Dietary Modulation of the Dynamics between Leukocyte Inflammation, Cholesterol Flux, and HDL function in Metabolic Syndrome

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Dietary Modulation of the Dynamics between Leukocyte Inflammation, Cholesterol Flux, and HDL Function in Metabolic Syndrome

Catherine J. Andersen, Ph.D.
University of Connecticut, 2013

Obesity is associated with dysfunctional lipoproteins, low-grade inflammation, and an increased risk of cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM). HDL and leukocytes play well documented roles in chronic disease development, whereas HDL-leukocyte interactions further modify cellular cholesterol flux and inflammatory potential. Therefore, therapeutic strategies that alter HDL-leukocyte dynamics may have profound effects on obesity-related disease progression.

We investigated the modulation of HDL-leukocyte dynamics by a dietary intervention known to favorably affect HDL metabolism and inflammation in metabolic syndrome (MetS) – a population at increased risk for CVD and T2DM. Thirty-seven men and women classified with MetS consumed a moderate carbohydrate-restricted diet in addition to either 3 whole eggs per day (EGG) or the equivalent amount of egg substitute (SUB) for 12 weeks. Egg yolks are a rich source of highly bioavailable, bioactive phospholipids and antioxidant carotenoids. After the 12-week intervention, all subjects increased HDL-cholesteryl ester/triglyceride ratios; however, whole egg intake resulted in enrichment of HDL in phosphatidylethanolamine and egg yolk-derived sphingomyelin species. The cholesterol-
accepting capacity of subject serum from macrophage foam cells was further increased by whole egg feeding, whereas no changes were observed from egg substitute intake.

We further assessed the effects of this intervention on peripheral blood mononuclear cell (PBMC) inflammation. While there were no changes in IL-1β, IL-6, and toll-like receptor 4 (TLR4) mRNA expression throughout the intervention, TLR4 mRNA expression was increased by whole egg intake, positively correlating with NF-κB p65 DNA binding activity. However, LPS-induced IL-1β and TNFα secretion was increased from baseline to week 12 in the SUB group only. Given these observations, we further sought to determine whether HDL-mediated cholesterol flux played a role in modulating PBMC inflammation. Compared to baseline, there was a trend toward a reduction in PBMC cholesterol content in the EGG group, whereas changes in PBMC cholesterol positively correlated with changes in lipid raft content. These observations corresponded to increases in ABCA1 and HMG-CoA reductase mRNA expression from whole egg intake only, in addition to a trend toward increased ABCA1 protein. Together, these findings suggest that HDL-leukocyte dynamics can be favorable modulated through diet in MetS.
Dietary Modulation of the Dynamics between Leukocyte Inflammation, Cholesterol Flux, and HDL Function in Metabolic Syndrome

Catherine J. Andersen

B.S., University of Connecticut, 2008
M.S., University of Connecticut, 2011

A Dissertation
Submitted in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy at the University of Connecticut 2013
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Catherine J. Andersen

2013
APPROVAL PAGE

Doctor of Philosophy Dissertation

Dietary Modulation of the Dynamics between Leukocyte Inflammation, Cholesterol Flux, and HDL Function in Metabolic Syndrome

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University of Connecticut

2013
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<td>Biological Term</td>
<td>Abbreviation</td>
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<tr>
<td>Protein inhibitor of activated signal transducer and activator of transcription 1</td>
<td>PIAS1</td>
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<tr>
<td>Protein kinase C</td>
<td>PKC</td>
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<tr>
<td>Reactive oxygen species</td>
<td>ROS</td>
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<tr>
<td>Receptor for advanced glycation end products</td>
<td>RAGE</td>
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<tr>
<td>Retinoid X Receptor</td>
<td>RXR</td>
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<tr>
<td>Reverse cholesterol transport</td>
<td>RCT</td>
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<tr>
<td>Ribosomal ribonucleic acid</td>
<td>rRNA</td>
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<tr>
<td>Saturated fatty acid</td>
<td>SFA</td>
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<tr>
<td>Scavenger receptor class B type 1</td>
<td>SR-BI</td>
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<td>Serum amyloid A</td>
<td>SAA</td>
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<tr>
<td>Silencing mediator for retinoid and thyroid hormone receptor</td>
<td>SMRT</td>
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<tr>
<td>Soluble intracellular adhesion molecule 1</td>
<td>sICAM-1</td>
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<td>Soluble vascular cell adhesion molecule 1</td>
<td>sVCAM-1</td>
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<td>Sphingomyelin</td>
<td>SM</td>
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<tr>
<td>Sterol Regulatory Element-Binding Protein</td>
<td>SREBP</td>
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<tr>
<td>T cell receptor</td>
<td>TCR</td>
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<tr>
<td>Thiazolidinedione</td>
<td>TZD</td>
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<td>Thromboxane</td>
<td>TX</td>
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<td>Toll-like receptor 4</td>
<td>TLR4</td>
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<td>Total cholesterol</td>
<td>TC</td>
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<tr>
<td>Transforming growth factor-β</td>
<td>TGF-β</td>
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<td>Transintestinal cholesterol efflux</td>
<td>TICE</td>
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<tr>
<td>Triglyceride</td>
<td>TG</td>
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<td>Term</td>
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<tr>
<td>Triglyceride-rich lipoprotein</td>
<td>TGRL</td>
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<tr>
<td>Trimethylamine-N-oxide</td>
<td>TMAO</td>
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<td>Tumor necrosis factor α</td>
<td>TNFα</td>
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<td>Type 2 diabetes mellitus</td>
<td>T2DM</td>
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<tr>
<td>Unfolded protein response</td>
<td>UPR</td>
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<tr>
<td>Unsaturated fatty acid</td>
<td>UFA</td>
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<tr>
<td>Vascular cell adhesion molecule 1</td>
<td>VCAM-1</td>
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<td>Vascular endothelial growth factor</td>
<td>VEGF</td>
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<td>White adipose tissue (WAT)</td>
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<td>White blood cell</td>
<td>WBC</td>
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<tr>
<td>Whole egg group</td>
<td>EGG</td>
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<tr>
<td>Wild type</td>
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Chapter 1

Introduction
Obesity is associated with an increased risk of developing chronic diseases, including cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), and cancer [1]. The prevalence of obesity has increased over the past few decades, as now more than one-third of adults in the United States are obese, in addition to 17% of children and adolescents [2]. This trend is now similarly observed in developing countries due to increased urbanization, transition from traditional food patterns to energy-dense “Western diets”, and a reduction in physical activity [3]. As of 2008, it was estimated that obesity-related medical costs had risen $147 billion per year in the United States, up from $78.5 billion in 1998 [4]. Together, obesity has been deemed the leading cause of preventable death [5], and it has risen to become a global economic and health burden.

The pathophysiology of obesity-related diseases stems from underlying metabolic disturbances that cause tissue stress and dysfunction. Excess nutrient intake leading to obesity requires adipose tissue expansion in order to accommodate the increased influx of nutrients, which is dependent upon insulin-mediated energy storage [6]. In adults, adipose expansion occurs primarily through adipocyte hypertrophy, rather than adipocyte hyperplasia [7]. Lipid-engorged, hypertrophic adipocytes are more prone to activation of endoplasmic reticulum (ER) and mitochondrial stress responses, in addition to inducing shear mechanical stress on the extracellular environment. Together, these factors promote the activation of chronic, pro-inflammatory state for as long as the cells remain stressed and engorged [6, 8].

Prolonged stress and inflammation within the adipose tissue can lead to adipocyte apoptosis and the release of chemotactic mediators such as monocyte chemoattractant protein-1 (MCP-1), resulting in inflammatory leukocyte infiltration [9]. While macrophages represent approximately 5-10% of cells in healthy adipose tissue, it has been demonstrated
that macrophages can represent up to 50% of all cells within hypertrophic obese adipose tissue [10]. Pulse-labeling studies conducted in vivo have demonstrated that the macrophages recruited into adipose tissue at the onset of obesity are highly pro-inflammatory, classically activated M1 macrophages, in contrast to resident macrophages in lean adipose, which are typically alternatively-activated, anti-inflammatory M2 macrophages [11-13]. Increased T cell infiltration is also observed in obese adipose tissue, and some evidence suggests that these lymphocytes may become activated in response to unique antigens generated in obese adipose tissue during high-fat feeding [14]. In addition, greater levels of pro-inflammatory T\textsubscript{H}1 cells have been observed in obese adipose, whereas levels of anti-inflammatory T\textsubscript{reg} cells are reduced, corresponding to greater impairments in insulin sensitivity [14, 15].

Inflammatory leukocytes within adipose tissue further perpetuate an inflammatory state through production of resistin and interleukin (IL)-1\textbeta, whereas both hypertrophic adipocytes and macrophages increase secretion of tumor necrosis factor \alpha (TNF\alpha), IL-6, and MCP-1 [16]. Elevated levels of TNF\alpha counteract insulin-mediated nutrient uptake, through inhibition of 1) glucose transporter type 4 (GLUT4) translocation to the adipocyte cell surface, 2) lipoprotein lipase (LPL)-mediated lipolysis of chylomicron triglycerides (TG), 3) peroxisome proliferator-activated receptor \gamma (PPAR\gamma)-mediated TG synthesis for free fatty acid (FFA) adipocyte storage, and 4) perilipin-mediated lipid droplet formation [6]. Together, these effects are associated with increased FFA mobilization from adipose into the circulation [17]. Hypertrophic adipocyte-derived retinol-binding protein 4 (RBP4) is also known to impair insulin signaling by reducing phosphatidylinositol-3 kinase (PI3K) signaling in muscle, while concomitantly increasing hepatic gluconeogenic enzyme expression [18, 19].
As hypertrophic adipocytes increase secretion of inflammatory mediators, production of adiponectin is decreased, potentially through TNFα-mediated inhibition [6]. Binding of adiponectin isoforms to their cognate receptors 1) induces AMP-activated protein kinase (AMPK)-mediated inhibition of sterol regulatory element-binding protein (SREBP)-1c-induced lipogenesis, 2) inhibits cytosolic nuclear factor κ B (NF-κB) translocation to induce pro-inflammatory gene transcription, and 3) promotes PPARα and PPARγ transcriptional activity to increase β-oxidation and GLUT4 translocation; overall exerting anti-inflammatory and insulin-sensitizing effects [20]. Adipocyte-derived leptin also stimulates fatty acid oxidation, however, high circulating levels found in obesity are typically indicative of leptin resistance [21]. Therefore, hypertrophic stress prevents adequate contribution of anti-inflammatory and insulin-sensitizing adipocyte mediators.

Overall, hypertrophic, inflammatory adipose loses insulin sensitivity, results in increased lipolysis and impaired lipid storage [6]. FFAs and TGs are mobilized to the circulation, leading to accumulation of their fatty acyl-CoA ester and diacylglycerol derivatives in the skeletal muscle, liver, and pancreatic β-cells, resulting in impaired tissue functioning and insulin resistance. The consequences of prolonged maintenance or worsening of this metabolically dysfunctional state further perpetuates dysregulation of lipid metabolism and immunity, thereby increasing an individual’s risk for developing a wide range of chronic diseases [6].

Clinical manifestation of these underlying conditions often present as the parameters of metabolic syndrome (MetS), a clinical classification characterized by a clustering of three or more components, including central adiposity, elevated blood glucose, plasma TG, and blood pressure, and low plasma HDL-cholesterol (HDL-C) [22]. In addition to these
qualifying parameters, obesity and MetS are associated with endothelial dysfunction, atherogenic dyslipidemia, insulin resistance, and chronic low-grade inflammation [23]. In line with national obesity trends, it has been estimated that approximately 34% of adults in the U.S. have MetS [24]. The high prevalence of MetS is a significant public health concern, as classification with MetS increases an individual’s risk of CVD and T2DM by 2- and 5-fold, respectively [22].

Despite the average 2- and 5-fold increased risk of CVD and T2DM [22], respectively, MetS classification can yield fairly diverse populations with varying degrees of disease risk severity due to the combinatory nature of qualifying parameters. Accordingly, particular combinations of MetS components within the MetS classification have been associated with greater risk for developing coronary heart disease (CHD) and premature death – including combinations that include low plasma HDL-C [25]. Independent of other MetS parameters, HDL-C has consistently been shown to be inversely associated with risk of developing CVD [26, 27]. Obesity-related diseases have also been shown to impair the multitude of atheroprotective function of HDL, antioxidant, anti-inflammatory, vasodilatory, and antithrombotic activities, in addition to the capacity of HDL to promote cholesterol efflux and reverse cholesterol transport (RCT). The effects of metabolic dysfunction on HDL hold significant implications for whole-body lipid metabolism and transport, atherosclerosis development, and CVD risk [28].

Similar trends have been observed with markers of inflammation. In men of the West of Scotland Coronary Prevention Study cohort, prediction of CHD and T2DM development over a 5-year period was strengthened by inclusion of C-reactive protein (CRP), levels of which were significantly higher in men with MetS [29]. CRP was additionally found to
predict both CHD and T2DM independent of MetS status. Ridker et al [30] reported a similar relationship between MetS and CRP, where CRP levels dose-dependently increased with greater number of MetS components, and CRP values added predictive power to the determination of CVD death risk. Further, peripheral blood mononuclear cells from obese subjects are often in a more pro-inflammatory state [31, 32], which has been associated with increased migration to metabolic tissues, greater vascular wall adhesion and extravasation, enhanced priming toward pro-inflammatory responses, and perpetuation of atherosclerosis and T2DM [14].

In addition to the significant independent roles of HDL and inflammatory leukocytes in chronic disease progression, a relatively recent area of research has demonstrated that the interaction between HDL and leukocytes concomitantly modulate cellular cholesterol flux and inflammatory potential. Elevated levels of cellular cholesterol favor the formation of lipid rafts, and have been associated with increased pro-inflammatory responses in macrophages and T lymphocytes due to lowered cellular activation thresholds [33-35]. Therefore, the capacity of HDL to function as cellular lipid acceptor – in conjunction with the activity of anti-inflammatory HDL-associated transporters ATP-binding cassette transporter A 1 (ABCA1) and ATP-binding cassette transporter G 1 (ABCG1) – promotes maintenance of appropriately controlled leukocyte responses [36]. Together, the dynamic between HDL and leukocytes represents a promising therapeutic target to reduced CVD and T2DM risk in obesity and MetS.

Therapeutic strategies for treating obesity and MetS often consist of modification of lifestyle and dietary patterns. Dietary therapies promoting weight loss and restoration of adipose tissue integrity are commonly prescribed to favor global improvements in metabolic
dysfunction [37]; however, based on recent research findings described above, the addition of functional foods that target HDL-leukocyte dynamics may provide further benefit. Previous intervention studies conducted in our laboratory have demonstrated that dietary carbohydrate restriction in conjunction with daily whole egg consumption promotes improvements in HDL profiles and inflammatory markers in obese and MetS populations [38-41]. These effects are thought to be attributable to the insulin sensitizing and anti-inflammatory weight loss effects of carbohydrate restriction, in addition to bioactive nutrients, phospholipids, and antioxidant carotenoids within egg yolk [42-45]. Therefore, we believe that this dietary strategy is a potential therapeutic candidate to favorably modulate the relationship between HDL function and the inflammatory potential of leukocytes in MetS – individuals at high risk for CVD and T2DM [22]. Within this context, this dissertation will address several important research questions.

**Research Questions**

**Question 1:** Does daily whole egg consumption during moderate carbohydrate restriction alter HDL lipid composition and cholesterol-accepting functions in MetS?

**Hypothesis 1:** We hypothesized that daily consumption of whole eggs would favorably alter HDL lipid composition due to habitual intake of highly bioavailable phospholipids within egg yolk. We further hypothesized that carbohydrate restriction would promote global metabolic improvements, that, in combination with HDL lipid modulation, would increase the cholesterol-accepting capacity of subject serum from macrophage foam cells *ex vivo.*
**Question 2:** Does daily whole egg consumption during moderate carbohydrate restriction alter the inflammatory potential of peripheral blood mononuclear cells (PBMC) in MetS?

**Hypothesis 2:** We hypothesized that carbohydrate restriction would reduce basal inflammatory gene expression and NF-κB activity in PBMC, in addition to LPS-mediated inflammatory cytokine production due to global improvements in metabolic dysfunction and inflammation. We further hypothesized that daily whole egg intake would show greater reductions in PBMC inflammatory potential.

**Question 3:** Does daily whole egg consumption during moderate carbohydrate restriction alter parameters of PBMC cholesterol flux in MetS?

**Hypothesis 3:** We hypothesized that daily consumption of whole eggs would reduce PBMC cholesterol content and lipid raft formation, and that these changes would be associated with increased expression of HDL-associated transporters ABCA1 and ABCG1.

**References**


Chapter 2

Literature Review
Given the scope of this dissertation project, the following literature review will cover mechanisms underlying HDL metabolism, inflammation, the dynamics between HDL and leukocytes, and dietary strategies aimed at modifying these pathways within the context of obesity-related disease.

Section 1

HDL

Introduction

HDL represents a heterogeneous class of lipoprotein particles that are diverse in size, structure, lipid species distribution, and apolipoprotein/enzyme profiles [1, 2]. Together, HDL is known to protect against cardiovascular disease (CVD) by facilitating the removal of excess cholesterol from the body via reverse cholesterol transport (RCT), in addition to possessing antioxidant, anti-inflammatory, vasodilatory, antithrombotic, and immunoregulatory activities [2]. The following section will review the dynamics of HDL formation, metabolism, and functions, as well as potential therapeutic strategies to optimize atheroprotective HDL functions.

HDL is the smallest class of lipoproteins, with particle diameters ranging from 5.0 to 14.5 nm [3]. Compared to larger, more lipid-rich apolipoprotein (apo) B-containing lipoproteins (i.e. chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoprotein (LDL)), HDL particles are also the most dense due to a relatively richer protein
content (~ 50% by weight) [1]. The primary protein component of HDL particles is apoA-I, which provides structural integrity to the particle [4], yet is also crucial to lipid acquisition, cellular cholesterol efflux, antioxidant properties, and HDL particle clearance [5, 6]. Recent proteomics research has identified over 100 additional HDL-associated proteins, including a variety of apolipoproteins, and antioxidant and antithrombotic enzymes - the distributions of which may further contribute to the overall function of HDL [1, 7, 8].

The composition of HDL is dependent upon the relative “maturity” of the particle. Approximately 5% to 10% of HDL in healthy human plasma is in the form of small, nascent HDL particles [9, 10], or preβ-1 HDL, based on its electrophoretic migration pattern. Preβ HDL-1 circulate in a mostly lipid-free, discoidal configuration made up of two apoA-I, and ~16 phospholipids (PL) that form a bilayer together with few free cholesterol (FC) molecules [1, 11]. Subsequent acquisition of PL, FC, and apoA-I results in the formation of more mature HDL particles (also termed α-HDL, based on its electrophoretic migration pattern), which are spherical in structure with a core of cholesteryl esters (CE) and TG surrounded by a PL and FC outer monolayer with 4 to 5 apoA-I [1]. αHDL represents 90% to 95% of HDL particles in human plasma [10]; however, constant plasma remodeling of HDL particles and interactions with peripheral tissues results in dynamic conversion between preβ and αHDL particles, as well as alterations in composition and size within preβ and αHDL subfractions [6].

*Formation of HDL*

HDL formation is initiated by the expression and secretion of apoA-I from either the liver or intestine as either lipid-poor, monomeric apoA-I, or as a nascent, cholesterol-poor HDL
particle [11]. In order to prevent rapid cubulin-mediated clearance of apoA-I by the kidneys, apoA-I must be 1) lipidated to form preβ-1 HDL, or 2) incorporated into pre-existing mature α-HDL particles [6]. Lipidation of lipid-poor apoA-I to form discoidal preβ-1 HDL particles requires acquisition of FC and PL via ATP-binding cassette transporter A 1 (ABCA1)-mediated unidirectional efflux. Preβ-1 HDL particles acquire additional FC and PL via ABCA1- or ATP-binding cassette transporter G 1 (ABCG1)-mediated efflux, resulting in formation of more cholesterol-rich, spherical α-HDL particles. α-HDL particles can acquire further FC and PL via ABCG1- and scavenger receptor class B type I (SR-BI)-mediated efflux, as well as from aqueous diffusion [12, 13].

**Physiological determinants of plasma HDL-cholesterol levels**

It has been established that the liver-derived HDL is the primary contributor to plasma HDL-C, and that hepatic ABCA1 plays a critical role in determining HDL-C levels [6]. Individuals with Tangier disease have ABCA1 gene mutations, and are known to have marked deficiency in HDL-C, apoA-I, and an increased risk of CVD [14, 15]. Similarly, liver-specific ABCA1−/− mice have an ~80% reduction in plasma HDL-C levels [8], whereas HDL-C levels in ABCG1−/− and SR-BI−/− mice are relatively unaffected or increased, respectively; although, the increases in HDL-C in SR-BI−/− mice are most likely due to decreased hepatic HDL-CE clearance [9]. Independent of plasma HDL-C levels, loss of SR-BI and macrophage-specific ABCA1 and ABCG1 results in greater development of atherosclerosis [16-18]. These findings demonstrate the critical role of HDL and HDL-associated transporters in atherosclerosis, while also supporting the notion that HDL-C levels may not truly capture CVD severity and risk. This concept has been further supported by the fact that multiple
pharmacological therapies specifically designed to increase HDL-C have failed to demonstrate reductions in the severity of CVD risk, progression, disease outcomes, or death [19, 20].

**Atheroprotective functions of HDL**

As mentioned above, HDL exhibits atheroprotective properties beyond HDL-C levels, including antioxidant, anti-inflammatory, vasodilatory, antithrombotic, and immunoregulatory activities [2]. Together, these properties confer a wide range of biological activities to HDL, while also affecting the capacity of HDL to accept cellular lipids and participate in RCT [2]. In general, it appears that many of these atheroprotective HDL properties are regulated independently of circulating HDL-C levels. However, much like plasma HDL-C levels, many of these HDL parameters are known to be impaired in conditions of obesity, metabolic syndrome, and CVD, thereby resulting in the formation of dysfunctional HDL that may perpetuate CVD rather than protect against it. Therefore, therapeutic strategies to reduce CVD must strive to improve overall HDL function [21].

**Antioxidant properties of HDL**

HDL is known to possess antioxidant properties which contribute to the atheroprotective nature of this lipoprotein class [22]. Atherosclerotic lesions contain abundant oxidized lipid and protein species which perpetuate plaque development and endothelial dysfunction [23]. Physiological environments of oxidative stress further promote the formation of oxidized LDL (oxLDL), which preferentially undergoes scavenger receptor-mediated uptake by macrophages, leading to the formation of lipid-laden foam cells – the hallmark characteristic of atherosclerosis [24]. OxLDL also triggers activation of pro-inflammatory, apoptotic, and
cytotoxic pathways to perpetuate the development and severity of coronary artery disease [25].

Antioxidant activity of HDL is predominantly related to various types of associated apolipoproteins and antioxidant enzymes that serve to hydrolyze and/or remove oxidized lipids [22]. For example, HDL-associated apolipoproteins (apo) apoA-I, apoA-II, apoA-IV, apoE, apoJ, and apoM are known to contribute to antioxidant activity [2, 26]. The antioxidant activity of both apoA-I and apoM is thought to be attributable to their ability to remove oxidized phospholipids from both LDL and peripheral cells – including those in the arterial wall – which may then be eliminated from the body through the liver [26-28]. HDL is also known to associate with enzymes possessing antioxidant activity, including paraoxonase 1 (PON1), platelet-activating factor acetylhydrolase (PAF-AH), and lecithin-cholesterol acyltransferase (LCAT) [22, 29]. These enzymes provide antioxidant activity to HDL by hydrolyzing LDL-derived oxidized phospholipid species, thereby inhibiting the formation of oxLDL [2, 30]. HDL has also been shown to bind glutathione selenoperoxidase (GSPx), which further serves to protect against oxidative stress by reducing lipid hydroperoxides [31].

Aside from antioxidant activity conferred from associated apolipoproteins and enzymes, HDL can prevent oxLDL formation by sequestering oxidizing transition metal ions [32]. HDL is also a known carrier of antioxidant nutrients, including vitamin E and carotenoids. However, the extent by which these nutrients contribute to the antioxidant activity of HDL remains unclear [22].

HDL with defective antioxidant activities have been identified in metabolic syndrome (MetS) [33], type 2 diabetics [34], and healthy postmenopausal women [35] when compared to healthy and premenopausal women, respectively. A reduced capacity of HDL to protect
against LDL oxidation in these populations may be attributed to reduced activities of antioxidant apolipoproteins and enzymes [36].

**Anti-inflammatory effects of HDL**

In addition to antioxidant activity, functional HDL is known to possess anti-inflammatory properties. Anti-inflammatory activity of HDL typically refers to the ability of HDL to inhibit endothelial cell expression of adhesion molecules in response to cytokines, thereby reducing monocyte adhesion to the arterial wall in the initial stages of atherosclerosis development [2, 37]. Arterial inflammatory pathways and adhesion molecule expression can be activated by the same accumulation of oxidized lipids that cause oxidative stress, as described above [23].

The anti-inflammatory activity of HDL is mostly attributed to the same enzymes that exert antioxidant activity, including HDL-associated apolipoproteins (i.e. apoA-I, apoA-II, apoA-IV, apoM) and oxidized lipid-hydrolyzing enzymes (PON1, PAF-AH, and LCAT) [2, 26]. Bioactive phospholipid species carried by HDL such as sphingosine-1-phosphate (S1P) – carried by apoM - may also play a role in HDL’s anti-inflammatory activity, in addition to antiapoptotic and immunoregulatory functions [38, 39].

Conversely, HDL can become more inflammatory during acute phase responses to stress, infection, or inflammation [40]. Under these conditions, HDL has been shown to have reduced apoA-I, PON, PAF-AH, and have be less able to prevent LDL-induced monocyte transmigration and monocyte chemoattractant protein-1 (MCP-1) expression in human aortic endothelial and smooth muscle cell co-cultures [41]. Further, HDL can acquire serum amyloid A (SAA) – an acute-phase pro-inflammatory protein that is known to associate with
HDL and displace apoA-I [42]. Similar to the acute-phase protein C-reactive protein (CRP), SAA has been shown to be predictive of coronary artery disease (CAD) development [43]. This may be due to the fact that pro-inflammatory HDL is also less able to participate in RCT function, as SAA increases the binding of HDL proteoglycans, thereby preventing HDL transport back to the liver to complete RCT [2].

As with other HDL parameters, HDL inflammatory profiles do not appear to be related to plasma HDL-C levels [44, 45]. Similar findings have been reported in animal models of atherosclerosis, where atherosclerotic lesion area in cholesterol-fed rabbits positively correlated with SAA and inflammatory indexes for HDL and LDL, but not total cholesterol or HDL-C [46].

**Vasodilatory properties of HDL**

In addition to antioxidant and anti-inflammatory functions, HDL possesses vasodilatory activity by promoting the production of vasodilatory mediators, including nitric oxide (NO) and prostacyclin (PGI₂) [2]. ApoA-I stimulates endothelial cell NO production through activation of nitric oxide synthase (NOS) [47], whereas HDL also induces expression of arachidonic acid derived-PGI₂ – a potent vasorelaxer - in vascular smooth muscle cells in a cyclooxygenase 2 (COX-2)-dependent mechanism [48-50].

**Antithrombotic properties of HDL**

HDL further exhibits antithrombotic activity by inhibiting platelet aggregation [51] and factors that promote blood coagulation, including factors X, Va, VIIIa, and tissue factor [52, 53]. One of the mechanisms by which HDL exerts antithrombotic activity is through
enrichment in cardiolipin and phosphatidylethanolamine (PE) – phospholipids with potent anticoagulant properties [54, 55]. PE is present on endothelial cells throughout the aorta under normal conditions, and is used as an essential cofactor in the protein C coagulant pathway. Conditions of PE deficiency or depletion increase severe risk of thrombosis, and are highly associated with fetal loss during pregnancy in humans and mice [56].

Reverse Cholesterol Transport

Considered to be one of the most important atheroprotective functions of HDL, RCT is the process of HDL-mediated lipid acquisition from peripheral cells – including arterial wall macrophage foam cells – for return to the liver and excretion from the body via the bile and feces [19]. RCT represents a diverse network of processes that encompasses 1) cellular cholesterol efflux from peripheral tissues to HDL, 2) intravascular remodeling of HDL particles through interactions with cell-surface receptors, lipid transfer proteins, lipolytic enzymes, and non-HDL lipoproteins, and 3) mechanisms involved in hepatic cholesterol uptake and HDL clearance [6, 57]. The efficiency of RCT, as well as CVD risk, is known not only to be dependent upon the atheroprotective quality of HDL, but also on numerous physiological and genetic factors that influence RCT dynamics [1, 58]. Similar to the HDL properties described above, RCT is thought to be impaired in individuals with metabolic dysfunction, although it has yet to be definitively confirmed in vivo in humans [59].

Cholesterol efflux capacity of HDL

Cellular cholesterol efflux from peripheral tissue to HDL is considered to be the first step of RCT [10]. The ability and efficacy of HDL to mediate cellular cholesterol efflux is
often referred to as the “cholesterol efflux capacity” or “cholesterol-accepting capacity” of HDL. The process of cholesterol efflux is not only dependent on the presence of a functional lipid acceptor, but also the expression of lipid transporters. Due to the clinical relevance in regard to atherosclerosis development [12, 57], the focus of this section will be on the dynamics of HDL-mediated cholesterol efflux from macrophages.

Macrophage FC and PL efflux to lipid-poor apoA-I/HDL occurs via aqueous diffusion, as well as the ABCA1-, ABCG1-, SR-BI-mediated pathways described above [60]. Accumulation of LDL-derived cholesterol in macrophages leads to increased formation of oxysterols, which are cholesterol derivatives that serve as endogenous ligands for sterol-sensing transcription factors, liver X receptor (LXR) α and β [61, 62]. Oxysterol-induced activation of LXRα/β leads to LXR-retinoid X receptor (RXR) hetero-dimerization and transcriptional activation of ABCA1 and ABCG1, which contain LXR response elements (LXREs) within their promoter gene regions. Synthetic LXR agonists have been shown to increase HDL-C and reduce atherosclerosis; however, their success as a pharmaceutical agent is currently limited by their adverse ability to increase sterol regulatory element binding protein 1-c (SREBP-1c)-mediated hepatic lipogenesis and plasma TG levels [63].

Despite accumulation of cellular cholesterol content, expression of ABCA1, ABCG1, and SR-BI has been shown to be impaired in individuals with chronic metabolic disease, including coronary artery disease, hypertension, and T2DM [64-67]. This may be due to the fact the expression of these cellular transporters can be inhibited by serum components known to be elevated in metabolic disease, including serum glucose, tumor necrosis factor α (TNFα), CRP, and oxLDL [68-72]. Fatty acids have additionally been shown to differentially
regulate ABCA1 and ABCG1 [73, 74], whereas weight loss can increase ABCG1- and SR-BI mediated efflux in obese women [75].

As mentioned above, receptor- and aqueous diffusion-mediated FC and PL efflux pathways have varying specificities for different HDL subclasses; therefore the individual contributions of each efflux pathway is somewhat dependent upon the distribution of HDL particle subclasses present in plasma [76, 77]. While loss of ABCA1 results in the greatest arterial cholesterol accumulation and increase in atherosclerosis development in vivo, in vitro findings suggest that ABCG1 and SR-BI-mediated efflux pathways may also be important contributors when cultured in complete serum [76, 78, 79]. In addition, aqueous diffusion has been reported to be responsible for 30% to 35% of macrophage FC efflux in both in vitro and in vivo studies [80, 81].

The cholesterol efflux capacity of HDL can further be affected by additional HDL characteristics, including lipid composition, associated protein and enzymes, antioxidant and inflammatory profiles, and disease status [2, 19]. For example, pro-inflammatory HDL is less able to promote efflux [82, 83], whereas PON1 can increase ABCA1-mediated HDL binding to promote enhanced cholesterol efflux from macrophages [84]. Enrichment of HDL in phospholipids – particularly phosphatidylcholine and sphingomyelin – has also been shown to increase the cholesterol-accepting capacity of HDL [85-87]. Compared to healthy subjects, cholesterol efflux is reduced in subjects with obesity [88, 89], hypertension [65], coronary artery disease, type 2 diabetes [90], and low HDL-C [91, 92].

*Intravascular remodeling of HDL particles*

A variety of proteins and enzymes are known to contribute to the intravascular
remodeling of HDL. In addition to the antioxidant and anti-inflammatory properties discussed above, LCAT plays an important role in HDL particle maturation and cholesterol acquisition. LCAT is a liver-derived, lipid-free or HDL-associated enzyme that is activated by apoA-I [93]. LCAT catalyzes the transfer of 2-acyl groups from lecithin to FC, resulting in the formation of HDL-CE [6, 76]. The accumulation of CE within the HDL core results in formation of larger, mature α-HDL particles [94]. LCAT is thought to maintain the FC concentration gradient between HDL and macrophages that allows for in the incorporation of incoming FC into HDL [13, 93-95]. Individuals with LCAT deficiency have significantly decreased plasma HDL-C, apoA-I, and the second most common HDL-associated apolipoprotein, apoA-II. In addition, levels of smaller, immature preβ-1 and α-4 HDL particles are increased, whereas larger, mature α-1 and α-2 levels decreased. Together, these data show that LCAT activity is necessary for proper HDL maturation [94].

Another key player in intravascular HDL remodeling is cholesteryl ester transfer protein (CETP). CETP is a liver and adipose-derived glycoprotein that binds lipoproteins in the circulation, where it mediates the exchange of HDL-CE for apoB-containing lipoproteins (chylomicron/chylomicron remnants, VLDL, intermediate-density lipoproteins (IDL), LDL) –TG [96]. CETP activity is known to increase with greater apoB lipoprotein-TG content, resulting in HDL-CE depletion and increased HDL-TG accumulation [97]. Elevated HDL-TG makes HDL a better substrate for hepatic lipase-mediated TG lipolysis, resulting in an overall reduction in HDL particle size and HDL-C levels [6]. Although elevated non-HDL-CE levels are typically associated with increased CVD risk, CETP-mediated transfer of HDL-CE to apoB-containing lipoproteins still contributes to the RCT process, as these lipoproteins may be taken up by the liver via LDL-receptor (LDL-R), LDL-R related protein
(LRP)-, and SR-BI-mediated pathways for biliary cholesterol excretion [98-100], as well as non-biliary secretion via the transintestinal cholesterol efflux (TICE) pathway [101, 102]. In general, CETP activity has been regarded as pro-atherogenic. CETP-deficient patients have extremely high HDL-C levels and reduced CVD incidence [103]; however, while synthetic CETP inhibitors have been shown to increase HDL-C in humans, they have also been shown to increase all-cause and CVD-related mortality [104, 105]. These adverse effects have since been attributed to compound-specific toxicity off-target activation of the renin-angiotensin-aldosterone system, prompting further investigation into development of safer and more classes of CETP inhibitors [106-108].

As with CETP, elevated apoB-containing lipoprotein-TG content increases activity of lipoprotein lipase (LPL). With primary activity in adipose, skeletal muscle, and endothelial cell surfaces, LPL mediates lipolysis of TG within TG-rich lipoprotein (TGRL); i.e. chylomicron and VLDL particles, resulting in formation of CR and IDL/LDL, respectively [109]. LPL-mediated lipolysis of TGRL simultaneously results in the release and transfer of FC, PL, and apolipoproteins from TGRL to HDL, resulting in the formation of larger, more cholesterol- and apoA-I-rich HDL [6]. HDL-bound phospholipid transfer protein (PLTP) also plays a key role in HDL remodeling activity during TGRL-TG lipolysis, as it serves to transfer surface PL from TGRL to HDL, resulting in the formation of larger, less dense HDL particles [110]. Larger HDL particles observed with greater PLTP activity are also known to be a consequence of PLTP-mediated HDL particle fusion [111]. Lack of PLTP has been shown to impair ABCA1-mediated FC efflux from macrophages to lipid-free apoA-I, all together suggesting that PLTP plays a prominent role in HDL maturation and formation [112].
As mentioned above, CETP-mediated HDL-TG enrichment results in greater hepatic lipase (HL) affinity for HDL particles [6]. HL is a hepatocyte-derived enzyme with both TG lipase and phospholipase A1 activity. HL has greater specificity for HDL-TG than TGRL-TG, resulting in conversion of larger HDL to smaller HDL particles, preβ HDL, and lipid-poor or –free apoA-I [113]. Overall, increased HL-mediated HDL particle catabolism is associated with lower HDL-C levels [114]. Endothelial lipase (EL) is another lipolytic enzyme with greater specificity for HDL-TG than TGRL-TG; however, this primarily endothelial cell-derived enzyme predominantly has phospholipase A1 activity. Similar to HL, increasing EL-mediated HDL-PL hydrolysis is associated with reduced HDL-C, apoA-I, and HDL particle size due to increased fractional catabolism [113].

Mechanisms involved in hepatic cholesterol uptake and HDL clearance

Following retrieval of cholesterol from peripheral tissues and subsequent intravascular remodeling processes, HDL may then transport CE back to the liver for biliary excretion, or be catabolized by the kidney or steroidogenic tissues [6, 115, 116]. Hepatic SR-BI-mediated HDL catabolism is thought to be one of the primary mechanisms by which HDL-C is cleared from the body [117]. Although it was originally considered, recent evidence suggests that HDL does not play a significant role in non-biliary cholesterol excretion via the TICE pathway [118]. Similar to macrophage SR-BI, hepatic SR-BI preferentially binds and induces lipid flux from larger, more cholesterol-rich α-1 and α-2 HDL (HDL2) particles [77]. While SR-BI expression on macrophages contributes to lipid efflux [119], hepatocyte SR-BI expression allows for selective uptake of HDL lipid components, resulting in formation of small, dense HDL particles and shedding of lipid-free apoA-I into the circulation. Newly
released apoA-I must be rapidly recycled and/or relipidated through cellular efflux or HDL remodeling pathways in order to avoid cubulin-mediated renal catabolism and lysosomal degradation [6, 116]. Additional proposed mechanisms of hepatic HDL uptake have also been identified, including those involving scavenger receptor CD36, as well as ATP synthase ectopic β-chain-mediated HDL holoparticle endocytosis [120, 121]. While these pathways have been implicated in contributing to HDL metabolism and clearance, further investigations in vivo are required to determine their physiological impact on RCT and CVD risk.

**Implications for HDL-based therapies**

The evidence presented above highlights the important atheroprotective roles of HDL. Under optimal physiological conditions, functional HDL will possess the capacity to exert antioxidant, anti-inflammatory, antithrombotic, vasodilatory activities, allowing it to effectively serve as a lipid acceptor for cellular cholesterol [2]. As demonstrated within the context of atherosclerosis, the dynamic between HDL and macrophages is a critical determinant of disease severity [19, 64, 122]. Interestingly, recent data suggests that HDL may play a significant role of modulating the activity of other leukocytes – particularly in regard to their inflammatory potential [123-125]. Given that inflammatory leukocytes play an increasingly well-documented role in obesity-related disease progression [126-128], optimization of HDL function may confer physiological effects beyond the scope of atherosclerosis.

In order to adequately assess the dynamics between HDL and leukocytes, it is important to first place the physiological role of inflammation into context – both in regard to
immunity and chronic metabolic disease. Therefore, the following section will review the mechanisms underlying inflammation under acute and chronic conditions.

Section 2

Inflammation

Introduction

Inflammation is a normal, adaptive physiological response to pathogenic insult, including microbial infection and tissue injury; however the incidence of chronic low-grade, systemic inflammation underlying multiple highly prevalent chronic metabolic diseases warrants reevaluation of inflammatory processes in disease pathogenesis. Acute inflammatory responses are considered beneficial if executed in a local, controlled manner, as they function to rapidly and effectively eliminate pathogenic stimuli and return the affected tissue to a normal, homeostatic state through coordinated activation and resolution of pro-inflammatory leukocyte activity [129]. However, failure of the body to appropriately execute and resolve acute inflammatory responses can lead to a detrimental chronic inflammatory tissue state, characterized by pathological tissue remodeling, fibrosis, and impaired functioning, and due to persistent inflammatory cell infiltration, activation, and leukocyte-mediated tissue damage [130, 131]. More recently, similar adverse physiological adaptations have been observed within the context of obesity-related disorders, where prolonged metabolic stress and tissue malfunction are believed to promote the development of CVD, T2DM, insulin resistance, and
hepatic steatosis – all of which coincide with a chronic state of systemic, low-grade inflammation [132-134].

**The acute inflammatory response**

Acute inflammatory responses are rapidly initiated in response to molecular alarm signals that are indicative of microbial invasion or tissue damage, allowing for innate immune cell recruitment and activation, elimination of the pathogenic stimuli, resolution of immune-mediated inflammation, and restoration of tissue homeostasis [135]. Infection and tissue injury leads to necrotic cell death, resulting in the disruption of cell membranes the release of cytoplasmic and nuclear components containing conserved damage-associated molecular patterns (DAMPs), which include DNA, nucleosomes, and high-mobility group box 1 protein (HMGB1) – a nuclear protein that modulates chromatin accessibility and one of the most well-characterized endogenous alarm signals [135-137]. Microbial pathogens provide similar signals termed pathogen-associated molecular patterns (PAMPs), which are molecular components that are conserved amongst specific classes of microorganisms, yet distinct from the host’s endogenous molecules (e.g. lipopolysaccharide (LPS) present in the cell membrane of gram negative bacteria) [138]. DAMPs and PAMPs induce leukocyte activation through binding of pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nucleotide-binding oligomerization-domain (NOD)-like receptors (NLRs), which are expressed on antigen-presenting cells of the innate immune system, including patrolling steady-state non-classical CD14<sup>low</sup>CD16<sup>+</sup> monocytes, tissue-resident macrophages, and dendritic cells [135, 138]. Differential expression has also been reported in certain lymphocyte subsets [139, 140].
In macrophages, LPS-mediated TLR activation leads to signaling through the adaptor molecule myeloid differentiation primary response gene 88 (MyD88) [141], and transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), both of which promote the transcriptional activation of pro-inflammatory cytokines and chemokines, such as TNFα, and CXC-chemokine ligand 1 (CXCL1) and CXCL2 [135, 142, 143]. Similarly, HMGB1 has been shown to bind and activate receptor for advanced glycation end products (RAGE), TLR2, TLR4, TLR9 and interleukin 1 receptor (IL-1R), leading to the activation of NF-κB and greater production of TNFα, interleukin (IL)-6 and phagocyte-attracting chemokines such as IL-8, chemokine ligand (CCL) 3 and CCL4 [138, 144, 145].

The role of NF-κB in acute inflammatory responses

NF-κB is a family of dimeric transcription factors that serves as one of the primary mechanisms by which macrophages induce pro-inflammatory gene expression in response to cellular stress and external inflammatory stimuli [146]. As mentioned above, NF-κB plays important roles in both acute and chronic inflammation. Transcriptionally active NF-κB consists of homo- and heterodimers of NF-κB/Rel family members, including p65 (RelA), c-Rel, RelB, p50/105, and p52/100; however, only p65, c-Rel, and RelB contain C-terminal transactivation domains, whereas p52 and p50 subunits rely on interactions with these factors to positively regulate transcription of target genes [147]. The p50-p65 dimer is the most well characterized, as it is the predominant NF-κB subunit pair that mediates LPS- and TNFα-induced expression of pro-inflammatory genes [148, 149]. The majority of NF-κB dimers are retained in the cytoplasm of resting cells by inhibitor of κ B (IκB) proteins, which contain strong nuclear export signals and physically mask the nuclear localization signal (NLS)
regions of Rel family subunits. Activation of transcriptionally active NF-κB is therefore dependent upon dissociation from IκB and nuclear translocation of the NF-κB dimer [150]. NF-κB can be activated in response to ligation of a variety cell surface immune receptors, including TNFα and IL-1β receptors, TLRs, NLRs, and T and B lymphocyte antigen receptors [148, 151, 152]. Receptor ligation triggers the activation of the trimeric IκB kinase (IKK) complex, containing catalytic subunits IKKα and IKKβ, as well as the regulatory scaffolding subunit NF-κB essential modulator (NEMO/IKKγ). Active IKK phosphorylates specific serine residues of IκB, resulting in dissociation from NF-κB subunits, proteosomal degradation of IκB, and translocation of NF-κB from the cytoplasmic compartment to the nucleus [146]. NF-κB dimers subsequently induce transcriptional activation of target genes containing NF-κB binding sites within their promoter regions, including TNFα, IL-6, MCP-1, and COX2 [153]. Low levels of NF-κB are constitutively present in the nucleus; however, in non-stimulated cells, transcriptional activity is actively repressed through binding of a co-repressor complex containing co-repressors such as nuclear receptor corepressor (NCoR), silencing mediator for retinoid and thyroid hormone receptor (SMRT), and histone deacetylase 3 (HDAC3) [149]. This basal state of active NF-κB repression allows for the inhibition of NF-κB by anti-inflammatory transcription factors LXR and peroxisome proliferator-activated receptor γ (PPARγ) in the presence of inflammatory stimuli, as discussed in more detail below [154].
**Neutrophil recruitment and activation**

Dendritic cell- and monocyte/macrophage-derived cytokines and chemokines facilitate neutrophil activation, recruitment, and extravasation into the affected tissue, where neutrophils release contents from cytosolic granules containing cytotoxic reactive oxygen species (ROS), and antimicrobial and matrix-degrading hydrolytic enzymes [155, 156]. Neutrophils also release soluble complexes of IL-6 and the IL-6 receptor (IL-6R), which activate endothelial cells to express CCL2 and vascular cell-adhesion molecule 1 (VCAM1), thereby promoting the adhesion and extravasation of circulating monocytes into the affected tissue, where they can subsequently differentiate into macrophages in response to macrophage-derived granulocyte-monocyte colony-stimulating factor (GM-CSF) [157, 158]. Activation of monocytes/macrophages leads to rapid cyclooxygenase or lipoxygenase (LOX)-mediated formation of pro-inflammatory lipid mediators from membrane-derived arachidonic acid (AA), including prostaglandins and leukotrienes. Leukotriene B4 serves as a potent chemoattractant of certain T cell subsets, monocytes, and neutrophils, while also promoting neutrophil granule release and ROS production [159-162].

Together, the combined pro-inflammatory, anti-microbial, and phagocytic activity of neutrophils and monocytes/macrophages ultimately leads to the elimination of pathogenic stimuli; however, neutrophil lifespan and activity must be tightly controlled due to the nonspecific nature of cytotoxic neutrophil granule contents, which have the potential to cause excessive damage to self-tissues [156]. Accordingly, the initiation of inflammatory response resolution is characterized by the onset of neutrophil apoptosis and inhibition of further neutrophil recruitment [163]. Macrophage-derived TNFα contributes to the regulation of neutrophil lifespan throughout the inflammatory response, as low TNFα levels extend the
lifespan of relatively short-lived neutrophils, whereas excessive levels facilitate neutrophil apoptosis [164]. Apoptotic neutrophils release lactoferrin, lysophosphatidylcholine (LPC), nucleotides, and CX3CL1, which serve macrophage chemoattractants [135]. Monocyte/macrophage- and neutrophil-derived lipid mediators also play a critical role in resolution, as accumulating levels of pro-inflammatory prostaglandin E2 (PGE2) and PGD2 gradually promote the synthesis of anti-inflammatory, pro-resolving lipoxins in a process termed eicosanoid class-switching [165]. Lipoxin A4 inhibits neutrophil recruitment, ROS production, and NF-κB activity, resulting in reduced production of pro-inflammatory cytokines and chemokines [166], while simultaneously promoting monocyte migration and macrophage phagocytosis of apoptotic neutrophils. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-derived lipid mediators, including resolvins, protectins, and maresins, exhibit similar anti-inflammatory and pro-resolving activity, as discussed in greater detail below [167]. Phagocytosis of apoptotic neutrophils further elicits anti-inflammatory macrophage responses by inducing increased secretion of transforming growth factor-β (TGF-β), IL-10, and vascular endothelial growth factor (VEGF), which serve to promote endothelium repair [135, 168]. Together, the coordinated efforts of innate immune cells allow for recognition and elimination of pathogenic insults, repair of tissue injury, and restoration of tissue homeostasis [167].

**Chronic inflammation in metabolic disease**

As opposed to acute inflammatory responses, which are triggered by specific events or noxious stimuli, the chronic low-grade inflammation, or “para-inflammation,” observed in obesity-related diseases is thought to be initiated in metabolic cells in response to excess nutrient overload, leading to prolonged tissue stress [129, 169]. While metabolic disturbances
in obesity, CVD, insulin resistance, and T2DM often span multiple metabolic organ systems (i.e. the liver, pancreas, and skeletal muscle), tissue stress and inflammation is thought to be initiated in white adipose tissue (WAT). The onset of obesity promotes excess nutrient-induced adipocyte hypertrophy and endoplasmic reticulum (ER) stress, leading to elevated secretion of pro-inflammatory cytokines and leukocyte infiltration. Dysfunctional WAT also has a diminished capacity to store lipids, resulting in ectopic lipid deposition in non-WAT tissues and perpetuation of systemic insulin resistance, atherogenic dyslipidemias, and para-inflammation [132, 169]. Clinical manifestation of these underlying conditions often present as the of parameters of MetS - a clinical classification characterized by a clustering of central adiposity, elevated blood glucose, high plasma TG, elevated blood pressure, and low plasma HDL-C, resulting in a 2- and 5-fold increased risk for CVD and T2DM, respectively [170].

**Adipose tissue dysfunction and endoplasmic reticulum (ER) stress**

Adipose tissue is a dynamic metabolic organ that contributes to the maintenance of nutrient homeostasis by serving as a source of readily accessible energy stores, while also functioning as an endocrine organ through the secretion of hormones and adipokines. Insulin-dependent storage of excess nutrients is dependent upon the rapid formation of lipid droplets [171], which are lipid storage organelles that predominantly consist of TG and CE. TG and CE are synthesized within the inner leaflets of the ER membrane, forming a lipid droplet core that buds off surrounded by a monolayer of ER membrane-derived phospholipids and associated PAT-domain proteins, including perilipin, adipose differentiation-related protein (ADRP), and TIP47-related protein, as well as the ER chaperone protein BiP [172]. In obesity, prolonged intake of excessive nutrients requires
increased lipid droplet formation coupled with excessive demand for PAT protein synthesis, ultimately leading to ER exhaustion and an accumulation of newly synthesized unfolded and misfolded protein aggregates that activate the unfolded protein response (UPR) [173]. Accumulating protein aggregates are sensed by ER stress proteins, including PKR-like eukaryotic initiation factor 2α kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF-6). Under normal, non-stressed conditions, BiP binds PERK, IRE1 and ATF6, rendering them inactive. However, increased lipid droplet formation limits BiP availability, leading to activation of PERK, IRE1, and ATF6 signaling [174]. IRE-1 signaling leads to mitogen-activated protein kinase (MAPK)/c-Jun N-terminal kinase (JNK)-mediated activation of AP-1, whereas PERK, IRE-1, and ATF6 signaling pathways facilitate NF-κB activation, overall resulting in an increased production of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-6, IL-8, and MCP-1 from both adipocytes and tissue-resident macrophages [146]. In high-fat diet-induced obese ob/ob mice, elevated ER stress promotes JNK-mediated serine phosphorylation of insulin receptor substrate-1 (IRS-1), leading to suppressed insulin receptor signaling and impaired insulin sensitivity. Prolonged UPR also triggers CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP) and caspase-12-mediated adipocyte apoptosis, which stimulates leukocyte influx into adipose tissue [174]. While macrophages represent approximately ~5-10% of cells in healthy adipose tissue, macrophages can represent up to 50% of all cells in obese WAT. Pulse-labeling studies conducted in vivo have demonstrated that the macrophages recruited into adipose tissue at the onset of obesity are highly pro-inflammatory, classically activated M1 macrophages, in contrast to resident macrophages in lean adipose, which are typically alternatively-activated, anti-inflammatory M2 macrophages.
Increased T cell infiltration is also observed in obese adipose tissue, and some evidence suggests that these lymphocytes may become activated in response to unique antigens generated in obese adipose tissue during high-fat feeding [126]. In addition, greater levels of pro-inflammatory T\(_{h1}\) cells have been observed in obese adipose, whereas levels of anti-inflammatory T\(_{reg}\) cells are reduced, corresponding to greater impairments in insulin sensitivity [126, 178].

**Impaired nutrient storage and insulin resistance**

The overall inflammatory state of dysfunctional obese WAT impairs the nutrient storage capacity of adipocytes. For example, TNF\(\alpha\) is known to counteract insulin-mediated nutrient uptake through inhibition of insulin-mediated glucose transporter type 4 (GLUT4) translocation to the adipocyte cell surface, LPL-mediated lipolysis of chylomicron TGs for adipocyte storage, and perilipin-mediated lipid droplet formation. In addition, TNF\(\alpha\) increases hormone sensitive lipase (HSL) activity, promoting FFA mobilization from adipose into the circulation [132, 179]. Elevated levels of circulating FFAs are commonly observed in obesity, and can be deposited in the liver, skeletal muscle, and pancreas, leading to impairments in tissue function, metabolic homeostasis, and increased risk of developing hepatic steatosis and T2DM [132, 180]. Obesity is also known to increase fat deposition in primary and secondary lymphoid organs, including the bone marrow, thymus, spleen and lymph nodes, leading to alterations in the distribution of leukocyte populations, lymphocyte activity, and overall immune defenses [181-185]. The lipotoxic effects of systemic lipid deposition contribute to the elevated levels of plasma pro-inflammatory mediators characteristic of obesity-related diseases, where increased levels of TNF\(\alpha\), IL-1\(\beta\), IL-6,
MCP-1, and CRP are commonly observed [186]. The combination of increased lipid deposition and inflammatory mediators further promotes insulin resistance, as insulin resistant diet-induced obese \textit{ob/ob} mice show greater insulin sensitivity when lacking either TNF\(\alpha\) or TNF receptors [187]. TNF\(\alpha\) receptor ligation induces JNK signaling and NF-\(\kappa\)B activation, which have further been shown to contribute to insulin resistance [186]. TNF\(\alpha\) signaling is also known to down-regulate peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) – a ligand-activated transcription factor that counteracts insulin resistance by promoting adipogenesis through hyperplastic vs. hypertrophic adipocyte expansion, TG biosynthesis for adipocyte storage, antagonism of NF-\(\kappa\)B, and increased expression of adipose-derived adiponectin, an adipokine that exerts systemic anti-inflammatory and insulin sensitizing effects [132, 188]. Accordingly, many of the most common and effective pharmacological therapies prescribed for T2DM belong to the thiazolidinedione (TZD) class of synthetic PPAR\(\gamma\) ligands [189]. However, effective reversal and/or improvement of para-inflammation and obesity-associated disease is most commonly observed with weight loss, as evidenced by improved resolution indices and decreased circulating levels of inflammatory mediators [190, 191].

\textit{Role of NF-\(\kappa\)B and AP-1 in obesity-related disease}

The role of NF-\(\kappa\)B and AP-1 in obesity-related chronic inflammation is becoming increasing well characterized [169]. ER stress-activated PERK has been shown to trigger degradation of I\(\kappa\)B, allowing for NF-\(\kappa\)B-mediated expression of TNF\(\alpha\) [192]. Similarly, AP-1 can be activated by IRE-1 as a consequence of the UPR, leading to adipocyte dysfunction, macrophage activation and recruitment, and insulin resistance [174]. In liver tissue from
obese NASH patients, both AP-1 and NF-κB DNA binding were found to be significantly greater than non-obese controls, corresponding to greater insulin resistance and oxidative stress as measured by HOMA-IR and ferric reducing ability of plasma (FRAP), respectively [193]. Together, NF-κB and AP-1 serve as two of the master regulators of pro-inflammatory gene expression in response to ER stress, and inflammatory stimuli within the context of acute and chronic inflammation.

**Relationship between acute and chronic inflammation**

Acute inflammatory responses and para-inflammation are similar in that both are characterized by leukocyte activation and infiltration into affected tissues, resulting in the generation of pro-inflammatory mediators; however, these responses differ in the triggers of instigation (noxious stimuli or tissue injury vs. metabolic tissue stress), and their capacity to resolve inflammation and restore tissue homeostasis [167, 169]. Following successful elimination of the pathogenic factor, acute inflammatory responses appropriately facilitate resolution of inflammatory mediators, tissue repair, and return to normal, healthy tissue homeostasis. Conversely, the onset of para-inflammation results in a shift in homeostatic set points of affected tissues to perpetuate a maladaptive state with no defined marker of resolution [129]. Para-inflammation can also lead to impairments in acute inflammatory responses, as murine models of diet-induced obesity exhibit delayed resolution of inflammatory responses [194]. Therefore, while triggers of initiation and resolution between acute and chronic inflammation differ, the shared leukocyte-mediated processes and molecular signaling underlying acute and chronic inflammation suggests that inflammatory
crosstalk may occur, perhaps further complicating the pathogenesis of acute and inflammatory disease.

**Leukocyte profiles in obesity**

Similar to what is observed in metabolic tissues, leukocytes tend to reside in elevated basal pro-inflammatory state in obesity. In human peripheral blood mononuclear cells (PBMCs) – a mixed leukocyte population containing approximately 60-70% T cells, 5-15% B cells, 5-10% natural killer cells, 0.5-2% dendritic cells, 15-30% monocyte/macrophages, and 2% granulocytes [195-198] – p50- and p65-DNA binding was found to be elevated in obese subjects when compared to normal-weight control, whereas IκBβ was significantly lower in the obese group. Increased NF-κB binding corresponded to increased mRNA expression of NF-κB target genes, including migration inhibition factor (MIF), IL-6, TNFα, and matrix metalloproteinase 9 (MMP9) in obese subjects, as well as significantly greater plasma FFA and insulin resistance, as measured by homeostasis model assessment of insulin resistance (HOMA-IR) [199]. Further, PBMC NF-κB-DNA binding, as well as mRNA expression of TNFα, IL-6, MIF, and MMP9 has been shown to decrease with 5% weight loss in non-diabetic, obese female subjects, indicative of potential steps toward resolution of obesity-associated para-inflammation [200].

Recently, elevations in the standard clinical white blood cell (WBC) measure has been used as a marker of leukocyte inflammation – particularly within the context of MetS. According to Japanese MetS criteria, WBC counts served as a positive predictor of MetS [201]. In addition to MetS being associated with higher white WBC counts in Japanese populations [202], MetS components are typically worsened in higher WBC quartiles.
Similarly, in an elderly (75 year old) Swedish population, WBC counts were found to be positively associated with parameters of MetS; however, this was more prevalent in women [203]. Conversely, long-term, moderate weight loss (5.4% of body weight over 3 years) has been shown to reduce WBC counts and serum inflammatory markers (IL-1β, IL-6, urinary isoprostanes) [204].

_Altered immunity in obesity_

Despite having an increased basal level of inflammation, obesity is associated with impaired immune responses, further suggesting that the elevated levels of leukocyte inflammation represent markers of metabolic dysfunction rather than an enhanced capacity to clear infection and mount organized immune responses. Both high-fat diet-fed and _ob/ob_ obese mice experience increased mortality in response to _Staphylococcus aureus_-induced sepsis, corresponding to several impaired innate immune functions [205]. Diet-induced obesity has been shown to impair memory CD8+ T cell responses to influenza virus infection, resulting in increased mortality, viral titers in lung, and worsened lung pathology [183]. These adverse effects were associated with an obesity-induced failure to maintain influenza-specific CBD8+ memory T cells, suggesting that obesity may reduce vaccine efficacy [183]. Obesity has additionally been associated with impaired T cell-mediated immune surveillance by promoting reductions in thymopoiesis and constriction of T cell receptor (TCR) diversity [206]. As mentioned above, the reduction in certain lymphocyte subsets may coincide with concomitant increases in T cell that respond to antigens unique to dysfunctional adipose, as well as lymphocytes that favor metabolic tissue infiltration and pro-inflammatory responses [126, 178]. In the National Health and Nutrition Examination Survey (NHANES) 2005-2006
cohort, obesity was associated with an increased prevalence of allergic disease in children, primarily driven by allergic sensitization to food [207]. These findings suggest that obesity impairs normal immune functioning, which may further perpetuate metabolic disease complications.

The role of inflammatory leukocytes in perpetuating obesity-related disease

Evidence for the role of pro-inflammatory leukocytes in perpetuating obesity-related metabolic dysfunction has been documented on both animal and human models. Winer et al. [126] demonstrated that the incidence of murine insulin resistance could be regulated through CD4⁺ T cell-based immunotherapy. Inflammatory monocytes and macrophages have additionally been associated with physiological complications in metabolic tissues – particularly in adipose and the arterial wall within the context of atherosclerosis. Certain subsets of “inflammatory” CD16⁺ monocytes have been shown to be more likely to interact with activated endothelium, extravasate into the arterial wall, and differentiate into macrophages [208, 209]. CD14⁺/CD16⁺ monocytes have been associated with hypercholesterolemia, as well as an increased incidence of CHD [127, 128]. Further, polarization of classically activated pro-inflammatory M1 or alternatively activated anti-inflammatory M2 macrophage subsets has been shown to affect atherosclerosis and adipose tissue dynamics [175, 210], a process which is further regulated by T cells [211]. Together, these findings demonstrate that obesity promotes a state of positive feedback between inflammatory leukocytes and adverse metabolic outcomes.
Exogenous and endogenous factors linking acute and chronic inflammatory responses

As described above, mechanisms underlying the activation of acute inflammatory responses overlap with pathways of obesity-related chronic inflammation. A variety of exogenous and endogenous factors similarly play a role in both acute and chronic inflammation, and may further perpetuate the dysregulated relationship between the two in obesity. Keeping the scope of this dissertation in mind, the following section will review the roles of LPS, fatty acids, and cholesterol in inflammation.

The role of lipopolysaccharide (LPS) in inflammation

LPS is immunostimulatory glycolipid located on the outer membrane of Gram-negative bacteria that activates innate immune responses via PRRs such as TLRs [135, 212]. LPS consists of an amphipathic lipid A component that serves as the conserved molecular pattern element, in addition to core hydrophilic polysaccharides and O-antigen [212]. As described above, LPS-mediated TLR activation leads to the initiation of pro-inflammatory signaling cascades, thereby promoting the transcriptional activation of pro-inflammatory cytokines and chemokines via transcription factors such as NF-κB and AP-1 [135, 142, 143]. Control of LPS-induced inflammatory responses is essential to prevent adverse physiological consequences, including organ dysfunction and failure, hypotension, and septic shock [213].

While the role of LPS in acute inflammation and infection has been well elucidated, recent research has demonstrated an increasingly significant role for LPS in obesity-related chronic inflammation [214, 215]. Aside from direct bacterial infection, LPS exposure can occur via the consumption of LPS-containing foods, as well as the presence of LPS-containing microbiota in the gut. Both exogenous and endogenous LPS has been shown to
contribute to plasma LPS levels, as gut- or diet-derived LPS is known to be absorbed into intestinal capillaries, then transported into circulation by lipoproteins [216-218]. Since obesity is associated with increased energy intake and adverse shifts in microbiota populations [219], it is plausible to expect that LPS plays a role in obesity-related inflammation.

Recent research findings have further corroborated this concept. In an observational study conducted in 201 healthy men, plasma LPS concentrations were found to be positively correlated with dietary fat and energy intake [220]. In C57B6/J mice, 4 weeks of high-fat feeding increased plasma LPS concentrations 2- to 3-fold, while also increasing the proportion of LPS-containing gut microbiota. Subcutaneous infusion of LPS further induced insulin resistance, adipose tissue inflammation, and hepatic lipid deposition [216]. Correspondingly, circulating levels of LPS have been shown to be elevated in T2DM patients when compared to healthy controls, whereas LPS has additionally been shown to activate inflammatory pathways in human adipose tissue \textit{ex vivo} [214]. Interestingly, 10-wk rosiglitazone treatment reduced LPS levels, along with glucose and insulin. Fasting insulin positively correlated with serum endotoxin levels in non-diabetic subjects [214]. Lipopolysaccharide-binding protein (LBP) - an indirect measure of LPS and systemic inflammation - is associated with many factors of obesity and MetS, including abdominal obesity, low HDL-C, and elevated levels of serum cytokines and liver enzymes (IL-6, IL-8, alanine aminotransferase (ALT), aspartate aminotransferase (AST) [215]. Together, these findings support the detrimental role of LPS in obesity-related inflammation and metabolic dysfunction.
The role of fatty acids in inflammation

Fatty acids are well known to influence both pro- and anti-inflammatory activity through a variety of cellular mechanisms. Fatty acids are key components of cell membrane phospholipids, and due to their variability in structure and functionality, alterations in membrane fatty acid composition can influence the physical properties of membranes in terms of fluidity and lipid raft structure, as well as the availability of fatty acids to be used as precursors for the generation of bioactive lipid mediators, and ligands for lipid-sensing transcription factors [221, 222]. The ability of fatty acids to differentially modulate inflammatory responses has significant implications for both dietary recommendations and within the context of chronic inflammation underlying metabolic disease [223]. Immune cell fatty acid membrane composition highly correlates with dietary intake, usually in a dose-dependent manner, whereas obesity is associated with excess amounts of adipose-derived plasma FFAs, resulting in exposure of body tissues to greater concentrations of fatty acids, while also resulting in lipid deposition in non-adipose tissues [132, 221]. Together, fatty acids predominantly exert pro- and anti-inflammatory effects by 1) serving as precursors to immunoregulatory eicosanoids and lipid mediators, 2) through activation of TLR4-mediated activation of NF-κB and AP-1-mediated pro-inflammatory gene expression, and 3) by acting as ligands for PPARs, which are able to suppress pro-inflammatory gene expression via SUMOylation-dependent transpression of NF-κB and AP-1 [154, 224, 225].

Immunoregulatory properties of fatty acid derived-lipid mediators

Omega-3 (ω-3) and omega-6 (ω-6) PUFAs serve as essential dietary precursors to eicosanoids, which are potent bioactive lipid mediators involved in both the propagation and
resolution of inflammatory responses [167, 225]. Human leukocytes are typically rich in the ω-6 PUFA arachidonic acid (AA), representing ~ 20% of membrane phospholipid fatty acid content [221]. AA is therefore the predominant substrate for eicosanoid synthesis, giving rise to multiple lipid mediators with variable inflammatory properties, including prostaglandins (PGs), thromboxanes (TX), leukotrienes (LTs), and lipoxins (LXs). Whereas LXs exhibit anti-inflammatory and pro-resolving activity, PGs, TXs, and LXs are most commonly associated with pro-inflammatory responses, with PGE$_2$ and LTB$_4$ being two of the most well characterized subclasses. In response to a wide variety of physical, chemical, hormonal, and inflammatory stimuli [226], AA is released from cell membrane phospholipids via hydrolysis by phospholipases such as phospholipase A$_2$, allowing for COX-1 and COX-2-mediated formation of PG and TX precursors, as well as 5-LOX-mediated formation of LT precursors [221]. LTB$_4$ binds G protein-coupled transmembrane receptors BLT1 and BLT2, inducing signaling to promote potent chemotaxis of certain T cell subsets, monocytes, and neutrophils, in addition to neutrophil granule release and ROS production [159-162]. Four E type prostanoid (EP1, -2, -3, -4) receptor subtypes have been characterized that bind PGE$_2$, each triggering the activation of different intracellular signaling pathways [226]. Although PGE$_2$ is known to induce macrophage and dendritic activation and inflammatory mediator expression, it has also been shown to decrease expression of TNFα, IFNγ, IL-1β, and MCP-1 in LPS-stimulated macrophages, suggesting that variation in EP receptor ligation may affect the pro-inflammatory activity traditionally associated with this eicosanoid [226-230]. In addition, accumulating levels of PGE$_2$ and PGD$_2$ in macrophages gradually promote the synthesis of anti-inflammatory, pro-resolving lipoxins in a process termed eicosanoid class-switching [165]. LXA$_4$ activates the LXA$_4$ receptor (ALX), a G-protein coupled receptor, whose
signaling has been shown to block components of the p38 MAPK pathway and attenuate NF-κB activation, resulting in reduced production of pro-inflammatory cytokines and chemokines [166]. ALX may also be activated by 15-epi-LXA₄, an “aspirin-triggered lipoxin,” contributing to this common, non-steroidal drug’s anti-inflammatory benefits [231]. Aspirin (acetyl-salicylic acid) induces acetylation of the COX2 active site, thereby altering its enzymatic activity to switch from PG precursor production, to that of 15-HpETE – a substrate for 5-lipoxygenase-mediated 15-epi-LXA₄ formation [131]. Together, LXA₄–ALX signaling is known to reduce neutrophil recruitment and degranulation, while promoting monocyte/macrophage migration and phagocytosis of apoptotic leukocytes and lymphocytes, overall promoting the resolution of inflammatory responses [131, 232].

ω-3 PUFAs also serve as precursors to anti-inflammatory, pro-resolving lipid mediators, including resolvins, protectins, and maresins. COX-2 and cytochrome P450 mediate the formation of EPA-derived E series Resolvin E1 (RvE1) and E2 (RvE2), whereas formation of DHA-derived D series resolvins (RvD1-4) is dependent upon LOX activity [131]. RvE1 and D series resolvins bind the G-protein coupled receptors CMKLR1 and GPR32/ALX, respectively, resulting in suppression of dendritic cell and macrophage inflammatory cytokine production, reduced neutrophil recruitment, and stimulation of macrophage phagocytosis [131, 233]. RvE1 is also known to displace LTB₄ by binding its receptor, BLT1, resulting in a dampening of LTB₄-mediated pro-inflammatory activity [234]. Intraperitoneal injection of RvE1 into ob/ob mice was shown to improve markers of high-fat diet-induced hepatic steatosis and insulin resistance, as evidenced by reduced hepatic lipid accumulation, as well as increased mRNA expression of adiponectin, GLUT4, IRS-1, and PPARγ in WAT [235]. DHA can also give rise to 15-LOX-generated protectin D1, or 12-
LOX-generated maresin 1, both of which share similar anti-inflammatory and pro-resolving effects of lipoxins and resolvins [236, 237].

Modulation of gene expression by fatty acids

In addition to serving as precursors to immunomodulatory eicosanoids, a variety of fatty acids and fatty acid derivatives have been shown to differentially modulate pro-inflammatory gene expression through 1) activation of TLR4-mediated NF-κB and AP-1, and 2) SUMOylation-dependent transrepression of NF-κB and AP-1 by PPARγ [154, 224, 225]. A medium-chain saturated fatty acid (SFA) component of LPS, lauric acid, has been shown to trigger TLR4 signaling in RAW 264.7 macrophages, corresponding to increased NF-κB activation and COX-2 expression [238]. Myristic (14:0), palmitic (16:0), and stearic (18:0) SFAs have also been shown to induce IL-6 expression in macrophages, whereas unsaturated fatty acids (UFAs) have no effect [224]. Originally believed to directly ligate TLR4, recent findings suggest that SFAs indirectly trigger TLR4 signaling through modulation of the cell surface receptor orientation and spacing within lipid raft structures [239, 240]. The ability of SFA to modulate TLR4 has potentially significant implications within the context of obesity and T2DM, where increased levels of circulating FFAs and para-inflammation are commonly observed [129, 132, 180]. In mice, in vivo lipid infusion and high-fat diet feeding resulted in TLR4-dependent increases in NF-κB-DNA binding and TNF-α, IL-6, and MCP-1 mRNA expression in adipose, corresponding to greater impairments in skeletal muscle and WAT glucose sensitivity [224]. LPL-mediated lipolysis of TGRLs results in the release of FFA fractions that promote increased TNF-α and intracellular adhesion molecule (ICAM) expression, as well as elevated ROS production in human aortic endothelial cells (HAECs)
Treatment of LPS-stimulated human monocyte/macrophages with physiological concentrations of SFAs resulted in greater IL-6 and IL-8 mRNA and protein expression, which was at least partially attributable to SFA metabolism to ceramide, and activation of protein kinase C (PKC)-ζ and MAPKs Erk, JNK, and p38 [242]. Further, in human U937 monocyte-derived macrophages, treatment of the SFAs palmitic and lauric acid resulted in significantly greater increases in the pro-atherogenic and lymphocyte migration inducing chemokine IP-10 mRNA, whereas UFAs oleic acid, EPA, linoleic acid, and AA had no effect. Palmitic acid also led to increased mRNA expression of COX-2, IL-8, MCP-1, and MIG, as well as greater NK-κB DNA binding activity when compared to untreated control cells [243].

In contrast to pro-inflammatory transcriptional responses induced by SFAs, a variety of UFAs and UFA derivatives have been shown to inhibit pro-inflammatory gene expression by promoting PPARγ-mediated transpression of NF-κB and AP-1 [154]. Multiple ω3- and ω6-PUFAs and PUFA derivatives serve as ligands for PPARα, -β/δ, and -γ, which are members of the nuclear receptor superfamily of transcription factors that serve well-characterized roles as master regulators of metabolic processes [244]. Upon ligand binding, PPARs heterodimerize with RXRs, allowing for PPAR-RXR binding to PPAR response elements (PPRE) within promoter regions of target genes, including those involved in mitochondrial and peroxisomal fatty acid β-oxidation in the liver (PPARα), fatty acid oxidation in skeletal muscle (PPARβ/δ), and activation of WAT fatty acid synthesis, storage, glucose homeostasis, and adipogenesis (PPARγ) [132, 244]. While anti-inflammatory roles for all PPAR subtypes have been reported, the most well-characterized is that of PPARγ, predominantly due to its ability to inhibit NF-κB- and AP-1-mediated pro-inflammatory gene
transcription via SUMOylation-dependent transrepression, similar to that of LXR [154]. PPARγ is predominantly expressed in adipose tissue, adrenal gland, and spleen, and its expression is rapidly induced upon differentiation of human monocytes to macrophages [245, 246]. In macrophages, endogenous PPARγ ligands include essential fatty acid metabolites such as 15-deoxy-Δ12,14-prostaglandinJ2 (15d-PGJ2), and oxidized fatty acids 9-HODE and 13-HODE, which are often found as components of oxidized LDL particles [244]. In addition, the insulin-sensitizing TZD class of antidiabetic drugs act as potent synthetic activators of PPARγ [189]. Upon ligand binding, PPARγ undergoes allosteric reconfiguration to reveal specific lysine residues that are targets for conjugation with SUMO1, which is dependent upon the activity of SUMO E3 ligase protein inhibitor of activated signal transducer and activator of transcription 1 (PIAS1). SUMOlyated PPARγ binds NCoR and SMRT-containing corepressor complexes on NF-κB and AP-1, preventing their dissociation, recruitment of coactivator complex, and transcriptional activation of target genes [154, 247]. Accordingly, PPARγ activation is known to suppress expression of TNFα, IL-15, MCP-1, vascular cell adhesion molecule 1 (VCAM1), and inducible nitric oxide synthase (iNOS) in LPS-stimulated macrophages in an NCoR-dependent transrepression [154]. In addition, recent findings have demonstrated that PPARγ activation primes primary human monocytes toward differentiation of alternatively activated M2 macrophages, which predominantly produce anti-inflammatory IL-4 and IL-13 cytokines over IFNγ and IL-1β [248]. PPARγ is also known to positively regulate transcriptional activation of PPRE-containing LXRα, resulting in greater ABCA1 activity and the addition LXR-mediated transrepression of pro-inflammatory gene expression [249, 250]. Activation of PPARγ in endothelial and vascular smooth muscle cells has also been shown to result in inhibition of NF-κB phosphorylation
and transcriptional activity [251, 252]. Lastly, PPARγ activation in adipose tissue is associated with reduced adipocyte hypertrophy, leukocyte infiltration, pro-inflammatory cytokine secretion, circulating free fatty acids levels, and overall improvements in insulin sensitivity and adipose dysfunction [169, 253-255].

Together, fatty acid-rich lipid membranes serve as vital sources of potential immunomodulatory lipids with varying capacities to promote pro- and anti-inflammatory activity through a variety of mechanisms. The presence of elevated FFAs in obesity-related diseases, as well as the ability to shift fatty acid-mediated inflammatory activity through diet suggest that modulation of fatty acid consumption and endogenous metabolism may serve as a mechanism to modulate para-inflammation underlying chronic metabolic disease [221].

The role of leukocyte cholesterol content in inflammation

In addition to LPS and fatty acids, an additional factor linking acute and chronic inflammation is the regulation of leukocyte cholesterol flux. This is predominantly due to the fact that cholesterol serves as an essential structural component of lipid rafts, which are dynamic cholesterol-rich microdomains within the exoplasmic leaflets of the phospholipid bilayer of plasma membranes where transmembrane proteins and receptors reside – including PRRs such TLR4 [256]. Elevated levels of cellular cholesterol favor the formation of lipid rafts, and have been associated with increased pro-inflammatory responses in macrophages and T lymphocytes due to lowered cellular activation thresholds [124, 257, 258]. Given the significant role of these cell types in immunity and chronic disease, these findings have important implications for the physiological consequences of obesity.
In line with its traditional atheroprotective role as an acceptor of lipids from arterial wall macrophage foam cells and peripheral tissues, a novel role for HDL has emerged in the regulation of leukocyte inflammatory potential via the modulation of cholesterol flux. The significance of this pathway has further been supported by the immunomodulatory functions of HDL-associated transporters, including ABCA1 and ABCG1 [123, 125, 258, 259]. This relationship may further uncover a dysfunctional HDL-leukocyte dynamic within the context of obesity and MetS – conditions associated with atherogenic dyslipidemia, chronic inflammation, and altered immunity [183, 260]. Therefore, the following section will review the evidence thus far elucidating the role of HDL-leukocyte interactions in relation to cellular inflammatory potential and cholesterol flux.

**Section 3**

**HDL-leukocyte dynamics**

*The role of cholesterol in hematopoiesis and leukocyte dynamics*

Cellular cholesterol availability and handling is also known to play a role in hematopoiesis, particularly in regards to maintenance of hematopoietic stem cells (HSC) and multipotent progenitor (MPP) populations [261]. Cholesterol is an essential component of cellular plasma membranes, and can be derived from either lipoprotein uptake or local synthesis, whereas cholesterol export mechanisms are known to maintain appropriate cellular cholesterol balance [262]. LXRα and LXRβ function as oxysterol-sensitive, ligand-activated transcription factors, which heterodimerize with RXR to increase expression of ABCA1 and
ABCG1, which serve to facilitate cholesterol efflux leukocytes to HDL. Mice lacking ABCA1 and ABCG1 display leukocytosis, a myeloproliferative disorder characterized by elevated leukocyte numbers – particularly monocytes. Abca1⁻/⁻Abcg1⁻/⁻ mice have elevated levels of bone marrow leukocyte progenitors, and circulating monocytes and neutrophils in blood, which is attributable to increased cell proliferation due to hyper-responsiveness to hematopoietic growth factors IL-3 and GM-CSF [261]. Cell-surface IL-3 and GM-CSF receptors are integrated into cholesterol- and glycoprotein-rich lipid rafts within the cellular plasma membrane, which allow for enhanced receptor oligomerization and signaling, resulting in rapid HSC and progenitor proliferation in response to growth factor signaling [262, 263]. High cholesterol-feeding and low plasma HDL contributes to excess accumulation of cellular cholesterol, resulting in greater lipid raft formation, and greater responsiveness to proliferative cues, whereas elevated HDL levels have been shown to suppress HSC and MPP proliferation [123, 261, 262, 264]. Greater expression of genes involved in cholesterol metabolism has been observed in more primitive progenitors, corresponding to greater proliferative capacity, whereas expression of these genes decreases with subsequent differentiation [265-267]. In addition, leukocytes derived from acute myeloid leukemia patients display altered cholesterol metabolism, in that they take up more LDL, fail to repress cholesterol synthesis, and may have impaired cholesterol efflux, all together promoting greater proliferation and cell survival [267]. Overall, these findings suggest that disruptions in cellular cholesterol metabolism may favor expansion of myeloid lineage cells.
Leukocyte-specific ABCA1 and ABCG1 play important roles in atherosclerosis, inflammation, and immunity, as recent data suggests that ABCA1- and ABCG1-mediated redistribution of cellular lipids to HDL modulates cellular membrane and lipid raft composition in a manner that diminishes inflammatory potential of leukocytes [123, 257]. In addition to near depletion of plasma HDL-C levels, global deletion of ABCA1 resulted in accumulation of macrophage CE in Ldlr\(^{-/-}\) mice, in addition to increased expression of scavenger receptors and chemotactic responses \textit{in vitro} [268]. Further, Abca1\(^{-/-}\)Ldlr\(^{-/-}\) additionally exhibited exacerbated LPS-induced endotoxemia. Interestingly, increases in circulating inflammatory markers (MCP-1, IL-1\(\beta\), macrophage colony-stimulating factor (m-CSF)) were observed without increases in total WBC, or specific leukocyte subsets (lymphocytes, monocytes, neutrophils, eosinophils, or basophils) when compared to Ldlr\(^{-/-}\) mice, suggesting an increased inflammatory potential of leukocytes without inducing hyperproliferation [268].

Macrophage-specific ABCA1 deletion leads to impaired lipid efflux to apoA-I, in addition to increased membrane free cholesterol and lipid raft content when compared to WT macrophages [258]. Further, ABCA1\(^{-/-}\) macrophages displayed greater expression of pro-inflammatory cytokines (IL-6, IL-12p40, TNF\(\alpha\)) in response to LPS, which was in part dependent upon TLR4/CD14-associated adaptor protein MyD88 signaling and NF-\(\kappa\)B and MAPK pathways [258]. No changes were observed in plasma lipids, corresponding to other studies that show macrophage-specific ABCA1 has little impact on plasma HDL-C levels [269, 270]. Interestingly, increased free cholesterol accumulation in ABCA1\(^{-/-}\) macrophages did not appear to induce ER stress despite greater inflammatory potential pathways [258].
As indicated above, much of the anti-inflammatory effects of ABCA1 and ABCG1 appear to be due to alterations in plasma membrane lipid raft content. ABCA1 expression leads to significant redistribution of cholesterol and sphingomyelin from lipid rafts to non-raft regions of cell membranes through its ATPase-related function [271]. ABCA1-mediated reductions in lipid raft content have been shown to increase ADAM17-mediated cleavage of TNF and TNF receptors, which may result in reduced TNFα signaling [272]. Lipid raft structure also affects TLR signaling, as TLR4 and MyD88/TRIF-mediated inflammatory gene expression was significantly increased in peritoneal macrophages isolated from ABCA1−/−, ABCG1−/−, and ABCA1−/−ABCG1−/− mice [259]. Loss of ABCG1 in CD4 T cells additionally increases total lipid raft content, resulting in enhanced TCR-mediated hyperproliferation [123]. ApoA-I-ABCA1 interactions have also been shown to trigger JAK2-mediated activation of STAT3, which can suppress LPS-induced pro-inflammatory gene expression of TNFα and IL-6 in macrophages [273, 274]. Interestingly, comparative analysis of mouse monocyte subsets revealed that ABCA1 expression is lower in inflammatory Ly6C\textsuperscript{hi} blood monocyte populations, which yield greater pro-inflammatory activity in response to LPS and IFNγ when compared to Ly6C\textsuperscript{lo} monocytes [275].

The role of ABCA1 and ABCG1 in immunity

The roles of ABCA1 and ABCG1 in modulating leukocyte activity have important implications for immunity. Myeloid cell-specific knockout of ABCA1 protected C57BL/6 mice against *Listeria monocytogenes* infection, as characterized by reductions in liver damage, inflammation, and lipid accumulation when compared to wild type mice [276]. Further, deletion of ABCA1 increased LPS-induced macrophage chemotaxis (*in vitro*) and
migration *in vivo*, which corresponded to findings of increased hepatic monocyte/macrophage infiltration to clear infection more efficiently. Interestingly, *Listeria monocytogenes* infection *in vitro* caused reductions in ABCA1 protein in wild type macrophages, in addition to LXR target genes related to lipid metabolism, including ABCG1 and apoE. (ABCA1 mRNA was not affected). These findings suggest that *L. monocytogenes* reduces ABCA1 as a compensatory mechanism to increase cellular free cholesterol and lipid raft content to increase cellular inflammatory potential to clear infection [276]. However, these findings differ from Kaplan et al. [277], who found that LPS increased ABCA1 mRNA and protein expression, whereas LPS had no effect on ABCG1 in THP-1 cells. *In vivo*, mice injected with LPS dose-dependently increased hepatic ABCA1 expression, whereas ABCG1 and cholesterol 7α-hydroxylase (Cyp7α1) was reduced. Regulation of LPS-induced ABCA1 expression appeared to be LXR-independent [277]. Similar findings were reported by Thompson et al. [278], where LPS induced ABCA1 protein and mRNA expression, whereas both LPS and the LXR-agonist T0901317 stimulated ABCA1-mediated efflux of cell-surface LPS as a potential mechanism to restore cellular homeostasis [278].

*The role of HDL in immunity*

Given that the anti-inflammatory properties of ABCA1 and ABCG1 are in part attributable to their capacity to efflux cellular cholesterol and LPS [123, 258, 278], their immunoregulatory activities are additionally dependent upon the presence of an extracellular lipid acceptor – or HDL [261]. As described in previous sections, HDL possesses various anti-inflammatory and immunoregulatory activities by serving as a carrier for oxidized lipid-neutralizing enzymes and bioactive lipid species [279]. In addition to these functions, HDL may alter inflammatory
responses through neutralization of LPS. This function is not exclusive to HDL, as other lipoproteins (including chylomicrons, VLDL, LDL) are capable of binding the bioactive lipid A portion of LPS within their phospholipid-rich surfaces, thereby preventing it from interacting with receptors molecules on LPS-responsive cells [280, 281]; however, LPS appears to preferentially bind to HDL in plasma [281, 282]. Lipoproteins serve an important function in expulsion of LPS from the body – which can occur via hepatic uptake and secretion into bile [280, 283] – as the turnover rates of LPS-bound lipoproteins are faster than the rates of dissociation of LPS from lipoproteins [284].

Some studies suggest that the binding of LPS to HDL has profound effects in protecting against endotoxin-induced inflammation and shock. In C57BL6/CBA mice, Levine et al. [284] found that doubling the plasma HDL level by human ApoA-I transgenic expression or infusion of reconstituted HDL resulted in a 3- to 4-fold increase in survival from a lethal dose of LPS [284]. Lipoprotein-bound LPS has additionally been shown to lose its bioactivity in septic patients [281]. The LPS-neutralizing properties of HDL may be particularly important in obesity and MetS, where blood LPS levels are often found to be elevated, and may perpetuate metabolic dysfunction and inflammation [214, 216]. However, the low levels and dysfunctional nature of HDL – in addition to reduced expression levels of ABCA1 and ABCG1 – in these populations may fail to effectively sequester LPS, thereby further exacerbating disease progression.
Section 4

The potential role of diet in modulating HDL-leukocyte dynamics

Introduction

Given the significant independent and interrelated roles of HDL function and leukocyte inflammation in obesity-related disease progression, modulation of HDL-leukocyte dynamics appears to be a novel and promising therapeutic target. While dietary therapies that promote weight loss are commonly prescribed to favor global improvements in metabolic dysfunction, the addition of functional foods that target specific physiological pathways may provide further benefit [279, 285]. Previous intervention studies conducted in our laboratory have demonstrated that dietary carbohydrate-restriction in conjunction with daily egg consumption promotes improvements in HDL profiles and inflammatory markers [286-290]. Therefore, we believe that this dietary strategy is a potential therapeutic candidate to favorably modulate the relationship between HDL function and the inflammatory potential of leukocytes.

Carbohydrate restriction

Carbohydrate-restricted diets (CRD), or consumption of approximately < 35% of kilocalories from carbohydrate sources [291], have been proven to be one of the most effective dietary strategies to promote weight loss and improve clinical parameters of MetS [292]. CRD have further been shown to effectively improve HDL profiles, elevated markers of inflammation, insulin resistance, and endothelial dysfunction.
Carbohydrate restriction and lipid metabolism

Carbohydrate restriction induces a metabolic state that favors increases in plasma HDL-C and atheroprotective large, CE-rich HDL particles. Due to the body’s limited capacity to store carbohydrates in excess of oxidative metabolism, excess energy from CHO sources promotes hepatic de novo lipogenesis [293]. Greater glucose and insulin responses following CHO consumption results in the activation of 1) carbohydrate response element binding protein (ChREBP) and 2) SREBP-1c, respectively, resulting in transcriptional activation of numerous lipogenic genes, including acyl CoA carboxylase (ACC) and fatty acid synthase (FAS) [294, 295]. Increased hepatic lipogenesis results in increased packaging and secretion of large, TG-rich VLDL particles, which serve as preferred substrates for CETP-mediated transfer of CE from large (α-1) HDL particles to TGRL in exchange for TG, resulting in decreased HDL-C [291, 296, 297].

By reducing dietary carbohydrate substrate, CRD have been shown to decrease de novo lipogenesis [298], plasma TG, as well as decrease total number of VLDL particles measured by nuclear magnetic resonance (NMR), with greater decreases observed in large and medium-sized VLDL. Although changes in CETP activity are typically not observed, CRD have been shown to increase plasma HDL-C concentrations and LCAT activity, corresponding to increases in large atheroprotective HDL particles, with decreases in medium-sized, potentially pro-atherogenic HDL particles [289, 293, 299, 300]. Therefore, based on these findings, it appears that CRD-induced increases in HDL-C are most likely the indirect result of decreasing TGRL-TG, resulting in modulation of HDL remodeling to favor the formation of larger, more cholesterol-rich HDL particles.
Some evidence from animal studies suggests that the beneficial effects of CRD on MetS parameters may occur at the expense of the liver. In a hereditary hypertriglyceridemic rat model of MetS, high-sucrose feeding promoted VLDL secretion, down-regulated FFA oxidation, and increased *de novo* FFA synthesis from glucose, whereas the reverse was observed with high-fat feeding [301]. These changes corresponded to greater plasma TG and FFA in the sucrose group, as well as increased liver TG deposition in both fed and fasting states in the high-fat-fed animals [301]. While these data suggest that more data may be needed to properly assess the apparent benefits of carbohydrate-restriction in obesity and MetS, human studies suggest that carbohydrate restriction may benefit hepatic steatosis and MetS [302-304]. Further, carbohydrate-restriction was shown to be more effective in reducing liver TG than calorie restriction (-55% vs. -28%) in non-alcoholic fatty liver disease (NAFLD) subjects, despite similar weight loss between groups [305]. Additionally, a 12-week hypocaloric CRD (12% carbohydrate, 59% fat, 28% protein) increased postprandial flow-mediated dilation (FMD) when compared to a hypocaloric low-fat diet (56% carbohydrate, 24% fat, 20% protein) in overweight men with moderate hypertriglyceridemia [306], thereby suggesting that CRD improve endothelial function.

*Carbohydrate restriction and inflammation*

In addition to improving parameters of HDL metabolism, CRD have also proven to be an effective dietary strategy to reduce markers of inflammation in obese and MetS populations [190, 290]. In overweight men, carbohydrate-restriction (17% of energy from carbohydrates) effectively reduced plasma MCP-1 over the course of 12 weeks. When compared to a low-fat diet, a very-low carbohydrate diet (12% of energy from carbohydrate) was more effective at
reducing plasma levels of TNFα, IL-6, IL-8, MCP-1, E-selectin, ICAM-1, and plasminogen-activator inhibitor-1 (PAI-1) in overweight men and women. Further, the CRD reduced serum SFA in both TG and CE fractions [190], which may further contribute to anti-inflammatory effects. In MetS subjects of the Functional Genomics and Nutrition (FUNGENUT) cohort, a 12-week diet of high postprandial insulin response up-regulated genes related to tissues stress, and cytokine/chemokine-related signaling in subcutaneous adipose tissue, whereas a 12-week diet consisting of low postprandial insulin response foods down-regulated expression of genes related to insulin signaling and apoptosis [307]. Together, these findings suggest that CRD are effective therapeutic strategies for mitigating obesity-related inflammation in high-risk population such as MetS.

Eggs

In addition to the beneficial changes in HDL and inflammation from carbohydrate restriction described above, daily consumption of whole eggs has similarly been shown to confer benefit to HDL profiles and plasma inflammatory markers [286-290]. Eggs contain numerous bioactive components, including phospholipids, antioxidant carotenoids, and cholesterol [288, 308]. Together, the combination of these nutrients appears to have the capacity to modulate HDL metabolism and inflammation.

*Effects of egg intake on HDL metabolism and reverse cholesterol transport*

Many therapeutic strategies aimed at reducing cardiovascular disease risk have targeted RCT – one the primary mechanism by which HDL protects against atherosclerosis development [116]. Numerous studies have investigated the effects of egg intake on various
parameters of RCT [286, 289, 309-311]. Egg consumption has consistently been shown to increase plasma HDL-C – particularly in conjunction with carbohydrate restriction [286, 289]. In addition to increased HDL-C, egg intake promotes greater increases in the number of large CE-rich HDL particles and LCAT activity when compared to intake of yolk-free egg substitute, while simultaneously decreasing number of medium-sized HDL particles [289, 312, 313]. Together, findings from these studies suggest that whole egg intake favorably promotes HDL function and RCT, and may therefore reduce the risk and severity of atherosclerosis.

The initial step of RCT involves the efflux of cellular cholesterol to HDL [19]. In healthy men, consumption of 2 eggs per day during a 24-day National Cholesterol Education Program (NCEP) Step I diet (<30% fat, <10% saturated fat) increased cholesterol efflux from Fu5AH cells to subject serum when compared to an egg-free diet. Conversely, egg intake had no effect on cholesterol efflux during consumption of an oleic acid-rich diet (22% of energy) [309]. Further, the effects of egg intake appear to be a result of habitual consumption, as a single-dose egg meal does not appear to affect postprandial cholesterol efflux in healthy men [310]. Murine macrophage-like J774 cells incubated with postprandial serum following consumption of 1, 2, or 4 eggs had no effect on cellular total, free, or esterified cholesterol. However, cellular free cholesterol was slightly higher when comparing all egg groups combined to the 0-egg meal [310].

Egg intake has additionally been shown to alter the activity of proteins involved in HDL metabolism and RCT, including LCAT and CETP. Intake of 3 eggs per day during carbohydrate restriction has been shown to increase LCAT activity in overweight men [289], as well as men and women with metabolic syndrome [286]. While it remains controversial as
to whether increased LCAT activity is beneficial, some studies suggest that LCAT promotes RCT and reduces atherosclerosis [314]. In addition, CETP activity was shown to be 6% higher in healthy male subjects following a 24-day diet of 4 eggs when compared to intake of 0, 1, or 2 eggs per day (combined) [310]. Although high CETP activity has been implicated development of atherosclerosis due to the transfer of HDL-cholesterol to apoB-containing lipoproteins, CETP has also been shown to be important for macrophage RCT and HDL-derived cholesterol excretion in human and animal models [315, 316].

Egg feeding has additionally been shown to promote sterol excretion – potentially due to a combination of decreased absorption and increased RCT [311]. In Sprague-Dawley rats, egg-enriched diets increased fecal neutral sterol and bile acid concentrations, as well as hepatic mRNA expression of LDL-receptor, cholesterol 7α-hydroxylase, and LCAT when compared to egg-free control. Further, egg feeding blunted increases in plasma total and LDL-cholesterol, as well as liver triglyceride and cholesterol levels that were observed in a pure cholesterol-fed group, while also promoting greater increases in plasma HDL-C. These observations corresponded to decreased apoB and increased apoA-I [311]. Overall, findings from the studies presented above suggest that egg intake favorably promotes RCT, and may therefore reduce cardiovascular disease risk.

Egg intake and leukocyte cholesterol
Egg intake has similarly been shown to modulate leukocyte mRNA expression of LDL-R and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG CoA Red) – the rate-limiting enzyme of cholesterol synthesis. Mononuclear cell HMG CoA Red activity was reduced within 2 hours of consuming 4 hard boiled eggs (~1g cholesterol) in healthy men, whereas depletion of
circulating cholesterol levels by plasmapheresis led to a two-fold increase in activity in monocytes [317]. Further, daily intake of whole eggs during carbohydrate restriction reduced HMG CoA Red and LDL-R mRNA expression in overweight men, whereas subjects consuming yolk-free egg substitute during carbohydrate restriction displayed increases in the expression of both genes [318]. These findings indicate that both carbohydrate restriction and egg intake are capable of modulating leukocyte cholesterol flux.

Eggs and Inflammation

Eggs contain a number of dietary factors with the potential to promote pro- and anti-inflammatory pathways. Egg white-derived hen egg lysozyme has been shown to reduce intestinal inflammation in an experimental model of colitis [319], whereas egg yolk serves as a rich, bioavailable source of antioxidant carotenoids lutein and zeaxanthin [320]. Eggs are also a good source of bioactive phospholipids – particularly phosphatidylcholine (PC) [321, 322]. While PC is known to have anti-inflammatory properties [323-325], PC has also been shown to promote gut microflora-mediated production of trimethylamine-N-oxide (TMAO) – a PC metabolite associated with an increased risk of adverse cardiovascular events [326]. Egg yolk is also a rich source of cholesterol, which can be cytotoxic in high doses [327]; however, adverse effects of dietary cholesterol are largely dependent on intestinal cholesterol absorption. Intestinal cholesterol absorption is highly variable amongst individuals, both within and between populations of different health statuses. While intestinal cholesterol absorption can range from 15% to 85% in healthy individuals [289, 328], obese [329] T2DM [330] and metabolic syndrome [331] subjects tend to have lower rates of fractional cholesterol absorption compared to healthy controls. Decreased capacity to absorb
cholesterol is often coupled with increased endogenous cholesterol synthesis, which most likely contributes to the dyslipidemias observed in these populations [332].

Multiple studies have demonstrated that habitual egg consumption can alter markers of inflammation, with effects varying across populations and metabolic disease status. In a subset of older adult (over 60 years old) taking cholesterol-lowering medication, consumption of 2 or 4 eggs per day for 4 weeks did not alter CRP [333] - an acute phase protein that is a strong predictor of cardiovascular disease risk [43]. However, in overweight men consuming an ad libitum carbohydrate-restricted diet for 12 weeks, the addition of three eggs per day resulted in decreases in plasma CRP [290]. Beneficial reductions in CRP were not observed in overweight men consuming an equivalent amount of yolk-free egg substitute, although pro-inflammatory MCP-1 was reduced in this group. Both egg and egg substitute groups displayed increases in adiponectin – an adipose-derived protein with anti-inflammatory and insulin-sensitizing properties [334]; however, changes in the whole egg group (+21%) were greater than those in the egg substitute group (+7%). Daily consumption of whole egg and egg substitute during carbohydrate restriction had no effect on plasma levels of other inflammatory markers in this population, including pro-inflammatory soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble intracellular adhesion molecule 1 (sICAM-1), TNFα, and IL-8 [290]. These data suggest that whole egg and egg white intake during carbohydrate restriction promotes reductions in systemic inflammation, with whole egg intake conferring slightly greater benefit.

Egg consumption has similarly led to improvements in circulating plasma inflammatory markers in men and women classified with metabolic syndrome [287]. While consuming a moderate carbohydrate-restricted diet, the addition of 3 eggs per day led to
decreases in TNFα and serum amyloid A (SAA), whereas no changes in these compounds were observed in MetS subjects consuming a yolk-free egg substitute. Differing from the results observed in overweight men, no changes in CRP or adiponectin were observed in either egg group in the MetS population, nor were changes observed in sVCAM-1, sICAM-1, IL-6 or anti-inflammatory IL-10 [287].

While numerous studies have demonstrated beneficial effects of eggs on markers of inflammation, additional evidence suggests that degree of insulin sensitivity is an important factor in determining the response to egg feeding. Tannock et al. [335] found that consumption of 4 eggs per day for 4 weeks increased CRP and SAA in lean insulin-sensitive subjects following an American Heart Association–NCEP step 1 diet, whereas no changes in these inflammatory markers were observed in lean or obese insulin resistant subjects. These observations may be due to the fact that lean and obese insulin resistant patients have a diminished capacity to absorb dietary cholesterol [336, 337], which may promote tissue inflammation if compensatory mechanisms to mitigate cholesterol-mediated cytotoxicity are inadequate [338].

Egg intake may also increase inflammatory gene expression in PBMCs by promoting cellular cholesterol loading, which can enhance leukocyte-mediated inflammatory responses [124]. The relationship between cellular cholesterol flux and inflammatory potential may have profound effects on both atherosclerosis development and immunity [124, 259]. Evidence for the role of dietary cholesterol in leukocyte-mediated inflammation and immunity has been demonstrated by Perez-Guzman et al. [339], where individuals consuming 800 mg cholesterol/day increased the sterilization rate of sputum cultures in pulmonary tuberculosis patients. However, in populations with impaired immunity, such as
those who are obese [183], egg intake during carbohydrate-restriction may rectify dysregulated HDL-leukocyte dynamics, as this intervention favors global metabolic improvements and reduction in factors that negatively regulate anti-inflammatory ABCA1 and ABCG1 expression, such as TNFα and CRP [287, 292]. Taken together, the extent by which egg intake influences leukocyte inflammation in relation to immune response and metabolic disease outcomes remains to be determined.

Conclusions

The data presented in this review highlights a significant role for HDL function and leukocyte inflammation in pathogenesis of obesity-related disease. These findings further support the potential for dietary modulation of HDL-leukocyte dynamics, which hold significant implications for the therapeutic role of diet in ameliorating chronic disease risk and progression.

References


244. Varga T, Czimmerer Z, Nagy L: PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochim Biophys Acta* 2011, 1812:1007-1022.


via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. 


318. Mutungi G, Torres-Gonzalez M, McGrane MM, Volek JS, Fernandez ML: Carbohydrate restriction and dietary cholesterol modulate the expression of HMG-
CoA reductase and the LDL receptor in mononuclear cells from adult men. *Lipids Health Dis* 2007, 6:34.


333. Goodrow EF, Vishwanathan R, Wilson T, Nicolosi R: C-reactive protein (CRP) levels are not affected in participants consuming the equivalent of 2 and 4 egg yolks/day while on cholesterol-lowering medication. *FASEB* 2007, 21.


Chapter 3

Intervention Study Overview
**Introduction**

Based on the evidence presented in the previous chapter, it is reasonable to predict that diet can exert effects on the dynamics between leukocyte inflammation, cholesterol flux, and HDL function. The relationship between diet and these physiological processes may be particularly relevant for individuals at high risk for metabolic disease and immune dysfunction, such as those classified with MetS. Based on results from previously conducted intervention studies in our laboratory [1-5], we hypothesize that egg intake during carbohydrate restriction may modulate these parameters in MetS.

Therefore, in order to assess the specific aims outlined in the introduction, the following intervention was performed as described below. This intervention was similarly designed to assess the clinical outcomes and implications on parameters of MetS, lipoprotein sizes, insulin resistance, and carotenoid status. The findings from these objectives have recently been published [4-6].

**Experimental Design**

*Subject recruitment*

Subjects were recruited from the University of Connecticut population and surrounding communities, with the goal of recruiting 40 men (n = 20) and women (n = 20). Inclusion criteria was defined as I) being between the age of 30-70 years old, and II) classification with MetS as defined by the NCEP:ATPIII criteria [7]. MetS parameters are presented in Table 3.1. Individuals were excluded from participation if they had a personal history of diabetes, stroke, cancer, liver, renal, or heart disease, endocrine disorder, or any known inborn errors of metabolism; if they were pregnant or lactating; if they had experienced weight loss of >10% of body weight in preceding 12 weeks; if they were taking any glucose-lowering
prescriptions or supplements; if they had fasting TG > 500 mg/dL, fasting glucose > 126 mg/dL, resting BP > 140/90 mmHg; or an allergy to eggs. Eligibility for participation was determined following a fasted screening session, in which MetS parameters will be assessed.

Table 3.1 NCEP:ATP III criteria for MetS

<table>
<thead>
<tr>
<th>MetS Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>≥ 102 cm</td>
</tr>
<tr>
<td>Women</td>
<td>≥ 88 cm</td>
</tr>
<tr>
<td>Fasting triglycerides</td>
<td>≥ 150 mg/dL</td>
</tr>
<tr>
<td>Fasting HDL-C</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>≤ 40 mg/dL</td>
</tr>
<tr>
<td>Women</td>
<td>≤ 50 mg/dL</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>≥ 100 mg/dL</td>
</tr>
<tr>
<td>Resting blood pressure</td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>≥ 130 mmHg</td>
</tr>
<tr>
<td>Diastolic</td>
<td>≥ 85 mmHg</td>
</tr>
<tr>
<td><strong>or</strong></td>
<td></td>
</tr>
<tr>
<td>Taking blood pressure medication</td>
<td></td>
</tr>
</tbody>
</table>

**Dietary Intervention**

We conducted a 12-week, parallel, single-blind intervention study in which subjects were instructed to follow a CR diet (25-30% of total calories), in addition to consuming either 3 whole eggs/day (EGG group) or the equivalent amount of egg yolk-free egg substitute (SUB group). An overview of the study intervention is depicted in Figure 3.1. EGG and SUB group assignment was determined randomly, and subjects were matched based on gender, age, and BMI following qualification for the study by screening. Subjects were not informed of their egg group assignment throughout the intervention; therefore, subjects were asked to refrain from eating any other egg products outside of what was provided to them during the study. Both whole egg and egg substitute products were commercially available, liquid egg
products (Sysco). Subjects were provided with egg products on a bi-weekly basis in coded containers. Ingredients in the whole egg product included: whole eggs, citric acid (to preserve color), and 0.15% water (added as a carrier for citric acid). Ingredients for the egg substitute product included: egg whites (99%), less than 1% vegetable gums (xanthan and guar gum) and β-carotene to provide color. The nutrient composition of both egg products is provided in Table 3.2. In addition to the dietary prescription, subjects were asked to maintain all medication, supplement, and exercise doses and regimens.

### Table 3.2 Egg product nutrient composition

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Whole egg (EGG)</th>
<th>Egg substitute (SUB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy, kcal</td>
<td>186</td>
<td>60</td>
</tr>
<tr>
<td>Carbohydrates, g</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Protein, g</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Total fat, g</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Saturated fat, g</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol, g</td>
<td>534</td>
<td>0</td>
</tr>
</tbody>
</table>

1Nutrient composition is representative of a daily serving of each egg product (1/2 cup).
Baseline characteristics

As described above, subjects were randomly assigned to either the EGG or SUB group on the basis of gender, age, and BMI. Additional baseline characteristics are presented in Table 3.3. No significant differences were observed between EGG and SUB groups in any parameter.

Table 3.3 Baseline characteristics of study subjects

<table>
<thead>
<tr>
<th></th>
<th>EGG</th>
<th>SUB</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>7</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Women</td>
<td>13</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>Age, y</td>
<td>50.6 ± 8.5</td>
<td>50.9 ± 8.3</td>
<td>0.894</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>87.3 ± 20.2</td>
<td>85.6 ± 16.2</td>
<td>0.765</td>
</tr>
<tr>
<td>Body mass index (BMI), kg/m²</td>
<td>30.4 ± 5.5</td>
<td>30.6 ± 5.1</td>
<td>0.926</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>105.0 ± 11.7</td>
<td>102.9 ± 11.7</td>
<td>0.599</td>
</tr>
<tr>
<td>Men</td>
<td>113.1 ± 9.6</td>
<td>104.9 ± 17.5</td>
<td>0.320</td>
</tr>
<tr>
<td>Women</td>
<td>100.6 ± 10.6</td>
<td>102.1 ± 9.3</td>
<td>0.715</td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic, mmHg</td>
<td>119.3 ± 14.3</td>
<td>117.9 ± 15.2</td>
<td>0.773</td>
</tr>
<tr>
<td>Diastolic, mmHg</td>
<td>80.5 ± 9.7</td>
<td>81.1 ± 9.9</td>
<td>0.836</td>
</tr>
<tr>
<td>Taking BP medication, n</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>192.4 ± 30.4</td>
<td>200.5 ± 38.5</td>
<td>0.477</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>114.2 ± 29.1</td>
<td>122.0 ± 35.1</td>
<td>0.468</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>49.9 ± 14.3</td>
<td>50.1 ± 13.8</td>
<td>0.981</td>
</tr>
<tr>
<td>Men</td>
<td>38.0 ± 5.0</td>
<td>36.4 ± 9.8</td>
<td>0.781</td>
</tr>
<tr>
<td>Women</td>
<td>56.4 ± 13.5</td>
<td>55.8 ± 11.0</td>
<td>0.899</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>140.9 ± 58.0</td>
<td>142.4 ± 63.5</td>
<td>0.938</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>96.8 ± 14.5</td>
<td>97.5 ± 12.0</td>
<td>0.871</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.3 ± 2.2</td>
<td>1.7 ± 1.0</td>
<td>0.242</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>0.35 ± 0.35</td>
<td>0.49 ± 0.60</td>
<td>0.374</td>
</tr>
</tbody>
</table>

Total cholesterol, HDL-C, triglycerides, and glucose were measured by Cobas c 111 analyzer by enzymatic assay as described by Blesso et al [4]. HOMA-IR was calculated using insulin values measured by ELISA [4]. P value represents independent t-test comparisons between EGG vs. SUB groups.
Dietary compliance

Subjects were asked to complete 5-day diet records at baseline, week 6, and week 12 of the intervention to assess dietary intake and compliance. Subjects received personalized dietary guidelines based on estimated energy expenditure, in addition to bi-weekly diet counseling and weigh-ins. Egg product compliance was assessed by return of empty egg containers provided in the previous dispersement, as well as by a daily egg compliance questionnaire. Daily average nutrient intakes for each group are presented in Table 3.4 and Figure 3.2. While all subjects experienced similar changes in macronutrient intake, differences were observed between groups in intake of egg yolk-derived nutrients cholesterol and choline.

Summary of clinical outcomes

In addition to the objectives outlined by this dissertation, this intervention was previously implemented to determine the effects of egg intake during moderate carbohydrate restriction on the clinical parameters of MetS, in addition to atherogenic lipoprotein profiles and carotenoid status. Notably, when compared to egg substitute intake, whole egg consumption induced more favorable effects on plasma HDL-C, lipoprotein size profiles, plasma inflammatory markers, insulin resistance and plasma and lipoprotein carotenoids, despite similar degrees of weight loss (~4% of body weight). Therefore, the findings presented in the subsequent chapters are within the context of these previously reported observations [4-6].
Table 3.4 Average daily nutrient intake

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy, kcal/day</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGG</td>
<td>1943 ± 520</td>
<td>1597 ± 341</td>
<td>1537 ± 419</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SUB</td>
<td>2121 ± 310</td>
<td>1603 ± 670</td>
<td>1560 ± 566</td>
<td></td>
</tr>
<tr>
<td><strong>Carbohydrate, % en</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGG</td>
<td>42.7 ± 6.3</td>
<td>30.4 ± 8.1</td>
<td>27.6 ± 10.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SUB</td>
<td>38.8 ± 8.2</td>
<td>28.0 ± 7.3</td>
<td>29.1 ± 9.1</td>
<td></td>
</tr>
<tr>
<td><strong>Fat, % en</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGG</td>
<td>37.3 ± 5.9</td>
<td>44.1 ± 7.0</td>
<td>46.0 ± 8.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SUB</td>
<td>40.2 ± 6.8</td>
<td>45.1 ± 7.7</td>
<td>45.4 ± 6.7</td>
<td></td>
</tr>
<tr>
<td><strong>Protein, % en</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGG</td>
<td>17.3 ± 3.0</td>
<td>22.7 ± 4.9</td>
<td>23.2 ± 4.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>SUB</td>
<td>17.3 ± 3.1</td>
<td>25.7 ± 5.0</td>
<td>24.0 ± 4.2</td>
<td></td>
</tr>
<tr>
<td><strong>Cholesterol, mg/day</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGG</td>
<td>359.9 ± 177.7</td>
<td>692.6 ± 103.9</td>
<td>740.8 ± 139.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>SUB</td>
<td>344.9 ± 133.1</td>
<td>219.9 ± 123.1</td>
<td>213.4 ± 83.3</td>
<td></td>
</tr>
<tr>
<td><strong>Choline, mg/day</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGG</td>
<td>332.1 ± 127.4</td>
<td>503.8 ± 90.7</td>
<td>505.5 ± 113.3</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>SUB</td>
<td>350.1 ± 90.3</td>
<td>278.2 ± 120.4</td>
<td>247.8 ± 99.9</td>
<td></td>
</tr>
</tbody>
</table>

Dietary data was collected from 5-day diet records collected at each time point, then analyzed by Nutrition Data System for Research (NDSR). All data are presented as mean ± SD. EGG: n = 19; SUB: n = 17. P values represent significance for time effects for all subjects determined by two-way repeated measures ANOVA. Different subscripts within each row represents differences between means as determined by post-hoc multiple comparisons with Bonferroni correction (P < 0.05). # Indicates significant time point differences between EGG and SUB group (P < 0.001).

Figure 3.2 Average daily macronutrient intake

Changes in total grams of dietary carbohydrate (CHO), fat (FAT) and protein (PTN) throughout the intervention. Data from all subjects are presented together. "***P < 0.0001 represented significance changes over time as determined by repeated measures ANOVA."
References


Chapter 4

Egg consumption modulates HDL lipid composition and increases the cholesterol-accepting capacity of serum in metabolic syndrome
A version of this chapter has recently been published in *Lipids*

Abstract

We recently demonstrated that daily whole egg consumption during moderate carbohydrate restriction leads to greater increases in plasma HDL-cholesterol (HDL-C) and improvements in HDL profiles in metabolic syndrome (MetS) when compared to intake of a yolk-free egg substitute. We further investigated the effects of this intervention on HDL composition and function, hypothesizing that the phospholipid species present in egg yolk modulate HDL lipid composition to increase the cholesterol-accepting capacity of subject serum. Men and women classified with MetS were randomly assigned to consume either 3 whole eggs (EGG, n = 20) per day or the equivalent amount of egg substitute (SUB, n = 17) throughout a 12-week moderate carbohydrate-restricted (25-30% of energy) diet. Relative to other HDL lipids, HDL-cholesteryl ester (CE) content increased in all subjects, with greater increases in the SUB group. Further, HDL-triglyceride (TG) content was reduced in EGG group subjects with normal baseline plasma HDL-C, resulting in increases in HDL-CE/TG ratios in both groups. Phospholipid analysis by mass spectrometry revealed that HDL became enriched in phosphatidylethanolamine in the EGG group, and that EGG group HDL better reflected sphingomyelin species present in the whole egg product at week 12 compared to baseline. Further, macrophage cholesterol efflux to EGG subject serum increased from baseline to week 12, whereas no changes were observed in the SUB group. Together, these findings suggest that daily egg consumption promotes favorable shifts in HDL lipid composition and function beyond increasing plasma HDL-C in MetS.
**Introduction**

HDL is a key cardioprotective biomarker in obesity-related metabolic diseases, including cardiovascular disease (CVD) and metabolic syndrome (MetS) [1]. The primary mechanism by which HDL promotes cardiovascular health is thought to be through mediating the acquisition of lipids from macrophage foam cells within the arterial wall for participation in reverse cholesterol transport (RCT) [2], while antithrombotic, anti-inflammatory, and antioxidant properties of HDL confer further protection against atherosclerosis [3]. It is well recognized, however, that HDL function is impaired under inflammatory conditions associated with obesity, and that standard clinical measures of steady-state plasma HDL-cholesterol (HDL-C) often fail to capture the true atheroprotective - or even atherogenic - potential of circulating HDL particles [3, 4]. Therefore, therapeutic strategies aimed at enhancing HDL-mediated cardioprotection should target mechanisms that go beyond simply increasing HDL-C, and rather improve the quality of HDL as a lipid acceptor [5, 6].

The capacity of HDL to facilitate cellular cholesterol efflux is differentially affected by the diverse, heterogeneous nature of HDL size and structure, as well as protein and lipid composition [6]. While various HDL components and pathways have been targeted, modulation of HDL lipid composition – particularly the HDL-phospholipid (HDL-PL) fraction – is an emerging and promising approach to improving HDL function [7, 8]. In the majority of circulating HDL, phospholipids represent ~ 45-50% of total lipids [9], with predominant phospholipid classes including phosphatidylcholine (PC; ~80%), sphingomyelin (SM; ~10%), phosphatidylethanolamine (PE; ~3%), lysophosphatidylcholine (LysoPC; ~1-2%) and phosphatidylinositol (PI; ~1-2%) [10, 11].
Total HDL-PL, as well as the distribution of HDL phospholipid classes, has been related to CVD risk and severity [12, 13], and can differentially affect the lipid-accepting capacity of HDL [8, 14]. Greater enrichment of HDL in phospholipids – such as PC and SM – has also been associated with a greater lipid-accepting capacity of HDL and/or human serum; however, the extent by which different phospholipid classes can promote efflux may be variable across cellular models and efflux pathways mediated by ATP-binding cassette transporter A I (ABCA1), ATP-binding cassette transporter G I (ABCG1), scavenger receptor B I (SR-BI), or aqueous diffusion [7, 8, 15].

Dietary intake of phospholipids derived from soy, safflower, dairy, marine, and egg sources are known to favorably modulate plasma lipid levels in both humans and animal models [16-20]; however, few studies have investigated how intake of phospholipid-rich foods or supplements affect the relative distribution HDL-PL classes or lipid-accepting functions. Dietary phospholipids are known to be highly bioavailable (>90%), can be partially absorbed intact in the intestine, and are preferentially incorporated into HDL following ingestion [21, 22]. Of all food sources to favorably modulate HDL-PL distribution and particle function, eggs represent one of the most promising candidates, since egg yolk is one of the richest dietary sources of PC, PE, and SM [23, 24]. Previous studies in our laboratory have demonstrated that consumption of 3 eggs per day as part of a carbohydrate-restricted diet improves atherogenic lipoprotein profiles and increases plasma HDL-C in overweight men [25] and men and women with MetS [26, 27] to a greater extent when compared to intake of a yolk-free egg substitute. In MetS, beneficial changes from whole egg intake included greater increases in large HDL particles, HDL and LDL diameters, and lecithin–cholesterol acyltransferase (LCAT) activity, as well as greater reductions in
atherogenic large and medium VLDL particles when compared to the intake of egg substitute [26]. These findings are significant since individuals with MetS can have dysfunctional HDL [28] and a 2-fold increased risk of developing CVD [29]. Therefore, the aim of this study was to determine whether improvements in HDL profiles from egg intake correspond to changes in HDL lipid composition and the cholesterol-accepting capacity of HDL. We hypothesized that daily consumption of phospholipid-rich egg yolk for 12 weeks would modulate HDL-PL distribution to reflect egg-derived phospholipid species, corresponding to greater cholesterol efflux to subject serum.

Materials and Methods

Study design and subjects

Experimental design and subject characteristics have previously been described [26, 27]. Briefly, 37 subjects (25 women; 12 men) classified with MetS were recruited to participate in a 12-week parallel, randomized, single-blind diet intervention. Eligibility for participation was dependent upon subjects falling within the age range of 30-70 years old, meeting the National Cholesterol Education Program Adult Treatment Panel III (NCEP:ATP III) revised criteria for MetS [30], and having no clinical diagnosis of chronic/metabolic disease [26, 27]. During the 12-week study, subjects were instructed to follow an ad libitum moderate carbohydrate-restricted diet (25-30% of energy from carbohydrates) in addition to consuming either 3 whole eggs (EGG group) or the equivalent amount of egg yolk-free egg substitute (SUB group) each day. The egg substitute product consisted of egg whites (99%), <1% xanthan and guar gums, beta-carotene for color, and provided 0 mg of cholesterol, whereas the daily serving of whole egg contained 534 mg of cholesterol. Dietary analysis and nutrient
composition of both egg products have been reported in greater detail elsewhere [26, 27]. This study was approved by the University of Connecticut Institutional Review Board; Protocol #: H10-173.

**Plasma and serum collection**

Fasting plasma and serum samples were collected at baseline and week 12. Plasma was obtained following blood collection into EDTA-coated tubes and centrifugation at 2200 x g for 20 minutes at 4°C. A preservative cocktail (1 ml/L sodium azide, 1 ml/L phenylmethylsulfonyl fluoride, and 5 mL/L aprotinin) was added to plasma prior to storage. Serum was obtained following collection of blood into anticoagulant-free tubes and processed under sterile conditions. Both plasma and serum were aliquoted and stored at -80°C until analysis.

**Plasma lipids and body weight**

Fasting plasma HDL-C, total cholesterol (TC) and triacylglycerides (TG) were measured by enzymatic methods using a Cobas c 111 clinical analyzer (Roche Diagnostics, Indianapolis, IN), as previously described [26]. LDL-C was estimated by the Friedewald equation [31]. Body weight was assessed biweekly from baseline to week 12.

**Isolation of LDL and HDL subfractions from plasma**

LDL ($d = 1.019-1.063$ g/mL) and HDL subfractions ($d = 1.063-1.21$ g/mL) were isolated from EGG ($n = 19$) and SUB ($n = 15$) subject plasma at baseline and week 12 by sequential ultracentrifugation as previously described [27]. Plasma yields from 3 subjects were not
adequate to perform isolation. Plasma density was first adjusted to 1.019 g/mL with KBr and centrifuged at 42,000 rpm for 19 hours to remove VLDL and IDL fractions \((d < 1.019 \text{ g/mL})\) using an Optima LE-80K ultracentrifuge. The remaining plasma fractions was sequentially adjusted to appropriate densities to isolate LDL \((d = 1.019-1.063 \text{ g/mL})\) and HDL \((d = 1.063-1.21 \text{ g/mL})\) with KBr, then layered beneath corresponding KBr-based density solutions [32] in Quickseal tubes (Beckman Coulter). LDL and HDL subfractions were isolated by centrifugation for 3 hours at 60,000 rpm in a vertical vTi-65 rotor. LDL was used for acetylation experiments described below, whereas HDL was then dialyzed for 24 hours (0.9% NaCl, 0.01% Na\(_2\)EDTA, pH 7.4), aliquoted, and stored at -80° C for further analysis of lipid components.

**Analysis of HDL lipid components**

Phospholipid (PL), free cholesterol (FC) (Wako Chemicals, Richmond, VA), TC and TAG (Pointe Scientific, Canton, MI) content of HDL subfractions \((d = 1.063-1.21)\) was determined by commercially available reagent kits according to the manufacturer’s instructions. Cholesteryl ester (CE) content was calculated as \((\text{TC} - \text{FC}) \times 1.67\).

**Analysis of HDL and egg product phospholipid classes and associated species**

Quantification of PC, LysoPC, PE, PI, and SM species in HDL samples and egg products was performed as described by Sorci-Thomas et al. [33]. Briefly, egg product and HDL lipids were extracted by the Bligh-Dyer method [34] containing internal standards, evaporated under an argon stream, and dissolved in chloroform/methanol (2:1). Lipid extracts were injected onto a 3.9 x 200 mm Waters µPorasil column (10 µm particle size) and analyzed by
tandem mass spectrometry utilizing a Finnigan TSQ Quantum Discovery Max mass spectrometer (MS/MS). Data for each phospholipid class in egg products are presented mg/g of egg protein or mole-percent (mol%) of total phospholipids. Data for HDL phospholipid classes are presented as mol%. Percent homology of phospholipid molecular species for the EGG group was calculated as: \[ \frac{[\text{HDL molecular species (nmol) identified in both HDL and egg product}]}{[\text{Total HDL molecular species (nmol) }]} \times 100 \]. Phospholipid species homology of the SUB group was similarly calculated by identifying the percentage of total HDL species (nmol) that were identified in the egg substitute product.

**LDL Acetylation**

LDL was isolated from combined fasting plasma samples from two healthy donors by sequential ultracentrifugation, as described previously [27]. Following isolation, LDL was dialyzed (0.15 M NaCl) for 24 hours at 4°C. Protein content was measured by bicinchoninic acid (BCA) assay (Thermo Scientific) modified to contain 0.2% sodium dodecyl sulfate (SDS) as recommended by the manufacturer for measurement of lipoprotein samples. The LDL sample was added in equal parts to a saturated sodium acetate solution with continuous stirring in an ice water bath while protected from light. Acetic anhydride was then slowly added over 1 hour (1.5 µl/mg LDL protein). The acetylated-LDL (acLDL) solution was dialyzed (0.15M NaCl, 0.3 mM EDTA, pH 7.4) for 24 hours at 4°C then filter-sterilized for use in cholesterol efflux assays.
**Cholesterol efflux assays**

RAW 264.7 macrophages were used to conduct cholesterol efflux assays for a subset of subjects. 24 hours after seeding on 12-well plates, cells were loaded with 100 µg/ml acLDL and 0.5 µCi [1,2-^3^H(N)]cholesterol (American Radiolabeled Chemicals, Inc., St. Louis, MO) for 24 hrs. Cells were then washed and treated with 10 µM T0901317 for 24 hours to stimulate liver X receptor (LXR)-mediated expression of lipid transporters, ABCA1 and ABCG1. Efflux media was then added containing 20% subject serum and 0.2% BSA in RPMI, with each serum sample ran in triplicate. Efflux was performed for 3 hours at 37°C, followed by collection of cell media and cell lysates. Cell lysates were obtained by washing cells with 0.1 N NaOH and collection of the supernatant. Aliquots of cell media and lysates were added to liquid scintillation cocktail and radioactivity was measured with a Beckman LS 6500 Scintillation Counter (Beckman Coulter Inc., Indianapolis, IN). Percent cholesterol efflux was calculated as \([\text{[(3H-cholesterol radioactivity in media) x 100]} / \text{[(3H-cholesterol radioactivity in media) + (3H-cholesterol radioactivity in cell lysate)]]}\).

**Statistical analysis**

All statistical analyses were performed using SPSS version 18. Repeated measures ANOVA was used to test the overall effects of the intervention between EGG vs. SUB groups (the between-subjects factor) and over time (the within-subjects factor). Paired \(t\) tests were used to test differences between baseline vs. week 12 values within EGG or SUB groups. Independent \(t\) tests were used to compare the differences in absolute or percent change in variables between groups. Bivariate Pearson correlations were used to determine
relationships between parameters. Data are reported as mean ± SD unless noted otherwise. \( P < 0.05 \) was considered significant.

**Results**

*Egg product phospholipid analysis*

Whole egg and yolk-free egg substitute products were analyzed by mass spectrometry to identify the predominant phospholipid classes and associated molecular species (Table 1). The relative distribution of phospholipid classes was similar between the two egg products, with PC representing the most abundant phospholipid class, followed by PE, and smaller amounts of SM, LysoPC, and PI. As expected, the whole egg product provided significantly greater amounts of phospholipid within each class, since the majority of egg phospholipids are found in the yolk [23]. Additionally, a greater number of molecular species within each class – with the exception of SM – were identified in the whole egg product. Percent similarity of molecular species detected within each phospholipid class (i.e. species homology) between whole egg and egg substitute products ranged from 62.5 – 100%.
Table 4.1 Phospholipid composition of whole egg and egg substitute products

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Whole Egg</th>
<th>Egg Substitute</th>
<th>Species homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phospholipids (mg/serving)</td>
<td>413.6</td>
<td>18.9</td>
<td>82.1</td>
</tr>
<tr>
<td>Molecular species (#)</td>
<td>65</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>PC (mg/serving)</td>
<td>308.8 (72.1%)</td>
<td>14.3 (73.2%)</td>
<td>82.0</td>
</tr>
<tr>
<td>Molecular species (#)</td>
<td>21</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>PE (mg/serving)</td>
<td>86.1 (20.5%)</td>
<td>3.4 (17.9%)</td>
<td>89.5</td>
</tr>
<tr>
<td>Molecular species (#)</td>
<td>19</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>SM (mg/serving)</td>
<td>10.4 (2.6%)</td>
<td>0.6 (3.3%)</td>
<td>100.0</td>
</tr>
<tr>
<td>Molecular species (#)</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>LysoPC (mg/serving)</td>
<td>8.2 (2.9%)</td>
<td>0.3 (2.6%)</td>
<td>62.5</td>
</tr>
<tr>
<td>Molecular species (#)</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>PI (mg/serving)</td>
<td>9.4 (1.9%)</td>
<td>0.7 (3.0%)</td>
<td>69.2</td>
</tr>
<tr>
<td>Molecular species (#)</td>
<td>13</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Total phospholipids and individual phospholipid classes are reported as mg/daily serving (1/2 cup) of whole egg or egg substitute products. Values in parentheses represent the contribution of each phospholipid class to total phospholipids, reported as mole percent (mol%). The total number of individual molecular species identified within each phospholipid class is shown for both egg products. Species homology represents the (%) similarity of individual molecular species within each phospholipid class between whole egg and egg substitute products.

Baseline characteristics and changes in body weight

As described in Chapter 3, there were no differences in plasma TC, LDL-C, HDL-C, weight, BMI, age, or the additional MetS parameters between EGG and SUB groups at baseline (Table 3.3) [26, 27]. As expected with moderate carbohydrate restriction, subjects in both groups significantly decreased body weight over the course of the 12-week intervention, with no differences between groups (EGG: -3.6 ± 2.6 kg vs. SUB: -3.4 ± 2.3 kg; \( P = 0.78 \)).

Plasma HDL-C classification and response

Changes in plasma lipids and lipoprotein particle profiles were briefly described in Chapter 3 and have been reported elsewhere [26, 27]. Upon admission into the study, 55% of EGG
subjects met the MetS criteria for low plasma HDL-C (men: < 40 mg/dL; women: < 50mg/dL), similar to 53% of SUB subjects enrolled. While no changes were observed in either TC or LDL-C throughout the 12-week intervention, plasma HDL-C increased in all subjects, with greater increases in the EGG group (+19.1%) when compared to the SUB group (+ 9.9%) \( (P < 0.05) \) (Figure 4.1) [26, 27]. Interestingly, the difference in HDL-C response between groups was primarily driven by subjects who entered the study with normal HDL-C levels (EGG (n = 9): +11.56 ± 8.0 mg/dL vs. SUB (n = 8): + 4.0 ± 3.5 mg/dL; \( P = 0.026 \)), rather than those with low baseline HDL-C (EGG (n = 11): +6.1 ± 6.3 mg/dL vs. SUB (n = 9): +5.3 ± 6.9 mg/dL; \( P = 0.79 \)). By the end of the intervention, only 15% of subjects in the EGG group still met the MetS criteria for low plasma HDL-C, compared to 23.5% of SUB subjects.

**Figure 4.1 Effects of egg intake and carbohydrate restriction on plasma HDL-C**

Plasma HDL-C was measured at baseline, week 6, and week 12 of the intervention. \( **P < 0.01 \), repeated measures ANOVA (time effect). \( \*P < 0.05 \), independent \( t \) test.
HDL lipid composition

HDL subfractions \( (d = 1.063-1.21) \) were analyzed to assess whether egg feeding could impact the relative distribution of HDL lipid fractions (Table 4.2). While there were no changes in HDL-FC or -PL content from baseline to week 12, HDL became relatively more enriched in CE in all subjects, with slightly greater increases in the SUB group. Reductions in HDL-TG \( (P = 0.078) \) and HDL-PL \( (P = 0.082) \) content from baseline to week 12 were observed in EGG and SUB groups, respectively, although these changes failed to reach statistical significance. Interestingly, however, comparison of subjects with normal baseline HDL-C revealed that HDL-TG content was reduced in the EGG group only (EGG: -2.2 ± 2.4\% vs. SUB: 0.0 ± 0.9\%; \( P = 0.03 \)). Together, these changes led to increases in HDL-CE/TG ratios in all subjects, whereas the slightly greater increases in the EGG group failed to reach significance when compared to the changes in the SUB group \( (P = 0.071) \). Baseline plasma HDL-C levels and gender did not appear to have an effect on changes in HDL lipid composition in either EGG or SUB group.

Distribution of HDL phospholipid classes

HDL subfractions \( (d = 1.063-1.21) \) were further analyzed by mass spectrometry to identify the distribution of predominant PL classes (Table 4.3). While the relative distribution of HDL-PC, –LysoPC, –PI, or –SM did not change throughout the intervention, enrichment of HDL in PE was observed in the EGG group only (EGG: +40.8 ± 16.3\% vs. SUB: -3.6 ± 25.5\%; \( P = 0.026 \)). Interestingly, changes in HDL-PE content were positively associated with increases in plasma HDL-C \( (r = 0.74; P = 0.038) \), and negatively associated with changes in HDL-TAG content \( (r = -0.833; P = 0.010) \).
### Table 4.2 Effects of egg feeding on HDL lipid composition during moderate carbohydrate restriction

<table>
<thead>
<tr>
<th></th>
<th>EGG</th>
<th>SUB</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 12</td>
<td>Change</td>
</tr>
<tr>
<td>CE, mass%</td>
<td>40.4 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.3 ± 4.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+ 1.00</td>
</tr>
<tr>
<td>FC, mass%</td>
<td>5.1 ± 1.5</td>
<td>5.6 ± 1.3</td>
<td>+ 0.51</td>
</tr>
<tr>
<td>PL, mass%</td>
<td>46.0 ± 3.8</td>
<td>45.4 ± 4.2</td>
<td>− 0.60</td>
</tr>
<tr>
<td>TAG, mass%</td>
<td>8.5 ± 2.7</td>
<td>7.6 ± 2.4</td>
<td>− 0.91</td>
</tr>
<tr>
<td>CE/TG, mass ratio</td>
<td>5.5 ± 2.9</td>
<td>6.1 ± 2.6</td>
<td>+ 0.6</td>
</tr>
</tbody>
</table>

Individual lipid components of HDL subfractions (d = 1.063–1.21) from EGG (n = 19) and SUB (n = 15) groups are presented as percent of total HDL lipid mass (mass%). CE/TG ratios are presented as mass ratios. Data are reported as mean ± SD. Different letters within the same row indicate significantly different mean values (P < 0.05).
Table 4.3 Effects of egg feeding on HDL-PL class distribution during moderate carbohydrate restriction

<table>
<thead>
<tr>
<th>mol%</th>
<th>EGG Baseline</th>
<th>EGG Week 12</th>
<th>Change</th>
<th>SUB Baseline</th>
<th>SUB Week 12</th>
<th>Change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Time</td>
</tr>
<tr>
<td>PC</td>
<td>79.1 ± 3.4</td>
<td>76.1 ± 4.3</td>
<td>− 3.0</td>
<td>78.6 ± 1.4</td>
<td>77.3 ± 2.8</td>
<td>− 1.3</td>
<td>0.070</td>
</tr>
<tr>
<td>PE</td>
<td>3.1 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+ 1.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.2 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.1 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>− 0.2</td>
<td>0.085</td>
</tr>
<tr>
<td>SM</td>
<td>11.8 ± 1.4</td>
<td>13.5 ± 3.6</td>
<td>+ 1.7</td>
<td>12.1 ± 0.4</td>
<td>12.5 ± 0.6</td>
<td>+ 0.5</td>
<td>0.28</td>
</tr>
<tr>
<td>LysoPC</td>
<td>3.2 ± 2.3</td>
<td>3.9 ± 1.7</td>
<td>+ 0.7</td>
<td>3.7 ± 1.7</td>
<td>4.4 ± 2.1</td>
<td>+ 0.7</td>
<td>0.15</td>
</tr>
<tr>
<td>PI</td>
<td>2.9 ± 0.5</td>
<td>2.3 ± 0.6</td>
<td>− 0.5</td>
<td>2.3 ± 0.4</td>
<td>2.7 ± 0.7</td>
<td>+ 0.5</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Phospholipid classes identified in HDL subfractions (d = 1.063–1.21) from EGG (n = 4) and SUB (n = 4) groups are presented as percent of total HDL-PL (mol%). Data are reported as mean ± SD. Different letters within the same row indicate significantly different mean values (\( P < 0.05 \)). *\( P < 0.05 \); independent \( t \) test comparing changes in %HDL-PtdEtn from baseline and week 12 between EGG vs. SUB.
Phospholipid species homology between HDL and egg products

We further aimed to identify whether HDL subfractions became relatively more enriched in phospholipid molecular species present in the egg products throughout the intervention. In calculating the homology between HDL and egg product phospholipids, we found that molecular species identified in egg products represented an average 85%, 84%, 95%, and 90% of total HDL species of PC, PE, LysoPC, and PI classes, respectively. This trend remained constant from baseline to week 12. The primary differences between PC and PE from egg and human HDL were the concentrations of 1-O-alkyl- or –alk-1-enyl species. On average, human HDL contained 3-fold more 1-O-alkyl- or –alk-1-enyl-phosphocholine and 9-fold more 1-O-alkyl- or –alk-1-enyl-phosphoethanolamine (data not shown). In contrast, the SM species identified in whole egg and egg substitute products represented only 23.2% and 24.5% of HDL-SM species in EGG and SUB groups at baseline, respectively. By the end of the 12-week intervention, HDL subfractions from the EGG group shared greater SM species homology with the whole egg product (Figure 4.2), whereas no changes in homology between SUB group HDL and the egg substitute product were observed. Further, changes in SM species homology were positively correlated with changes in HDL-C ($r = 0.80$, $P = 0.017$). Increases in SM species homology in the EGG group were due to increases in the appearance of egg product SM species in HDL subfractions rather than non-specific reductions in non-egg product-derived SM species (data not shown). These findings suggest that EGG-derived SM species may be incorporated into HDL in a manner that can significantly alter HDL phospholipid species distribution.
Figure 4.2 SM species homology between egg products and HDL

Percent homology of SM species in HDL and egg products. SM species homology represents the percentage of total HDL-SM species (nmol) that were similarly identified in the whole egg (EGG group) or egg substitute (SUB group) products. N = 4 per group; data are represented as mean ± SEM. *P < 0.05; NS: non-significant.

**Cholesterol efflux**

Cholesterol efflux to subject serum was measured to assess whether changes in HDL lipid composition from egg feeding affected the cholesterol-accepting capacity of HDL. While no changes in cholesterol efflux were observed in the SUB group, cholesterol efflux significantly increased in the EGG group (+2.4%) from baseline to week 12 (Figure 4.3). There was a trend toward a positive association between changes in cholesterol efflux and changes in SM homology between HDL and egg product SM species ($r = 0.69; P = 0.060$), as well as a trend toward a negative correlation between changes in cholesterol efflux and changes in fasting plasma glucose (reported in [26])($r = -0.69; P = 0.060$). No correlations were found between changes in cholesterol efflux and any other HDL lipids.
Figure 4.3 Effects of egg intake on the cholesterol-accepting capacity of serum

Percent cholesterol efflux from macrophage foam cells to subject serum. Cholesterol efflux was performed over a 3-hour period to assess the total cholesterol-accepting capacity of serum obtained from EGG (n = 4) and SUB (n = 4) subjects. Data are represented as mean ± SEM. *P < 0.05; NS: non-significant.

Discussion

While previous studies have found that egg consumption increases HDL-C and improves HDL particle profiles [25-27, 35], it has yet to be determined whether these changes correspond to alterations in HDL composition or HDL function. Since eggs are a rich source of dietary phospholipids which known to exert plasma lipid-modulating effects, we hypothesized that daily egg consumption may additionally alter HDL lipid composition and PL class distribution to improve the ability of HDL to function as a lipid acceptor in MetS - individuals often characterized with low HDL-C, dysfunctional HDL [6], and an overall 2-fold increased risk of developing CVD [29]. Compared to intake of a yolk-free egg substitute, consumption of 3 eggs per day for 12 weeks resulted in greater enrichment of
HDL in CE, PE, and SM species present in whole eggs, as well as greater cholesterol efflux from cholesterol-loaded macrophages to subject serum. These changes correspond to previously reported improvements in HDL particle profiles in this population, along with beneficial effects on additional markers of atherogenic dyslipidemia and insulin resistance [26]. To our knowledge, this is the first study to demonstrate that increases in HDL-C from egg consumption during carbohydrate restriction correspond to modulation of HDL lipid composition and increase in the overall lipid-accepting capacity of serum in MetS.

The classification of MetS by the NCEP:ATP III criteria is diverse in nature due to the requirement of any 3 or more qualifying parameters [29], thereby creating a spectrum of MetS severity. Severity of MetS may be related to the degree of metabolic disturbances that affect lipoprotein metabolism, including insulin resistance and low-grade systemic inflammation [1]. Prospective cohort studies have further demonstrated that individuals who meet a greater number of MetS parameters have an increased risk of CVD [36] – particularly when low plasma HDL-C is one of the factors [37]. In this population, over half of the subjects randomized to each group had low plasma HDL-C levels. While we have observed greater increases in HDL-C from whole egg feeding in previous studies [18, 26, 27], it is interesting to note that the greater HDL-C response to whole egg feeding in MetS was driven by subjects who began the intervention with normal HDL-C levels. As we did not identify any differences in HDL lipid composition between subjects with low and normal baseline plasma HDL-C levels, this observation may be reflective of a greater responsiveness to the intervention in subjects falling within the healthier end of the MetS spectrum, as 7 out of 8 EGG group subjects with normal baseline HDL-C met only 3 MetS parameters, whereas EGG group subjects with low baseline HDL-C more commonly met 3, 4, and 5 MetS
parameters (data not shown). Although the pattern of MetS parameters between subjects with low and normal baseline HDL-C were similarly observed in the SUB group, these factors did not appear to impact the overall reduced HDL-C response in the SUB group when compared to the EGG group [26, 27]. While it is important to note that the dietary cholesterol provided by whole eggs may play a role in HDL-C responses [18], the overall pattern of plasma lipid changes in the EGG group (i.e. increases in HDL-C without adversely affecting TC or LDL-C levels [26, 27]) is similar to findings reported from dietary phospholipid supplementation studies, suggesting that the yolk-derived phospholipids are responsible for the observed effects [16, 17]. This concept is further supported by the fact that egg PC and SM has been shown to decrease lymphatic absorption of cholesterol [64, 65]. Although further investigation is warranted, it is reasonable to hypothesize that phospholipid-rich foods other than egg may confer benefits to HDL lipid composition and function similar to those observed in the current study.

HDL lipid composition has been shown to be an important factor in determining HDL function, metabolism, and CVD risk beyond plasma HDL-C levels. Individuals with low HDL-C [38] and MetS [39] often have greater enrichment in HDL-TG and reduction in HDL-CE and HDL-PL. Enrichment of HDL in TG has been associated with greater cholesteryl ester transfer protein (CETP) activity [40], enhanced clearance of apoA-I from circulation [41], and impaired LCAT-mediated esterification of free cholesterol and HepG2 cell uptake of HDL-CE [42]. Conversely, CE enrichment of HDL promotes greater SR-BI-mediated HDL-CE uptake [43], and is indicative of larger, more buoyant HDL particles that often correlate with reduced risk of CVD [44]. We observed relative increases in HDL-CE content in all subjects, concurrent with the previously described increases in HDL particle
size in this population [26]. Although there were slightly greater relative increases in HDL-CE in the SUB group, this is most likely reflective of greater non-significant reductions in HDL-FC and HDL-PL compared to the EGG group. Interestingly, whole egg feeding led to greater reductions in HDL-TG content in subjects who entered the study with normal plasma HDL-C levels, further suggesting that these subjects were metabolically healthier and more responsive to the dietary intervention. HDL-CE/TG ratios increased all subjects, suggesting an overall improvement in HDL lipid composition regardless of group assignment, as would be expected due to the hypotriglyceridemic effects of carbohydrate-restricted diets [45].

Although changes in total HDL-PL were not found as we hypothesized, we did observe changes in the distribution of HDL phospholipids classes and SM species, including an increase in relative HDL-PE content and SM species homology to the whole egg product in the EGG group only. Alterations in HDL-PE content may affect the cholesterol-accepting capacity of patient serum, as well as pathways of HDL metabolism and the anti-thrombotic properties of HDL [6, 8, 46]. In hypercholesterolemic men with exceptionally high plasma HDL-C (>1.75 mmol/L, or 68 mg/dL), HDL was found to be enriched in PE, yet HDL-PE content was negatively correlated with cholesterol efflux from rat Fu5AH hepatoma cells [8]. Conversely, the relationship between HDL-PE and cholesterol efflux was positive in men with normal HDL (1.10 to < 1.50 mmol/L, or 43 - 58 mg/dl), although this pattern did not reach statistical significance. Our findings suggest that greater HDL-PE content may be associated with greater cholesterol efflux in a macrophage cell model in men and women with MetS – a population predominantly with low plasma HDL-C.

Enrichment in HDL with specific phospholipids may also confer atheroprotective benefits beyond that of modulating cholesterol efflux capacity. PE is also known to promote
activated protein C-mediated anticoagulant activity [46]; therefore, PE-enriched HDL may exert greater antithrombotic activity [6, 47]. Oxidation of HDL has also been shown to promote depletion of PE [48], suggesting that enrichment of HDL in PE may be indicative of a less oxidized HDL profile.

In addition to increases in HDL-PE content, we observed that daily whole egg consumption for 12 weeks led to the enrichment of HDL in SM species present in the egg product, potentially reflecting egg-derived SM that became incorporated into HDL. Egg yolk serves as a uniquely rich and highly bioavailable (>90%) source of dietary phospholipids, which can be absorbed partially intact in the intestine and have been shown to be preferentially incorporated into HDL particles following ingestion [21, 22]. Further, in various animal models, feeding of natural PC and synthetic dimyristoylphosphatidylcholine (DMPC) has been shown to increase jejunal apoA-I synthesis [49, 50], which may promote intestinal secretion of HDL - a process which is thought to contribute ~30% of total circulating HDL [51, 52]. Further, the presence of egg SM has been shown to lower lymphatic absorption of other phospholipids (PC, PE, lysoPC, and PI), without affecting SM absorption [66]. This finding may explain the increase in % SM species homology from baseline to week 12 observed in the EGG group. Together, these concepts suggest that consumption of egg-derived phospholipids may serve as a relatively direct mechanism to modulate HDL lipid composition and intestinal HDL production.

We further demonstrated that cholesterol efflux to subject serum increased from baseline to week 12 in the EGG group only (+2.4%). Due to the variability in experimental methodology for efflux assays and the relatively recent application of this approach to assessing CVD risk in response to therapeutic intervention, it is yet to be determined how the
observed changes in efflux affect long-term CVD risk and development. However, through implementation of statistical modeling that adjusted for standard cardiovascular disease risk factors, Khera et al. [5] found that coronary artery disease (CAD) risk (as measured by carotid intima-media thickness) was reduced by 30% for every 1-SD increase in cholesterol efflux capacity. These findings suggest that CAD risk could be significantly reduced with relatively small increases in cholesterol efflux. Following this logic, we believe that the +2.4% (or +0.5 SD) increase in efflux observed in the EGG group is a clinically relevant improvement in CVD status.

The increases in cholesterol efflux associated with whole egg consumption may be attributed to an increased lipid-accepting capacity of HDL, as well as changes in other serum components. Various factors related to MetS have been associated with impaired cellular cholesterol efflux, including hypertension [53], overweight/obesity [54], low HDL-C [55], elevated blood glucose [56], and markers of inflammation [57]; however, it remains unclear whether cholesterol efflux is impaired in MetS. Conflicting results may be due to variability of different cellular efflux models [15], as well as the innate diversity of MetS populations in regard to qualifying parameters [58]. Cholesterol and phospholipid efflux from fibroblasts to subject plasma was shown to be similar between healthy individuals and those classified as non-diabetic MetS [59, 60]; however, fibroblasts express minimal SR-BI – a pathway that is more responsive to shifts in HDL-PL, larger HDL particles, and a greater contributor to total efflux in macrophages [61, 62]. Although few clinical trials have examined the effects of diet on cholesterol efflux, weight loss has been shown to increase cholesterol efflux to plasma via SR-BI- and ABCG1-mediated pathways, whereas ABCA1-mediated cholesterol efflux was reduced – presumably due to a reduction in more nascent preβ HDL [63]. These findings
corresponded to increases in the larger HDL$_2$ subfractions, which additionally become more phospholipid rich [63]. Cholesterol efflux was also increased in MetS subjects following a 12-week treatment of insulin-sensitizing drug pioglitazone [5]. Accordingly, we observed a trend toward a negative correlation between changes in fasting plasma glucose and cholesterol efflux, suggesting that improvements in insulin resistance promote greater efflux in MetS.

In summary, we have demonstrated that increases in HDL-C from daily whole egg intake during moderate carbohydrate restriction coincide with clinically relevant changes in HDL lipid composition and the lipid-accepting capacity of subject serum in MetS. Further, it appears that changes in HDL-C and lipid composition in response to egg feeding vary within the MetS classification, whereas subjects with normal plasma HDL-C levels at baseline displayed more favorable increases in HDL-C and a reduction in HDL-TG content. Similar to the changes in cholesterol efflux, these observations may be related to variation in insulin sensitivity within this MetS population. Overall, these findings indicate that eggs may serve as a functional food to promote beneficial shifts in HDL composition, metabolism, and function in MetS.
References


Chapter 5

Egg intake during carbohydrate restriction alters the inflammatory potential of peripheral blood mononuclear cells in metabolic syndrome
Abstract

Leukocyte inflammation under basal, unstimulated conditions has been linked to adverse metabolic outcomes in relation to CVD and T2DM. Therefore, it is important to address this issue in populations at high risk of chronic metabolic disease, such as individuals with metabolic syndrome (MetS). We set out to determine whether egg intake during carbohydrate-restriction – an intervention that has previously been shown to reduce plasma inflammatory markers – could effectively reduce the inflammatory potential of peripheral blood mononuclear cells (PBMCs) in MetS. Thirty-seven men and women classified with MetS consumed a moderate carbohydrate-restricted diet (25-30% of energy) for 12 weeks, in addition to consuming either 3 whole eggs per day (EGG group) or the equivalent amount of yolk-free egg substitute (SUB group). PBMCs were isolated at baseline and week 12 of the intervention. Body weight was reduced by an average ~4% in all subjects from baseline to week 12 of the intervention. While there were no changes in PBMC mRNA expression of IL-1β, IL-6, and TLR4 throughout the intervention, contrary to our expectations, TLR4 mRNA expression was increased in the EGG group only. Changes in TLR4 mRNA expression positively correlated with changes in NF-κB p65 subunit DNA binding activity, in addition to changes in dietary cholesterol and choline intake. However, upon stimulation with LPS, PBMC TNFα and IL-1β production increased from baseline to week 12 in the SUB group, whereas no changes were observed in the EGG group. Together, these findings suggest that daily whole egg consumption during carbohydrate restriction does not increase the inflammatory potential of PBMC in response to LPS, despite increases in TLR4 expression. Conversely, moderate carbohydrate-restriction and egg substitute intake increases LPS-induced inflammatory responsiveness in the MetS.
Introduction

Chronic, low-grade inflammation is commonly associated with obesity-related metabolic diseases, and is often indicative of organ stress and insufficient resolution of inflammatory immune responses [1-3]. Elevated levels of plasma inflammatory markers have been reported in CVD, T2DM, and MetS [4, 5]. These cytokines, chemokines, adhesion molecules, and acute phase proteins may be derived from metabolically stressed adipose tissue, liver, and skeletal muscle, in addition to activated leukocytes [1-3].

Recent focus has been turned to the role of leukocytes in contributing to metabolic disease progression [6, 7], as well as how obesity can affect basic leukocyte properties and function and immunity [8]. Multiple studies have demonstrated that factors related to obesity increase the basal inflammatory profile of lymphocytes [9], in addition to promoting greater pro-inflammatory responses upon stimulation and activation [10, 11]. The consequences of these actions can include altered monocyte and lymphocyte migration and adhesion patterns, impaired immunity, prolonged systemic inflammation, and greater difficulty in resolving acute inflammatory responses. These changes have further been linked to greater progression of atherosclerosis, arterial dysfunction, adipose tissue inflammation and dysfunction, and insulin resistance [7, 10, 11]. Therefore, it is important that therapeutic intervention strategies targeting markers of chronic low-grade inflammation similarly address dysregulated leukocyte signaling.

Dietary and lifestyle therapies that promote weight loss and improved diet quality have been shown to effectively ameliorate markers of systemic inflammation, including inflammatory cytokines and acute phase proteins such as TNFα, CRP and lipoprotein-associated SAA [12-14]. Moderate weight loss (~5% of body weight) has additionally been
shown to reduce inflammatory gene expression and NF-κB DNA binding activity in PBMC from obese women [9].

It has previously been established that carbohydrate restriction effectively reduces plasma inflammatory markers in overweight populations [14]. We have further demonstrated that daily intake of whole eggs during carbohydrate restriction reduces CRP in overweight men [13], as well as TNFα and SAA in men and women with MetS [12] – individuals at increased risk for developing CVD and T2DM [15]. Eggs contain various anti-inflammatory factors, including the antioxidant carotenoids lutein and zeaxanthin, as well as various bioactive phospholipid species [16, 17]. Therefore we sought to determine whether whole egg intake during carbohydrate restriction could further modulate inflammatory properties of PBMC in MetS, as PBMC subsets (predominantly lymphocytes and monocytes) play important roles in the progression of CVD and T2DM [7, 18-20].

Materials and Methods

Study Design

Men and women (n = 37) classified with MetS according to the NCEP:ATP III criteria [15] were recruited to participate in a 12-wk moderate carbohydrate-restricted (25-30% of total energy) diet intervention. Subjects were randomly assigned to consume either 3 whole eggs per day (EGG group) or the equivalent amount of yolk-free egg substitute (SUB group). Details of this intervention are described in Chapter 3.
**Dietary analysis**

Dietary intake was determined by analysis of 5-day food records collected at baseline, week 6, and week 12 of the intervention. Nutrient analysis was performed using Nutrition Data Systems for Research (NDSR).

**Sample collection**

Fasting blood samples were collected at baseline and week 12 of the intervention for isolation of PBMC and serum for cell culture assays. All samples were processed under sterile conditions. Serum was aliquoted and stored at -80°C.

**Peripheral blood mononuclear cell isolation**

Fasting blood (50ml) was collected into EDTA vacutainer tubes, diluted with PBS, and layered over Ficoll Paque (GE Healthcare) according to the manufacturer’s instructions. Samples were centrifuged at 400 x g for 35 minutes using a Beckman Coulter centrifuge with swing bucket rotors allowing for separation of the buffy coat. The buffy coats were collected, washed twice with PBS, and resuspended in RPMI. Aliquots of freshly isolated PBMCs were taken for collection of nuclear extracts and RNA extraction as described below. Remaining cells were diluted 1:1 with cryopreservation media (RPMI containing 20% FBS, 10% DMSO) and frozen at a controlled rate in CoolCell containers (BioCision) at -80°C for at least 24 hrs. PBMC samples were then transferred to liquid nitrogen for long-term storage.
Quantitative real-time RT-PCR

PBMC mRNA expression of inflammatory genes was determined by quantitative real-time RT-PCR (qRT-PCR) [21, 22]. RNA from freshly isolated PBMCs was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. 1 µg of RNA was treated with DNase I (Promega, Madison, WI) and reverse transcribed by MMLV reverse transcriptase (Promega, Madison, WI) using a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories). qRT-PCR analysis was performed using the Sybr Green procedure and a Bio-Rad CFX96 system (Bio-Rad Laboratories). Primer sequences were designed according to the GenBank database, and are presented in Table 5.1. Expression of mRNA values was calculated using the threshold cycle (Ct) value. Relative expression levels of each target gene were calculated using the comparative $2^{-\Delta\Delta Ct}$ method following normalization to 18S rRNA expression [21-23].

Table 5.1 Quantitative real-time RT-PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tr>
<td>IL-1β</td>
<td>5’-ACGATGACCTGTACGATCACT-3’</td>
<td>5’-ACACCAAGCTTTTTTGCTTGAG-3’</td>
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<td>5’-GCTGGAATTACCAGCGGCT-3’</td>
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NF-κB p65 DNA binding activity

Nuclear fractions were collected from freshly isolated PBMCs using the Nuclear Extraction kit (Active Motif, Carlsbad, CA) following the manufacturer’s instructions [22, 24]. Nuclear
fractions were stored at -80°C until analysis. Total nuclear protein was determined by BCA assay (Thermo Scientific), then used to determine the DNA binding activity of the NF-κB p65 subunit using the TransAM® NFκB p65 Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, PBMC nuclear extracts were added to the 96-stripwell ELISA plate. Each well was coated in multiple copies of immobilized specific double-stranded oligonucleotides, which allow for binding of activated NF-κB. NF-κB p65 binding was then detected by addition of a primary NF-κB antibody, followed by a horseradish peroxidase-conjugated secondary antibody. Following color development, p65 DNA binding activity was quantified spectrophotometrically at 450 nm with a reference wavelength of 655nm. Data are presented as the spectrophotometric reading at optical density (OD) 450 nm.

**PBMC stimulation assays**

Inflammatory responses to LPS were assessed in a subset of subject PBMCs (EGG: \( n = 5 \); SUB: \( n = 5 \)). 2 x 10⁶ PBMCs were plated in 12-well plates, then treated with or without 500 ng lipopolysaccharide (LPS) per well for 6 hrs in serum-free media. Media was collected, then assayed for TNFα (BD Bioscience), IL-1β, and IL-10 (Abcam) by ELISA.

**Statistical analysis**

All statistical analyses were performed using SPSS version 18. Repeated measures ANOVA was used to test the overall effects of the intervention between EGG vs. SUB groups (the between-subjects factor) and over time (the within-subjects factor). Paired \( t \) tests were used to test differences between baseline vs. week 12 values within EGG or SUB groups.
Independent *t* tests were used to compare the differences in absolute or percent change in variables between groups. Bivariate Pearson correlations were used to determine relationships between parameters. Data are reported as mean ± SEM unless noted otherwise. *P* < 0.05 was considered significant.

**Results**

*Moderate carbohydrate restriction reduces body weight regardless of egg group assignment*

As described in previous chapters, all subjects adhered to the moderate carbohydrate dietary prescription, thus increasing percent of daily energy coming from fat and protein sources, while reducing percent of energy from carbohydrates (Figure 5.1 A). While no differences in macronutrient intake between groups was observed, the EGG group increased daily intake of egg yolk nutrients, including dietary cholesterol and choline intake throughout the 12-week intervention (Figure 5.1 B). Conversely, the SUB group reduced daily dietary intake of cholesterol and choline. Regardless of group assignment, subjects in both EGG and SUB groups experienced an average ~4% reduction in body weight by the end of the intervention (Figure 5.1 C).
Figure 5.1 Moderate carbohydrate restriction reduces body weight regardless of egg product assignment

Moderate carbohydrate restriction reduces body weight regardless of daily whole egg or egg substitute intake. A) Macronutrient intake as % of daily energy. Data from both EGG and SUB groups are combined, as there were no statistical differences in macronutrient intake between groups. B) Average daily intake of dietary cholesterol and choline in EGG (yellow bars) and SUB (orange bars) groups. C) Changes in body weight throughout the intervention. All data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.0001 (time effect) and +++P < 0.0001 (group effect) as determined by repeated measures ANOVA. ###P < 0.001, paired t test comparing baseline to week 12 in each group. P = 0.6 represents independent t test comparing the difference between changes in % weight loss between EGG and SUB groups.
Inflammatory gene expression

Pro-inflammatory gene expression in PBMCs is known to be elevated in conditions of obesity and metabolic dysfunction, and reduced by weight loss [9]. Therefore, we sought to determine whether our dietary intervention could modulate inflammatory PBMC gene expression. Interestingly, although we did not observe changes in IL-6, IL-1β, or TNFα mRNA expression in either group, TLR4 mRNA expression increased from baseline to week 12 in the EGG group only (Figure 5.2 A). Correspondingly, changes in TLR4 mRNA expression positively correlated with changes in % dietary fat ($r = 0.649, P < 0.0001$), as well as with changes in daily dietary intake of cholesterol ($r = 0.433; P = 0.012$) and choline ($r = 0.394; P = 0.026$) (Figure 5.2 B) – nutrients that are abundant in egg yolk.
Figure 5.2 Egg intake during moderate carbohydrate restriction is associated with increased PBMC mRNA expression of TLR4

Changes in pro-inflammatory gene expression from egg intake. A) All data are expression as mean ± SEM. mRNA expression was determined by qRT-PCR, with data normalized to 18S rRNA. *P values represent paired t-tests between baseline and week 12 for each respective group. B) Pearson correlation representing the relationship between changes in PBMC TLR4 mRNA expression and changes in dietary fat (as % of total energy), cholesterol, and choline from baseline to week 12 of the intervention.
**NF-κB p65 DNA binding activity**

Increased PBMC NF-κB activity has been implicated in the increased pro-inflammatory gene expression observed in obese individuals, while it has also been shown to be decreased by weight loss [9]. Therefore, we assessed the DNA binding activity of the active NF-κB subunit p65 in response to our dietary intervention. Changes from baseline to week 12 did not reach significance in either EGG or SUB group (Figure 5.3 A), nor did the difference in changes between groups ($P = 0.085$) (Figure 5.3 B). However, changes in NF-κB p65 DNA binding activity positively correlated with changes in TLR4 mRNA expression ($r = 0.394; P = 0.026$), as well as with changes in daily dietary choline intake ($r = 0.442; P = 0.035$) (Figure 5.3 C).
Figure 5.3 Increased PBMC TLR4 mRNA expression is from egg intake is associated with NF-κB activity

Effects of egg intake on NF-κB p65 DNA binding activity. NF-κB p65 DNA binding activity was assessed in nuclear extracts obtained from freshly isolated PBMCs. A) NF-κB p65 DNA binding activity at baseline and week 12 of the intervention in EGG and SUB groups. B) Changes in NF-κB p65 DNA binding activity in EGG and SUB group subject PBMCs. C) Pearson correlation between changes in NF-κB p65 DNA binding activity and changes in PBMC TLR4 mRNA expression and dietary choline.

Inflammatory challenge

Contrary to our original hypothesis, the findings thus far suggest that whole egg intake during moderate carbohydrate restriction tended to increase the basal inflammatory state of PBMC in MetS. Further, moderate carbohydrate restriction in the SUB group did not appear to have a significant effect on PBMC inflammation, despite its favorable effects on body
weight and composition. Therefore, we sought to determine whether our dietary treatment had an effect on responsiveness of PBMCs to inflammatory stimuli. PBMCs were cultured and assessed for their capacity to secrete pro-inflammatory IL-1β and TNFα, or anti-inflammatory IL-10, either under basal conditions or in response to LPS.

Under basal, non-stimulated conditions, no significant changes in PBMC IL-1β or TNFα production from baseline to week 12 were observed in either group (Figure 5.4 A). This may be in part due to a large variability in subject response and a relatively small subset sample size. However, a weak trend for reduced IL-1β was observed over time in the SUB group (P = 0.086), as well as for TNFα when all subjects were combined (P = 0.089) (Figure 5.4 A). IL-10 levels were below the range of assay detection for the majority of subjects under resting conditions (data not shown).

Conversely, surprising group differences were detected upon stimulation with LPS. While no changes in IL-1β or TNFα were observed in the EGG group, LPS-induced PBMC production of IL-1β and TNFα were increased from baseline to week 12 in the SUB group (Figure 5.4 A). Changes in both IL-1β and TNFα were significantly greater in the SUB group when compared to the EGG group (Figure 5.4 B). Changes in IL-10 from baseline to week 12 failed to reach significance in either group, although a weak trend suggested an increase overall when all subjects were combined (P = 0.087). Changes between EGG and SUB groups were not statistically different (P = 0.245) (Figure 5.4 C).
Figure 5.4 Egg substitute intake during carbohydrate restriction increases PBMC inflammatory responsiveness to LPS

Effects of egg intake during carbohydrate restriction on LPS-induced cytokine secretion. A) PBMC TNFα and IL-1β secretion with or without stimulation with LPS. B) Changes in LPS-induced TNFα and IL-1β secretion from baseline to week 12. C) LPS-induced PBMC IL-10 secretion. IL-10 was generally non-detectable under non-stimulated conditions. All data are represented as mean ± SEM. EGG: n = 5, SUB: n = 5.
Discussion

Elevated levels of basal leukocyte inflammation have been demonstrated in obesity and metabolic syndrome [9, 25], and have been implicated in leukocyte-driven progression of metabolic disease [6, 7]. Therefore, dietary strategies represent a promising strategy to mitigate inappropriate activation of pro-inflammatory leukocyte responses in order to prevent adverse metabolic disease outcomes.

In this study, we demonstrate that, contrary to our original hypothesis, mRNA expression of TLR4 is increased by daily intake egg intake during moderate carbohydrate restriction, correlating with NF-κB p65 DNA binding activity. However, these changes did not correspond with alterations in inflammatory PBMC responsive to LPS, whereas LPS-induced PBMC TNFα and IL-1β secretion was increased in by moderate carbohydrate restriction and egg substitute intake. Together, these findings further exemplify a novel role for dietary egg consumption and carbohydrate restriction in modulating the inflammatory potential of leukocytes.

Previous studies [9] have reported reductions in inflammatory gene expression in PBMCs following moderate weight loss. This phenomenon is thought to be indicative of global improvements in metabolic tissue stress and function, thereby reducing the presence of pathogenic factors capable of activating leukocytes, while also promoting the resolution of inflammatory responses. However, despite moderate weight loss and decreases in plasma TNFα and SAA from whole egg consumption in this population [12], we did not observe reductions in PBMC mRNA expression of TNFα, IL-1β, or IL-6 from baseline to week 12. Conversely, we observed significant increases in TLR4 mRNA expression in subjects consuming whole eggs.
TLR4 is a member of the TLR family of pattern recognition receptors that recognizes exogenous and endogenous ligands, including Gram-negative bacteria-derived LPS and fatty acids [26]. Elevated expression of TLR4 may be particularly influential in obesity-related disease, as obesity has been associated with increased intake of dietary fatty acids, increased gut permeability and serum levels of LPS, as well as elevated levels of circulating FFA capable of activating TLR4 signaling [26, 27]. Increased TLR4 activity has been associated with high-fat feeding, elevated cellular cholesterol levels, and activation of NF-κB transcriptional activity, corresponding to the correlations observed in this study [27-30].

Despite the role of TLR4 in obesity, our study is limited in that only mRNA levels of TLR4 were measured. While various inflammatory factors differentially regulate TLR4 mRNA expression, functional activity of this receptor is further regulated at the level of protein translation, cell surface localization, accessory molecule availability and expression, and plasma membrane composition [27]. Interestingly, omega-3 fatty acids have been shown to inhibit LPS- or SFA-induced TLR4 activation by altering plasma membrane lipid raft composition, thereby preventing assembly of TLR4 homodimers and signaling component complexes within lipid rafts to allow for subsequent inflammatory signal transduction [31]. Similar impairments in TLR4 signaling have been demonstrated via depletion of cellular cholesterol – an important structural component of lipid rafts [29, 30]. Further, TLR4 expression can also promote different types of cellular signaling depending on the cell type in which it is expressed. This is an important consideration with regard to the present study, since PBMCs consist of mixed leukocyte population containing approximately 60-70% T cells, 5-15% B cells, 5-10% natural killer cells, 0.5-2% dendritic cells, 15-30% monocyte/macrophages, and 2% granulocytes [32-35]. For example, TLR4 protein is
expressed intracellularly in naïve human CD4\(^+\) T cells, whereas cell surface TLR4 expression is found in activated CD4\(^+\) T cells [36]. Cell surface expression of TLR4 is associated with increased anti-inflammatory T\(_{\text{reg}}\) cell activation, as well as an enhancement of T\(_{\text{reg}}\) cell-mediated suppressive functions [37]. Therefore, simply observing changes in TLR4 mRNA expression does not necessarily signify changes in cellular inflammatory potential without further analyzing surface protein expression or plasma membrane and lipid raft composition, and additional measures of inflammatory potential.

Correspondingly, the fact that TLR4 mRNA expression does not necessarily translate to increased cellular inflammatory responses was further exemplified by our findings from PBMC stimulation assays. Despite increased mRNA expression of TLR4 that correlated NF-κB p65 DNA binding activity, daily whole egg intake for 12 weeks rendered PBMCs less responsive to LPS-induced cytokine secretion when compared to responses from egg substitute consumption. These findings suggest that 1) a component of the egg substitute product (in conjunction with carbohydrate restriction) was inducing greater inflammatory responses that were only observed upon LPS stimulation of PBMC (and not under basal conditions or in plasma); and/or 2) a component of the egg product was blunting increased PBMC responsiveness to LPS induced by carbohydrate restriction.

One possible explanation could be that whole eggs provide dietary oxidized phospholipids (OxPL) that impair responsiveness of PBMCs to LPS. Compared to other dietary phospholipid sources, egg-derived phospholipids have been shown to more susceptible to copper- and pH-induced oxidation due to its relatively higher content of long-chain PUFAs [38]. Generation of oxidized phospholipid metabolites by gut microflora has also been reported in response to egg feeding [39]. Although OxPL have been linked to pro-
inflammatory responses and atherosclerosis in some cases [40, 41], the oxidized phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (Ox-PAPC) and associated phospholipid oxidation products have been shown to inhibit LPS-mediated TLR4 activation [42, 43]. OxPL species have been shown to inhibit LPS-induced inflammatory signaling in macrophages, endothelial cells, and smooth muscle cells through alteration of lipid raft and caveolar processing and through competitive interaction with TLR accessory proteins, such as MD-2, CD14, and LPS-binding protein [42-44]. Together, the presence of OxPL inhibits LPS-induced membrane translocation of LPS receptor complexes that are required for signaling and propagation of pro-inflammatory cytokine production [42, 43]. The capacity of OxPL to inhibit bacterial pathogen-induced inflammatory signaling is thought to serve as an endogenous feedback mechanism to potentiate oxidative damage and promote resolution of tissue inflammation [44, 45]. Although we did not assess the effects of this intervention on oxidized species in serum, PBMCs, or lipoproteins, we did observe significant changes in HDL phospholipid composition from egg intake (see Chapter 4), suggesting the possibility of incorporating egg yolk-derived OxPL into blood components if present in the whole egg product.

Another explanation for the differential PBMC responses to LPS between EGG and SUB groups could be that PBMCs from the SUB group were more primed toward pro-inflammatory responses due to the relatively greater inflammatory environment when compared to the EGG group (which displayed decreases in plasma TNFα and SAA) [12]. Bosisio et al. [46] found that in human monocytes and macrophages, LPS induced TLR4 mRNA expression, while reducing its surface protein expression. However, priming with interferon γ (IFNγ) increased the responsiveness of cells to LPS-induced NF-κB DNA
binding activity and production of TNFα and IL-12 [46]. Following our 12-week intervention, the SUB group did not display reductions in plasma TNFα and SAA as was observed in the EGG group [12], suggesting that the SUB cells could remain more primed for inflammatory responses following stimulation.

Given the changes in HDL composition and the cholesterol-accepting capacity of serum from egg intake described in the previous chapter, it is additionally plausible that the differences in PBMC inflammatory potential between the EGG and SUB groups could be due to changes in cellular cholesterol content and lipid raft formation. As described above, TLR4 signaling is dependent upon plasma membrane composition and lipid raft integrity to facilitate the convergence of LPS receptor complex components required for LPS/TLR4-mediated inflammatory signaling [27, 43]. The inflammatory potential of leukocytes has further been shown to be regulated through modulation of the HDL-associated lipid transporter ABCA1, where greater expression of these membrane transporters is known to deplete lipid raft cholesterol and suppress TLR4-mediated inflammatory signaling [29, 30]. Therefore, it is possible that the effects of egg intake during carbohydrate restriction on PBMC inflammatory potential may be attributable to alterations in HDL-leukocyte dynamics.

Taken together, the data presented in this study highlights a novel perspective on the anti-inflammatory properties of egg intake during carbohydrate restriction. These findings further raise interesting questions regarding the role of HDL-mediated efflux in modulating PBMC inflammation, as well as implication for immunity.
References


44. Erridge C, Kennedy S, Spickett CM, Webb DJ: Oxidized phospholipid inhibition of toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4: roles for CD14,


Chapter 6

Egg intake during carbohydrate restriction alters peripheral blood mononuclear cell cholesterol flux in metabolic syndrome
Abstract

Leukocyte cholesterol flux has been linked to the regulation of cellular inflammatory potential. Therefore, we sought to determine whether peripheral blood mononuclear cell (PBMC) cholesterol content and distribution could be modulated through diet in metabolic syndrome (MetS) – a population associated with chronic low-grade inflammation and dyslipidemias. Thirty-seven men and women classified with MetS participated in a 12-week moderate carbohydrate-restricted diet (25-30% of energy), in addition to consuming either 3 whole eggs per day (EGG group) or the equivalent amount of yolk-free egg substitute (SUB). Compared to baseline, there was a trend toward a reduction in PBMC cholesterol content in the EGG group, whereas no changes were observed in the SUB group. Although total PBMC lipid raft content was not altered in either group, changes in lipid raft content positively correlated with changes in total PBMC cholesterol. To determine the cellular pathways involved in the changes observed in cholesterol flux, expression of genes related to cholesterol efflux (ABCA1 and ABCG1) and cholesterol synthesis and uptake (HMG-CoA reductase and LDL-receptor (LDL-R)) were measured. While no changes in ABCG1 or LDL-R were observed in either group, ABCA1 and HMG-CoA Red mRNA expression increased from baseline to week 12 in the EGG group only. A trend toward an increase in ABCA1 protein was similarly observed in the EGG group. Together, these findings suggest that whole egg intake during carbohydrate-restriction reduces PBMC cholesterol content, most likely in part by efflux via ABCA1. Coupled with the changes in HDL lipid composition, the cholesterol-accepting capacity of serum, and PBMC inflammatory potential presented in previous chapters, these findings may serve as further evidence that whole egg
intake during carbohydrate restriction favorably modulates HDL-leukocyte dynamics in MetS.

**Introduction**

Regulation of leukocyte cholesterol flux has recently garnered significant attention due to its implications for inflammation, immunity, atherosclerosis, and metabolic disease [1-3]. Leukocytes with elevated levels of cholesterol have been shown to be more inflammatory and hyperproliferative [1, 4, 5], which may lead to inappropriate inflammatory hyper-responsiveness to stimuli, impaired resolution of inflammation, or misguided immune responses.

In line with this area of research, HDL-associated lipid transporters, including ABCA1 and ABCG1, have been shown to play significant roles in the modulation of leukocyte cholesterol content. While the anti-atherogenic properties of both ABCA1 and ABCG1 have been well documented due to their capacity to participate in the initiation of reverse cholesterol transport through efflux of macrophage foam cell lipids to HDL [6-8], these proteins additionally possess the capacity to reduce inflammatory responsiveness through depletion of lymphocyte cholesterol content and lipid raft formation [9, 10]. Cellular cholesterol depletion impairs the formation lipid rafts within the plasma membrane where transmembrane proteins and receptors such as TLR4 reside, thereby blunting the activation of inflammatory signaling [9-11]. ABCA1 expression in leukocytes has additionally been implicated in cellular recruitment and migration, as deletion of leukocyte-specific ABCA1 results in more advanced atherosclerotic lesions, as well as increased leukocyte counts in the liver, spleen, and peripheral blood [12]. Further, ABCA1 is thought to efflux LPS to HDL as
a means of neutralizing it inflammatory effects, while also serving as a transports system for excretion of LPS from the body via the bile [13-15].

In accordance with their role in atherosclerosis and leukocyte inflammation, expression of ABCA1, ABCG1, and SR-BI has been shown to be impaired in individuals with chronic metabolic disease, including coronary artery disease (CAD), hypertension, and T2DM [16-19]. Expression of these cellular transporters are also known to be impaired by specific serum components that are commonly found to be elevated in metabolic disease, including serum glucose, tumor necrosis factor α (TNFα), C-reactive protein (CRP), and oxidized LDL (oxLDL). Inhibition of HDL-associated transporters by these factors has been further linked to reductions in macrophage lipid efflux to HDL [20-24]. Dietary factors have also been demonstrated to differentially regulate ABCA1 expression in human PBMC [25], suggesting that therapeutic intervention aimed at treating metabolic dysfunction may have the capacity to alter leukocyte inflammatory potential through modulation of cellular cholesterol levels.

Given that 12 weeks of daily whole egg intake during moderate carbohydrate restriction has concomitantly modulated HDL composition, cholesterol efflux from macrophages to serum, and PBMC inflammatory potential in MetS, we further hypothesized that daily egg consumption alters cellular cholesterol content via expression of HDL-associated transporters.

Materials and Methods

Study Design
Men and women (n = 37) classified with MetS according to the NCEP:ATP III criteria [26] were recruited to participate in a 12-wk moderate carbohydrate-restricted (25-30% of total energy) diet intervention. Subjects were randomly assigned to consume either 3 whole eggs per day (EGG group) or the equivalent amount of yolk-free egg substitute (SUB group). Details of this intervention are described in Chapter 3.

*Dietary analysis*

Dietary intake of was determined by analysis of 5-day food records collected at baseline, week 6, and week 12 of the intervention to ensure dietary compliance. Nutrient analysis was performed using Nutrition Data Systems for Research (NDSR). All subjects adhered to the experimental dietary regimen as described in previous chapters.

*Sample collection*

Fasting blood samples were collected at baseline and week 12 of the intervention for isolation of PBMC and plasma. PBMC samples were processed under sterile conditions. Plasma was obtained following blood collection into EDTA-coated tubes and centrifugation at 2200 x g for 20 minutes at 4°C. A preservative cocktail (1 ml/L sodium azide, 1 ml/L phenylmethylsulfonyl fluoride, and 5 mL/L aprotinin) was added to plasma prior to storage at -80°C.

*Peripheral blood mononuclear cell isolation*

Fasting blood (50ml) was collected into EDTA vacutainer tubes, diluted with PBS, and layered over Ficoll Paque (GE Healthcare) according to the manufacturer’s instructions.
Samples were centrifuged at 400 x \( g \) for 35 minutes using a Beckman Coulter centrifuge with swing bucket rotors allowing for separation of the buffy coat. The buffy coats were collected, washed twice with PBS, and resuspended in RPMI. Aliquots of freshly isolated PBMCs were taken for collection of nuclear extracts and RNA extraction as described below. Remaining cells were diluted 1:1 with cryopreservation media (RPMI containing 20% FBS, 10% DMSO) and frozen at a controlled rate in CoolCell Chambers (BioCision) at -80°C for at least 24 hrs. PBMC samples were then transferred to liquid nitrogen for long-term storage.

**Quantification of PBMC cholesterol content**

PBMC cholesterol content was measured by gas chromatography/mass spectrometry (GC/MS) using an Agilent 7890 GC/MS equipped with an Agilent HP-5MS capillary column with dimensions: 30 m x 0.25 mm (0.25 µm film thickness). PBMC cholesterol was extracted by isopropyl alcohol following the addition of 5α-cholestane as an internal standard. PBMC lipids were dissolved in hexane prior to injection. Run conditions were: Initial temperature = 150 °C; Temp ramp = 15 °C/min to 225 °C (hold for 5 min); Temp ramp = 15 °C/min to 300 °C (hold for 10 min); with a total run time of 25 min. Helium was used as the carrier gas. Cholesterol values were normalized to total cell protein as determined by BCA assay.

**Quantitative real-time RT-PCR**

PBMC mRNA expression of inflammatory genes was determined by quantitative real-time RT-PCR (qRT-PCR) as described in Chapter 5 [27, 28]. RNA from freshly isolated PBMCs was extracted using TRIZol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. 1 µg of RNA was treated with DNase I (Promega, Madison, WI)
and reverse transcribed by MMLV reverse transcriptase (Promega, Madison, WI) using a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories). qRT-PCR analysis was performed using the Sybr Green procedure and a Bio-Rad CFX96 system (Bio-Rad Laboratories). Primer sequences were designed according to the GenBank database, and are presented in Table 6.1. Expression of mRNA values was calculated using the threshold cycle (Ct) value. Relative expression levels of each target gene were calculated using the comparative 2-ΔΔCt method following normalization to 18S rRNA expression [27-29].

Table 6.1 Quantitative Real-time PCR primer sequences

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<td>HMGR</td>
<td>5'- CCCAGTTGTGCGTCTTCCA-3'</td>
<td>5'- TTCGAGCCAGGCTTTACCT-3'</td>
</tr>
<tr>
<td>LDL-R</td>
<td>5'-ACTGGGTGACTCCAAACTTCAC-3'</td>
<td>5'-GGTTGCCCGCTTGACA-3'</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5'- CGGCTACCACATCCAGGA-3'</td>
<td>5'-GCTGGAATTACCGCGGCT-3'</td>
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</table>

ABCA1 protein

Whole cell lysates were collected from freshly isolated PBMC as described by Rasmussen et al [27]. Briefly, following isolation, PBMCs were pelleted and resuspended in whole cell lysis buffer (150 mmol/L NaCl, 25 mmol/L Tris-HCL, pH 7.4, 1% Triton X-100) containing Protease Inhibitor Cocktail set III (Calbiochem) for 20 minutes on ice. Cells were repelleted to collect the supernatant for storage at -80°C until analysis. ABCA1 protein content was determined by ELISA (Biotang Inc). Data are presented as ng protein per 1 x 10^6 cells.

Lipid raft staining and microscopy

Lipid rafts were quantified using the Vybrant Alexa Fluor 488 Lipid Raft Labeling Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Briefly, 1 x
$10^6$ cryopreserved PBMCs were rapidly thawed at 37 °C, then labeled with a fluorescent cholera toxin subunit B (CT-B)-Alexa Fluor 488 conjugate, which binds to the pentasaccharide chain of plasma membrane ganglioside $G_{M1}$ localized within lipid raft domains. An anti-CT-B antibody was then added to induce crosslinking of the fluorescent conjugate. PBMC were loaded onto chamber slides to visualize lipid raft fluorescence using a Leica TCS SP2 Laser Scanning Confocal microscope. Total lipid raft fluorescence was quantified using a fluorescence plate reader at excitation 488 and absorbance/emission 495/519.

*Homeostatic model assessment-insulin resistance (HOMA-IR)*

HOMA-IR was calculated as follows: fasting insulin (µIU/mL) \times fasting glucose (mmol/mL)/22.5 [30]. Fasting glucose was measured enzymatically using a Cobas c 111 analyzer (Roche Diagnostics, Indianapolis, IN). Insulin levels were determined by ELISA (Mercodia AB, Uppsala, Sweden) [31].

*Statistical analysis*

All statistical analyses were performed using SPSS version 18. Repeated measures ANOVA was used to test the overall effects of the intervention between EGG vs. SUB groups (the between-subjects factor) and over time (the within-subjects factor). Paired $t$ tests were used to test differences between baseline vs. week 12 values within EGG or SUB groups. Independent $t$ tests were used to compare the differences in absolute or percent change in variables between groups. Bivariate Pearson correlations were used to determine
relationships between parameters. Data are reported as mean ± SEM unless noted otherwise. 

*P* < 0.05 was considered significant.

**Results**

*Egg intake induces a trend toward a reduction in PBMC cholesterol content*

Total PBMC cholesterol content was measured to determine whether egg consumption altered leukocyte cholesterol levels. Interestingly, there was a trend for a decrease in PBMC cholesterol content from baseline to week 12 in the EGG group, whereas no changes in cellular cholesterol levels were observed in the SUB group (Figure 6.1).

**Figure 6.1 Effects of egg intake and carbohydrate restriction on PBMC cholesterol content**

![Graph showing PBMC cholesterol content](image)

Relationship between PBMC cholesterol content and inflammatory gene expression from egg intake. A) Values are presented as mean ± SEM. *P* = 0.057 for paired *t* test between baseline and week 12 in the EGG group. EGG: *n* = 15; SUB: *n* = 13.
Changes in PBMC cholesterol content correlate with changes in lipid raft content

As we observed a trend toward a decrease in total PBMC cholesterol, we further sought to determine whether these changes corresponded to reductions in lipid raft formation. Potentially due to a lack of available samples, we failed to observe any changes in lipid raft formation from baseline to week 12 in either EGG or SUB group (Figure 6.2 A and B). However, changes in total PBMC cholesterol positively correlated with changes in lipid raft content (Figure 6.2 C). Although it is speculative, this relationship suggests that we may have seen a decrease in lipid raft fluorescence in the EGG group that would mimic the trend in cellular cholesterol levels if given a larger sample size. Interestingly, changes in PBMC lipid rafts were positively correlated with changes in HOMA-IR – an estimate of basal insulin resistance [32] (Figure 6.2 C), corresponding to the concept that inflammatory leukocytes promote insulin resistance [33].
Figure 6.2 Effects of egg intake and carbohydrate restriction on PBMC lipid rafts

PBMC lipid rafts were labeled with the Vybrant Alexa Fluor 488 Lipid Raft Labeling Kit. A) Labeled PBMCs were visualized using a Leica TCS SP2 Laser Scanning Confocal microscope. B) Total lipid raft fluorescence was quantified using a fluorescence plate reader. $P = 0.5$ comparing the change in total fluorescence between EGG and SUB groups. C) The relationship between changes in total lipid raft fluorescence and PBMC cholesterol content, and HOMA-IR as determined by Pearson correlation.
**Cholesterol gene expression**

Given the trend toward a reduction in cellular cholesterol content observed in the EGG group, we further aimed to identify whether this change corresponded to alterations in the expression of genes related to cholesterol efflux (ABCA1 and ABCG1), synthesis (HMG CoA Red), and uptake (LDL-R). Interestingly, while no changes in ABCG1 mRNA expression were observed in either group (**Figure 6.3 B**), ABCA1 mRNA expression significantly increased from baseline to week 12 in the EGG group only (**Figure 6.3 A**). This change corresponded to a trend toward greater increases in ABCA1 protein from baseline when compared to the SUB group (**Figure 6.3 E**), as well as a trend toward significant change compared to the SUB group (**Figure 6.3 F**). Similar EGG group-specific increases in HMG CoA Red mRNA expression were observed from baseline to week 12 (**Figure 6.3 C**), whereas no changes in LDL-R mRNA expression were observed in either group (**Figure 6.3 D**).
Figure 6.3 Egg intake during carbohydrate restriction alters PBMC cholesterol gene expression

Effects of egg intake during carbohydrate restriction on PBMC cholesterol gene. Values are presented as mean ± SEM. mRNA expression was determined by real time PCR, with data normalized to 18S rRNA. ABCA1 protein was determined by ELISA.

A) ABCA1 mRNA (P = 0.013, *P = 0.9)

B) ABCG1 mRNA (P = 0.9, *P = 1.0)

C) HMG CoA Reductase (P = 0.010, *P = 0.9)

D) LDL-R (P = 0.4, *P = 0.7)

E) ABCA1 protein (ng/1 x 10^6 cells) (P = 0.078, *P = 0.6)

F) % Change in PBMC ABCA1 protein (ng/million cells) from baseline (P = 0.078, #P = 0.57, *P = 0.6)
Discussion

Leukocyte cholesterol flux plays an important role in the regulation of cellular inflammatory potential, with important implications for metabolic disease and immunity. In this study, we have provided evidence that 12 weeks of daily whole egg consumption during moderate carbohydrate restriction alters leukocyte cholesterol flux, corresponding to the changes in HDL composition, cholesterol efflux, and PBMC inflammatory potential described in previous chapters. These findings highlight a novel and exciting role for diet in modulating the relationship between cellular inflammation and HDL metabolism.

In this study, we observed that egg consumption trended toward decreasing PBMC cholesterol content after 12 weeks. Previous studies have similarly reported changes in leukocyte cholesterol content through diet, although results have differed. In healthy men and women following an isolcaloric diet while maintaining dietary cholesterol < 250 mg/day, increasing dietary fat from an average of 17% of energy (“low-fat”) to 34% (“high-fat”) for 10 days resulted in enrichment of leukocytes in cholesterol (3-fold) and phospholipids [34]. The cholesterol:phospholipid ratios further increased, as did leukocyte cholesterol ester synthase (CES) activity. Changes in leukocyte lipid fractions were evident within 5 days of consuming the 10-day “high-fat” diet, and lasted for an additional 40 days following a reversal to the “low-fat” dietary guidelines. Interestingly, changes in leukocyte cholesterol corresponded with reductions in cholesterol within CE-rich HDL$_2$ particle fractions, and concomitant increases in cholesterol within smaller, denser HDL$_3$ fractions [34]. While our intervention provided considerably more dietary fat as a percent of total energy (~45% on average), differences between the studies may be attributable to the fact that our subjects
were actively undergoing weight loss, restricting carbohydrates, and consuming greater daily doses of egg-derived cholesterol and bioactive phospholipids [35].

Although no changes over time were observed – potentially due to a lack of available samples – changes in PBMC cholesterol content positively correlated with PBMC lipid raft content. To date, this is the first study to our knowledge that has investigated the effects of diet on leukocyte lipid rafts within the context of human intervention trials. Cholesterol serves as an essential structural component of lipid rafts, which are dynamic cholesterol-rich microdomains within the exoplasmic leaflets of the phospholipid bilayer of plasma membranes where transmembrane proteins and receptors reside – including PRRs such TLR4 [11]. Elevated levels of cellular cholesterol favor the formation of lipid rafts, and have been associated with increased pro-inflammatory responses in macrophages and T lymphocytes due to lowered cellular activation thresholds [1, 4, 5]. Given the significant role of leukocytes in immunity and chronic disease, regulation of cellular cholesterol flux and lipid raft formation may have important implications for the physiological consequences of obesity. Interestingly, changes in PBMC lipid rafts were positively correlated with changes in HOMA-IR – an estimate of basal insulin resistance [32]. This finding may be related to the role of inflammatory lymphocytes in the progression of T2DM [33], in addition to impaired ABCA1-mediated cholesterol flux and expression under conditions of insulin resistance [36], as discussed in further detail below.

In addition to changes in PBMC cholesterol content, we similarly observed changes in the expression of genes related to cholesterol flux, including ABCA1 and HMG CoA Red. Whereas expression of ABCA1 is traditionally regulated via LXR-mediated transcriptional activation under conditions of elevated cellular cholesterol levels, reduced cellular
cholesterol levels promote sterol regulatory element binding protein-2 (SREBP2)-mediated expression of HMG CoA Red – the rate-limiting enzyme for cholesterol synthesis. This pathway serves as a mechanism to replenish cellular cholesterol when it has become depleted [37]. Therefore, the increase in PBMC HMG CoA Red mRNA expression observed in our study corresponds to lower cellular cholesterol levels.

Conversely, the increased levels of ABCA1 expression observed in this study may be due to the improved metabolic and inflammatory milieu from egg intake during moderate carbohydrate restriction. As mentioned above, ABCA1 expression is reduced in patients with obesity-related metabolic disease [16-19], models of insulin resistance and diabetes [36], and by pro-inflammatory mediators such as TNFα and CRP [21, 22]. We have previously demonstrated the whole egg intake during moderate carbohydrate restriction reduces TNFα and SAA in this same MetS population [38], whereas no changes were observed in the group consuming egg substitute. Further, while HOMA-IR was reduced in all subjects over time, it was reduced to a greater extent in subjects consuming whole eggs [31]. Together, these findings suggest that whole egg intake during moderate carbohydrate restriction promotes global metabolic improvements that favors ABCA1 expression.

In line with previous findings within the literature, the findings from our study and previous chapters demonstrate the dynamic relationship between PBMC inflammatory potential, cholesterol flux, and ABCA1 expression. As mentioned above, ABCA1 has repeatedly been shown to serve as a link between cellular cholesterol flux and inflammatory potential. Landry et al. [39] demonstrated that ABCA1 expression leads to significant redistribution of cholesterol and sphingomyelin from lipid rafts to non-raft regions of cell membranes through its ATPase-related and efflux functions [39]. ABCA1-mediated
reductions in lipid raft content have been shown to increase ADAM17-mediated cleavage of TNF and TNF receptors, which may result in reduced TNFα signaling [40]. Lipid raft structure also affects TLR signaling, as TLR4 and MyD88/TRIF-mediated inflammatory gene expression was significantly increased in peritoneal macrophages isolated from ABCA1−/−, ABCG1−/−, and ABCA1−/−ABCG1−/− mice [2]. ApoA-I-ABCA1 interactions have also been shown to trigger JAK2-mediated activation of STAT3, which can suppress LPS-induced pro-inflammatory gene expression of TNFα and IL-6 in macrophages [41, 42]. Together, these findings support our observation that egg intake concomitantly increases ABCA1 expression while blunting increases in LPS-induced cytokine secretion observed from carbohydrate restriction.

In conclusion, we have demonstrated that habitual whole egg intake during moderate carbohydrate restriction modulates PBMC cholesterol flux – a phenomenon that is most likely attributable to increased ABCA1 expression. These findings correspond to the changes observed in PBMC inflammatory potential, HDL lipid composition, and cholesterol-accepting capacity of serum described in previous chapters. While future studies are needed to determine the functional consequences of these changes in relation to metabolic disease progression, these findings highlight a novel and powerful role for diet in the treatment of inflammation and dyslipidemia.

References

transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. 


Chapter 7

Significance and future directions
Summary of dissertation findings

Obesity is associated with dysfunctional lipoproteins, low-grade inflammation, and an increased risk of chronic diseases such as CVD, T2DM, and cancer [1]. As outlined in this dissertation, HDL and leukocytes play well-documented independent roles in chronic disease development, whereas HDL-leukocyte interactions further modify cellular cholesterol flux and inflammatory potential [2, 3]. Therefore, therapeutic strategies that alter HDL-leukocyte dynamics may have profound effects on obesity-related disease progression.

In line with this theory, the purpose of this dissertation was to evaluate the effects of dietary manipulation on the dynamics between leukocyte inflammation, cholesterol flux, and HDL function in MetS – a population at high risk for CVD and T2DM [4]. Given the capacity of dietary carbohydrate restriction and daily whole egg consumption to promote improvements in HDL profiles and inflammatory markers [5-8], we hypothesized that this dietary strategy was a potential therapeutic candidate to favorably modulate the relationship between HDL function and the inflammatory potential of leukocytes.

Thirty-seven men and women classified with MetS consumed a moderate carbohydrate-restricted diet in addition to either 3 whole eggs per day (EGG) or the equivalent amount of egg substitute (SUB) for 12 weeks. In regard to HDL function, we discovered that carbohydrate restriction increased HDL-CE/TG ratios, whereas the addition of daily whole egg intake resulted in enrichment of HDL in phosphatidylethanolamine and egg yolk-derived sphingomyelin species. Whole egg feeding further increased the cholesterol-accepting capacity of subject serum from macrophage foam cells [9].

We further assessed the effects of this intervention on PBMC inflammation. While no changes in IL-1β, IL-6, and TLR4 mRNA expression were observed throughout the
intervention, TLR4 mRNA expression was increased by whole egg intake, positively correlating with NF-κB p65 DNA binding activity. However, LPS-induced IL-1β and TNFα secretion was increased from baseline to week 12 in the egg substitute group only. To determine whether HDL-mediated cholesterol flux played a role in PBMC inflammation, we assessed parameters of cholesterol flux. Compared to baseline, there was a trend toward a reduction in PBMC cholesterol content in the whole egg group, whereas changes in PBMC cholesterol positively correlated with changes in lipid raft content. These observations corresponded to increases in ABCA1 and HMG-CoA Red mRNA expression from whole egg intake only, in addition to a trend toward increased ABCA1 protein. Together, these findings suggest that HDL-leukocyte dynamics can be favorable modulated through diet in MetS.

Significance and novelty of dissertation work

This dissertation has explored multiple novel areas of research within the context of human nutrition, with significant implications for obesity-related disease risk and treatment. In Chapter 4, we demonstrated the egg feeding during carbohydrate restriction modulates HDL lipid composition and increases the cholesterol-accepting capacity of human serum [9]. We have previously observed presumably favorable shifts in HDL particle sizes and increases in HDL-C from habitual egg feeding [5, 7, 10, 11]. However, as data presented in the literature suggests, the true atheroprotective nature of HDL is best determined via measures of functional capacity – including its ability to serve as a cellular cholesterol acceptor [12]. Therefore, this study is significant and the first to our knowledge to demonstrate that increases in HDL-C and HDL particle size from egg consumption during carbohydrate
restriction corresponds to modulation of HDL lipid composition and increase in the overall lipid-accepting capacity of serum in MetS.

The findings from this first study not only have significant implications for the role of diet in atherosclerosis development and treatment, but, when coupled with findings presented in Chapters 5 and 6, also further support the role of HDL in mitigating leukocyte inflammation. To the best of our knowledge, this is the first comprehensive human intervention study to have examined the role of diet in modulating dynamics between leukocyte inflammation, cholesterol flux and lipid rafts, and HDL function. Overall, these findings may have profound implications for the capacity of diet to regulate metabolic disease progression and immunity.

Future directions

The findings presented throughout this dissertation have raised additional questions worthy of further research. While we have demonstrated that whole egg intake during carbohydrate restriction appears to increase the cholesterol-accepting capacity of HDL in serum [9], it could be beneficial to assess the effects of this intervention on additional atheroprotective functions of HDL – including antioxidant, anti-inflammatory, and vasodilatory activity. Given the known anti-thrombotic properties of PE, it may be reasonable to hypothesize that egg feeding may further promote anti-thrombotic HDL activity as it promotes HDL-PE enrichment [13].

Given the capacity of egg feeding to promote incorporation of yolk-derived phospholipids into HDL [9], it may be further beneficial to assess the presence of oxidized phospholipid (OxPL) species in HDL. As mentioned in previous chapters, OxPL and
associated metabolites have been shown to inhibit LPS-induced inflammatory signaling in macrophages, endothelial cells, and smooth muscle cells through alteration of lipid raft and caveolar processing and through competitive interaction with TLR accessory proteins, such as MD-2, CD14, LPS-binding protein [14-16]. Together, the presence of OxPL inhibits LPS-induced membrane translocation of LPS receptor complexes that are required for signaling and propagation of pro-inflammatory cytokine production [15, 16]. Compared to other dietary phospholipid sources, egg-derived phospholipids have been shown to more susceptible to copper- and pH-induced oxidation due to its relatively higher content of long-chain PUFAs [17]. Generation of oxidized phospholipid metabolites by gut microflora has also been reported in response to egg feeding [18]. The presence of HDL-OxPL from our intervention may help explain the increases in LCAT activity from egg feeding [5, 7], as well as the decreased responsiveness of PBMC to LPS [14].

Future studies may also benefit from investigating the effects of this intervention on specific cell types and subsets within PBMC fractions. As mentioned in previous chapters, PBMCs consist of mixed leukocyte population containing approximately 60-70% T cells, 5-15% B cells, 5-10% natural killer cells, 0.5-2% dendritic cells, 15-30% monocyte/macrophages, and 2% granulocytes [19-21]. Evidence suggests that the effects observed from this intervention may be variable across cell populations, and may have different implications for immunity and disease. For example, TLR4 protein is expressed intracellularly in naïve human CD4⁺ T cells, whereas cell surface TLR4 expression is found in activated CD4⁺ T cells [22]. Cell surface expression of TLR4 is associated with increased anti-inflammatory T_{reg} cell activation, as well as an enhancement of T_{reg} cell-mediated suppressive functions [23]. Further, certain subsets of “inflammatory” CD16⁺ monocytes
have been shown to be more likely to interact with activated endothelium, extravasate into
the arterial wall, and differentiate into macrophages [24, 25]. CD14⁺/CD16⁺ monocytes have
been associated with hypercholesterolemia, as well as an increased incidence of CHD [26, 27]. Therefore, it would be interesting to determine whether this intervention has differential
effects on leukocyte subsets.

Finally, given the observed effects on PBMC cholesterol flux and inflammatory
potential in response to LPS, it is important to further investigate the effects of this
intervention on immunity. Obesity has been associated with impaired immune responses to
infection and influenza [28], in addition to increased risk of allergy and autoimmune disease
[29, 30]. Calorie restriction and weight loss have been shown to enhance T cell-mediated
immune responses in overweight adults, whereas dietary lutein – a carotenoid abundant in
egg yolk – was shown to possess immunomodulatory activity in various animal models [31, 32]. As highlighted in previous chapters, ABCA1-mediated cholesterol flux and expression,
have been shown to possess immunomodulatory activity that alters an organism’s capacity to
fight bacterial infection [33]. Therefore, it is plausible to hypothesize that future studies may
uncover an effect of egg intake during carbohydrate restriction on immunity.

Taken together, the work presented in this dissertation has highlighted a novel an
exciting role for diet in the modulation of leukocyte inflammation, cholesterol flux, and HDL
function in MetS. These findings have significant implications for the role of dietary
intervention in the treatment of obesity-related metabolic disease and immunity.
References


14. Erridge C, Kennedy S, Spickett CM, Webb DJ: Oxidized phospholipid inhibition of toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4: roles for CD14,


