DISSE RTATION

LIPIDS AND OXIDATIVE STRESS AS MEDIATORS OF ENDO THELIAL PATHOPHYSIOLOGY IN OBESITY

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ABSTRACT

LIPIDS AND OXIDATIVE STRESS AS MEDIATORS OF ENDOTHELIAL PATHOPHYSIOLOGY IN OBESITY

Because obesity is a well established independent risk factor for diabetes and coronary artery disease (CAD), it is important to identify factors associated with obesity that are responsible for disease progression and interventions to decrease risk of developing obesity associated co-morbidities. Two of the many mediators of obesity associated risk for diabetes and CAD are oxidative stress and oxidized phospholipids, which have been implicated in vascular disease initiation and progression through endothelial cell activation, macrophage recruitment and advanced plaque rupture.

Plasma platelet activating factor acetylhydrolase (Lp-PLA₂) is an enzyme that circulates bound to LDL cholesterol and degrades platelet activating factor (PAF), a potent inducer of the platelet coagulation cascade and thrombosis. In addition, Lp-PLA₂ degrades oxidized phospholipids to lysophospholipid products and fatty acids that may also induce inflammatory changes in multiple cell types, including vascular endothelial cells.

Exogenous antioxidant supplementation has been examined as a means of decreasing vascular oxidative stress, however, exogenous antioxidants have little or no
effect on CVD outcomes, and in some cases it may increase mortality. A novel approach to protecting cells from oxidative stress is to increase cellular endogenous antioxidant defenses. NF-E2-related factor 2 (Nrf2) is a transcription factor that binds to the antioxidant response element (ARE) promoter region of many genes including phase II antioxidant enzymes. Protandim is a combination of phytochemicals that is thought to induce Nrf2 stabilization and translocation to the nucleus, with subsequent increases in phase II antioxidant enzymes and protection against oxidative stress.

The overall objectives of the three studies we performed were to 1) globally analyze obesity associated lipid and oxidative stress using lipidomics techniques 2) determine the effects of identified obesity associated oxidative and lipid stress on the vascular endothelium 3) determine whether Protandim treatment could protect vascular endothelial cells from an oxidative challenge, and 4) characterize Lp-PLA₂ in human adipose and skeletal muscle.

Experiment 1 tested the hypothesis that oxidized phospholipids would be greater in morbidly obese gastric bypass patients compared to lean surgical controls, and that global lipid profiles would differ between groups. To test this hypothesis we performed a combined targeted and global lipidomic analysis of plasma lipids from morbidly obese gastric bypass patients and lean controls. We identified a group of ether-linked lipids that were greater in obese subjects compared to lean, and further examined whether a representative lipid from this group induced pathophysiological phenotypic changes in vascular endothelial cells.
Experiment 2 tested the hypothesis that Protandim would protect human coronary artery endothelial cells (HCAEC) against an oxidative challenge by increasing phase II antioxidant enzymes in a Nrf2 dependent manner. To do this we performed a series of in vitro experiments treating HCAEC with Protandim and determined that Protandim induced Nrf2 nuclear localization, increased phase II antioxidant enzyme expression, and protected cells from undergoing apoptosis in response to an oxidative challenge. Silencing Nrf2 prior to the oxidative challenge inhibited the Protandim induced protection.

Experiment 3 tested the hypothesis that Lp-PLA₂ would be detectable in human adipose tissue and that Lp-PLA₂ would be greater in adipose from morbidly obese gastric bypass patients compared to lean. In addition, we examined whether adipose Lp-PLA₂ may be related to circulating Lp-PLA₂ activity, inflammation, and glucose intolerance.

We have identified ether-linked lipids that are elevated in obese subjects compared to lean. We found that Lp-PLA₂ is expressed in human adipose for the first time, adipose Lp-PLA₂ is co-localized with macrophages, and report relationships between Lp-PLA₂ and indices of glucose homeostasis and inflammation. Lastly, we found that Protandim protects endothelial cells from an oxidative challenge in a Nrf2 dependent manner. Collectively, these data provide insight into the oxidative and lipid stress milieu that occurs in obese subjects.
# TABLE OF CONTENTS

Abstract ......................................................................................................................................................... ii

Table of Contents .......................................................................................................................................... v

Chapter I – Introduction and Experimental Hypotheses .............................................................................. 1

Chapter II - Manuscript I ............................................................................................................................ 21

   Lipidomic analysis of human plasma reveals novel lipids of interest that are elevated in morbidly obese humans compared to lean.

Chapter III – Manuscript II ......................................................................................................................... 64

   Protandim induced Nrf2 activation protects human coronary artery endothelial cells against an oxidative challenge

Chapter IV – Manuscript III ......................................................................................................................... 96

   Lipoprotein-associated Phospholipase A(2) is expressed in human adipose tissue and correlates with HbA1C

Chapter V – Conclusions ............................................................................................................................. 124

Appendix and Supplementary Material ..................................................................................................... 127

References

Manuscript 1 CSU and PVH Informed Consents, and HIPPA Clearance

Medical and Exercise History
CHAPTER I – INTRODUCTION AND EXPERIMENTAL HYPOTHESES

Introduction and Experimental Hypotheses

With rates of obesity increasing, and with an associated increased incidence of diabetes and cardiovascular disease (CVD), research in the area of obesity associated CVD pathology is a public health necessity. CVD is the leading cause of death in the United States, and coronary artery disease (CAD) is responsible for 68% of all CVD deaths [2]. Currently 65% of Americans are overweight, with over 30% of those individuals qualifying as obese [3]. The Framingham Heart Study has identified risk factors for CAD, including age, obesity, hypercholesterolemia, diabetes and hypertension. Obesity related hypertension, elevated low density lipoprotein (LDL) cholesterol, reduced high density lipoprotein (HDL) cholesterol, dyslipidemia (elevated triglycerides and ectopic fat distribution), and oxidative stress all contribute to CVD pathology, and specifically to atherosclerosis and cardiovascular remodeling. Increased carotid artery intima thickness (CIT), impaired ventricular function, and increased ventricular wall thickness have also all been observed in obese subjects compared to lean [4]. Insulin resistance, hyperglycemia, advanced glycation end product formation, and diabetes associated oxidative stress all also contribute to vascular disease [5-7]. While obesity associated pathophysiology and co-morbidities are complicated and
multi-faceted, two significant contributors to obesity-related CAD risk are reactive oxygen species (ROS) and dyslipidemia [4, 8-17]. The purpose of the projects described in this dissertation was to characterize the pathophysiology of obesity associated lipid and oxidative stress, and identify potential interventions to protect vascular endothelial cells against disease initiation and progression.

**Lipid and Oxidative Stress**

*Oxidized phospholipids*

Lipid oxidation can be ROS mediated or can occur enzymatically [18-20], resulting in a variety of products [19, 20]. Oxidized phospholipid components of LDL, which are increased in human obesity, can induce endothelial cell activation and pro-atherosclerotic changes in vascular endothelial cells [15]. Endothelial cell activation is a phenotypic shift characterized by expression of inflammatory mediators and cell adhesion molecules. Phospholipids containing arachidonate and linoleate, as well as unesterified arachidonate and linoleate, can be enzymatically oxidized by lipoxygenases (LOX), which may also cause endothelial cell activation [21-24] [19].

Phosphatidylcholine (PC) is the most abundant phospholipid product in mammalian cells, and PC oxidation products have been widely examined [25]. PCs that have polyunsaturated fatty acids, particularly arachidonate, at the SN-2 position are especially susceptible to oxidation, and this results in a variety of potential oxidized products. One such lipid is 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC), which is a major cell membrane constituent susceptible to oxidative damage.
PAPC and several products of PAPC oxidation including 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC), 1-palmitoyl-2-(5,6-epoxyisoprostane E₂)-sn-glycero-3-phosphorylcholine (PEIPC), 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC), and lysophosphatidylcholine (lyso-PC) have been shown to activate endothelial cells and are implicated in atherosclerotic progression [15, 17, 26, 27]. These same oxidized phospholipids are also found deposited in atherosclerotic plaques [15, 17]. PAPC, POVPC, PGPC, and PEIPC are increased 10-25 fold in circulating modified LDL, and 3-6 fold in vascular lesions in rabbits fed an atherogenic diet. This increase in oxidized phospholipids in the vascular lesions is 10-20 times the amount needed to activate endothelial cells [15].

Oxidized phospholipids induce endothelial cell activation, in particular increasing expression of cell adhesion molecules and chemoattractant proteins involved in monocyte and neutrophil recruitment and binding. [9, 10, 12, 18, 28, 29]. For example, POVPC increases monocyte chemoattractant protein 1 (MCP-1) expression and monocyte binding to endothelial cells [27]. PGPC induces monocyte and neutrophil binding and vascular cell adhesion molecule-1 (VCAM-1) [15, 27]. PEIPC can stimulate an increase in MCP-1 in a dose dependent manner in human aortic endothelial cells (HAEC) [16]. Lyso-PC also stimulates MCP-1, as well as VCAM-1 and intracellular cell adhesion molecule (ICAM-1) [26]. PAPC alone does not induce ICAM-1, or VCAM-1, but does stimulate MCP-1 and monocyte binding [30].
Enzymatically oxidized lipids

Both non-esterified polyunsaturated fatty acids (PUFA), particularly arachidonate and linoleate and PUFA as components of phospholipids, are susceptible to oxidation by LOX [19]. The resulting products include hydroperoxyeicosatetraenoates (H(p)ETEs), hydroperoxyoctadecadienoates (H(p)ODEs), PL-HETEs and PL-HODEs which are formed in multiple cell types including macrophages, adipocytes, and endothelial cells. LOX can also directly oxidize LDL although the mechanisms by which this occurs are not fully understood [31]. LOX products may play a role in the beginning stages of atherosclerosis. A mouse model over-expressing 12/15 LOX, which resulted in elevated production of 12SHETE, also had increased MCP-1 and ICAM expression and increased monocyte binding to the endothelium [32]. A subsequent study found that 12/15LOX knockout mice crossbred to apoE-deficient mice, compared to atherosclerotic apoE deficient mice, showed a decrease in monocyte adhesion to the aorta, an effect that was mimicked by a LOX inhibitor [33]. HETE Isomer type may be important, as evidenced by 12SHETE, but not its stereoisomer 12RHETE (which is not a product of LOX peroxidation), increasing MCP-1 RNA and protein in macrophages [23]. In addition, LOX protein and products have been found localized in atherosclerotic lesions from humans [34]. Thus, whether formed via direct oxidation or enzymatically, it is clear that oxidized lipids in the endothelium contribute to the pathophysiology of atherosclerosis.
Platelet activating factor and Lp-PLA₂

Platelet activating factor (PAF) and the acylhydrolases that degrade PAF have been implicated in diabetes and CAD. PAF, a pro-inflammatory ether-linked lipid, and

plasma platelet activating factor acetylhydrolase/ lipoprotein associated phospholipase A₂ (Lp-PLA₂) may be involved in vascular cell pathology. Currently there is a lack of consensus as to whether Lp-PLA₂ is pro- or anti-inflammatory. PAF acts on multiple cell types, is a potent stimulator of the platelet coagulation cascade, inflammation, and thrombosis, and is constitutively synthesized through a de novo mechanism as well as

Figure 1. PAF metabolism including de novo and remodeling pathways.
through a remodeling pathway (Figure 1 [1]). However, it is through the remodeling pathway that most inducible PAF synthesis occurs [35, 36]. Pro-inflammatory and pro-thrombotic mediators including TNF-α stimulate PAF synthesis through the remodeling pathway [37]. In the remodeling pathway, an sn-1 ether-linked phosphocholine, usually with an arachidonate or other polyunsaturated fatty acid at the sn-2 position, is acted on by a phospolipase resulting in the removal of the sn-2 chain. If the sn-2 chain is arachidonate, the arachidonate is then available for eicosanoid synthesis or other biological processes. The lyso-PAF that is left is acetylated by a lyso-PAF acetylhydrolase that transfers an acetyl group from acetyl CoA to the sn-2 position of the lyso-PAF [1]. Thus ether-linked phosphatidylcholine species function as substrates in the formation of PAF. Increased PAF also induces the expression of PAF-acetylhydrolases, however to what degree has yet to be fully determined [37].

Lp-PLA₂ is one member of a family of PAF acetylhydrolases (PAF-AH) that degrade PAF. Specifically Lp-PLA₂ specifically refers to the circulating form of PAF-AH [38]. While Lp-PLA₂ has been detected in several cell types including macrophages, rat hepatocytes and rat adipocytes in culture, circulating Lp-PLA₂ is generally thought to come from cells of hematopoetic origin, primarily macrophages [38]. Lp-PLA₂ is present in the circulation bound to either LDL or HDL. While it can transfer between LDL and HDL, Lp-PLA₂ activity is significantly decreased when bound to HDL [39]. Only approximately 1 in 1,000 LDL particles carries Lp-PLA₂ and apolipoprotein B-100 is essential for interaction between LDL and Lp-PLA₂. Lp-PLA₂ associates most strongly with small dense LDL, but activity is 2-4 fold lower when associated with small dense LDL.
compared to other LDL fractions [40]. Lp-PLA₂ can also be glycosylated [41], though the function of this modification has not been thoroughly examined. The single study to examine the role of glycosylation found that it likely is involved in association of the enzyme with HDL; removal of Lp-PLA₂ glycosylation led to increased association of Lp-PLA₂ with HDL [41].

In addition to degrading PAF, Lp-PLA₂ also hydrolyzes glycerophospholipids resulting in lysophospholipid and free fatty acid products. Lp-PLA₂ affinity is specific to species containing sn-2 fatty acid chains of 6 carbons or fewer, and glycerophospholipids with longer sn-2 fatty acid side chains that have undergone oxidative modification [40]. The products of this breakdown are lysophospholipids and in some cases oxidized fatty acid chains including arachidonic acid and its derivatives [40, 42-44]. Thus, whether Lp-PLA₂ degrading oxidized phospholipids is pro- or anti-inflammatory is debated as the lysophospholipid species that result from this breakdown also have detrimental effects on the endothelium. Lysophosphatidylcholine (LPC) is a component of oxidized LDL and its presence in oxidized LDL particles is 5-fold higher than in native LDL [44]. LPCs have been shown to increase plasminogen activator inhibitor-1, a potent prothrombotic and proatherogenic protein, in adipocytes [45]. In addition, LPCs can induce endothelial cell expression of cell adhesion molecules, an important step in endothelial cell activation and atherosclerotic progression [26] [46].

Several studies have shown an association between increased plasma Lp-PLA₂ activity and CAD, and drugs to inhibit Lp-PLA₂ activity in CAD patients are in phase III
clinical trials [47, 48]. Kinney et al. showed that Lp-PLA₂ activity is independently associated with coronary artery calcification [49], and Herrmann et al. found that elevated Lp-PLA₂ in carotid artery plaques results in higher risk for cardiac events [50]. Inhibition of Lp-PLA₂ in a swine model showed that inhibiting Lp-PLA₂ in diabetic and hypercholesterolemic animals decreased atherosclerotic lesion content of LPC and plaque area, and reduced the incidence of necrotic and unstable plaque phenotypes [51]. Decreasing necrotic core size and stabilizing lesions is of particular interest as necrotic core rupture induces thrombosis, and Lp-PLA₂ is elevated in advanced, necrotic plaques, co-localized with macrophages [52]. In addition, inhibition of Lp-PLA₂ reduced expression of inflammatory genes in whole coronary artery homogenates [51].

Other data suggest Lp-PLA₂ may be protective against CAD [53, 54]. Delivery of liver directed Lp-PLA₂ into apoE null mice increased circulating Lp-PLA₂ activity, and reduced macrophage recruitment and adhesion through decreasing PAF like lipids in VLDL. This effect is attributed to increased association of Lp-PLA₂ with HDL [54]. An additional study in apoE null mice using the same method for increasing Lp-PLA₂ showed an increase in plasma activity accompanied by a decrease in modified-LDL and LPC/PC ratio [53]. Following carotid artery denudation, animals that had received exogenous Lp-PLA₂ had less oxidized LDL, macrophages, and smooth muscle cells in the intimal space, in addition to a decreased lesion area and volume [53]. The decrease in lesion size was only significant in males suggesting a sex specific effect. Studies in humans have also shown sex differences in Lp-PLA₂ activity, with men having higher plasma activity than women. Post-menopausal women generally have increased activity
compared to pre-menopausal women, which can be decreased with some types of hormone therapy [55, 56]. Whether Lp-PLA$_2$ activity attenuates or exacerbates CAD may depend on other factors including total cholesterol, and whether inhibiting Lp-PLA$_2$ is beneficial or not may depend on the stage and severity of the plaque. It may be that higher Lp-PLA$_2$ activity is beneficial in preventing CAD initiation, but in advanced plaques it promotes rupture and thrombosis in mature plaques.

Data now show that Lp-PLA$_2$ activity may be predictive of diabetes in humans (in review), though mechanisms to explain this have yet to be examined. Both Lp-PLA$_2$ activity in insulin sensitive tissues and circulating Lp-PLA$_2$ may be involved. If increased Lp-PLA$_2$ activity results in increased degradation of phospholipids to lysophospholipids that induce inflammation in insulin sensitive tissues and the vascular endothelium, then inhibiting Lp-PLA$_2$ may be a beneficial intervention in diabetes and CVD. Two major insulin sensitive tissues involved in development of diabetes are skeletal muscle and adipose. It has been demonstrated that LPC can induce insulin resistance in skeletal muscle, an effect that was ameliorated by inhibiting LPC formation [57]. While Lp-PLA$_2$ has been found in rodent adipocytes [58], its role in adipose tissue and skeletal muscle has not been examined. As noted earlier, most circulating Lp-PLA$_2$ is thought to be macrophage derived, and macrophage infiltration of adipose tissue is a hallmark of obesity. Thus it is likely that Lp-PLA$_2$ is present and active in human adipose tissue though this has yet to be examined, and it is unknown whether tissue Lp-PLA$_2$ could be implicated in obesity associated diabetes.
Reactive oxygen species and antioxidant defense

Oxidation of lipids is one of the ways by which increased ROS production may contribute to chronic disease states. Broadly, the term ROS refers to compounds containing oxygen molecules that are chemically reactive [59]. ROS function normally as cell signaling molecules and are generated through multiple mechanisms including the mitochondrial electron transport chain, uncoupled nitric oxide synthase, xanthine oxidase, and NADPH oxidase. When production of ROS exceeds antioxidant defense oxidative stress occurs. The most commonly described ROS include the hydroxyl radical (\(\text{OH}^-\)), superoxide (\(\text{O}_2^-\)) and hydrogen peroxide (\(\text{H}_2\text{O}_2\)). Production of ROS is normally tightly countered by extensive intracellular antioxidant systems that include iron containing species, thiols, and antioxidant enzymes. Well-characterized antioxidant enzymes include but are not limited to superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, peroxiredoxins and heme oxygenase. Oxidized LDL is a common systemic marker of oxidative stress, while several other markers are commonly used as indicators of oxidative damage to lipids, proteins, and nucleotides. Malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) are commonly used as indicators of lipid peroxidation. Protein carbonyls are a common marker of oxidative damage to proteins, and 8-hydroxy-2’-deoxyguanosine (8-OHdG) is used as a marker of DNA oxidation. There is still a lack of consensus on the utility of circulating markers of oxidative stress such as oxidized-LDL and MDA as clinical predictors of future coronary events [60].
**Oxidative stress in obesity**

There is a significant body of literature demonstrating increased oxidative stress with obesity. As visceral adipose mass increases, secretion of inflammatory adipokines by adipocytes and resident macrophages increases. Proinflammatory cytokines such as TNF-α, IL-6, and IL-8 have been shown to increase ROS production in multiple tissues and cell types including adipose, monocytes/macrophages, and endothelial cells [61]. Furukawa et al. showed that increased obesity was strongly associated with markers of systemic oxidative stress [62]. In addition, adipose tissue ROS production increased with obesity and elevated circulating free fatty acids. The increase in adipose tissue ROS occurred through increased NADPH oxidase expression, and this increase in oxidative stress led to increased secretion of inflammatory adipokines including IL-6, plasminogen activator inhibitor-1 (PAI-1), and MCP-1 [62]. Importantly, in obese mice, inhibition of NADPH oxidase led to decreased adipose ROS production, attenuated ROS-induced increases in inflammatory cytokines and improved blood glucose and insulin concentrations [62]. Increased vascular NADPH oxidase expression has been observed in obese subjects compared to lean with concomitant elevation in markers of protein oxidative damage and inflammation [14]. Collectively these data support a scenario where increased adipose tissue secretes proinflammatory cytokines, which increase adipose and systemic oxidative stress. This oxidative stress leads to additional inflammatory cytokine production and a continued vicious cycle occurs leading to progression of systemic metabolic disease. Weight loss can improve the systemic and tissue pro-oxidative profile. In bariatric surgery patients, oxidative stress and
inflammation markers improve with significant weight loss [63]. In addition, one year post-surgery after significant weight loss, plasma MDA was significantly decreased while total serum antioxidants were increased, and indicators of systemic inflammation and glucose status were improved [63].

*Obesity associated oxidative stress, lipid stress, and the endothelium*

Increased oxidative stress leads to oxidized LDL cholesterol, which has a well-established role in the initiation and progression of atherosclerosis and endothelial cell activation [64]. LDL cholesterol is oxidized and taken up by macrophages in the vessel wall leading to increased inflammation, foam cell production, and macrophage and endothelial cell apoptosis. Oxidized LDL adversely affects adipose tissue as well. Oxidized LDL, and specifically the LPC component of oxidized LDL, induces adipokine production in adipose. Specifically, LPC induces PAI-1, which is a potent prothrombotic cytokine, in a ROS dependent manner [45].

In the endothelium, the role of NOX superoxide production is currently a topic of research because NOX expression and activity might be causally related to vascular disease. NOX enzymes are comprised of multiple subunits, which assemble in plasma membranes and generate O$_2^-$ radicals [65]. To date, seven NOX isoforms have been identified [65]. In vascular endothelial cells NOX2 and NOX4 are the primary isoforms [65]. Superoxide generated by these isoforms plays a role in regulating cellular function, including proliferation and angiogenesis, as well as entering the circulation and
permeating the sub-endothelial space where it can induce oxidative damage [65]. In obese rats, superoxide production by NOX precedes development of myocardial dysfunction [66]. In primates with diet-induced atherosclerosis, lesion severity was related to increased superoxide production [67]. When these primates were returned to a normal diet, superoxide production returned to normal with concomitant decreases in NOX activity and NOX subunit expression [67].

The increase in oxidized phospholipids with obesity may contribute to CVD pathology through increasing NOX activity and superoxide production. Oxidized phospholipids can increase superoxide generated by NOX, and can increase NOX expression and localization to membranes in endothelial cells [13]. Treatment of endothelial cells with ox-PAPC resulted in increased NOX activity, which was abrogated by RNA silencing of NOX, and by pharmacological inhibition of NOX [11]. Ox-PAPC has also been shown to increase recruitment of cytosolic NOX subunits to the membrane [68], superoxide production by NOX4, and NOX4 subunit expression in bovine aortic endothelial cells [13]. Ox-PAPC induces increases in MCP-1 and expression of other pro-inflammatory genes through NOX4 activation and subsequent up-regulation of $\mathrm{O}_2^-$ signaling pathways \textit{in vitro} [11]. \textit{In vivo}, a knockout mouse model for the p47 subunit of NOX and ApoE had smaller atherosclerotic lesion areas than ApoE(-/-) only mice, regardless of whether animals were fed an atherosclerotic diet. [69]. In addition, atherosclerotic progression in apoE null mice is attenuated by inhibiting both macrophage and endothelial cell NOX dependent superoxide production [70].
There is also evidence indicating a relationship between HETE products and NOX in endothelial cells [71, 72]. 20-HETE activates NOX by stimulating subunit translocation to the membrane, and increases NOX superoxide production in bovine pulmonary artery endothelial cells [72]. In addition, 20-HETE has been shown to increase superoxide in pulmonary artery endothelial cells via NADPH oxidase activation [72], and increases in monocyte MCP-1 expression and secretion stimulated by 12-HETE were attenuated by NOX inhibition [23].

Studies in humans confirm the relationship between oxidized phospholipids, NOX, obesity and CVD [6, 14, 73, 74]. Compared to non-obese controls, overweight and obese subjects demonstrate increased NOX subunit expression in the vascular endothelium, as well as augmented oxidative stress [14]. NOX subunit expression and NOX activity are greater in vessels of diabetics undergoing coronary artery bypass [6]. Increased NOX activity is associated with clinical risk factors for atherosclerosis in humans including hypercholesterolemia and diabetes [73]. NOX subunit expression is elevated in lesions of coronary arteries in bypass graft patients, particularly in the vicinity of macrophages, and NOX expression levels correlate with severity of atherosclerosis [74].

Impaired antioxidant defense systems may contribute to a pro-oxidant environment in endothelial cells. SOD activity is decreased in coronary arteries of humans with clinical CAD compared to controls [75]. Heme oxygenase and glutathione peroxidase are also decreased in the aortas of high fat diet-fed animals [76]. Total
plasma antioxidant capacity is decreased in morbidly obese subjects compared to lean, but improves after bariatric surgery [77]. Antioxidant defenses increase in response to increased ROS production, however with chronic overproduction of ROS the capacity of antioxidant systems to respond is decreased, diminishing overall antioxidant capacity.

In summary, there is a role for oxidized lipids and ROS in endothelial cell activation and pathogenesis of vascular disease. However, much research is still needed to fully understand how obesity-related oxidative and lipid stress leads to diabetes and CVD, and thus understand how to intervene to decrease obesity and CAD-related mortality.

**Obesity and Oxidative Stress Interventions**

*Bariatric surgery*

With the increasing prevalence of obesity and the subsequent risk of co-morbidities, bariatric surgeries, including Roux-en-Y gastric bypass and laparoscopic banding, have emerged over the past decade as a safe and relatively effective obesity treatment. Current data indicate that successful surgeries result in an average loss of 60% of excess weight [78]. Epidemiological reports show that CVD risk, as classified by Framingham criteria, decreases significantly as early as six months following surgery [79]. To qualify for Roux-en-Y gastric bypass, a patient must have a body mass index greater than 40 kg/m² or a BMI greater than 35 kg/m² with co-morbidities. The bariatric population is a useful model for studying the pathophysiology of morbid obesity.
because of the unique physical characteristics of the subjects, rapid and predictable weight loss, and the ability to obtain valuable tissue during surgery that is not accessible in a standard clinical laboratory.

Antioxidant interventions

Interventions to combat oxidative stress have included supplementation with exogenous antioxidants that have been largely ineffective. Clarke et al. performed a meta-analysis on eight trials examining beta-carotene or vitamin E supplementation in healthy individuals and those at risk for CVD, and reported no protective effects of supplementation on future development of CVD [80]. Other studies report similar findings as well as data indicating that vitamin A, vitamin E and beta carotene may increase mortality in some cases [81-83], and that vitamin C can be pro-oxidant [84].

An alternative approach to combat oxidative stress is to activate the antioxidant response element (ARE) binding transcription factor NF-E2-related factor 2 (Nrf2). Nrf2 activation leads to induction of endogenous phase II antioxidant enzymes and protection from oxidative stress. Nrf2 binds to the ARE in the promoter regions of several hundred genes including many phase II antioxidant enzymes [85, 86]. Nrf2 is constitutively expressed but is marked for ubiquitination by association with Kelch-like ECH-associated protein 1 (Keap1). Activation of Nrf2 occurs when it is released from Keap1, translocates to the nucleus, heterodimerizes with small Maf or Jun proteins, binds the ARE, and initiates transcription of the associated genes. Multiple mechanisms
for Nrf2 activation have been proposed (reviewed in [85]). Electrophilic or oxidative modification to Keap1 cysteine thiol groups leads to dissociation of Nrf2 binding by Keap1 [87]. Direct phosphorylation of Nrf2 by a number of kinases, including several from the MAPK family, can also cause dissociation of Nrf2 from Keap1 [88-90]. The exact role of direct Nrf2 phosphorylation is not completely understood, as others have reported that phosphorylation of Nrf2 by multiple MAPKs may have a limited contribution to Nrf2 activation [91].

Protandim is a commercially available dietary supplement consisting of phytochemicals derived from five widely studied medicinal plants including silymarin from milk thistle, curcumin from turmeric, bacopa extract, ashwaganda, and green tea extract. It is thought that the five components of Protandim act synergistically to induce Nrf2 [92]. Phytochemical compounds, including the combination in Protandim, likely activate Nrf2 and induce phase II antioxidant enzyme expression through activation of various kinases with subsequent Nrf2 phosphorylation [93]. In humans, supplementation with Protandim significantly decreased circulating TBARS, and significantly increased erythrocyte SOD and catalase activity [94]. Data from in situ experiments in human saphenous veins show that Protandim increased SOD and catalase activity in addition to HO-1 activity, while decreasing superoxide and markers of lipid peroxidation [95]. Significant Nrf-2 dependent HO-1 induction by Protandim has also been shown in multiple cell lines in vitro, with increases in HO-1 being as high as 8.5 fold over baseline [92]. Thus, phytochemical induced Nrf2 activation is a potential
therapeutic intervention against endothelial cell oxidative stress and associated vascular disease initiation and progression.

Hypotheses and Experiments

The overall objectives of these projects were to investigate obesity associated lipid and oxidative stress, and examine strategies to protect coronary artery endothelial cells against these stressors.

Experiment 1- We hypothesized that lipid profiles would be significantly different in plasma from morbidly obese humans compared to lean, and that lipids that are elevated in obese subjects would induce inflammatory changes in cultured endothelial cells. In addition, we hypothesized that oxidized phospholipids would be greater in plasma from obese subjects compared to lean.

The first experiment was designed to use a lipidomic analysis to compare lipid distribution and oxidation in samples from lean control subjects and morbidly obese gastric bypass patients. The analysis was a combined targeted and shotgun approach. The targeted portion examined oxidized phospholipids that are known to induce endothelial cell pathogenic phenotypes. The shotgun portion involved a sample wide examination of lipid species to determine what is different between lean and obese, to identify biomarkers or pathological lipids that have not been characterized yet in this capacity.
**Experiment 2**- We hypothesized that Protandim would activate Nrf2 and induce phase II antioxidant enzyme expression in human coronary artery endothelial cells (HCAEC), and that Protandim treatment prior to an oxidative challenge would protect HCAEC in a Nrf2 dependent manner.

The second experiment was designed to examine whether Protandim can protect human coronary endothelial cells (HCAEC) from an oxidative challenge, and if that protection is Nrf2 mediated. We first examined Nrf2 nuclear localization and antioxidant enzyme protein expression in response to Protandim. To determine whether Protandim effects on Nrf2 and antioxidant enzymes protected cells from an oxidative challenge, we examined cell death in response to an oxidative challenge with and without Protandim treatment. Last, to determine if Nrf2 mediated protection, we knocked down Nrf2 prior to Protandim treatment, then examined cell death in response to an oxidative challenge.

**Experiment 3**- We hypothesized that Lp-PLA₂ would be expressed in human adipose tissue, and that expression would be greater in tissue from obese subjects compared to lean. We also hypothesized that Lp-PLA₂ expression would correlate with plasma Lp-PLA₂ activity, plasma inflammatory markers and indices of glucose tolerance.

The third experiment was designed to examine the presence of Lp-PLA₂ in different adipose depots from lean and obese subjects, and determine cell type distribution of Lp-PLA₂ within those adipose depots. In addition, plasma Lp-PLA₂ activity,
plasma TNF-α and HbA1C were measured to determine if there is a relationship between adipose Lp-PLA₂ and markers of inflammation and glucose intolerance.

**Main conclusions**

These projects describe novel investigations into lipid and oxidative stress in obesity, how these factors affect endothelial cells, and how phytochemicals in Protandim can protect endothelial cells from oxidative stressors. Experiment 1 showed that while ox-PC is not elevated in obese subjects compared to lean, a group of ether-linked phospholipids are greater in the serum of obese subjects compared to lean. In addition, these lipids induce cell adhesion molecule protein expression in endothelial cells. Experiment 2 showed that Protandim induces Nrf2 nuclear localization and phase II antioxidant enzyme protein expression, and protects endothelial cells from an oxidative challenge in a Nrf2 dependent manner. Experiment 3 showed that Lp-PLA₂ is expressed in adipose tissue, that adipose tissue Lp-PLA₂ is preferentially localized to adipose resident macrophages, and that glycosylation of adipose Lp-PLA₂ correlates with indices of glucose homeostasis.

Collectively, these data provide insight into the oxidative and lipid stress milieu that exists in obese subjects, novel information regarding tissue Lp-PLA₂ expression and relation to indices of glucose homeostasis and inflammation, and evidence supporting Protandim as an intervention to protect against endothelial oxidative stress.
CHAPTER II- MANUSCRIPT I

LIPIDOMIC ANALYSIS OF HUMAN PLASMA REVEALS NOVEL LIPIDS OF INTEREST THAT ARE ELEVATED IN MORBIDLY OBESE HUMANS COMPARED TO LEAN.

Title: Lipidomic analysis of human plasma reveals novel lipids that are greater in morbidly obese humans compared to lean.

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Dyslipidemia and oxidative stress are characteristic of obesity and diabetes, and lipid peroxidation appears to play a major role in cardiovascular disease. We performed a lipidomic analysis to explore differences in lipid profiles between plasma from lean and obese subjects, and used in vitro methods to examine a role for the identified lipids in endothelial cell pathophysiology. Plasma was collected from 15 morbidly obese and 13 lean control subjects. Lipids were extracted from plasma and analyzed using LC/MS, and MS/MS to characterize lipid profiles and identify lipids that are elevated in obese subjects compared to lean. Orthogonal partial least squares- discriminant analysis (OPLS-DA) modeling showed that lipid profiles were significantly different in obese subjects compared to lean. Analysis of lipids that were driving group separation in the OPLS-DA model and that were significantly elevated in the obese group led to identification of a group of ether-linked phosphatidylcholine and phosphatidylethanolamine lipids of interest. Treatment of human coronary artery endothelial cells with the ether-linked phosphatidylethanolamine induced expression of cell adhesion molecules, a hallmark of endothelial cell activation. We also scanned our data for oxidized phospholipids that have previously been shown to induce endothelial cell activation in vitro, and did not find any significant differences between groups in vivo. These data suggest a role for ether-linked lipids in obesity associated dyslipidemia and vascular disease.

**Keywords:** Lipidomics, obesity, dyslipidemia, endothelial cells, oxidized phospholipids.
Introduction

Dyslipidemia and oxidative stress are characteristic of obesity and diabetes, and lipid peroxidation appears to play a major role in diabetes, cardiovascular disease, and cancer [1, 2]. Lipid oxidation can be mediated by reactive oxygen species (ROS) or occur enzymatically, resulting in a variety of potential products. The mechanisms of oxidized phospholipid formation have been extensively reviewed [3, 4]. Obesity is associated with increased oxidized LDL that leads to inflammation and oxidative stress in vascular endothelial cells and contributes to the pathophysiology of the metabolic syndrome and atherosclerosis [5, 6]. The chronic inflammatory state associated with morbid obesity and the metabolic syndrome further increases the oxidative modification of phospholipids [7]. Collectively, this creates a “vicious cycle” of inflammation, oxidative stress, and lipid stress, which contributes to obesity-associated pathology. Ectopic fat deposition in morbidly obese individuals also contributes to the pathogenesis of vascular and metabolic disturbance by inhibiting insulin action and disrupting lipid metabolism in the liver, pancreas, kidney and skeletal, smooth and cardiac muscle[8]. This “lipotoxicity”, also including the sequelae associated with abnormal oxidation of lipids, contributes to inflammation and oxidative stress in multiple tissues [9] [10, 11].

Phosphatidylcholine (PC) species that contain polyunsaturated fatty acids, particularly arachidonate, at the SN-2 position are especially susceptible to free radical oxidation resulting in a variety of potential oxidized products. One such lipid
is 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC), which is a common cell membrane constituent and circulates within cholesterol particles. PAPC and several products of PAPC oxidation including 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC), 1-palmitoyl-2-(5,6-epoxyisoprostane E₂)-sn-glycero-3-phosphorylcholine (PEIPC), 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC), and lysophosphatidylcholine (lyso-PC) have been shown to induce cell adhesion molecule expression and inflammatory mediator secretion in endothelial cells and have been implicated in atherosclerotic progression [12-17]. In addition, oxidized phospholipids are found deposited in atherosclerotic plaques [18, 19]. PAPC, POVPC, PGPC, and PEIPC are increased 10-25 fold in modified LDL and 3-6 fold in lesions in rabbits fed an atherogenic diet [19]. This increase in oxidized phospholipids is 10-20 times the amount needed to induce pro-atherogenic phenotypic shifts in endothelial cells [19]. Additionally, endothelial cell apoptosis may be an independent risk factor for thrombosis [20], and PAPC, PGPC, and lyso-PC induce endothelial cell apoptosis [21-23] thus contributing to plaque progression and rupture. In addition to up-regulating expression of inflammatory mediators and cell adhesion molecules, oxidized phospholipids can induce expression of NADPH oxidase and production of reactive oxygen species (ROS), directly increasing cellular oxidative stress [24] [25]. Because PC is the most abundant cellular phospholipid, most oxidation products detected contain the choline head group [26]. However, other lipid classes and additional oxidized phospholipids are likely involved in vascular pathology, and it is
likely that interactive effects between oxidized phospholipids contribute to CVD/metabolic disease pathophysiology.

Lipidomics is broadly defined as the large-scale study of pathways and networks of cellular lipids in biological systems. Lipidomics can be used to examine the presence and structure of the range of lipid species within different tissues making it an attractive method for examining distribution of phospholipids in tissue, as well as for globally examining differences in lipids between subject groups. A broad examination of oxidized lipid species that are elevated and potentially contribute to metabolic and cardiovascular disease pathology in morbidly obese humans has not been performed. A lipidomic study of this nature could provide insight into the link between obesity and pathogenesis of atherosclerosis and facilitate discovery of lipid biomarkers that increase disease risk.

The purpose of this study was to: 1) use a shotgun lipidomics approach to examine global lipid distribution in plasma from lean and morbidly obese humans to identify lipids that are greater in the morbidly obese population that may contribute to metabolic and cardiovascular pathology, 2) examine the effects of lipids identified in our shotgun analysis as elevated in obese subjects in an *in vitro* endothelial cell model to determine whether they induce disease related phenotypic changes, and 3) use a targeted lipidomics approach to determine if there is a difference in distribution of oxidized phospholipids in plasma from lean and morbidly obese humans. We hypothesized that lipid profiles would be significantly different in plasma from morbidly obese humans compared to lean, and that lipids identified in
our shotgun approach that are elevated in obese subjects compared to lean would
induce inflammatory changes in endothelial cells. In addition, we hypothesized that
oxidized phospholipids shown previously \textit{in vitro} to cause endothelial cell
production of inflammatory mediators and cell adhesion molecules would be
greater in plasma from obese humans compared to lean.
Materials and Methods

Ethics approval

The Institutional Review Boards of Colorado State University and Poudre Valley Hospital approved this protocol (CSU protocol #-05-116H, PVH protocol #-07-874). Each volunteer was informed of the potential risks and written consent was obtained prior to enrollment. The study followed the guidelines set forth by the Declaration of Helsinki.

Study overview

A total of 15 morbidly obese gastric bypass patients, and 13 lean controls age 18-60 were recruited to participate through the Northern Colorado Surgical Associates (NCSA) of the Bariatric Center of the Rockies. Bypass patients were required to have a body mass index (BMI) > 40 kg/m², while controls were required to have a BMI < 30 kg/m². There were no other exclusion criteria allowing the subject population to be heterogeneous, and thus differences in lipid profile between groups would more closely represent the general clinical population. Subjects completed medical and exercise questionnaires prior to undergoing a venipuncture blood draw following a 12-hour fast. Blood was collected in vacutainer tubes containing EDTA, 0.5ml was aliquoted for HbA1C analysis, the remainder was centrifuged (1200g, 15 min, 4°C) and stored at -80°C until lipid extractions were performed.
Subject characteristics

Participant characteristics are shown in Table 1. We were unable to obtain body composition data because subject size exceeded the capacity of the available dual energy x-ray absorptiometry (DEXA) equipment. Height and weight were measured at the surgical center, while other demographic and health information was obtained through a health history questionnaire. Whole blood samples were sent to the University of Colorado Denver Clinical Translational Research Center for analysis of hemoglobin A1C (HbA1C) using a DCA Vantage analyzer (Siemens, Deerfield, IL).

Lipid extraction

Lipid extractions were performed using the methyl-tert butyl ether (MTBE) method described by Matyash et al. [27], with all steps performed in the Captair Pyramid glove box (Erlab, Rowley, MA) under argon gas to prevent exposure to oxygen and subsequent oxidation of lipids. The MTBE method is analogous to the traditional Folch and Bligh Dyer methods, except the organic lipid containing phase is present above the aqueous phase eliminating the need to collect the organic layer from beneath the aqueous layer. We ran a Bligh Dyer extraction and an MTBE extraction side by side to confirm that lipid yields and content were indeed equivalent prior to proceeding with the MTBE method. Briefly, 200μl plasma was added to 80μl of mass spec grade H₂O in a glass tube with a Teflon lined cap, followed by addition of 1.5ml molecular grade methanol. The tubes were then vortexed, followed by the addition of 5ml molecular grade MTBE, and then rocked for 1hr at room temperature. Following the incubation, 1.25ml mass spec grade H₂O
was added and the tubes were vortexed and centrifuged at 1,000 x g for 10min. The upper organic phase was collected, placed in another glass tube with a Teflon lined cap, and dried under nitrogen gas. Samples were re-suspended in 1ml LC-MS grade methanol, and topped with high purity argon gas. Samples were stored for no longer than one week at -20°C prior to chromatographic analysis.

**Liquid chromatography/mass spectroscopy (LC/MS)**

Both the targeted and shotgun analysis were performed on lipid extracts were separated by reversed phase ultra performance liquid chromatography (UPLC) on an Acquity instrument (Waters, Milford MA). Solvent A consisted of an 89:5:5:1 mixture of water, isopropyl alcohol, acetonitrile and 500mM ammonium acetate respectively. Solvent B consisted of a 50:49:1 mixture of isopropyl alcohol, acetonitrile, and 500mM ammonium acetate, respectively. All solvents were LC-MS grade (Fisher, Optima LCMS). One microliter injections were loaded to a 1.0x100mm Acquity BEH C8 column held at 50°C with a 140 μl/min flow of 100% solvent A. Solvent A was held at 100% for 0.1 min, followed by a linear gradient to 40% Solvent A over 0.9 min. A second linear gradient to 100% Solvent B was achieved over 10 minutes, followed by a 3 min hold at 100% Solvent B. The chromatographic system was returned to initial conditions via a 0.1 min linear gradient to 100% Solvent A, followed by a 5.9 min equilibration prior to the subsequent injection. Total run time was 20 min. Eluate was introduced to a Q-ToF Micro mass spectrometer (Waters/Micromass, Manchester, UK) via electrospray in either the positive or negative ionization mode. Capillary voltages were held at 3000 V and 2200 V in positive and negative ionization modes, respectively. In both
modes the sample cone voltage was held at 30 V, the collision energy was held at 7 V, the source temperature was held at 130°C, and the desolvation temperature was held at 300°C. Data were acquired over the 100-1200 m/z range in all analyses, at a rate of 2 scans/sec. The sample set was analysed in independently randomized technical duplicate for each ionization mode. Both the UPLC and mass spectrometer were operated using MassLynx software version 4.1 (Waters, Milford, MA).

Peak detection and integration were performed using MarkerLynx software (Waters, Milford MA). Chromatographic peaks eluting between 0 and 14 min with m/z values between 100 and 1200 were detected using a mass window of 0.07 Daltons and a retention time window of 0.1 min. Apex Track peak parameter settings were used, with peak width and baseline noise set for automatic calculation. A threshold of 10 counts per scan and a noise elimination level of 6 were implemented to minimize the detection of spectral noise, and the deisotope option was enabled to exclude isotopic peaks from the final data matrix of detected features and their intensities across all samples.

**OPLS modeling and lipid identification**

For the shotgun analysis, multivariate statistical analysis was performed using SIMCA-P+ software (Umetrics Kinnelton, NJ). We used principal components analysis (PCA), and combined PLS and orthogonal single correction discriminant analysis (OPLS-DA) to analyze the LC/MS data. In short, PCA is an unbiased analysis of the total metabolite content (in our analysis the total plasma lipid profile) within a set of samples. PCA detects variance among samples and provides sample clusters
based on similarity of molecular profile. OPLS-DA is an extended form of partial least squares-discriminant analysis (PLS-DA) that, in addition to explaining overall differences between classes, separates predictive and non-predictive variation [28]. Data were modeled to visualize discrimination between the obese and lean control groups using the OPLS-DA model and scores plot of the first and second components. Goodness of fit was quantified by $R^2X$ and $R^2Y$, and predictability by $Q^2Y$.

There were factors in addition to obesity that could influence and potentially weaken our model because of the heterogeneity of the subject population. However, lipids that emerge as significantly different between lean and obese despite the heterogeneity best represent the general clinical population. To account for potential model weakness, a strict analysis and reduction to specific lipid ions of interest was performed using the models and scores plots from the OPLS-DA analysis, along with the raw LC/MS data. This analysis allowed us to identify lipids that were significantly different between lean and morbidly obese subjects as well as those lipids driving the difference between the groups. First, a list was compiled of all the ions falling between 0.5 and 1 on the scores plots from both positive and negative mode because these ions were driving the difference between groups in the OPLS-DA model. An additional list was compiled containing all ions that were significantly different between groups based on t-tests comparing feature abundance. All ions on the t-test list with a difference of less than 10 fold between obese and lean control groups were excluded, as were the features with abundances too low to be distinguished as actual lipid ions, allowing us to narrow the list to
approximately the most significantly different 10% of the ions. The remaining ion features on the t-test list were cross-referenced with the ion features on the scores plot list. A final list was compiled of ion features that were present on both the scores plot list and final t-test list. These were the lipid ions that were both driving the difference between groups observed in the OPLS modeling, and were statistically significantly different between groups. Ions on this list were further examined using tandem mass spectroscopy (MS/MS).

Tandem mass spectroscopy and lipid identification

Samples were analyzed by MS/MS as described above for the initial LC/MS analysis with the exception of selective mass filtering with the quadrupole for our ions of interest, and a collision energy of 40 V. Fragmentation patterns were analyzed and cross referenced to Lipid Maps mass spectrometry peak prediction resources [29], and matched with published spectra where available for identification of lipids of interest. Standards were commercially available (Avanti Polar Lipids, Alabaster, AL) for three of the tentatively identified lipids of interest, 1,0,1’-(Z)-Octadecenyl-2-Arachidonoyl-sn-Glycero-3-Phosphoethanolamine, 1-Palmitoyl-2-Linoleoyl-sn-Glycero-3-Phosphocholine, and 1-octadecanoyl-2-(5Z, 8Z, 11Z, 14Z-eicosatetraenoyl)-sn-Glycero-3-Phospho-(1’-myo-inositol)) and we performed MS/MS on these to confirm our tentative identifications. Following confirmation of our tentative identifications, we used the ether-linked phosphoethanolamine standard in cell culture treatments to determine if it induced pathological phenotypic changes in endothelial cells. Treatment was done with this standard because it was the ether-linked standard that was available.
Cell culture and lipid treatments

Primary human coronary artery endothelial cells (HCAEC) (Lonza, Walkersville, MD) were grown in endothelial cell growth medium (EBM-2) containing 5% FBS and manufacturer recommended supplemental growth factors, antibiotics and antifungals. All assays were performed on cells at 80-100% confluence, between passages 3 and 9. Lipid treatments included normal medium controls, and the 1-0-1’(Z)-Octadecenyl-2-Arachidonoyl-sn-Glycero-3-Phosphoethanolamine (PE) standard. This PE standard was used for endothelial cell treatments as a representative ether-linked lipid. Treatment concentration for the PE standard was 25µg/ml, with all treatments performed for 4 hrs and repeated a minimum of 3 times in duplicate or triplicate.

Western blot analyses

HCAEC were seeded in 65mm polystyrene cell culture dishes and grown to at least 80% confluence prior to lipid treatment. Following treatment, cells were scraped in RIPA buffer (50mM Tris, 0.15M NaCl, 1% Na deoxycholic acid, 1mM EGTA, 1% NP40) containing protease and phosphatase inhibitors, and sonicated 3 x 10 sec. Protein concentrations were determined using a BCA assay, and samples were diluted to equal concentrations with Laemmli sample buffer. Samples were separated on 7.5 or 10% polyacrylamide gels (depending on the molecular weight of the protein of interest) at 125 V, and transferred to nitrocellulose membranes (BioRad, Hercules, CA) for 1 hr at 50 V. Membranes were blocked for 1 hr in Superblock (Thermo Scientific, Rockford, IL), then incubated with primary antibodies against VCAM(1:200) and ICAM(1:200) followed by the appropriate
HRP-conjugated secondary antibodies. Membranes were developed by chemiluminescence using SuperSignal West Dura substrate (Thermo Scientific, Rockford, IL), with digital images obtained using the Biospectrum UVP system (Upland, CA). All signals were normalized to β-actin obtained from the same blot, and expressed as the percent of the normal medium control condition.

**ELISA analysis**

Monocyte chemoattractant protein-1 (MCP-1) in the cell culture medium was determined using a sandwich ELISA (R & D, Minneapolis, MN). The analysis was performed according to manufacturer instructions. The lower limit of detection was 31.2 pg/ml and average CV was 2.2%.

**Statistical analysis**

Modeling and analysis of LC/MS data are described above. Comparison of ion abundance between lean and control groups was performed using unpaired t-tests. Lipid treatment effects on HCAEC were compared using one-way ANOVA with Tukey’s post-hoc where applicable. Significance was set *a priori* at p<0.05.
Results

Subject characteristics

There was no difference in average age between groups, but average BMI was significantly higher (p<0.01) in the morbidly obese group (49.87±11.27 kg/m²) compared to lean (25.76±4.39 kg/m²) and HbA1C was significantly higher (p<0.01) in the morbidly obese group (6.41±0.35 % of total) compared to lean (5.15±0.10 % of total) (Table 1).

Global lipidomic analysis

For both the shotgun and targeted analysis we analyzed lipid extracts from lean and obese subjects using LC/MS. Representative chromatography and spectroscopy are presented in Supplementary Figure 1. We then began the analysis for the shotgun approach with the principal component analysis (PCA). PCA models (Figure 1) for positive and negative mode poorly classified the lean and obese groups, which was not unexpected given the heterogeneity of the groups. The PCA model eliminated outliers prior to the biased OPLS-DA analysis. OPLS-DA modeling was performed on the remaining lean and obese lipid profile data obtained in both positive and negative mode. The positive mode model and scores plot are shown in Figure 2A, the negative mode model and scores plot are shown in Figure 2B. R²X, R²Y, and Q²Y of the positive mode model were 0.298, 0.839, and 0.259 respectively, while R²X, R²Y, and Q²Y of the negative mode model were 0.49, 0.658, and 0.216 respectively. The R values indicate the goodness of fit of the x and y axis.
components, the Q value indicates the predictability of the model. While the greater the R and Q values the better, our values are reasonable based on other analyses in heterogeneous diseased human populations [30]. Based on the OPLS-DA models and statistical analysis as described above, a list of 43 lipid ions that were significantly different between groups and driving the group separation in the OPLS-DA model was compiled for further examination, 26 of those were higher in obese subjects (Supplementary Table 1).

*Lipid ion of interest identification*

MS/MS analysis was performed on all lipid ions of interest. Following MS/MS analysis, screening of spectra, comparison of fragments in Lipid Maps, comparison to published spectra, and elimination of ions that were unidentifiable or became obsolete, 22 ions of interest were tentatively identified. Numerous tentatively identified ions were present as multiple adducts. Table 2 shows the final list of ions of interest that were greater in obese subjects and of interest for further study. We discovered that multiple ether-linked phospholipids were significantly higher in the obese group compared to lean and were driving group separation in the OPLS-DA model. Figure 3 shows a representative MS/MS spectrum of PE(P-18:0/20:4(5Z,8Z,11Z,14Z)) an ether-linked phosphatidylethanolamine (PE) that was identified in negative mode, and a previously published spectrum of the same ion.

To confirm our tentative ion identifications, we purchased the three standards that were available from our list to analyze via MS/MS and compare the
spectra of our unknown ions to the spectra of the standards. Spectra from all three standards matched the spectra of our unknowns confirming the identity of those three ions. Figure 4A shows the negative mode MS/MS spectrum of PI(18:0/20:4(5Z,8Z,11Z,14Z)) from our sample and Figure 4B the negative mode MS/MS spectrum of the PI(18:0/20:4(5Z,8Z,11Z,14Z)) standard. Major identifying peaks include 885 (parent ion), 303 (20:4 arachidonic acid), 283 (18:0 steric acid), and 241 (inositol head group). Because the exceptional finding from the shotgun analysis was the emergence of the group of ether-linked lipids, we next sought to examine what potential effects ether-linked lipids may have on endothelial cells, to obtain insight into the relationship between obesity associated dyslipidemia and vascular disease. From the list of ether-linked lipids, we were able to perform in vitro cell culture treatments using the p-PE standard.

*Endothelial cell response to ether-linked phosphatidylethanolamine*

We used PE-(P-18:0/20:4(5Z,8Z,11Z,14Z)) (p-PE) to treat HCAEC. p-PE induced increases in the cell adhesion molecules ICAM (158% of control, p<0.05) and VCAM (144% of control, p<0.05) (Figure 5). Cell culture medium was collected for analysis of the secreted inflammatory mediator MCP-1. MCP-1 did not differ in medium collected from cells incubated in normal medium, with PAPC or oxidized PAPC, and those that were exposed to p-PE (Figure 6).

*Targeted analysis of oxidized phosphatidylcholine*

Because previous in vitro data indicate that oxidized phosphatidylcholine (ox-pc) products cause pathological phenotypic changes in endothelial cells, we
targeted pc and ox-pc in our LC/MS data from morbidly obese and lean plasma using a targeted approach. We scanned all ions in the positive mode analysis for any that could be PC, PGPC, POVPC, or PEIPC based on mass. While none of the best potential matches for these lipids were between 0.5 and 1 in our OPLS-DA models, PC appeared to be significantly higher in obese subjects compared to lean (p=0.040). Of the best matches for the ox-pc species, only those for PGPC were close to significantly elevated in the obese compared to lean (p=0.052, and 0.055), while the best potential matches for PEIPC were not significant (p=0.707, and 0.661) and no ions that could represent POVPC were detected (Table 3).
Discussion

Because obesity is a major independent risk factor for coronary artery disease, and a major component of that risk is dyslipidemia, we sought to characterize the lipid profiles from morbidly obese human subjects compared to lean. We were able to combine *in vivo* and *in vitro* methods and further examine the role of lipids that were identified and elevated in obese subjects in pathophysiology of endothelial cells. The major findings of this study were that a group of ether-linked lipids was elevated in plasma from morbidly obese subjects compared to lean, and an ether-linked phosphatidylethanolamine increased cell adhesion molecule protein but not the inflammatory mediator MCP-1 in cultured endothelial cells. In addition, targeted oxidized PAPC products previously shown to alter endothelial cell phenotype were not elevated in our obese group compared to lean.

*Ether-linked lipids*

Importantly, there was a significant difference in the lipid profiles between our lean and obese subjects. Following our conservative and strict approach to narrowing down the total lipid profile to a small list of lipids of interest, a group of ether-linked phospholipids that were elevated in obese subject plasma compared to lean that were driving the separation of groups in our model emerged. Ether-linked lipids are characterized by an ether linkage between the glycerol backbone and one or both fatty acid side chains (usually the sn-1 position) as opposed to an ester linkage. A subclass of ether-linked lipids are plasmalogens which are characterized
by a vinyl ether linkage at the sn-1 position with an ester linkage at the sn-2 position.

Ether-linked lipids have been recently characterized in macrophages, lens cells, adipose, liver, and serum [31-33]. Kotronen et al. performed a lipidomic analysis on serum, liver, subcutaneous adipose, and intraabdominal adipose from a group of obese subjects [32]. Results showed that ether-linked PEs were higher in liver compared to both adipose depots, but ether-linked PCs were higher in adipose depots. In this analysis ester PCs were more abundant in all tissues compared to ether-linked, but ether-linked PEs were more abundant than esters in all tissues. Ether-linked PCs and PEs have also been characterized in macrophages, along with ether-linked lipids with other head groups, though this analysis did not attempt to examine the role of such lipids [33]. Other recent work profiling lipid composition of LDL found that plasmalogen PEs are decreased in metabolic syndrome patients compared to lean, as well as in diabetics compared to lean, and the proportion of plasmalogen PE negatively correlates with waist circumference. [34] In the present study we did not have enough subjects to stratify results based on diabetes in our obese group. Collectively, these data and ours support further examination of why ether-linked phospholipids are altered between lean and obese subjects and what the role of ether-linked lipids in metabolic disease may be.

*Physiological roles of ether-linked lipids*

Lipidomics often functions as a hypothesis generating technique where biomarkers of interest emerge from large data sets indicating further analysis of
physiological roles of identified features. Our findings and those described above support further investigation of the discrepancies in ether-linked lipids between lean and obese subjects, as well as diabetics and non-diabetics, as there is a lack of consensus about whether ether-linked lipids are pro- or anti-oxidant and inflammatory. Ether-linked phospholipids are present in most cell membranes and studies of their function have ranged from signaling in brain cells to antioxidant properties [35-38]. Ether-linked lipids are more susceptible to oxidation compared to ester-linked lipids ([37, 38]). Khaselev and Murphy found that ether-linked plasmalogens are preferentially oxidized over ester-linked phospholipids, possibly due to structural differences leaving the polyunsaturated sn2 side chains more exposed [39], and lower bond dissociation energies of ether linkages [36]. Decreased plasmalogens in LDL from patients with the metabolic syndrome and diabetes have been observed and suggested as an indicator of oxidative stress [34]. Plasmalogen deficiency has also been linked to Alzheimers disease [35]. Whether ether-linked lipids function as ROS scavengers or are simply more susceptible to oxidation remains to be seen. However if ether-linked lipids are scavengers and are preferentially synthesized in response to a pro-oxidant environment or altered membrane fluidity, this may be of interest in reference to obese and diabetic populations where increased oxidative stress is often a problem.

Ether-linked lipids may be pro-inflammatory. Ether-linked lipids can serve as arachidonic acid reservoirs. Oxidation products of ether-linked lipids include proinflammatory lysophospholipids and ether-linked lipids are platelet activating factor (PAF) precursors. Ether-linked lipids, particularly plasmalogens have a high
proportion of arachidonic acid at the sn-2 position, and are involved in membrane remodeling as well as intracellular arachidonic acid metabolism [40, 41]. Arachidonic acid released from the sn-2 position of ether and ester lipids can be enzymatically oxidized to form prostaglandins, prostacyclins, thromboxanes, and leukotrienes, all of which are pro-inflammatory [42, 43]. In addition, the remaining lysophospholipids serve as precursors to PAF formation and can be pro-inflammatory and pathological. Lysophosphocholines (LPCs) have been shown to increase plasminogen activator inhibitor-1, a potent prothrombotic and proatherogenic protein [11]. In addition, LPCs can induce endothelial cell expression of cell adhesion molecules, an important step in endothelial cell activation and atherosclerotic progression [9] [44]. Ether-linked lipids with arachidonic acid at the sn-2 position serve as PAF synthesis precursors, as removal of the sn-2 fatty acid chain is the first step in the PAF formation remodeling pathway. PAF is an ether-linked species with a choline headgroup, an ether-linked fatty acid at the sn-1 position and an acetyl group at the sn-2 position. PAF is a potent stimulator of the platelet coagulation cascade, thrombosis, and inflammation. PAF is synthesized in response to external pro-inflammatory and pro-thrombotic mediators including TNF-α [45]. PAF has been implicated in atherogenesis because it activates and recruits inflammatory cells, and in advanced plaque rupture because it initiates the coagulation cascade in platelets (reviewed in [46]). PAF synthesis occurs through the remodeling of sn-1 ether-linked phospholipids where through a series of enzymatic reactions the sn-2 side chain is removed and an acetyl group is attached in its place. What is unknown is whether PAF formation through the
remodeling pathway only occurs in response to external stimuli, or if remodeling formation of PAF can be driven by excess substrate availability. The ether-linked phospholipids, particularly those with arachidonic acid at the sn-2 position and choline head groups may serve as substrates for PAF formation and thus be characterized as pro-inflammatory.

\textbf{pPE and HCAEC}

To address whether ether-linked lipids can induce phenotypic changes in endothelial cells, we performed \textit{in vitro} experiments using a commercially available ether-linked PE that we identified as elevated in obese subjects. The pPE that we tested stimulated cell adhesion molecule expression in human coronary artery endothelial cells, a key step in atherogenesis. We cannot conclude based on these data that the effects of this plasmalogen phosphatidylethanolamine are representative of other ether-linked lipids, including those ether-linked phosphatidylcholines that were also identified during our analysis. However, our data suggest that this lipid may induce changes in endothelial cells indicative of vascular disease, and support further analysis of the role of ether linked lipids in the vascular endothelium.

\textit{Oxidized phospholipids}

The finding that phosphatidylcholine oxidation products that have been shown \textit{in vitro} to induce disease related phenotypic changes in endothelial cells were not elevated in our obese subjects was unexpected. Because oxidized phosphatidylcholine products have been found in modified LDL and atherosclerotic
lesions from high fat fed rabbits [19] and oxidized LDL has been found in lesions isolated from human vessels [47], we hypothesized they would be elevated in morbidly obese subjects compared to lean. Our findings do not discredit the data indicating detrimental effects of oxidized PAPC on endothelial cells, however oxidized PAPC products did not drive the separation between lean and obese groups in our model. It could be that a modest difference is enough to be biologically relevant. Our data support the importance of examining other classes of lipids and presence of oxidized phospholipids in human plasma and LDL.

Lipidomic and metabolomic analyses

Several recent studies have used lipidomic and metabolomic techniques to examine differences between diseased subject groups and healthy controls. As noted above Kotronen et al. performed lipidomic analysis on multiple tissues in subjects with metabolic syndrome and diabetes [32]. Wang et al. also examined plasma from diabetics using lipidomic techniques [48]. Kim et al performed metabolic profiling of overweight and obese subjects compared to lean and identified several lysophospholipids in overweight and obese subjects in addition to differences in fatty acid profiles between groups that generate questions related to altered metabolism in obesity [49]. Additional metabolic profiling in gastric bypass subjects before and after surgery has provided insight into the metabolic changes that occur with surgically induced weight loss [50].

While lipidomics and metabolomics studies don’t necessarily address mechanistic questions, information obtained using these techniques provides
information on global differences between groups and often drives hypothesis development for mechanistic questions. Each lipidomic and metabolomic analysis is performed using unique methods and parameters, and generates unique results contributing to global understanding of group differences and metabolism.

Limitations in our analysis include a small sample size, and a model with weaker than ideal predictability. In addition, while all preparation and analysis of samples following collection was performed under inert gas, we did not use an internal antioxidant. Regardless, the emergence of the ether-linked lipids of interest is strongly supported. Data in our lipidomic analysis show elevated ether-linked lipids in morbidly obese subjects compared to lean and support further investigation of the role this class may play in obesity associated vascular and metabolic disease.

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CSU Proteomics and Metabolomics Facility Academic Enrichment Program
Figure Legends

Figure 1. Principal components analysis models for all ions for all subjects detected in positive and negative mode.

Figure 2. OPLS-DA models and representative scores plots from positive mode analysis (A) and negative mode analysis (B). Black circles in the OPLS model represent lean controls, red squares represent obese pre-operative subjects. Each triangle on the scores plot represents an ion that was detected. The red boxes show the ions that were screened for further analysis.

Figure 3. Representative spectrogram of the ion m/z 750.5399 from MS/MS analysis that was identified as the plasmalogen phosphatidylethanolamine PE(P-18:0/20:4(5Z,8Z,11Z,14Z)), along with a previously published MS/MS spectrogram of the same species. Peak patterns match with the exception of a fragment of 331 in the previously published spectra resulting from presence of a minor additional isomer.

Figure 4. MS/MS spectrogram of ion m/z 885.5499 (PI(18:0/20:4(5Z,8Z,11Z,14Z)) from our samples (A), and the spectrogram of the standard purchased based on the initial tentative identification of the ion (B). Fragmentation patterns match at major peaks 885-parent ion, 581- parent ion with loss of sn-2 arachidonic acid side chain m/z 303, 303- liberated arachidonic acid, 283- liberated stearic acid, and 241- inositol head group.

Figure 5. Effects of lipid treatment on HCAEC cell adhesion molecule protein expression. pPE increased ICAM protein (158% of control, p< 0.05) and VCAM
protein (144% of control, p<0.05) in HCAEC. Data are presented as Mean ± SE. * = p<0.05 compared to control.

Figure 6. Effects of lipid treatment on HCAEC MCP-1 secretion. No differences were detected between treatments. Data are presented as Mean ± SE.

Supplementary Figure 1. Representative LC/MS chromatogram and spectrogram. The chromatogram x-axis shows minutes 6 to 14 of total run time, while the spectrogram x-axis shows the m/z of ions detected between 8 and 8.5 minutes.
### Tables

Table 1. Subject Characteristics

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<tr>
<th></th>
<th>Control</th>
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<tr>
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<td>n=13 (F=11, M=2)</td>
<td>n=15 (F=10, M=5)</td>
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<tr>
<td>Age (yr)</td>
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<td>44</td>
</tr>
<tr>
<td>Height (in)</td>
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<td>67±3</td>
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<tr>
<td>Weight (kg)</td>
<td>71±14</td>
<td>142 ± 33 *</td>
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<tr>
<td>BMI (kg/m(^2))</td>
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<td>49.87± 11.27 *</td>
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<tr>
<td>BMI max</td>
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<tr>
<td>HbA1C (% of total)</td>
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<td>6.41±0.35 *</td>
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<td>Anti-anxiety and anti-depressants</td>
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* = p<0.01 compared to control. Data are presented as Mean ± SE
Table 2. Identifications of ions from MS/MS analysis

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<th>Ion of Interest from Original LC/MS analysis</th>
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<td>750.5399 PE(P-18:0/20:4(5Z,8Z,11Z,14Z))</td>
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<tr>
<td>838.5599 PE(18:2(9Z,12Z)/20:2(11Z,14Z))</td>
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</tr>
<tr>
<td>829.57 PC(16:0/18:2(9Z,12Z))</td>
<td></td>
</tr>
<tr>
<td>802.5599 PC(O-16:0/18:2(9Z,12Z))</td>
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<td>816.57 PC(O-16:0/18:2(9Z,12Z))</td>
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<tr>
<td>830.59 PC(O-18:0/18:2(9Z,12Z))</td>
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<td>844.5999 PC(O-18:0/18:2(9Z,12Z))</td>
<td></td>
</tr>
<tr>
<td>832.5999 PC(O-18:0/18:1(9Z))</td>
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</tr>
<tr>
<td>868.5999 PC(O-18:0/20:4(5Z,8Z,11Z,14Z))</td>
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<td>870.6199 PC(O-18:0/20:3(8Z,11Z,14Z))</td>
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<td>885.5499 PI(18:0/20:4(5Z,8Z,11Z,14Z))</td>
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Table 3. Oxidized PAPC products from positive mode analysis

p-values were calculated using an unpaired t-test comparison of group means for each ion. Ave [c] (lean control) and [pre] (obese pre-surgical) values are average relative abundance of each ion for each group expressed in arbitrary units. C= control, O= obese pre-surgical. Scores plot locations represent the coordinates of the location of each ion on the positive mode scores plot shown in Figure 2.

<table>
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<tr>
<th>Phospholipid</th>
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<th>Best Match</th>
<th>P-value</th>
<th>Scores Plot Location</th>
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<tr>
<td>PAPC</td>
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<td>11.10_782.6904</td>
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<tr>
<td>PGPC</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>POVPC</td>
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<tr>
<td>PEIPC</td>
<td>828.6</td>
<td>8.31_828.5536</td>
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<tr>
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<td></td>
<td>8.32_828.6107</td>
<td>0.661</td>
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Figure 1.

Positive Mode

Negative Mode
Figure 2.

A.  

B.
Figure 3.

The LIT MS² spectrum of the ion at m/z 750.

p18h/20-4-4-CPEm structure together with two major p18h/20-3-CPEm and p16h/22-4-CPEm isomers.
Figure 4.

A.

B.
Figure 5.
Figure 6.

MCP-1

pg/ml

Ctr

p-PE
Supplementary Data

Figure 1.
Supplementary Table 1. Ions of interest identified in LC/MS analysis that were selected for MS/MS. P-value was calculated using an unpaired t-test comparison of group means for each ion. Ave [c] (lean control) and [pre] (obese pre-surgical) values are average relative abundance of each ion for each group expressed in arbitrary units. C= control, O= obese pre-surgical

<table>
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References


CHAPTER III – MANUSCRIPT II

PROTANDIM INDUCED NRF2 ACTIVATION PROTECTS HUMAN CORONARY ARTERY ENDOTHELIAL CELLS AGAINST AN OXIDATIVE CHALLENGE

Title: Protandim induced Nrf2 activation protects human coronary artery endothelial cells against an oxidative challenge

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Protandim treatment is a potential therapeutic intervention against endothelial cell oxidative stress and associated vascular disease initiation and progression. We hypothesized that Protandim treatment would induce NF-E2-related factor 2 (Nrf2) nuclear localization and phase II antioxidant enzyme protein expression in human coronary artery endothelial cells (HCAEC), providing protection against an oxidant challenge in a Nrf2 dependent manner. Protandim treatment induced Nrf2 nuclear localization, and increased HO-1 (778 % of control, p < 0.01), SOD1 (125.9 % of control, p = 0.015), NQO1 (126 % of control, p < 0.01) and Grx (119.5 % of control p = 0.07) in HCAEC. Treatment of HCAEC with H2O2 induced apoptosis in 34% of cells while pre-treatment with Protandim resulted in protection against the H2O2 challenge with only 6% apoptotic cells (p < 0.01). Nrf2 silencing significantly decreased the Protandim induced increase in HO-1 protein expression (p<0.01).

Nrf2 silencing also significantly decreased the protection afforded by Protandim against H2O2 induced apoptosis (p<0.01 compared to no RNA, and p<0.05 compared to control RNA). These results show that the phytochemicals in Protandim induce Nrf2 nuclear localization and antioxidant enzyme expression, and protect HCAEC from an oxidative challenge in a Nrf2 dependent manner.

**Keywords:** Protandim, NF-E2-related factor 2 (Nrf2), oxidative stress, endothelial cells
Introduction

Oxidative stress has been implicated in many chronic diseases including Alzheimer’s, diabetes, and coronary artery disease (CAD) [1-4]. In CAD, increased production of ROS and oxidative damage in the vascular endothelium contribute to disease initiation and progression. Specifically, increased vascular superoxide causes oxidation of lipids, decreased nitric oxide availability, increased expression of adhesion molecules and inflammatory mediators, and recruitment of monocytes to the endothelium[5-8]. Endothelium bound superoxide dismutase is also decreased in CAD patients compared to healthy controls, impairing the cellular response to excessive ROS production [9]. Atherosclerotic coronary arteries isolated from humans display increased superoxide production compared to non-atherosclerotic human coronary arteries, and in a mouse model of atherosclerosis, attenuation of superoxide production by decreased expression of NADPH oxidase results in a decrease in atherosclerotic lesion size [10, 11].

Initial studies examining the effects of decreasing oxidative stress in several different diseases, including cardiovascular disease, have used exogenous antioxidant supplements such as vitamins C and E. However, data showed no protective effects of exogenous antioxidants, and in some cases supplementation increased mortality [12-14].

A novel approach to decreasing disease associated oxidative stress involves bolstering endogenous antioxidant defense systems rather than relying on exogenous antioxidant supplementation. Protandim is a commercially available
dietary supplement consisting of phytochemicals derived from five widely studied medicinal plants including silymarin from milk thistle, curcumin from turmeric, bacopa extract, ashwaganda, and green tea extract. It is thought that the five phytochemical components of Protandim have a synergistic effect to induce phase II antioxidant enzymes and protect cells from oxidative stress through activation of the transcription factor NF-E2-related factor 2 (Nrf2). Nrf2 binds to the antioxidant response element (ARE) in the promoter region of several hundred genes including many phase II antioxidant enzymes [15, 16].

Nrf2 is constitutively expressed but is marked for ubiquitination by association with Kelch-like ECH-associated protein 1 (Keap1). Activation of Nrf2 occurs when it is released from Keap1, translocates to the nucleus, heterodimerizes with small Maf or Jun proteins, binds the ARE, and initiates transcription of the associated genes. Multiple mechanisms for Nrf2 activation have been proposed including oxidation of Keap1 and phosphorylation of Nrf2 itself (reviewed in [15]). Electrophilic or oxidative modification to Keap1 cysteine thiol groups leads to dissociation of Nrf2 from Keap1 [17]. Direct phosphorylation of Nrf2 by a number of kinases, including several from the MAPK family can also cause dissociation of Nrf2 from Keap1[18-20]. Phytochemical compounds including those in Protandim likely activate Nrf2 and induce phase II antioxidant enzyme expression through activation of various kinases with subsequent Nrf2 phosphorylation [21].

Although acute activation of Nrf2 occurs in vivo in response to oxidized phospholipid signaling, increased ROS production, hyperglycemia, and shear stress
[22-24], in chronic disease states the antioxidant response is often insufficient to maintain redox balance and prevent disease progression [24-26]. For example, Landmesser et al. report increased SOD activity in young hypercholesterolemic subjects compared to age matched controls[9]. In contrast, decreased SOD activity was observed in coronary arteries from CAD patients compared to age matched controls. It was recently reported that Protandim protected a human saphenous vein ex vivo culture from oxidative stress induced hyperplasia and vessel wall thickening [27]. Thus, phytochemical induced Nrf2 activation is a potential therapeutic intervention against endothelial cell oxidative stress and associated vascular disease initiation and progression. However the effects of Protandim on Nrf2 in human coronary artery endothelial cells are not known.

The purpose of this study was to determine 1) if treatment with Protandim induces Nrf2 nuclear translocation and phase II antioxidant enzyme protein expression in human coronary artery endothelial cells (HCAEC), 2) if treatment with Protandim protects HCAEC from apoptosis induced by an oxidant challenge, and 3) if Nrf2 mediates Protandim induced protection from an oxidative challenge. We hypothesized that Protandim treatment would induce Nrf2 nuclear localization and phase II antioxidant enzyme protein expression, and Protandim treatment prior to an oxidant challenge would afford cells protection in a Nrf2 dependent manner.
Materials and methods

Materials

HCAEC and cell culture reagents were purchased from Lonza (Walkersville, MD). Heme oxygenase-1 (HO-1) antibody was from Affinity Bioreagents (Golden, CO), nitroquinone oxidoreductase (NQO1) and glutathione reductase (Grx) antibodies and appropriate HRP conjugated secondary antibodies were purchased from AbCam, (Cambridge, MA). Nrf2, Cu-Zn superoxide dismutase (SOD1), and actin antibodies, HRP and FITC conjugated appropriate secondary antibodies, and Nrf2 siRNA and control RNA were purchased from Santa Cruz Biotech (Santa Cruz, CA). PrimeFect transfection reagent and PrimeFect diluent were from Lonza (Walkersville, MD). The Pierce BCA assay kit for determining protein concentrations, and protease and phosphatase inhibitors were from Thermo Scientific (Rockford, IL). TUNEL assay kits were from Roche (Indianapolis, IN).

Culture of HCAEC

Primary HCAEC were grown in endothelial cell growth medium (EBM-2) containing 5% FBS and manufacturer recommended supplemental growth factors, antibiotics and antimycotics. All assays were performed on cells at 80-100% confluence, between passages 3 and 9.

Protandim and H$_2$O$_2$ preparation and treatment

Protandim is a phytochemical composition containing *W. somnifera, B. monniera* (45% bacosides), *S. marianum, Ca. sinesis* (98% polyphenols and 45% (-)
epigallocatechin-3-gallate), and curcumin (95%) from turmeric (*Cu. longa*) (LifeVantage Corp., Littleton, CO). Protandim extract was prepared by mixing 500mg Protandim in 5ml 95% ethanol. The mixture was rocked overnight at room temperature, and then centrifuged for 15 min at 3,000 x g. The resulting supernatant contains ethanol extracted Protandim at a concentration of 100mg/ml. The Protandim extract was diluted to 20μg/ml with complete cell culture medium for all treatments following initial concentration response experiments that showed 20μg/ml was the lowest concentration that significantly increased phase II antioxidant enzyme protein expression. Cells not treated with Protandim were treated with 95% ethanol as a vehicle control, with a maximum ethanol concentration in the growth medium of 0.02% (2μl in 10ml). H₂O₂ (30% W/W) was diluted in complete cell growth medium to a final concentration of 1.25 μM for all treatments. Protandim treatments ranged from 1 hr to 12 hrs as indicated, and H₂O₂ treatments were 4 hrs unless otherwise indicated. In Protandim removal experiments, Protandim was removed, cells were washed with PBS, and then incubated in normal cell culture medium for the indicated duration.

*Western blot analyses*

HCAEC were seeded in 65mm polystyrene cell culture dishes and grown to at least 80% confluence prior to Protandim treatment. Following treatment, cells were scraped in RIPA buffer (50mM Tris, 0.15M NaCl, 1% Na deoxycholic acid, 1mM EGTA, 1% NP40) containing protease and phosphatase inhibitors, and sonicated 3 x 10 seconds. Protein concentrations were determined using a BCA assay, and
samples were diluted to equal concentrations with Laemmli sample buffer. Samples were separated on 10% polyacrylamide gels at 125 volts, and transferred to nitrocellulose membranes (BioRad, Hercules, CA) for 1 hr at 50 volts. Membranes were blocked for 1 hr in Superblock (Thermo Scientific, Rockford, IL), then incubated with primary antibodies against HO-1 (1:500), SOD1(1:500), NQO1(1:1000), and Grx(1:5000) followed by the appropriate HRP-conjugated secondary antibodies. Membranes were developed by chemiluminescence using SuperSignal West Dura substrate (Thermo Scientific, Rockford, IL), with digital images obtained using the Biospectrum UVP system. All signals were normalized to β-actin obtained from the same blot, and expressed as the percent of the vehicle control (no Protandim) condition.

*Immunocytochemistry for Nrf2 nuclear localization*

HCAEC were grown to confluence on coverslips in 35mm polystyrene cell culture plates coated with 2μg/cm² fibronectin prior to Protandim treatment. Cells were initially treated with Protandim for 1 hr, 2 hrs, 4 hrs, 8 hrs, and 12 hrs to determine the optimal duration of treatment for visualizing Nrf2 nuclear localization. Following time course experiments, all Protandim treatments were for 1 hr. Cells were washed with PBS, fixed for 30 min in 4% paraformaldehyde, washed with PBS, and then permeabilized in cold acetone for 30 min. Cells were blocked for 1 hr in 5% bovine serum albumin with 0.5% goat serum, and then incubated with Nrf2 primary antibody (1:100) for 1 hr at room temperature. Cells were washed with PBS then incubated for 45 min in FITC conjugated secondary antibody at room
temperature in the dark. The coverslips were mounted on slides using DAPI containing mounting medium for identification of cell nuclei, and visualized by fluorescence microscopy (Nikon TE2000).

*TUNEL assay to assess apoptosis*

A TdT-mediated dUTP nick end labeling (TUNEL) assay was used to assess HCAEC apoptosis in response to an oxidative challenge. Cells were grown to confluence on fibronectin coated coverslips prior to Protandim and H$_2$O$_2$ treatment. Cells were washed with PBS, fixed for 30 min in 4% paraformaldehyde, washed again with PBS, then permeabilized for two min in 0.1% Triton X-100 with 0.1% sodium citrate. Cells were washed again with PBS and incubated in TUNEL reagent for 1.5 hrs. The coverslips were then mounted on slides using DAPI containing mounting medium to identify cell nuclei. Signals were visualized using fluorescence microscopy (Nikon TE2000).

*Nrf2 silencing*

Prior to transfection, 250μL PrimeFect Diluent was mixed with 5μL PrimeFect transfection reagent and incubated at room temperature for 15 min. Nrf2 siRNA or control RNA was added to the transfection solution for a final concentration of 50nM, and incubated on the bench for 15 min. The transfection solution was applied to cells grown in antibiotic free media to 70-80% confluence, along with 1.25mL antibiotic free growth medium. The volume of transfection reagent used caused minimal distress to the cells as assessed by minimal changes to cell
morphology. After 24 hrs, the transfection solution was removed and the cells were rinsed with PBS, treated with Protandim, and assayed as indicated.

**Statistical analysis**

Unpaired t-tests were used to compare control vs. Protandim treatments. A two by three treatment (Protandim and no Protandim) by condition (normal medium, control RNA, and NRf2 siRNA) ANOVA with *a priori* linear contrasts of means was used to analyze Nrf2 silencing experiments. Percent protection was analyzed using one-way ANOVA. Statistical significance was set at p<0.05
Results

Antioxidant enzyme protein expression is increased in response to Protandim treatment.

HCAEC were treated with Protandim concentrations of 0 to 50μg/ml in 5μg/ml increments to determine a profile of Nrf2 activation as measured by an increase in HO-1 protein content. HO-1 protein increased from non-detectable in control conditions to 8-10 fold at 20-30μg/ml, while concentrations higher than 30μg/ml induced morphological changes (Figure 1A). We used the 20μg/ml concentration in all subsequent treatments. HO-1 protein was visible after 1 hr, and became significant and sustained from 4 hrs through the longest treatment period of 12 hrs (Data not shown). To confirm treatment concentration and duration on multiple antioxidant enzymes we determined that treatment with 20μg/ml Protandim for 12 hrs increased HO-1 (778 % of control + 82.25 p < 0.01), SOD1 (125.9 % of control + 6.05 p < 0.01), NQO1 (126 % of control + 6.5 p < 0.01), and Grx trended towards significance (119.5 % of control + 7.00 p < 0.05) (Figure 1B). All subsequent treatments used Protandim at a concentration of 20μg/ml for 12 hrs unless otherwise noted.

Protandim stimulates Nrf2 nuclear localization

HCAEC were treated with Protandim for 12 hrs and subsequently visualized with immunocytochemistry to determine Nrf2 nuclear localization. Nrf2 content and nuclear localization increased as soon as 1 hr of Protandim treatment. These
increases remained at 2 hrs, 4 hrs, 8 hrs, and 12 hrs (12 hrs was the longest treatment duration examined) (Figure 2). After removal of Protandim Nrf2 content and nuclear localization were decreased within 15 min (Figure 2).

*Protandim protects HCAEC from oxidative challenge induced apoptosis*

HCAECs were treated for 12 hrs with Protandim or vehicle control followed by a 4 hr exposure to 1.25 µM hydrogen peroxide and induction of apoptosis as measured by TUNEL assay. Importantly, Protandim was removed prior to hydrogen peroxide exposure. In vehicle controls apoptosis was induced in 34% of cells (Figure 3A and 3B) while pre-treatment with Protandim resulted in only 6% apoptotic cells (p < 0.01 compared to vehicle control) (Figure 3A and 3B).

*Nrf2 silencing diminishes Protandim induced increases in HO-1 and protection from an oxidative challenge.*

We used siRNA to silence Nrf2 prior to Protandim treatment to determine if Protandim induced increases in antioxidant enzyme expression and protection occur through Nrf2 activation. Nrf2 silencing prior to Protandim treatment significantly decreased (p<0.01) Protandim induced increases in HO-1 expression compared to both the no RNA conditions, and control RNA treatment. (Figure 4A and 4B). There were no differences between no RNA and control RNA conditions with or without Protandim.

We then silenced Nrf2 prior to Protandim treatment and an oxidative challenge. In cells receiving no RNA and cells that received control RNA, 30 - 40% of
cells underwent apoptosis (Figure 5 and 6A). Nrf2 siRNA treatment prior to H₂O₂ resulted in apoptosis in 80% of cells (p<0.0001) compared to no RNA conditions and control RNA (Figure 5 and 6A). In both no RNA and control RNA conditions, Protandim treatment prior to H₂O₂ resulted in significantly fewer apoptotic cells (p<0.0001 compared to normal conditions and control) (Figure 5 and 6A). The number of apoptotic cells in the no RNA condition compared to the control RNA condition was not significantly different with or without Protandim (p=0.413 no Protandim, p=0.093 with Protandim). Protandim prior to Nrf2 siRNA also significantly protected cells from apoptosis (p=0.023), however the amount of protection afforded by the Protandim in this condition was significantly less than in no RNA and control RNA conditions (p<0.01 and p<0.05) (Figure 5, 6A and 6B).
Discussion

The major findings of this study were that Protandim treatment of HCAEC induced Nrf2 nuclear localization and phase II antioxidant enzyme expression, and Protandim treatment prior to an oxidative challenge was protective against apoptosis in a Nrf2 dependent manner.

Nrf2 nuclear localization was observed following 1 hour of Protandim treatment, and remained elevated for all treatment durations up to twelve hours. In cardiac myocytes we have observed increases in Nrf2 nuclear localization as quickly as 15 minutes after initiation of Protandim treatment (unpublished data). Experiments determining how long increases in Nrf2 localization remain following removal of Protandim have shown the Protandim effect is diminished as early as 15 minutes following removal. Experiments to determine how long Protandim induced increases in antioxidant enzymes persist are ongoing. These data suggest that Protandim has an immediate and robust effect on Nrf2 nuclear localization that remains for a limited time after the removal of Protandim. The transient effects of Protandim on Nrf2 may be important because chronic Nrf2 activation has been shown to be carcinogenic [28].

The components in Protandim work synergistically to activate Nrf2. HO-1 RNA was increased to 1,000 percent of control in response to 40μg/ml Protandim in MIN6 cells, and 20μg/ml Protandim in SK-N-MC cells [29]. However, when each phytochemical component of Protandim was tested individually at the low
concentration found in Protandim extract, the maximum increase in HO-1 RNA was less than 200 percent of control [29].

Subjects taking Protandim report no adverse side effects [29, 30]. It has been shown that with oral Protandim supplementation in humans circulating TBARs decrease in 5-12 days, an effect that persists with continued supplementation as measured at 30 and 120 days [30]. In addition, erythrocytes isolated from subjects who ingested Protandim for 120 days demonstrated increased SOD and catalase activity, [30]. However, in each of these studies how long these effects persisted after Protandim supplementation was stopped was not assessed. Thus, while our data are promising, studies in vivo will need to be performed to determine if these results are translatable.

Protandim treatment protected HCAEC from H2O2 induced apoptosis, an effect that was diminished but not abrogated with silencing of Nrf2 prior to Protandim treatment. There are multiple potential explanations for this observation. First, Nrf2 may have only been partially silenced. This could be due to limitations in the amount of transfection reagent the cells tolerate, and thus the amount of Nrf2 siRNA that could be delivered. Second, diminished but incomplete protection may also be due to the rapid turnover rate in Nrf2. Nrf2 is constitutively expressed and rapidly degraded [21] The amount of siRNA that can be delivered is limited by the ability of the cells to tolerate the transfection procedure, therefore the capacity of the delivered Nrf2 siRNA to silence Nrf2 may be exceeded by the continuous turnover and subsequent increases induced by Protandim. Finally, Nrf2
independent mechanisms for induction of antioxidant enzymes and protection against oxidative stress may exist; possible candidates that can increase antioxidant defenses independently of Nrf2 include p53 and sirtuins [31, 32].

Increased apoptosis as an outcome in endothelial cells has direct translation to vascular disease development and outcomes. Endothelial cell apoptosis contributes to plaque progression and rupture, and may be an independent risk factor for thrombosis [33]. Endothelial cell apoptosis can be induced by oxidized lipids as well as by ROS, and the role of endothelial cell apoptosis in atherosclerosis has been extensively reviewed [34-36].

While we observed significant increases in expression of multiple antioxidant enzymes (approximately 125 % of control for SOD1, Grx, and NQO1), the Protandim induced increase in HO-1 was over 700 % of control. Until recently, the antioxidant properties of HO-1 were thought to be through production of the ROS scavenger bilirubin, but data now suggest HO-1 may have other antioxidant qualities and be important in multiple cell types in the vasculature. Kadl et al. found that cell death in macrophages was exacerbated in the absence of Nrf2 or with inhibition of HO-1 activity [23]. It has also been demonstrated in macrophages that HO-1 functions as an antioxidant by decreasing heme availability, which decreases expression of the NADPH oxidase heme containing subunit gp91phox and subsequently decreases superoxide production [37].

Decreasing NADPH oxidase superoxide production has important implications in both macrophages and endothelial cells. Substantial evidence exists
demonstrating a role for NADPH oxidase and increased superoxide production in obesity and atherosclerosis. Compared to non-obese controls, overweight and obese subjects demonstrate increased NADPH oxidase subunit expression and augmented oxidative stress in the vascular endothelium [38]. NADPH oxidase subunit expression is elevated in lesions of coronary arteries in bypass graft patients, particularly in the vicinity of macrophages, and NADPH oxidase expression levels correlate with severity of atherosclerosis [10]. Superoxide produced by any mechanism including NADPH oxidase is rapidly converted to H$_2$O$_2$. Our experiments used H$_2$O$_2$ as an oxidative stress to induce apoptosis in HCAEC. While the concentration of H$_2$O$_2$ used in our experiments was chosen based on consistent induction of apoptosis in 30-50% of untreated cells, H$_2$O$_2$ is a relevant oxidant in vivo [41]. Experiments in our lab to examine other relevant stressors are ongoing.

Whether HO-1 also functions as a protective antioxidant in endothelial cells through decreasing heme availability, inhibiting NADPH oxidase, and decreasing superoxide production, or via an alternative mechanism also is yet to be determined. Greater baseline HO-1 expression in aortic endothelial cells also results in decreased effects of oxidized phospholipids on inflammatory genes [39], suggesting that presence of HO-1 may delay progression of disease related phenotypic changes when cells are faced with chronic lipid and oxidative stresses. While it was beyond the scope of this study, determining whether HO-1 is essential to the Nrf2 dependent Protandim induced protection against an oxidative challenge is warranted.
Grx, SOD1, NQO1 were also significantly increased in HCAEC by Protandim, though the effect was not as large as that seen with HO-1. These are the first data indicating increases in Grx and NQO1 in response to Protandim; increases in SOD1 expression and activity have been previously reported [27, 30]. In erythrocytes isolated from human subjects following 120 days of Protandim supplementation, SOD activity was increased by 30% [30]. In an ex vivo preparation of human saphenous veins, Protandim treatment increased SOD activity 3-fold, HO-1 activity 7-fold, and catalase activity 12-fold [27]. Catalase has been shown to be increased by Protandim and to mediate protective effects in erythrocytes and saphenous vein preparations, as well as protect against skin cancer development in an animal model [27, 30, 40]. Our data in HCAEC show an approximate 10-fold increase in HO-1 expression, however catalase is undetectable in our model. Collectively, these data suggest that the antioxidant enzymes increase following Protandim treatment in a cell type specific manner.

Our data suggest positive effects of Protandim in healthy cells supporting the future examination of how Protandim may affect endothelial cells that have been chronically exposed to oxidative and lipid challenges. Most individuals experience transient lipid and oxidative stress in the vasculature throughout life, and it is the accumulated effects that eventually lead to overt vascular disease. Therefore it is of interest to determine if Protandim can slow or reverse disease related endothelial cell phenotypic changes using in vivo models of chronic oxidative stress.
Our current investigation shows for the first time that Protandim treatment in HCAEC induces Nrf2 nuclear localization, phase II antioxidant enzyme expression, and Nrf2 dependent protection from an oxidant stress. Oxidative stress has a well-established role in CAD disease initiation and progression, and our data support further research on phytochemical activation of Nrf2 and the endogenous antioxidant response as a potential therapeutic approach.

Acknowledgments

The authors thank Danielle Reuland and Laurie Biela for their previous work and technical assistance, and Dr. Manfred Diehl for his assistance with statistical analysis. Funding was provided by CSU Proteomics and Metabolomics Facility Academic Enrichment Program, and a Colorado State University CAHS mini grant.

List of Abbreviations:

HO-1, Heme oxygenase-1; Nrf 2, NF-E2-related factor 2; ROS, reactive oxygen species; SOD, superoxide dismutase; Grx, glutathione reductase; NQO1, NAD(P)H dehydrogenase [quinone] 1; Keap-1, Kelch-like ECH-associated protein 1; CAD, coronary artery disease; HCAEC, human coronary artery endothelial cells; Ptd, Protandim.
**Figure Legends**

Figure 1. Ptd treatment induces phase II antioxidant enzyme protein expression in HCAEC. (A) HCAEC were treated with increasing concentrations of Ptd for 12 hrs, HO-1 protein expression was measured. Concentrations of Ptd ranged from 1 μg/ml to 50 μg/ml. (B) Following determination of the appropriate concentration of Ptd, HCAEC were treated with 20μg/ml Ptd for 12hrs then protein expression of HO-1, Grx, SOD-1, and NQO1 was determined by Western blot. All bands were normalized to β-Actin as a loading control and are expressed as a percent of the control condition. *= p<0.01 compared to Ptd, **=p<0.05 compared to Ptd. Data are presented as mean ± SE.

Figure 2. Ptd induced Nrf2 expression and nuclear localization. HCAEC were treated with Ptd for 1 hr, following which immunofluorescence was used to visualize changes in Nrf2 expression and localization. Following Ptd treatment Nrf2 signal was increased and became visible in the nucleus. Increases in Ptd expression and nuclear localization were diminished within 15 min of removal of Ptd.

Figure 3. Ptd treatment protects HCAEC against an oxidative stress. HCAEC were treated with Ptd for 12 hrs, followed by H₂O₂ 4hrs. (A) Treatment of HCAEC with Ptd prior to an oxidative challenge resulted in significantly fewer apoptotic nuclei as determined by TUNEL assay. (B) Approximately 35% of cells underwent apoptosis without Ptd treatment prior to an oxidative challenge while pre-treatment with Ptd resulted in approximately 5% of cells undergoing apoptosis in response to the same
oxidative challenge. * = p<0.01 compared to control (no Ptd). Data are presented as mean ± SE percent of TUNEL positive (+) nuclei.

Figure 4. Silencing of Nrf2 abrogated Ptd induced increases in HO-1 expression. (A) Cells were treated with no RNA, control RNA or Nrf2 siRNA prior to 12hrs Ptd, then HO-1 expression determined by Western blotting. (B) HO-1 expression in response to Ptd treatment increased to over 1,000 % of control in the no RNA and control RNA conditions, while increases in HO-1 expression in the Nrf2 siRNA condition were negligible. Data are presented as mean ± SE percent of control (no RNA, no Ptd) condition. * = p<0.01  # = p<0.01 compared to within RNA condition Ptd treatment.

Figure 5. Silencing of Nrf2 resulted in a significant increase in the number of cells undergoing apoptosis in response to an oxidative challenge regardless of Ptd.

Figure 6. Ptd treatment following Nrf2 silencing protected HCAEC against an oxidative challenge, however, protection following Nrf2 silencing was significantly diminished compared to controls. (A) In no RNA, control RNA, and Nrf2 RNA conditions, significantly fewer cells underwent apoptosis when treated with Ptd prior to an oxidative stress. No RNA conditions and control RNA were not significantly different with or without Ptd. In Nrf2 siRNA conditions, % TUNEL positive (+) nuclei was significantly higher than no RNA and control RNA conditions.
with and without Ptd. (B) The percent protection afforded by Ptd was not significantly different between no RNA and control RNA conditions. The protection afforded by Ptd in the Nrf2 siRNA condition was significantly less compared to both no RNA and control RNA conditions. Data are presented as means ± SE. *= p<0.01 between indicated groups, **= p< 0.05 between indicated groups. # = p<0.01 compared to within RNA condition Ctr(no Ptd), ## = p<0.05 compared to within RNA condition Ctr(no Ptd)
Figure 1.

A.

Increasing Ptd Concentration from 0µg/ml to 50µg/ml

B.
Figure 2.

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<th>DAPI (nuclei)</th>
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<td>1 hr Ptd</td>
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<td>1 hr Ptd, 15 min removed</td>
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Figure 3.

A.

![Image of DAPI (nuclei), FITC (apoptosis), and Overlay for Ctr and Ptd conditions]

B.

![Bar graph showing % TUNEL + Nuclei for Ctr and Ptd conditions]
Figure 4.

A.

Actin
HO-1

Ptd

- - + + - - + + - - + +

No RNA Control RNA Nrf2 siRNA

B.

% of Control

No RNA Control RNA Nrf2 siRNA

# #

* *
Figure 5.

A.

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Figure 6.

A.

**% TUNEL + Nuclei**

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<td>Nrf2 siRNA</td>
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B.

**% Protection by Ptd**

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References


CHAPTER IV – MANUSCRIPT III

LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A(2) IS EXPRESSED IN HUMAN ADIPOSE TISSUE AND CORRELATES WITH HBA1C IN OBESITY

Lipoprotein-associated Phospholipase A(2) is expressed in human adipose tissue and correlates with HbA1C.

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Lipoprotein-associated phospholipase A2 (Lp-PLA2) catalyses the hydrolysis of platelet activating factor (PAF) and oxidized phospholipids. Lp-PLA2 activity is elevated in plasma of type 2 diabetics and potentially predicts diabetes. The purpose of this study was to examine Lp-PLA2 protein in adipose and skeletal muscle from obese subjects and lean controls, and determine how adipose tissue Lp-PLA2 relates to plasma Lp-PLA2 activity and HbA1C. We found unmodified and glycosylated Lp-PLA2 in adipose tissue and skeletal muscle from lean and obese subjects. In adipose tissue Lp-PLA2 co-localized predominantly with macrophages. Adipose tissue total and glycosylated Lp-PLA2 were not different between lean and obese groups (p=0.084, and p=0.1872). Correlations between obese subject adipose tissue total Lp-PLA2 and HbA1C, and obese subject adipose tissue glycosylated Lp-PLA2 and HbA1C trended towards significant (r=-0.517, p=0.126) and (r=0.491, p=0.125), respectively. When these correlations were re-calculated excluding subjects taking glycemic control medication, the correlation between adipose tissue total Lp-PLA2 and HbA1C became significant (r=-0.834, p=0.039), but the correlation between adipose tissue glycosylated Lp-PLA2 and HbA1C was weakened and became negative (r=-0.306, p=0.556). The presence of Lp-PLA2 in adipose tissue, along with associations between adipose Lp-PLA2 and HbA1C in obese subjects, suggests future research should examine a role for Lp-PLA2 in tissue insulin resistance.

**Keywords:** Lipoprotein-associated phospholipase A2 (Lp-PLA2), adipose, obesity, diabetes, macrophages
Introduction

Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is a macrophage-derived member of the platelet activating factor acetylhydrolase family that catalyses the hydrolysis of platelet activating factor (PAF) to inactive lyso-PAF and a free fatty acid. Lp-PLA₂ can be glycosylated but only a single study has examined the function of glycosylation suggesting it may be related to Lp-PLA₂ association with HDL versus LDL cholesterol in circulation [1]. Lp-PLA₂ is unique compared to other PAF acetylhydrolases because it also has affinity for short chain and oxidized phospholipids. Hydrolysis of oxidized phospholipids by Lp-PLA₂ results in potentially inflammatory lysophospholipids and oxidized fatty acids [2-5]. The role of Lp-PLA₂ has been studied more in relation to hydrolysis of oxidized phospholipids than PAF, because other PAF-acetylhydrolases also degrade PAF, while only Lp-PLA₂ has affinity for oxidized phospholipids.

Lp-PLA₂ is now recognized as a biomarker of cardiovascular disease (CVD) risk [6]. While some data indicate that increasing Lp-PLA₂ can protect against atherosclerotic lesion development [7, 8], most data associate increased plasma Lp-PLA₂ activity with CAD risk. Kolodgie et al. examined coronary arteries from patients that experienced sudden coronary death patients and found that in early plaques, Lp-PLA₂ was barely detectable. However, in advanced and ruptured plaques, Lp-PLA₂ was strongly expressed and co-localized with macrophages in necrotic cores [9]. Research continues to determine if Lp-PLA₂ is truly a risk factor
for CAD as opposed to just a biomarker, with drugs to inhibit Lp-PLA\textsubscript{2} activity in CAD patients are in clinical trials [10, 11].

Most epidemiological data show that Lp-PLA\textsubscript{2} is elevated in patients with the metabolic syndrome [12, 13]. Lp-PLA\textsubscript{2} activity is positively correlated with LDL cholesterol and negatively correlated with HDL cholesterol [14]. Data examining the relationship between fat distribution and Lp-PLA\textsubscript{2} are inconsistent. Pou \textit{et al.} found no relationship between Lp-PLA\textsubscript{2} and subcutaneous or visceral adipose volume [15], but Detopoulou \textit{et al.} found that plasma Lp-PLA\textsubscript{2} activity is associated with trunk adiposity [16]. Further, Lp-PLA\textsubscript{2} activity is elevated in diabetics [17], further Lp-PLA\textsubscript{2} activity may be predictive of diabetes regardless of BMI (in review).

Inflammatory mediators and excess fatty acids in the adipose tissue are known to play a role in insulin resistance [18-21]. The effects of lysophospholipids and oxidized fatty acids derived from Lp-PLA\textsubscript{2} hydrolysis of oxidized phospholipids on adipose tissue are not known, though these products can induce insulin resistance in skeletal muscle [22]. Lp-PLA\textsubscript{2} is predominantly macrophage derived, and a hallmark of obesity is macrophage infiltration into the adipose tissue in response to inflammatory mediators and necrotic adipocytes [23, 24]. Lp-PLA\textsubscript{2} protein has been detected in rodent adipocytes, but the same has not been investigated in human adipocytes or whole adipose tissue [25, 26]. Based on these collective data it is reasonable to hypothesize that Lp-PLA\textsubscript{2} is expressed in human adipose tissue, and may play a role in tissue insulin resistance and development of diabetes.
The purpose of this study was to: 1) examine Lp-PLA₂ expression in adipose and skeletal muscle from morbidly obese subjects and lean controls, and 2) determine how adipose tissue Lp-PLA₂ expression relates to plasma Lp-PLA₂ activity, markers of glucose homeostasis and circulating inflammatory markers. We hypothesized that Lp-PLA₂ would be expressed in human adipose but not skeletal muscle, and that expression would be greater in adipose tissue from morbidly obese subjects compared to that from lean subjects. In addition, we hypothesized that visceral adipose tissue Lp-PLA₂ protein content would correlate with circulating Lp-PLA₂ activity, markers of inflammation and markers of glucose homeostasis.
**Methods**

*Ethics approval*

The Institutional Review Boards of Colorado State University and Poudre Valley Hospital approved this protocol (CSU protocol # 05-116H, PVH protocol # 07-874). Each volunteer was informed of the potential risks and written consent was obtained prior to enrollment. The study followed the guidelines set forth by the Declaration of Helsinki.

*Study overview*

A total of 15 morbidly obese gastric bypass patients, and 13 lean controls age 18-60 were recruited to participate through the Northern Colorado Surgical Associates (NCSA) of the Bariatric Center of the Rockies. Lean controls were patients undergoing an abdominal surgery of comparable invasiveness to gastric bypass surgery. Bypass patients were required to have a body mass index (BMI) > 40 kg/m², while controls were required to have a BMI < 35 kg/m². Subjects completed medical and exercise questionnaires prior to undergoing a venipuncture blood draw following a 12-hour fast. Blood was collected in vacutainer tubes containing EDTA, 0.5ml were aliquoted for HbA1C analysis, and the remainder was centrifuged (1200g, 15 min, 4°C). Plasma was collected and stored at -80°C until assayed. Whole blood samples were sent to the University of Colorado Denver Clinical Translational Research Center for analysis of hemoglobin A1C (HbA1C) using a DCA Vantage analyzer (Siemens, Deerfield, IL). During surgery, omental
adipose and abdominal muscle were collected, frozen in liquid nitrogen, and stored at -80°C until assayed.

*Plasma Lp-PLA₂ activity and TNF-α*

Plasma Lp-PLA2 concentration was measured by an enzyme linked immunosorbent assay (ELISA) and activity determined by a colorimetric activity method, both performed by diaDexus, Inc (South San Francisco, CA) (mass, intra-assay coefficient of variation (CV)% <0.1%; activity, intra-assay CV% <2%). Plasma TNF-α was determined using a high sensitivity sandwich ELISA (R & D Systems, Minneapolis, MN). The lower limit of detection was 0.5pg/ml, average coefficient of variation was 3.96%.

*Western blot analyses of adipose and muscle*

Adipose tissue was weighed and homogenized in RIPA buffer (50mM Tris, 0.15M NaCl, 1% Na deoxycholic acid, 1mM EGTA, 1% NP40). The homogenate was centrifuged for 10 min at 10,000 x g at 4°C. The supernatant was collected from between the fat cake and pellet, transferred to a second tube, and centrifuged again for 3 min at 10,000 x g at 4°C. The resulting supernatant was collected, sonicated, and protein concentrations were determined using a BCA assay. Abdominal muscle was weighed, homogenized in RIPA buffer, and centrifuged at 800 x g for 10 min. The supernatant was collected and centrifuged at 1500 x g for 5 min. Again the supernatant was collected, sonicated and protein concentrations determined using a BCA assay. Adipose and muscle homogenates were diluted to equal concentrations with Laemmli sample buffer. A total of 20 μg protein from each sample was
separated on 10% polyacrylamide gels at 100-125 volts, and transferred to nitrocellulose membranes (BioRad, Hercules, CA) for 1 hr at 50 volts. Membranes were blocked for 1 hr in Superblock (Thermo Scientific, Rockford, IL), then incubated with primary antibody against Lp-PLA$_2$ at a dilution of 1-200 (Cayman Chemical, Ann Arbor, MI) followed by the appropriate HRP-conjugated secondary antibody. Membranes were developed by chemiluminescence using SuperSignal West Dura substrate (Thermo Scientific, Rockford, IL), with digital images obtained using the Biospectrum UVP system. All signals were normalized to β-actin obtained from the same blot.

Adipose tissue immunohistochemistry

Adipose tissue was fixed for 24 hrs in 10% neutral buffered formalin, paraffin embedded, sliced, and mounted. Slides were dewaxed by washing 2 x 5 min in xylene, 2 x 5 min in 100% ethanol, 2 x 5 min in 95% ethanol, 5 min in diH$_2$O, and 10 min in TBS with 0.5% Tween 20. Slides were blocked in 5% BSA with 0.5% goat serum in PBS, then incubated overnight in primary antibodies against Lp-PLA$_2$ 1:50 (Cayman Chemical), and F4/80 1:100 (Santa Cruz Biotech, Santa Cruz, CA). Slides were then washed 3 x 5 min and incubated with appropriate FITC conjugated and Texas Red conjugated secondary antibodies. Slides were again washed 3 x 5 min each, mounted with DAPI containing mounting medium for identification of cell nuclei, then visualized by fluorescence microscopy (Nikon TE2000).
Statistics

Lean and obese groups, group means were compared using un-paired t-tests. Comparisons between tissue parameters within subjects were done using paired t-tests. Relationships between all factors within and between groups were analyzed using Pearson's correlations. Significance was set a priori at p<0.05.
Results

Subject characteristics

BMI was significantly higher in the obese group (49.9 ± 11.3) compared to lean group (25.8 ± 4.4) (p<0.01), and HbA1C was significantly greater in the obese group (6.41±0.35) compared to lean group (5.15±0.10) (p<0.01) (Figure 1). There were no significant differences between the lean and obese groups for plasma Lp-PLA2 activity (p=0.40) or TNF-α (p=0.20)

Tissue Lp-PLA2

To determine if Lp-PLA2 was present in adipose tissue, we performed western blotting on visceral adipose tissue homogenates. We found that Lp-PLA2 is present in adipose tissue from lean and obese subjects, though total adipose Lp-PLA2 was not significantly different between groups (p= 0.084)(Figure 2A). We observed a band corresponding to unmodified Lp-PLA2 at approximately 45kDa, as well as bands corresponding to glycosylated Lp-PLA2 at approximately 56kDa as shown previously [1](Figure 2A). For further analyses we considered both total Lp-PLA2 and the ratio of glycosylated to unmodified Lp-PLA2. The ratio of glycosylated to unmodified was also not different in adipose between lean and obese (p=0.1872). Lp-PLA2 was also present in whole muscle homogenates from a subset of subjects, though there was no difference between lean and obese total Lp-PLA2 (p=0.3893) or glycosylated Lp-PLA2 (p=0.7552) (Figure 2B). We compared total Lp-PLA2 pair wise in adipose and muscle in lean and obese subjects combined, as well as in obese subjects analyzed alone. Total Lp-PLA2 and glycosylated Lp-PLA2 were significantly
higher in adipose compared to muscle in all subjects (<0.0001), and (p=0.01), respectively (Figure 2C).

Adipose Lp-PLA₂, plasma Lp-PLA₂, and glucose homeostasis

We derived multiple correlations between all measured factors in obese subjects. A significant correlation was found between age and adipose glycosylated Lp-PLA₂ (r=0.648, p=0.012). Other relationships trending towards significance (r>0.4 or p<0.2) were found between HbA1C and adipose total Lp-PLA₂ (r=-0.517, p=0.126), age and HbA1C (r=0.408, p=0.213), and HbA1C and adipose glycosylated Lp-PLA₂, (r=0.491, p=0.125). Scatter plots for each correlation are shown in Figure 3.

The correlations were re-calculated in obese subjects with data from subjects taking glycemic control medication excluded. Data from the remaining obese subjects revealed a significant correlation between HbA1C and adipose total Lp-PLA₂ (r=-0.834, p=0.039). Trends toward significance(r>0.4 or p<0.2) were found between age and glycosylated Lp-PLA₂ (r=0.647, p=0.060), age and HbA1C (r=0.572, p=0.236). With subjects taking glycemic control medication excluded, the correlation between HbA1C and adipose Lp-PLA₂ glycosylation became negative and non-significant (r=-0.306, p= 0.556). Scatter plots for each correlation are shown in Figure 4.

The same correlations were then examined in lean subjects. The correlation between HbA1C and total adipose Lp-PLA₂ (r=0.674, p=0.067) approached significance. This positive correlation was in contrast to the negative correlation
measured in obese subjects. The relationship between plasma TNF-α and total adipose Lp-PLA₂ in lean subjects also trended towards significance (r=0.529, p=0.094). Scatter plots are shown in Figure 5. We did not observe a correlation between plasma Lp-PLA₂ activity and adipose Lp-PLA₂ in any group of subjects.

Lp-PLA₂ adipose tissue immunohistochemistry

Because Lp-PLA₂ was present in total adipose tissue homogenates, and we observed relationships between adipose Lp-PLA₂ and the circulating factors described above, we performed immunohistochemical analysis (n=8/group) to determine whether adipose tissue Lp-PLA₂ is localized within resident macrophages, adipocytes, or both. We found that Lp-PLA₂ is found almost exclusively in macrophages. A representative image is presented in Figure 6 showing a field at 30x magnification and a region within that field at 60x.
Discussion

Because it has recently been demonstrated that circulating LpPLA₂ is predictive of diabetes (in review), and obesity is an established risk factor for diabetes, we sought to characterize Lp-PLA₂ in human visceral adipose tissue and determine if tissue Lp-PLA₂ was related to HbA1C. The major findings of this study were that Lp-PLA₂ is expressed in human visceral adipose tissue, and adipose tissue Lp-PLA₂ is predominantly localized in macrophages. We also measured a non-significant negative correlation between HbA1C and adipose tissue total Lp-PLA₂ in obese subjects, but a positive non-significant correlation between HbA1C and glycosylated Lp-PLA₂ in adipose tissue. When subjects taking glycemic control medication were removed from our analysis of obese subjects, the negative relationship between HbA1C and adipose total Lp-PLA₂ became significant. However, the correlation between HbA1C and adipose glycosylated Lp-PLA₂ was weakened and became negative.

To our knowledge, this is the first time that Lp-PLA₂ has been characterized in human adipose tissue. While we hypothesized that Lp-PLA₂ would be greater in adipose tissue from obese subjects compared to lean, there was in fact no difference. This finding is paradoxical. We would expect Lp-PLA₂ to be greater in adipose tissue from obese subjects because we also found that Lp-PLA₂ was localized within macrophages resident in adipose tissue, and macrophage infiltration of adipose is a hallmark of obesity [23]. However, we did not quantify differences in adipose resident macrophages between our lean and obese subjects. It may be that the
absolute amount of Lp-PLA$_2$ is less important than post-translational modifications and activity. Indeed, we found a positive association between HbA1C and glycosylated adipose tissue Lp-PLA$_2$ in obese subjects, though this did not reach significance. This relationship was significantly weakened when subjects taking glycemic control medications were removed from the analysis. While glycosylation of Lp-PLA$_2$ is well established [1, 27], whether Lp-PLA$_2$ glycosylation occurs spontaneously or enzymatically is unknown, and the function of Lp-PLA$_2$ glycosylation is not well characterized. The single study that examined a role for Lp-PLA$_2$ glycosylation found that in macrophages glycosylation is not related to secretion of Lp-PLA$_2$ but it does play a role in Lp-PLA$_2$ association with HDL [1, 27]. We now submit that glycosylation of adipose Lp-PLA$_2$ may be associated with HbA1C, that this relationship is potentially affected by diabetes medications, but that total adipose Lp-PLA$_2$ is negatively correlated with HbA1C. Further studies should examine the function of Lp-PLA$_2$ and Lp-PLA$_2$ glycosylation in adipose tissue.

It is well established that TNF-α and other inflammatory cytokines produced by both macrophages and adipocytes impair insulin signaling in adipose as well as other tissues, contributing to systemic insulin resistance [19, 28, 29]. It is possible that Lp-PLA$_2$ from adipose tissue macrophages adversely affects insulin signaling as products of Lp-PLA$_2$ hydrolysis of oxidized phospholipids can induce inflammation and insulin resistance [22, 30, 31]. Macrophages are known to exist in different activation states in adipose tissue [32]. M1 macrophages are pro-inflammatory, secrete cytokines including TNF-α and IL-6, and produce increased quantities of reactive oxygen species. M2 macrophages produce anti-inflammatory cytokines
such as IL-10, and their role in wound healing and immunoregulation is well characterized (reviewed in [28]). Obesity is associated with increased M1 macrophages in adipose tissue and signals from M1 macrophages are related to insulin resistance [32], however it is unknown whether Lp-PLA₂ expression or activity are increased in M1 macrophages specifically. Collectively, these data support a hypothetical scenario that could in part explain the relationship between Lp-PLA₂ and diabetes. If adipose macrophage Lp-PLA₂ increases LPCs and free fatty acids that contribute to adipocyte insulin resistance, secretion of TNF-α and other cytokines that impair insulin signaling, adipose tissue Lp-PLA₂ may be part of the pathology of diabetes (Figure 7).

Finding Lp-PLA₂ in skeletal muscle, albeit at significantly lower levels than in adipose, was unexpected. A role for Lp-PLA₂ in muscle has not been examined. While we detected Lp-PLA₂ in muscle this was in a subset of subjects too small to use for examination of correlations with other measured factors. In addition, due to limited tissue availability we were unable to perform immunohistochemical analysis on muscle samples to determine localization of Lp-PLA₂. It is possible that Lp-PLA₂ also has a physiological role in skeletal muscle, but this cannot be determined by the data collected in our study.

While the data from these experiments are significant and hypothesis generating, limitations must be considered. The major limitation is a small number of subjects. In addition, conclusions must be drawn with caution because these are retrospective cross sectional data. It is of interest to determine if adipose
macrophages contribute to circulating Lp-PLA₂, however we did not see a strong association between adipose tissue Lp-PLA₂ and plasma Lp-PLA₂, which may be due to the small subject numbers.

In conclusion, we detected Lp-PLA₂ in human adipose tissue, and showed that adipose Lp-PLA₂ is localized within adipose tissue macrophages. We also found associations between HbA1C and adipose tissue Lp-PLA₂ glycosylation. Presence of Lp-PLA₂ in adipose tissue along with associations between adipose Lp-PLA₂ and HbA1C in obese subjects suggest future research should examine a role for Lp-PLA₂ in adipose tissue insulin resistance.
Figure 1. BMI and HbA1C are significantly greater in obese subjects compared to lean. * = p<0.01 compared to obese.

Figure 2. Lp-PLA₂ is expressed in human visceral adipose and skeletal muscle. Whole tissue homogenates were probed for Lp-PLA₂ and expression levels normalized to β-actin or expressed as the ratio of glycosylated to unmodified. A- Representative western blot showing Lp-PLA₂ in visceral adipose tissue. B- Representative western blot showing Lp-PLA₂ in skeletal muscle. C- Total Lp-PLA₂ and glycosylated Lp-PLA₂ are significantly greater in adipose than muscle. There were no differences in total Lp-PLA₂ of glycosylated Lp-PLA₂ within lean or obese tissues. Data are presented as mean ± SE, *= p<0.01.

Figure 3. Scatter plots showing the relationships found in obese subjects related to tissue Lp-PLA₂ with Pearson’s correlation coefficients and p-values.

Figure 4. Scatter plots showing the relationships found in obese non-diabetic subjects related to tissue Lp-PLA₂ with Pearson’s correlation coefficients and p-values.

Figure 5. Scatter plots showing the relationships found in lean subjects related to tissue Lp-PLA₂ with Pearson’s correlation coefficients and p-values.

Figure 6. Representative immunofluorescent images showing that adipose Lp-PLA₂ co-localizes with resident macrophages. Green (FITC) = Lp-PLA₂, red (Texas Red)=
macrophage marker F4/80, blue (DAPI)= nuclei. The region denoted by the red box in the 30x FITC image shows the region magnified for 60x images.

Figure 7. Schematic depicting the hypothesized role of adipose tissue macrophage Lp-PLA$_2$ in the pathology of insulin resistance where macrophage Lp-PLA$_2$ in the adipose tissue and skeletal muscle increases LPCs and free fatty acids, which are one of the stimuli increasing secretion of TNF-α and other cytokines that impair local and systemic insulin signaling. Remaining question included whether Lp-PLA$_2$ directly or through downstream products influences insulin resistance within the respective tissue, or in other peripheral tissues, and whether tissue production of Lp-PLA$_2$ contributes to circulating Lp-PLA$_2$ activity.
Figures

Figure 1.

![BMI and HBA1C graphs comparing Lean and Obese individuals.](image-url)
Figure 2

A.

Visceral Adipose

Lp-PLA₂
Actin

Lean control Obese

B.

Muscle

Lp-PLA₂
Actin

Lean control Obese

C.

Total Lp-PLA₂

Glycosylated Lp-PLA₂

Lean
Obese

Adipose Muscle

Adipose Muscle

Lean
Obese
Figure 3.

- For the first graph:
  \[ r = -0.517 \]
  \[ p = 0.126 \]

- For the second graph:
  \[ r = 0.648 \]
  \[ p = 0.012 \]

- For the third graph:
  \[ r = 0.408 \]
  \[ p = 0.213 \]

- For the fourth graph:
  \[ r = 0.491 \]
  \[ p = 0.125 \]
Figure 4.

1. $r = -0.834$
   $p = 0.039$

2. $r = 0.647$
   $p = 0.060$

3. $r = 0.572$
   $p = 0.236$

4. $r = 0.306$
   $p = 0.556$
Figure 5.

![Graph 1: Pearson correlation coefficient (r) = 0.674, p-value (p) = 0.067.]

![Graph 2: Pearson correlation coefficient (r) = 0.529, p-value (p) = 0.094.]

r = 0.674  
$p = 0.067$

r = 0.529  
$p = 0.094$
Figure 6.
Figure 7.
References


CHAPTER V- CONCLUSIONS

Conclusions

Because obesity is a well established independent risk factor for diabetes and coronary artery disease (CAD), it is important to identify factors associated with obesity that are responsible for disease progression, and interventions to decrease risk of developing obesity associated co-morbidities. In order to meaningfully decrease diabetes and CAD incidence, we must increase our understanding of the mechanisms responsible for the disease sequelae. Our analyses focused on obesity associated oxidative and lipid stress, and how these factors contribute to the pathophysiology of diabetes and CAD.

We first performed a lipidomic analysis of plasma from lean and obese humans to determine which lipid species are elevated in obese subjects that may contribute to vascular pathology. A group of ether-linked lipids was identified in the obese subjects, and a representative lipid from the identified group induced endothelial cell expression of cell adhesion molecules. In addition, we screened our data for oxidized phospholipids that are known to induce endothelial cell activation and did not find major differences between lean and obese subjects. Ether-linked lipids such as those identified as greater in our obese subjects may contribute to obesity associated vascular pathology, and warrant further investigation.
We also sought to characterize Lp-PLA₂ in human adipose tissue. Lp-PLA₂ hydrolyzes platelet-activating factor and oxidized phospholipids, is a risk factor for CAD and is predictive of diabetes. We found Lp-PLA₂ in human adipose in both unmodified and glycosylated forms, predominantly co-localized with macrophages. In addition, HbA1C in obese subjects was negatively associated with total adipose Lp-PAL₂ but positively with glycosylated Lp-PLA₂. These descriptive data support further investigation of the role in adipose tissue insulin resistance.

Last, we examined whether the phytochemicals in Protandim could induce Nrf2 and antioxidant enzymes in HCAEC, and protect HCAEC from an oxidative challenge. Protandim treatment induced Nrf2 nuclear localization and increased expression of HO-1, SOD1, Grx, and NQO1 in HCAEC. Protandim treatment also reduced HCAEC apoptosis in response to an oxidative challenge, and protection was significantly decreased when Nrf2 was silenced prior to Protandim treatment. These data suggest that Protandim may be an effective intervention against oxidative stress and associated vascular pathology. These results have important implications for CAD disease intervention, as exogenous antioxidant treatments have been largely ineffective.

Understanding the milieu of changes in lipids and oxidative stress is important to understanding disease pathology and designing effective interventions to treat co-morbidities associated with obesity. Collectively, our data provide insight into dyslipidemia in obesity, and the connection between dyslipidemia in obesity and CAD. In addition, our data suggest that investigation of the role of Lp-PLA₂ is warranted.
Finally, we provide insight into the mechanism and efficacy of a potential intervention against oxidative stress in vascular disease. Future investigations should further consider the role of ether-linked lipids in vascular pathology and the role of Lp-PLA₂ in obesity and diabetes. As our understanding of chronic metabolic disease characteristics and mechanisms improves, interventions will become targeted, and therefore more effective, which has significant implications for public health status and recommendations.
APPENDIX AND SUPPLEMENTARY MATERIAL

References


88. Apopa, P.L., X. He, and Q. Ma, Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells. J Biochem Mol Toxicol, 2008. 22(1): p. 63-76.


Consent to Participate in a Research Study At Colorado State University

TITLE OF STUDY: Oxidized Phospholipids, Weight Loss, and Metabolic Function

PRINCIPAL INVESTIGATOR:
Dr. Benjamin F. Miller PhD, Assistant Professor, Department of Health and Exercise Science, Colorado State University. bfmiller@cahs.colostate.edu

CO-PRINCIPAL INVESTIGATORS:
Dr. Karyn Hamilton PhD, Assistant Professor, Department of Health and Exercise Science, Colorado State University. Karyn.Hamilton@colostate.edu
Dr. Matthew Hickey PhD, Associate Professor, Department of Health and Exercise Science, Colorado State University. Matthew.Hickey@colostate.edu
Dr. Christopher Bell PhD, Assistant Professor, Department of Health and Exercise Science, Colorado State University. Christopher.Bell@colostate.edu
Dr. Christopher Gentile, Post-doctoral Fellow, Department of Food Science and Human Nutrition, Colorado State University. CGentile@CAHS.colostate.edu
Dr. Stephan Pettine MD, Bariatric Center for the Rockies

WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH? If you are a surgical patient of Dr. Stefan Pettine, Dr. Quaid, or Dr. Roller we are interested in you taking part in this study. WHO IS DOING THE STUDY? Dr’s. Miller, Hamilton, Hickey, and Bell are professors in the Department of Health and Exercise Science at CSU, and along with Chris Gentile, a Post-doctoral Fellow and Ms. Donovan (a doctoral student in the department), are interested in the positive changes that occur in fat metabolism and insulin sensitivity following gastric bypass surgery.

WHAT IS THE PURPOSE OF THIS STUDY? Following gastric bypass surgery, positive changes in the body’s ability to regulate blood sugar and process fats occur independently of weight loss. We are interested in studying these changes and using what we learn to assist in treatment of other patients. To understand the differences and changes in gastric patients, it is necessary to compare their measurements to a group of patients not at the same level of obesity, and not undergoing a bypass but still experiencing an operation.
WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST?
The study will take place at the Bariatric Center of the Rockies, and Colorado State University. All testing will coincide as closely as possible with regularly scheduled pre-operative and follow-up appointments with your physician through the one year post-operative appointment. If we are only collecting a blood sample we will do this at the doctor’s office. For bypass patients, pre-operatively, and three times post-operatively we will be performing a clamp procedure (described below), and on these days, blood draws will be done concurrently with the clamp at the Colorado State University Human Performance and Clinical Research Laboratory.

WHAT WILL I BE ASKED TO DO?

If you are a bypass patient:

- We will ask you questions about your past and current health status, as well as personal information including birth date, current state of health, and any medications you are taking. In addition, we will ask you to keep a 3 day food diary before the operation.

- Consent to a liver biopsy (removal of a small sample of liver tissue), abdominal muscle biopsy, and an abdominal fat biopsy being taken during the operation. Details of the biopsies will be provided by the surgeon

- Provide venous blood samples at your pre-operative appointment, and your 5 follow-up appointments (this will take approximately 20 minutes per visit).

- Undergo a clamp to measure your insulin (hormone involved in blood sugar regulation) sensitivity at the CSU HPCRL the same day as your pre-operative appointment, and 6 week, 6, and 12 month follow-up appointments.

The “clamp”: This is the name commonly given to the procedure formally known as the hyperinsulinemic euglycemic clamp. This procedure measures the ability of your body to handle sugar. We will inject sugar (glucose) into one of your veins and insulin (a naturally occurring substance produced by your body) into a different vein. We will continue to inject insulin and sugar into your veins to try to keep the concentration of sugar in your blood the same. We will measure this concentration every 5-minutes. This test lasts approximately 3 hours and we will ask you to perform it four times. The test will be administered by the research team at CSU.
If you are a patient scheduled for a surgery other than gastric bypass:

- We will ask you questions about your past and current health status, as well as personal information including birth date, current state of health, and any medications you are taken.

- Consent to a blood sample, liver biopsy (removal of a small sample of liver tissue), abdominal muscle biopsy, and an abdominal fat biopsy being taken during the operation. Details of the biopsies will be provided by the surgeon

**ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY?**
If you are over age 50 or under age 18 you will be excluded from this study, and if you have uncontrolled diabetes.

**WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?**

**Surgical Risks**
- There are slight risks associated with tissue sampling during surgery including bleeding, and infection. These risks do not increase the risk associated with the operation were you not participating in the study. They can be addressed further by the operating physician if you have additional questions or concerns.

**Research Risks**
- Venous blood sampling, can cause pain, fainting, could result in bruising, and poses a slight risk of infection. General risks will be minimized by using sterile technique, and applying direct pressure to the venous sites for five minutes following removal of sampling needle. The risks associated with fainting will be minimized by conducting all blood sampling procedures with the subject in the supine position.
- The clamp procedure involves placement of a catheter (hollow plastic needle) inside a vein thus the usual risks of blood collection apply (minor discomfort, bruising, fainting and blood clot (rare)). In addition there is a risk of hypoglycemia (low blood sugar); symptoms include hunger, nervousness and shakiness, perspiration, dizziness or light-headedness, sleepiness, confusion, difficulty speaking, and feeling anxious or weak. Although hypoglycemia can happen suddenly it can usually be treated very quickly by terminating the insulin infusion and continuing the glucose infusion, returning blood sugar concentration back to normal.
- It is not possible to identify all potential risks in research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known and potential, but unknown, risks.

**ARE THERE ANY BENEFITS FROM TAKING PART IN THIS STUDY?** You will receive information regarding your changes in weight, body composition, insulin sensitivity, and
blood lipid levels periodically throughout the study period. You will also receive a diet analysis from a nutritional expert. In addition, information generated in this study may be able assist in treatment of future patients.

**DO I HAVE TO TAKE PART IN THE STUDY?** Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled. No decision regarding this research will impact your medical care in any way.

**WHAT WILL IT COST ME TO PARTICIPATE?** It will not cost you anything to participate in this study. Any treatment or medical costs that arise as a result of your participation in this study are your responsibility.

**WHO WILL SEE THE INFORMATION THAT I GIVE?**
We will keep private all research records that identify you, to the extent allowed by law. Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. We may publish the results of this study; however, we will keep you name and other identifying information private.

We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. For example, your name will be kept separate from your research records and these two things will be stored in different places under lock and key. You should know, however, that there are some circumstances in which we may have to show your information to other people. For example, the law may require us to show your information to a court.

**CAN MY TAKING PART IN THE STUDY END EARLY?**
If you have difficulty tolerating the procedures or you miss multiple appointments you may be removed at any time. Also, if you begin taking medications or develop a condition that interferes with the study outcomes you may be removed. We will explain any reason why you would be removed from the study to you should such circumstances arise.

**WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY?**
You will receive $60.00 per visit where the clamp procedure is performed.
WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH? The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Claims against the University must be filed within 180 days of the injury.

WHAT IF I HAVE QUESTIONS?

Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the PhD student, Elise Donovan at 970-491-6702. If you have any questions about your rights as a volunteer in this research, contact Janell Barker, Human Research Administrator at 970-491-1655. We will give you a copy of this consent form to take with you.

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing ___ pages.

Please initial each line indicating you understand and consent to all portions of the study.

I have read the consent form and agree to keep a 3 day food diary
Yes___
No____

I have read the consent form and agree to a liver biopsy
Yes___
No____

I have read the consent form and agree to a muscle biopsy
Yes___
No____

I have read the consent form and agree to a fat biopsy
Yes___
No____

I have read the consent form and agree to blood sampling
Yes___
No____

I have read the consent form and agree to the glucose clamp procedure
Yes___
No____
(If you are a control the clamp consent is not necessary)

________________________________________________________________________
Signature of person agreeing to take part in the study  Date

________________________________________________________________________
Printed name of person agreeing to take part in the study

________________________________________________________________________
Name of person providing information to participant  Date

________________________________________________________________________
Signature of Research Staff
POUDRE VALLEY HOSPITAL
POUDRE VALLEY HEALTH SYSTEM
Fort Collins, Colorado

SURGERY OR OTHER PROCEDURE: I ____________________________ (as needed) permit Dr. ____________________________ to perform the surgery/procedure I have been recommended by my primary physician. If an assistant is named, they may be assisting in performing one or all of the following tasks under the direct supervision of my primary surgeon: Opening and closing, harvesting grafts, dissecting tissue, removing tissue, implanting devices, and altering tissues. The surgery/procedure I have been recommended is ____________________________.

THIS SURGERY OR PROCEDURE HAS BEEN RECOMMENDED BECAUSE: ______________________________________

MY OTHER TREATMENT OPTIONS INCLUDE: ____________________________________________________________

I acknowledge that I have read and understand the following risks related to anesthetics. By signing this consent, I allow the use of any anesthetics, sedatives or other medications as directed by my surgeon, anesthesiologist, or certified nurse anesthetist working under the direct supervision of an anesthesiologist, that may be necessary. I understand that the administration of anesthetics, including sedation, carries with it certain risks above and beyond those relating to the procedure itself. These risks include but are not limited to: respiratory (breathing) problems; blood pressure problems; irregular heart beat; intubability; nausea and vomiting; prolonged drowsiness; damage to teeth and/or dental work; unsteadiness; failure to achieve adequate sedation and/or possible awareness memory of the procedure; allergic or other unexpected and possibly severe drug reactions; nerve damage; extended hospital stay and death.

I UNDERSTAND THAT:
- Any surgery or procedure and the use of anesthetics have some risks. These risks can be serious and in rare cases result in death.
- Treatment results are not guaranteed and may not cure the condition.
- The risks listed above are the most common risks but are not all the possible risks associated with this operation or procedure.
- As is deemed necessary by the surgeon of record, medical product representatives may be present in the Operating Room solely to observe the use of that supplier's surgical and/or medical equipment.

RISKS: The most common risks are bleeding, infection, nerve injury, blood clots, heart attack, allergic reactions and pneumonia. Other risks of this particular operation or procedure include: __________________________________________________________

SURGERY PATIENTS:
If during my surgery the doctor finds an unexpected medical need, I permit him/her to provide the necessary treatment(s). My doctor has fully explained the surgical procedure in words I understand. I have read and fully understand this consent form and all my questions have been answered. Do not sign unless you have read and thoroughly understand this form.

Patient/Responsible party________________________________________ Date ________ Time ________
Witness________________________________________________________
Doctor________________________________________________________
Anesthesia Provider (if other than surgeon)________________________

CONSENT FOR SURGERY OR OTHER PROCEDURE
TRANFUSION CONSENT

BLOOD TRANSFUSIONS
I also understand that I may need to receive blood or blood products while I am in the hospital. I understand there are several options I may consider in receiving blood. I may have:

- Blood donated by myself for my own use (self-donated blood).
- Blood donated by a friend or family for my own use (directed donors).
- Blood currently available to the hospital from other donors (banked blood).
- Other sources are: ____________________________

I UNDERSTAND SOME OF THE RISKS OF RECEIVING BLOOD OR BLOOD PRODUCTS ARE:
- Getting an infectious disease (such as hepatitis or AIDS)
- Bad reactions such as: fever, hives, high blood pressure, shortness of breath, and heart or kidney problems.

I UNDERSTAND THAT:
- Results of blood transfusions are not always successful and that guarantees cannot be made that the transfusion will help me.
- Losing large amounts of blood may result in death, if blood is not replaced.
- All donors are carefully screened, and all blood is tested thoroughly and properly.
- Blood donated directly for my use by friends or family has NOT been shown to be safer than banked blood.
- If blood I donated to be given back to myself tests positive for hepatitis, it will be discarded to protect the health care workers in the hospital.
- If there is not enough donated blood for my use (either self or directed donations), blood from the Blood Bank will be used.
- This consent to blood transfusion(s) is effective throughout this hospitalization.

My doctor has fully explained the possibility of blood transfusion in words I can understand. I have read and fully understand this consent form, and all of my questions have been answered. Do not sign unless you have read and thoroughly understand this form.

Patient/Responsible Party__________________________________________________________Date__________________________Time__________________________

Witness__________________________________________________________

Doctor__________________________________________________________

I refuse to receive blood or blood products for any reason. My doctor has explained the likely complications which may occur if I need blood and do not receive it. I will accept all risks associated with my refusal to receive blood and will release my doctor and Poudre Valley Hospital from any responsibility for any bad results, including my death, which may occur because I refused to accept blood or a blood product. ____________________________

(Please put your initials here if refusing blood or blood products.)

Patient Identification

CONSENT FOR SURGERY
OR OTHER PROCEDURE
Research Authorization Form
for Use and Disclosure of Health Information

"Please type your response in the gray-box area. The box will expand to accommodate your full text.

1. Patient Name:
   Date of Birth:

2. **Title of Protocol**: Oxidized Phospholipids, Weight Loss and Metabolic Function

3. **General Purpose of Research**: Following gastric bypass surgery, positive changes in the body’s ability to regulate blood sugar and process fats occur. We are interested in studying these changes and using what we learn to assist in treatment of other patients.

4. **What personal health information do researchers want to use?** The researchers want to copy and use the portions of your medical record that they will need for their research. Research study information used and/or released may include the following:
   - Name, address, phone number, age, sex
   - The history and diagnosis of your disease;
   - Specific information about the treatments you received, including previous treatment(s) you may have had;
   - Information about other medical conditions that may affect your treatment;
   - Medical data may include laboratory test results, tumor and heart measurements, x-rays, CT scans, photographs of radiation therapy target areas, and pathology results;
   - Information on side effects (adverse events) you may experience, and how these were treated;
   - Long-term information about your general health status and the status of your disease;
   - Data that may be released to tissue and/or blood samples that may be collected from you
   - Codes that will identify you, such as your social security number and medical record number.

   You may request a blank copy of data forms from the study doctor or his/her research staff to learn what information might be shared.

5. **How will my personal health information be used?**
The hospital will use your health information for research. As part of this research, they may give your information to the following sponsor(s)/group(s) taking part in the research. The hospital may also permit staff from these sponsor(s)/group(s) to review your original records as required by law for audit purposes.

**RELEASE/DISCLOSE TO:**
"Please type your response in the gray-box area. The box will expand to accommodate your full text.
Elise Donovan, PhD candidate Dept. of Health and Exercise Scienc Colorado State University

Name/Title: Poudre Valley Health System Institutional Review Board (IRB) - (A group of people who review the research to protect your rights.)
Phone: (970) 495-7333 IRB Manager FAX: (970) 495-7625

Rev. 10/22/07 KWM/tw
6. **How will my health information be kept private?**
The research sponsor will keep all patient information private to the extent possible, even though it is not required to follow the federal privacy laws. Only researchers working with the hospital will have access to your information. The research sponsor will not release personal health information about you to others except as authorized or required by law. However, once your information is given to other organizations that are not required to follow federal privacy laws, we cannot assure that the information will remain protected.

7. **What if I do not sign this permission form?**
If you do not sign this permission form you cannot participate in the research study for which you are being considered.

8. **If I sign this form, will I automatically be in the research study?**
No, there will be further discussion and a separate consent to sign. After discussion, you may decide to take part in the research study. At that time, you will be asked to sign a specific research consent form.

9. **What happens if I want to withdraw my authorization?**
You can change your mind at any time and withdraw your permission to allow your health information to be used in the research. If this happens, you must withdraw your authorization in writing. Beginning on the date you withdraw your permission, no new health information will be used for research. However, researchers may continue to use the health information that was provided before you withdrew your authorization.

If you sign this form and enter the research study, but later change your mind and withdraw your authorization you will be removed from the research study at that time.

To withdraw your permission, please contact the person below. He/she will make sure your written request to withdraw your permission is processed correctly.

**Title of Contact Person:** Elise Donovan  
**Address:** 220 Moby-B Complex Fort Collins, CO 80523  
**Phone:** 970-491-6702  
**FAX Number:** 970-491-0445

10. **How long will this authorization last?**
If you agree by signing this form that researchers can use your personal health information, this permission has no expiration date. However, as stated above, you can change your mind and withdraw your authorization at any time.

11. **What are my rights regarding access to my personal health information?**
You have the right to refuse to sign this permission form. You have the right to review and/or copy records of your health information kept by PVHS. You do not have the right to review and/or copy records kept by the research sponsor or other researchers associated with the research study.

*(Signature Page on Next Page)*
Authorization Approval and Receipt Acknowledgement:

I hereby authorize the use or disclosure of the health information described in this authorization and acknowledges receiving a signed copy of this authorization. I understand that if anyone who receives my health information is not a health care provider or a health plan, my health information may not be protected by federal privacy laws if my health information is re-disclosed by that recipient person or PVHS.

Signature: ______________________________ Phone: ______________
Print Name: _____________________________________________
Address: __________________________________________________________________________

Date: ____________________

Authorization must be signed by the patient or by the parent/legal guardian or a minor, or by the legal representative when the patient lacks decisional capacity, or if the patient is physically unable to sign but mentally understands and consents.

Basis for legal authority to sign this authorization by a personal representative:

Parent, Guardian, Etc: ____________________________________________

Witness:

Signature: ____________________________
Print Name: ___________________________
Date: _______________________________
MEDICAL AND EXERCISE HISTORY

NAME__________________________ GENDER________ DATE________

BIRTHDATE___________________ AGE____ HEIGHT_______ WEIGHT____

ADDRESS______________________________________________________________

TELEPHONE____________________________ EMAIL______________________

1. Are you Diabetic? _____________

2. Are you insulin resistant__________
   Are you hyperglycemic________
   Are you hypoglycemic__________

3. Do you exercise?__________

4. How much time per week do you spend exercising? ____________ hours/week

5. Do you or have you ever smoked? _________
   If yes: How long ago?__________ For how many years?__________ How many packs/day?__________
6. Do you have a history of heart disease? _________
   Do you have a history of high blood pressure? _________

7. Have you ever had your cholesterol measured?
   1 = yes
   2 = no

   If yes- write the date and value (or if it was normal or abnormal)
   ___________________________________________________________

8. Any medical complaints now (illness, injury, limitations)?
   1 = yes    If yes, describe completely________________________
   2 = no     ________________________________________________
   ___________________________________________________________

9. Any bleeding disorders?
   1 = yes    If yes, describe completely________________________
   2 = no     ________________________________________________
   ___________________________________________________________

10. Any major illness in the past?
    1 = yes    If yes, describe completely_______________________
11. Any surgery or hospitalization in the past?
   1 = yes  If yes, describe completely
   2 = no

12. Are you currently taking any medications (prescription or over-the-counter:
    including birth control)?
   1 = yes  If yes, list drugs and dosages
   2 = no

13. Are you allergic to any medications?
   1 = yes  If yes, list medications
   2 = no

14. How many drinks per week do you consume on average? ________
15. Have you or anyone else in your immediate family ever been diagnosed with liver disease?

1=yes If yes, what family member(s), and what diagnosis?________________________

2=no ______________________________________________________________

________________________________________________________