
ddH20 (1 mL per sample)
TG working reagent (800 uL per sample, blanks and standards) (prepared with Glycerol reagent, 4:1- glycerol reagent to TG reagent)
    Sigma #s: TG: T2449-10ML, Glycerol: F6428-40ML *do not shake reagents (make up in water)
Glycerol Standard (~50 ul per assay) (in serial dilutions: 100%, 50%, 25%, 12.5%, 6.25%, 3.125%) Sigma #: G7793-5ML

Equipment:

Pipettes:
    10-200uL with tips for samples, and buffer
    repeat pipetter with 800 uL capacity,
    glass pipette tips for transfer which can measure 900 uL w/ manual rubber tops (you'll have to make these with a cell culture aspirator)
    beckman pipette with tips that can measure at least 2 mL, and one 25 mL tip for water
1 ml polyethylene squirter (1 per sample)
16x100 mm glass vials (1 per sample), rinsed hexane
4 mL glass vials with screw-on tops (1 per sample), rinsed hexane
Vortex, Water bath with shaking, Nitrogen manifold, Plate reader
360 uL volume 96 well plate (#9017, Corning, Corning, NY)
Parafilm, Aluminum Foil, Scissors

TG quantification:

****Work is done in the hood in the antioxidant lab room 504**** (incubation step at 37 degrees C done in OBMet 604)

1. Water bath to 37 degrees, rinse glassware, make IHW + Hex-diET, thaw samples
2. Add sample to 16x100, in quantity determined by BCA assay equal to 25-100 ug of protein (500mg protein for cells), add PBS-10 mM EDTA (pH 7.4) to make a **total volume of 200 ul**
3. Add **2 ml** of isopropanol-hexane-water (IHW- 80:20:2 v/v/v), mix

* If you want to include 400 ul of sample in the buffer of the step above, you can by omitting the water from the IHW-the organic phase can only hold 400 ul of water before phase separation.

4. Cover tubes with aluminum foil and incubate for **30 minutes at room temp**
5. Add 500 ul of hexane-diethyl ether (1:1) to each tube and mix by vortexing—follow this step with an incubation of **about 10 minutes**, by covering the tubes with aluminum foil **at room temp**.
6. Add **1 ml** of water to separate phases, and mix by vortexing. Cover samples with aluminum foil and incubate for **~20 minutes at room temp.**
7. Pipette ~900 uL of the top, organic, phase into the corresponding 4 mL **glass vial**. **(Use polyethylene tips, below "mL" writing)** with screw on top
8. Evaporate the organic phase with Nitrogen at **100-kPa/14.0 p.s.i. At < 100 degrees C,** using manifold in antioxidant lab. This takes about 45-60minutes.
9. Steps 10+11 should be completed simultaneously and quickly, since the reaction can be carried out at room temp. Just be sure you prepare samples, standards, and blanks first, then add TG reagent to all at the same time.
10. Generate a standard curve: make serial dilutions by pipetting 15 uL of 100% standard into 15 uL of PBS-10mM EDTA, to make a 50% dilution, and so forth--making a total of 6 serially diluted standards.
    a. Pipette 8 ul of each diluted std into new eppi
    b. Make blank by using Glycerol Reagent mixed with PBS-10mM EDTA (4:1) (**800 uL** glycerol, **200 uL** buffer)
    c. Make TG working solution (4glyc:1TG):

    800 x (6 std + 8samples + 1extra) = 12 ml = (10glyc:2.5TG)
    3 samples = 7.5 mL (6glyc:1.5TG)
    8 samples = 12 mL (10glyc:2.5TG)
    11 samples = 15 mL (12glyc:3TG)
    14 samples = 17.5 mL (14glyc:3.5TG)
    17 samples = 20 mL (16glyc:4TG)
    20 samples = 22.5 mL (18glyc:4.5TG)
    23 samples = 25 mL (20glyc:5TG)

Standard Curve			
	Glycerol (ug)	Add (ul)	1st make dilutions
A	8.75	8ul (stock)0.26ug/ul std+800ul TGwork	←
B	4.38	8ul of 0.13 ug/ul std + 800 ul TGwork	15ul std+15ul buff
C	2.19	8ul of 0.065 ug/ul std + 800 ul TGwork	15ul B +15ul buff
D	1.09	8ul of 0.0325 ug/ul std + 800ul TGwork	15ul C +15ul buff
E	0.55	8ul of 0.01625 ug/ul std+800ul TGwork	15ul D +15ul buff
F	0.27	8ul of0.008125ug/ul std+800ul TGwork	15ul E +15ul buff
G	0	(800ul glyc reag + 200ul EDTA buff)	

11. With a repeat pipetter, pipette **800 uL** of TG working solution to each sample, then cover vials with screw on tops and cover them on the rack with parafilm, allowing them to incubate for **5 minutes at 37 degrees C with shaking at 360 rpm (set on 6-8).** Incubate blanks, standard curve and samples all together.
12. Then transfer **350 ul** of TG work/sample mixture from each glass vial, to a flat bottom 96-well plate, as well as curve and blanks.

13. Use plate reader set to 540 nm to determine absorbance
14. Express data as mg triglyceride/g protein by multiplying ug of glycerol by 40 to take into account starting protein was 25 ug.

$$\frac{\text{ug}}{25\text{ ug}} = \frac{\text{mg}}{25\text{ mg}} = \frac{1000\text{mg}}{25\text{ g}} = \frac{40\text{mg}}{\text{g}}$$

*N.B. Solvents used in this assay are **flammable** and **volatile** even at a low temperature. Storage, in non-explosion proof refrigerators or freezers may result in **explosion and/or fire! It is recommended that when samples are collected at different times, but within a few days, samples be added to IHW and stored covered (with glass) in a cooler on dry ice**

Buffers and Reagents

-BSA 2 ug/ul, 100 % and a 1:4 dilution (5ul of BSA and 15 ul of ddH_20)

-BCA buffer (200 ul/well)

Calculation for 96 wells:

- ddH2O 12 ml
- Reagent A 8 ml
- Reagent B 400 µl

PBS-10mM EDTA
To make 500 mL of this buffer, take 500 mL of 1x PBS and 2.261 g of EDTA (MW 452.2) 4.5 mg per mL of diluent for 10 mM concentration

8. **Analysis of total cholesterol (TC) and free cholesterol (FC) from BMDM** (adapted from Folch et al. [4] and Matthan et al. [5])

Materials:

Chemicals

- 5α Cholestane (Sigma C-8003; Store at room temperature in dessicator)
- Cholesterol Standard (Supelco 47127-U; 10mg/mL; Store at -20°C)
- Potassium Hydroxide Pellets (Sigma P-1767)
- Chloroform, 99.9+%, A.C.S. HPLC Grade (Sigma 366927)
- Methanol, Chromasolv, for HPLC, ≥99.9% (Sigma 34860)
- Petroleum Ether, Spectrophotometric Grade (Sigma 261734)
- Deionized Ultra Filtered (DIUF) Water (Fisher W2-4)

Glassware

- 50mL Round-bottom Volumetric Glass Flask with Glass Stopper
- 250mL Glass Bottle with Teflon Stopper, 50mL Glass Beakers, 100mL Graduated Glass Cylinder
- Watch Glasses (50mm) (Fisher 02-610A)
- 16x100mm Glass Tubes with Teflon Caps (Fisher 14-930-10B)
- 16x125mm Glass Tubes with Rubber Caps (Fisher 14-959-25C)
- 25x150mm Glass Tube with Teflon Cap (Fisher 14-930-10J)

Pipettes & Vials

- Eppendorf Pipette (100-1000μL) with Plastic Tips (Fisher 05-402-50)
- Digital Microdispenser Pipette (100μL) with Teflon Plunger (Fisher 21-169-20D)
- Microdispenser Replacement Glass Tips (Fisher 21-169F)
- 10mL Graduated Glass Pipettes (Fisher 13-675M)
- 6mL Volumetric Glass Pipettes (Fisher 13-650-2G)
- Brinkmann Dispensettes (1-10mL) (Fisher 13-706-32)
- Borosilicate Glass/Disposable Pasteur Pipettes (5 3/4″) (Fisher 13-678-20B)
- Pipet-Aid (Fisher 13-681-15)
- Amber Vials with Inserts (0.3mL) (Fisher 03-337-47; Wheaton 225221-01)
- Aluminum Seal PTFE/SIL/PTFE Liner (11mm) (Supelco 27360-U)

Miscellaneous

- Aluminum Weigh Boat (KHK Enterprises, Inc. C029-0412)
- Weighing Paper (4x4") (Fisher 09-898-12B)
- Teflon Tape, Microcentrifuge tubes (Fisher 02-681-320)
- Rubber Bulb 1cc (Fisher 03-448-25)
- Corning Cell Lifter (Fisher 07-200-364)

Gases

- 99.998% Pre-Purified Compressed Nitrogen (Medical-Technical Gases, Inc.)
- Regulator CGA 380 (Fisher, catalog No. E11215 D580)

Equipment

- Micro Balance (A x 26 DeltaRange, Mettler Toledo)
- PM200 Scale (Mettler Toledo)
- Vortexer (Genie 2, Fisher)
- N-EVAP (Organomation Associates, Inc.)
- Home-made GC Vial Holders (5mL plastic pipette tip surrounded by glass beads in a 10mL glass bottle)
- Dri-Heat Block (Lab-Line Multi-Blok Heater)
- Rocker (Speci-Mix, Thermolyne, Model No. M26125)
- Refrigerated Centrifuge (Sorvall RT6000B, Dupont)
- Crimper (Fisher 06-406-18F), Decrimper (Fisher 03-340-57B)

Preparation

Waste containers

- Methanol/KOH waste container for methanolic KOH and the lower layer from step #28 in the protocol.
- Chloroform and methanol waste container.

Making the internal standard (5α Cholestane)

(Concentration should be 0.4μg/μL. The amount to be made up depends upon the number of samples to be run for the study; internal standard is very stable.)

Example: For 250 samples,

- Weigh out 20mg of 5α cholestane into an aluminum weigh boat; use microbalance.
- Carefully transfer into a clean 50mL round-bottom volumetric glass flask.
- Using a Pasteur pipette, add chloroform up to the 50mL mark on the flask.
- Cap and vortex (speed 6) for 10 seconds, or until the 5α cholestane fully dissolves.
- Label with date and concentration (1μg/μL).
- Store the internal standard in the refrigerator. *(For future use, allow solution to reach room temperature before its addition to the 16x100mm test tubes.)*

Making the external standard (Cholesterol)

(Concentration should be 0.4μg/μL, external standard is very stable.)

Preparation and storage as above

Chloroform Methanol (CM) Mixture[1] (no BF3)

Into a 1000mL glass bottle with a teflon stopper, add chloroform and methanol in a 2:1(v/v) ratio. (Example- 500mL: 250mL).

Methanol/ DiH_20

Into a 1000mL glass bottle with a teflon stopper, add methanol and DiH_20 in a 1:1 (v/v) ratio. (Example- 100mL:100mL)

Making the 50% methanolic KOH (hydroxide removes FA from the cholesterol)
(This solution must be prepared fresh every day. Before preparing solution, rinse a 25x150mm glass tube with DIUF water, and a 250mL glass bottle and 100mL graduated glass cylinder with methanol.)

Example: For 12 samples,

- Weigh out 5g of potassium hydroxide (KOH) into a weight boat using the PM200 Scale. Transfer into a 25x150mm glass tube.
- Using the Brinkmann Dispensette, add 10mL of DIUF water.
- Cap and shake gently as the reaction is exothermic (i.e. it gives off heat).
- After the majority of the potassium hydroxide has gone into solution, vortex for 10 seconds. *(Should get a clear solution.)*
- Using a 6mL volumetric pipette, add 6mL of the KOH solution into a 250mL glass bottle (remaining 4mL can be poured down drain with lots of water)
- Add 94mL of methanol into a 100mL graduated glass cylinder
- Pour this methanol into the 250mL glass bottle containing the KOH solution
- Cap, shake and vortex for 20 seconds
- After each day, remaining MeOH KOH goes into MeOH KOH waste bottle under hood

Procedures

Rinse all necessary glassware with chloroform or methanol before use

- **need 7 tubes per sample, rinsed and labeled (6 16x100 and 1 16x125)**
- **2x 6mL volumetric glass pipet rinsed with $CHCl_3$**
- **1x 6mL volumetric pipet rinsed with MeOH**
- **10mL pipets**
- **100mL graduated cylinder**
- **250mL bottle with Teflon top**

LIPID EXTRACTION

1. Scrape PBS-washed cells ($1x10^6$ cells in 6-well plate) with 500µL of PBS (+ repeat) and collect into a microcentrifuge tube. Vortex the tube, split into 300ul for protein and 600ul for cholesterol.
2. Spin both at 2500rpm for 5 min to pellet cells. Remove PBS supernatant.
    a. Protein sample: add 50 ul PImix, scrape on rack, sit on ice 20 min. → protein protocol
    b. Cholesterol sample: add 200ul water, store -20
3. Protein calculations: 300 ul cells in PBS → bring up in 50ul PImix → use 5ul for BCA
    a. (__ug protein per 5ul) (x10) = ug protein for the 50ul PImix (same as 300ul PBS)
    b. Hopefully you will have at least 30ug per 5ul (or 300ug/300ul PBS) so that you can use at least 250ug for FC and 250ug for TC determinations
4. **After at least 24 hours**, thaw internal std and 12 samples (2 plates).

5. Pipette 250ug protein (<100 µL) of cells into each of 2 short chloroform-rinsed 16x100mm glass tube (one for TC, one for FC).
6. Add 10ul of internal standard (make sure at RT):
    a. 5-alpha (5-a) stock = 0.5mg/ml chloroform
    b. 10ul of stock = 5ug 5-a/sample
    c. Bring up in 25 ul chloroform at end = 5ug 5-a/25ul = 0.2ug/ul = 0.4ug 5-a/2ul injection
7. Add 3ml of CM mixture to each tube (TC+FC), flush with nitrogen, cap tightly
8. Place tubes on multi-tube vortexer for 10 minutes at level 1
9. Leave tubes in refrigerator overnight

LONG DAY

10. Next morning, switch on the dri-heat bath. Set the temperature knob to 100°C *(Safe temperatures for this procedure range from 95℃ to101℃)*
11. Let samples sit at RT for 15-20 min to warm up (if samples do not reach RT, pellet will not settle in spin, step 8)
12. Spin at 1800 x g for 20min at 4°C
13. Pour contents (supernatant) into new chloroform-rinsed tubes (tall for TC, short for FC)
    a. Then leave the short (FC) tube in 4°C until ready to extract FC

Total cholesterol (TC) measurement – tall tubes

14. Make sure dri-heat block is on to ~95°C
15. Dry down TC lipid extraction under nitrogen, takes at least 45 minutes, (cap when dry)
16. Make 50% methanolic KOH, cut teflon
17. Using a 10mL pipette, add 7mL of 50% freshly prepared methanolic KOH solution (made daily) *(Should get a milky white colored solution)*
18. Line the mouth of the test tubes with Teflon tape and cap tightly. Make a mark on the tubes corresponding to the level of liquid.
19. Vortex each tube for 10 seconds.
20. Place the tubes on the dri-heat block for 2 hours (this is when I extract FC)
21. Check every half hour to make sure that the level in each tube has not fallen below the mark. If it has, remove the tube, cool under water for 20-30 sec and refill to the mark with MeOH, **not methanolic KOH**. Recap and put it back in the dri-heat block.

Free cholesterol (FC) measurement – short tubes

22. Take FC tubes out of refrigerator and add 1mL of methanol: DiH_20 and cap
    a. (These steps will remove pellet. Even if you were using a diff protocol, as long as you do same for all FC tubes it should be fine to compare samples… continue)
23. Shake by hand for 30 seconds (solution will be milky white upon shaking, sometimes leaks).
24. Let the layers settle for 15 minutes (top layer should now be clear)

25. Pipette the upper layer (pellet will now be here in upper layer) and put into waste beaker (at end put all waste from these steps into MeOH:H_2O bottle waste container under hood in 439)
26. Use a new pipette and transfer the lower layer into a new 16x100 tube avoiding any remaining upper layer
27. Repeat steps 22-26 in new tube (with lower layer)
    a. First time, suck ALL bottom into pipette and separate (with some upper layer)
    b. Second time, leave small bubble in bottom (if you get upper layer into pipette, discard and get new one)
28. Evaporate the lower layer under nitrogen at 30℃.
29. Using the Digital Microdispenser pipette, resuspend FC by adding 100µL of chloroform
30. Rinse the sides of the test tubes from top to bottom with this chloroform
31. Carefully using Pasteur pipet transfer solution into designated crimp vial with insert
32. Repeat steps 29-31 for a total of 200µL in GC crimp vial
33. Place the vials in the N-EVAP using the GC vial holders.
34. Dry down under nitrogen, do not walk away from this dry down *(takes ~ 5 minutes)*
35. Resuspend in 25µL chloroform
36. Cap, vortex and store vials in –20°C freezer (Stable for at least one month)
37. Used glass tubes and all used glass goes into regular glass waste

contin. Total cholesterol (TC) measurement – tall tubes

38. After the 2 hours, let the tubes cool in a rack in the fume hood, or by running them under cold water. *(Should get a clear yellow colored solution if working with plasma – do not get it working with THP-1 macrophages)*
39. Using the Brinkmann Dispensettes, into each tube, add 2mL of DIUF water, followed by 3mL of petroleum ether **(MUST be in this order, water than petroleum ether)**. **SLOWLY!** Cap tightly.
40. Vortex each tube for 10 seconds and rock on rocker for 5 minutes
41. Centrifuge the tubes for 15 minutes at 1500 x g at 4°C.
42. Using a Pasteur pipette, very carefully transfer the supernatant (upper layer) into clean chloroform-rinsed 16x100mm tubes. Try not to disturb the lower layer *(If the lower layer is disturbed, re-spin tube in centrifuge for 15 minutes at 1500 x g at 4℃, then continue transfer)*
43. Repeat steps 39-43 **two** more times **only adding 3mL petroleum ether** to lower fraction in large 16x125mm tube, **do not add the DIUF water again**. Keep upper layer in hood, uncapped with pipet in it is ok *(After third transfer can put tubes in refrigerator(must be capped tightly))*
44. After 3 transfers of supernatant, dry down the supernatant under nitrogen. *(Dry down takes approximately 25-30 minutes. Should get a whitish-yellow residue if doing plasma, do not see this in THP-1 macrophages).* You do not want spots on the sides or you got some lower layer from transfers
45. Using the Digital Microdispenser pipette, resuspend the TC by adding 100µL of chloroform.

46. Rinse the sides of the test tubes from top to bottom with this chloroform. Very carefully transfer solution using a Pasteur pipet into each test tube's respective crimp vial with insert
47. Repeat steps 46 and 47 again for a total of 200µL in GC crimp vial.
48. Place the vials in the N-EVAP using the GC vial holders.
49. Dry down under nitrogen, do not walk away from this dry down *(takes ~ 5 minutes)*
50. Resuspend in 25µL chloroform
51. Cap, vortex and store vials in –20°C freezer (Stable for at least one month)
52. Used tubes from TC go into biohazard glass waste, all other glass goes in regular glass waste

Calculations

$$\text{RRF (external standard)} = \frac{\text{Cholesterol area/cholesterol amount}}{\text{5}\alpha\text{-Cholestane area/5}\alpha\text{-Cholestane amount}}$$

Internal standard concentration = 0.4µg/µL
Internal standard volume = 10µL
Internal standard amount = 0.4µg/µL x 10µL = 10µg (half for FC and the other half for TC)

Vial volume = 25µL
Injected volume = 2.5µL
ISTD = (0.4µg/µL x10µL) /2 x 2.5µL/25µL = 0.2µg (I add internal standard at the beginning, they divide equally total lipid extraction and internal standard into 2, therefore we need divided by 2)
TC (µg/100µg protein) = (TC area/5α-Cholestane area) x (ISTD/RRF/Injected volume) x Vial volume x 2 x 100
FC (µg/100µg protein) = (FC area/5α-Cholestane area) x (ISTD/RRF/Injected volume) x Vial volume x 2 x 100
CE (µg/100µg protein) = TC (µg/100µg protein) –FC (µg/100µg protein)

9. Nuclear and Membrane Protein Extraction (adapted from [6])

Materials
HEPES, Sigma # H3375-100G
KOH, Sigma #P1767-500G
KCl, Sigma #P3911-500G
Sucrose, Sigma #S0389-1KG
MgCl2, Sigma # M8266-100G
Na-EDTA, Sigma #E7889-100ML
Na-EGTA, Sigma #E8145-10G
Glycerol, Sigma # G7893-500ML
NaCl, Sigma # S7653-1KG
25 gauge needle
15 ml conical tubes
1.5 ml eppendorf tubes
Rotor Beckman Coulter TLA 120.2
Rotor tubes polycarbonate thickwall Beckman 343778
Ultracentrifuge and centrifuges

Pre-preparation (may be done a few days ahead of time)
Make up "*stocks*" described below, bringing them up in diH2O
Add stock solutions together to make Buffer A and B

Buffer A (100 mL working solution)
10mM HEPES-KOH, pH 7.4: 10 ml of 100mM stock
stock = 2.833g in 100mL + ~4 pellets, one at a time to get pH to 7.4
10mM KCl: 10mL of 100mM stock
stock = 0.7455g in 100mL
250mM sucrose: 40 mL of 0.625M stock
stock = 42.75g in 200mL
1.5mMMgCl2: 10 mL of 15mM stock
*stock = 285.63 mg in 200 mL *can also use for B*
1mM Na-EDTA/1mM Na-EGTA: 20 mL of 5mM Na-EDTA/5mM Na-EGTA stock
stock = 4mL of 0.5M Na-EDTA + 936.36 mg Na-EGTA in 400mL
10 mL water

Buffer B (100 mL working solution)
20mMHEPES-KOH, pH7.6: 10mL of 200mM stock
Stock = 5.667g HEPES in 100 mL + ~8 KOH pellets, 1 at a time to get pH to 7.6
2.5% glycerol: 10 mL 25% stock
Stock = 25 mL glycerol in 100mL
0.42M NaCl: 20 mL of 2.1M stock
Stock = 12.27g in 100mL
1.5mM MgCl2 * *see Buffer A ingredients*
1mM Na-EDTA/1mM Na-EGTA * *see Buffer A ingredients*
30 mL water

Preparation (day of extraction)
Turn on ultra/centrifuges, set to correct temps
Small centrifuge, 4^0C
Large centrifuge, 4^0C
Ultracentrifuge, 4^0C

Extraction Protocol:
1. Collect confluent cells from 100mm dishes into 15 mL conical tube:
    - wash plate 2 x with 5ml PBS
    - scrape in 2ml PBS, rinse plate with 2ml PBS
2. Centrifuge at 500 x g, 5min, 4^0C, discard supernatant
3. Bring up in 400ul Buffer A (PI) transfer to 1.5ml tube (don't forget blank)
4. Pass through 25 gauge needle 30x
5. Centrifuge 1000 x g, 7 min, 4^0C
6. Transfer "post nuclear supernatant" into thickwall ultra tube
    -Ultracentrifuge at 170,000 x g (70,000 rpm), 15 min, 4^0C
    -discard supernatant
    -bring up pellet in 50 ul RIPA (PI/DTT), vortex well
7. Bring up nuclear pellet in 50 ul Buffer B (PI/DTT), set rocking in cold room 1 hour
    -Ultracentrifuge at 100,000 x g (53,000 rpm), 30 min, 4^0C
    -keep supernatant

Ultracentrifuge tips:
- make sure condensation is wiped dry
- button on top of rotor should be clicked down to secure rotor onto spindle
- close door, choose time/temp/speed type in numbers and hit "enter"
- hit "door" to release vacuum and open door
- If error message occurs, wait until speed is back to 0, then press "ce" to clear (you should be able to open the door and try cleaning and re-aligning your rotor)

10. Western blot

Materials
Criterion Tri-HCl Precast gel (7.5%, 45ul well), BioRad #345-0005,
10% #345-0009, 15% #345-0019, 4-15% gradient #345-0027
10x Tris/Glycine ("Transfer Buffer"), BioRad #161-0771
10x Tris/Glycine/SDS Buffer ("Running Buffer"), BioRad #161-0772
Methanol, VWR #9093-03
Laemmli Sample Buffer, BioRad #161-0737
Beta Mercapto Ethanol, Sigma #M7154-25mL
Precision Plus Prot Kaleidoscope standards, BioRad #161-0375
Thick blot paper, Bio-Rad #1704085
Nitrocellulose Membrane, Bio-Rad #162-0094
Ponceau S. Stain, Sigma #P7170-1L
Nonfat Dry Milk or Powder Infant Formula, CVS #691968
Tween 20, sigma # P1379
10mM Tris (pH 8)

- 1 L diH2O, 1.2g trizma base, Sigma #T1378

Sodium Chloride (NaCl), Sigma #S7653-1KG
Primary antibodies from abcam (santa cruz is cheaper and gives more volume, lower quality)
Secondary antibodies from Santa Cruz
SuperSignal West Pico Chemiluminescent Substrate (ECL), ThermoScientific) #34080
kodak x-omat LS film, sigma # F1149-50EA

Equipment
Hot plate
Criterion Pre-Cast System (Running and Transfer containers, electrical source), Bio-Rad
Sponges, Stirbars, Razor blade
Ice container
Mini blotting containers, Research Products International Corp #248709, #248715
Rocker
Cold Room
Cassette for film exposure
Darkroom, developer
QuantOne scanner and software

Running Buffer (can reuse)

200 ml buffer (box)	or	200 ml buffer (SDS "Running Buffer" box)
10 ml 10% SDS		800 ml diH2O
790 ml diH2O		

Transfer Buffer (must be new)

10% MeOH:	100 ml buffer (box)	20% MeOH: 100 ml buffer ("Transfer Buffer" box)
	100 ml MeOH	200 ml MeOH
	800 ml diH2O	700 ml diH2O

TBS-T (can make up 10L stock in big Nalgene – minus the Tween)

	1 L diH2O	x10	10 L diH2O
(10mM)	10 ml Tris (pH 8)	x10	100 ml Tris (pH 8)
(150mM)	8.76 g NaCl	x10	87.66 g NaCl

1010 ml of stock + 5 ml Tween (shake well)

Electrophoresis

1. Turn on hotplate to boil samples (15-40 ug each)
2. Set up apparatus – peel off sticker, remove comb, wash with diH2O, set into box
3. Prepare loading buffer: (475ul Laemeli +25ul BME)**********look up best practices
   - May want to use a more concentrated Laemeli solution
4. Prepare samples:
   - Thaw on ice
   - Remove from ice and add exact same amt (ul) of 2x loading buffer as sample, mix well
   - Boil for 5 minutes, allow to cool at RT 5 min
5. Load samples and precision plus ladder (5ul)
6. Run at 80-100 volts until dye is near bottom (1-2hr)
7. Soak 4 sponges, 4 sheets filter paper, 2 nitrocellulose membranes in Transfer Buffer
8. Remove gels, pry open, cut off wells with razor blade, soak gel
9. Assemble transfer:
   a. Black away from me
      Sponge
      Filter
      Gel
      Membrane
      Filter
      Sponge
      On Red side
   b. Put in ice and stirbar first, black side toward the back
10. Transfer at 20v overnight in cold room (or 1-2 h at 100v)

Western Blotting (Block and Probe)

11. Rinse with TBS-T, pour some dilute Ponceau S (1-5% in diH2O), remove and rinse with diH2O
12. Rock in 5% Milk in TBS-T (the rest of Ponceau stain will come off) – 1 hr, remove milk
    a. 1g milk in 20ml TBS-T, 2.5g in 50ml
13. Rock in 5% milk with primary antibody
14. Wash x 3 with TBS-T, 5 min each, remove
15. Rock in 5% milk with secondary antibody
16. Wash x 3 with TBS-T, 5 min each, remove, wipe excess TBS-T with kimwipe
    a. During wash, turn on light in darkroom (it takes a few minutes to warm up) and sign name

17. ECL: Mix buffer 1 and buffer 2 (1:1) – 3ml per whole membrane, let it sit on membrane for 2 min
18. Use tweezers to pick up membrane, blot off excess ECL onto kimwipe
19. Place face down on saran wrap, tape into cassette – take to darkroom

Darkroom

1. Light should already be on and you should be signed up (so that no one turns off light)
2. Turn on developer
3. Do not cut film smaller than half of a full sheet because it will get stuck in developer
4. Quickly place film in cassette on top of membranes – no readjusting
5. May want to push button on machine to get it moving again
6. Place film in machine
    a. If it takes more than 3 minutes then your film is probably stuck and you will need to press button to move it through or open machine and find your film (pressing button while film is inside will likely over expose your film so only do this if it really is stuck!)

Scan into computer

20. Turn on, warm up 15 min
21. Open QuantOne
    a. File GS-710
22. Put on film face down
23. Click blue (?)
    a. Transmissive
24. Click preview
    a. Stop when image comes up
    b. Move box over image
    c. Click acquire
25. Save image
    a. File export to Tiff
    b. Publishing
    c. Same as scan
    d. Export
26. Subtract background
    a. Move background/click autoscale
    b. Click ok
27. Volume rect.
    a. Cover band
    b. Shift and move to rotate
    c. Control and move to get another box
    d. Want all the boxes to be the same size
28. Volume contour
29. Label by double clicking
30. Volume rectangle box around some background, double click, label as background
31. Volume analysis report export data (box, bottom rt) excel
    a. Adj volume ODmm2

11. References

1. Nguyen, M.T., et al., *A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways.* J Biol Chem, 2007. **282**(48): p. 35279-92.
2. Terpstra, A.H., *Isolation of serum chylomicrons prior to density gradient ultracentrifugation of other serum lipoprotein classes.* Anal Biochem, 1985. **150**(1): p. 221-7.
3. Schwartz, D.M. and N.E. Wolins, *A simple and rapid method to assay triacylglycerol in cells and tissues.* J Lipid Res, 2007. **48**(11): p. 2514-20.
4. Folch, J., M. Lees, and G.H. Sloane Stanley, *A simple method for the isolation and purification of total lipides from animal tissues.* J Biol Chem, 1957. **226**(1): p. 497-509.
5. Matthan, N.R., et al., *Deuterium uptake and plasma cholesterol precursor levels correspond as methods for measurement of endogenous cholesterol synthesis in hypercholesterolemic women.* Lipids, 2000. **35**(9): p. 1037-44.
6. DeBose-Boyd, R.A., et al., *Transport-dependent proteolysis of SREBP: relocation of site-1 protease from Golgi to ER obviates the need for SREBP transport to Golgi.* Cell, 1999. **99**(7): p. 703-12.