Unconventional Secretion of Proteins from Adipocytes

A DISSERTATION
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Ajeetha Josephrajan

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Advisor: Dr. David A Bernlohr

October 2018
Acknowledgements

I would like to thank my advisor and mentor Dr. David Bernlohr for his constant support and motivation for the past 5 years. Even during times when things were looking bleak in the research side of things, I could persevere due to his support, his words of advice and wisdom. His guidance has helped me mature as a researcher and for that I am very thankful.

I would like to thank my PhD advisory committee for their time and valuable comments on my research. I would also like to thank my lab members who were always there to discuss ideas, experiments and teach me new things every day. I enjoyed going to work because of them. Especially, the past graduate students Hongliang Xu, Kaylee Steen, and Amy Hauck with whom I shared many memorable experiences and I am very glad to have gained their friendship. They motivated me and inspired me to always give my best.

I am also thankful of other past and current Bernlohr lab members Ann Hertzel, Dalay Olson, Cyrus Jahansouz, Scott Kizy and Keith Wirth for making research fun and a great experience. I would also like to thank my friends Rocio Foncea and Wendy Hahn for standing by me and helping me with reach both my career and life goals.

I am very grateful to all the teachers and mentors whom I have had in the past, who had helped me achieve my goals and guide me along both from UMN or my past schools, especially my previous mentors Dr. Anja Bielinsky and Dr. Bangyan Stiles.
Dedication

This dissertation is dedicated to my family and friends from India and the US, who have stood by me. I am very thankful to my parents, Josephrajan and Vani, my sister Anirudhya for their constant encouragement and support and my husband Nagendra Prasad, for sharing all my highs and lows.
Abstract

Endocrine function of the adipose tissue plays a major role in maintaining energy balance and glucose homeostasis by releasing a large number of bioactive proteins. Any dysfunction of the endocrine function of the adipose tissue caused due to obesity will initiate pathophysiological changes and hasten disease progression. In this thesis, I focus on the secretion of leaderless proteins from the primary cells of the adipose tissue, the adipocytes. This secretion process is called unconventional protein secretion (UPS) and as shown here for the first time, our results indicate that the UPS is highly regulated and a variety of proteins are secreted upon the adipocyte receiving a lipolytic stimuli. To characterize the UPS, we followed the secretion pathway of unconventionally secreted adipocyte fatty acid binding protein (FABP4). FABP4 is one of the majorly expressed protein in mature adipocytes whose intracellular function is lipid storage and trafficking. Increasing evidence indicates that FABP4 has multiple functions extracellularly and is strongly associated with metabolic disease progression. Our results elucidate the regulation and mechanism of UPS/FABP4 secretion pathway. Unraveling the role of UPS proteins in the circulation and integrating them as a systemic response will be central to our understanding of the balance between healthy and unhealthy states. Such a study will be more insightful in predicting metabolic diseases than analyzing different individual marker proteins in the blood stream at a time for various pathologies.
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CHAPTER 1

METABOLIC FUNCTIONS AND PATHOPHYSIOLOGICAL IMPACTS OF ADIPOCYTE FATTY ACID BINDING PROTEIN (FABP4)

This chapter was written by Ajeetha Josephrajan
1. Adipose tissue morphology and function:

Adipose tissue (AT) is localized in two compartments namely the subcutaneous depot, that is underneath the skin and visceral depot, that is present in the trunk of the body. AT is therefore considered a multi-depot organ. AT is composed of the primary cell type adipocytes and its progenitors, stem cells, fibroblasts, endothelial cells and a range of inflammatory cell types including macrophages, neutrophils, lymphocytes. Adipose tissue is categorized into white (WAT) or brown adipose tissue (BAT). WAT is composed of adipocytes with large unilocular lipid droplet wherein the lipids are stored as triglycerides which can be utilized in time of energy demand. This concept of fat storage is conserved from unicellular to multicellular organisms. WAT is functionally important for maintaining systemic energy homeostasis whereas BAT is characterized by adipocytes with multilocular lipid droplets and play a major role in adaptive thermogenesis for maintaining body temperature. AT was originally considered as an inert organ with a major function of lipid storage, however, accumulating evidence clearly indicates that the tissue has diverse biological functions that varies based on their location, composition and environmental cues. Some of the functions include glucose and lipid metabolism, appetite regulation, coagulation, angiogenesis, reproduction, body weight homeostasis and vascular tone control.

1.1. Adipose tissue, an endocrine organ:

White adipose tissue is one of the largest endocrine tissues of the human body. The bioactive peptides secreted by WAT are collectively called adipokines and they can act in an endocrine, paracrine or autocrine manner causing effects on other organs via
circulation. Leptin, often referred to as the satiety hormone, was the first discovered hormone that was secreted from adipose tissue. Ever since that discovery, multiple secreted factors that control neuroendocrine and immune functions have extensively been identified. Additionally, the AT not only secretes, but is also responsive to other afferent signals from the different hormonal pathways or central neural system (CNS) due to the availability of various cell surface receptors. The adipokines function as immunologic, cardiovascular, metabolic and endocrine regulators. For example, secreted factors such as tumor necrosis factor (TNFa), interleukin-6 (IL-6) and complement factors B, C3 and D are immunologic. Renin–angiotensin axis molecules and plasminogen activator inhibitor-1 (PAI-1) have cardiovascular functions by regulating blood pressure or controlling fibrinolysis, respectively. Adiponectin, resistin, agouti related peptide, visfatin, lipoprotein lipase (LPL), cholesterol ester transfer protein (CETP), apolipoprotein E (ApoE) are molecules that have major roles in metabolic pathways and are extensively studied. Dysregulation of the endocrine function of the AT is one of the primary reasons for metabolic diseases in humans.

1.2 Adipose tissue lipolysis:
Dietary fatty acids are utilized by our body for energy production, membrane building and as signaling molecules. However, increased levels of non-esterified FAs (NEFA) can lead to multiple deleterious effects including, but not limiting to, ectopic fat deposition in organs and tissues, mitochondrial dysfunction and inflammation leading to metabolic disorders. This condition is often termed lipotoxicity. To counteract excess NEFA, AT esterifies these fatty acids with glycerol and stores them as triglycerides (TG).
Additionally, during times of nutrient excess the AT can undergo de novo lipogenesis (DNL) to synthesize fatty acids that can be stored, to be mobilized during energy demand.

Upon starvation or energy demand the TG are hydrolyzed by a process called lipolysis. Lipolysis refers to the hydrolysis of the stored triglyceride (TG) in the lipid droplet into three free fatty acid (FFA) and a glycerol. This process occurs sequentially with the help of hydrolytic enzymes called lipases. Three lipases act sequentially to break down the TG; first the TG is metabolized to diacyl glycerol (DG), followed by DG into monoacyl glycerol (MG) and the MG is hydrolyzed into glycerol and in each step, there is a concomitant release of one FFA. Adipose triglyceride lipase (ATGL) is the first enzyme of this process that converts TG into DG and is a rate-limiting step. DG is hydrolyzed into MG by hormone sensitive lipase (HSL). Monoglyceride lipase (MGL) breaks down MG into glycerol. Lipolysis is positively regulated by beta-adrenergic receptors signaling (β-AR).

The β-AR is a G-protein coupled receptor (GPCR) that is activated by catecholamines such as glucagon or epinephrine. Once activated, the Gs subunit of the GPCR in turn interacts with and activates adenylyl cyclase (AC). AC enzyme converts ATP to cAMP, the cAMP thus formed will activate protein kinase A (PKA). PKA phosphorylates cytosolic HSL and lipid droplet associated protein PLIN-1 and activates them. PLIN-1 phosphorylation enables downstream activation of ATGL. Together, ATGL and HSL
promote lipolysis. This FA cycling between lipogenesis and lipolysis is crucial for maintaining energy homeostasis in the body.

**1.3 Dysfunctional adipose tissue causing metabolic syndrome:**

During times of chronic caloric excess, AT gets enlarged due to nutrients being directed to them, thus overall protecting the body from increased blood glucose and NEFA levels. However, there is a limit to how much the AT can expand in a healthy manner. After which, the AT reaches the maximal capacity for excess fatty acid storage and consequently elevates the systemic lipid levels in the circulation which can reach other organs and tissue, thereby, causing deleterious effects such as liver steatosis or fibrosis.\(^{19,20}\) This AT enlargement defines obesity, wherein the tissue expansion can be due to pathological hyperplasia (increased cell number) or hypertrophy (increased cell size). Consequently, there is dysfunction in proliferation and differentiation of adipocytes and tissue plasticity and increased oxidative stress including hypoxia\(^ {21}\). As such, this condition causes multiple downstream effects including increased ROS production, ER stress, mitochondrial dysfunction, lipid aldehyde formation and unfolded protein response. Indeed, obesity driven lipid peroxidation and subsequent protein carbonylation can cause protein loss of function. For example, carbonylation of the nuclear protein estrogen-related receptor gamma (ERR-γ) results in the loss of its DNA-binding capacity leading to impaired mitochondrial biogenesis\(^ {22}\).

Additionally, AT also produces chemokines that recruit macrophages to infiltrate the expanding tissue. Macrophage infiltration causes another whole set of complications
leading to increased inflammation both in a local and systemic manner. These changes drive pathophysiological conditions such as dyslipidemia, hyperglycemia, hyperinsulinemia and insulin resistance. These multiple metabolic abnormalities encompass the basis for a condition collectively named metabolic syndrome (MetS). The MetS in turn drives the pathology for other diseases including Type-2-diabetes (T2D), cardiovascular diseases (CVD) and renal dysfunctions.

This thesis will specifically focus on the endocrine nature of adipocytes in driving systemic pathologies and metabolic syndrome. When the concentration of adipokines such as the ones mentioned earlier are changed during obesity, it can lead to beta cell dysfunction, insulin resistance, respiratory disorders, cancer, infertility, hepatic and renal dysfunction and cardiovascular complications as illustrated in Manna P et al 2015. This list of adipokines are expanding as we understand the adipose tissue biology better with improved technologies.

2. FABPs – Structure and function:

Fatty acid binding protein 4 belongs to a super family of small cytosolic proteins that is highly conserved across different species, these proteins can bind long chain fatty acids and other hydrophobic ligands. The first FABP was reported in 1972, since then other types of FABPs have been identified in different tissues namely liver FABP (FABP1), intestinal FABP (FABP2), heart FABP (FABP3), adipocyte FABP (FABP4), epidermal FABP (E-FABP5), ileal FABP (FABP6), brain FABP (FABP7), myelin FABP (FABP8), and testis FABP (FABP9). Though FABP family members have a
sequence similarity of 15-70% \(^{24}\), their three-dimensional structures have very high similarity with a 10-stranded anti-parallel \(\beta\)-barrel structure forming a cavity that is highly charged and polar and not different from the surface charge of the protein \(^{27}\). Each five strands form sheets that run orthogonal to each other, forming a pocket that is closed at the bottom. The top has a helix-turn-helix motif between \(\beta\)-strands 1 and 2 forming a lid to the cavity allowing entry and exit of lipid molecules as shown on Figure 1. Each FABP can bind at least one fatty acid except for LFABP which can bind more than one FA \(^{28}\). It should also be noted that different subtypes have different binding affinity and selectivity for ligands due to their structural differences. When a fatty acid binds to, for example FABP4, it forms salt bridge with Arg106, Arg126 and Tyr128 and mutations in these amino acids can lead to ligand binding deficient protein \(^{29}\).

Figure 1: X-ray crystallographic image showing FABP4 complexed with arachidonic acid The structure of FABP4 is depicted as a ribbon and arachidonic acid as space-filling spheres (RCSB PDB:1ADL) \(^{30}\).
2.1 FABP4 - Protein-protein interaction and function:

The focus of this thesis is the protein FABP4, which is primarily expressed in mature adipocytes and contributes to 1% of protein in AT \textsuperscript{31}. FABP4 expression can be controlled by adipocyte differentiation, and by chemical and hormones such as peroxisome proliferator-activated receptor (PPAR) \( \gamma \) agonists, dexamethasone, and insulin \textsuperscript{32,33}. FABP4 is also expressed in macrophages albeit 10,000-fold lesser than adipocytes \textsuperscript{34}.

FABP4 functions in fatty acid storage, and solubilizing and transporting ligands to different organelles for utilization. For example, for fatty acid oxidation, FABP4 can carry lipid to mitochondria or peroxisomes for oxidation or to ER, facilitating membrane synthesis, trafficking and signaling. They also influence transcriptional regulation in the nucleus \textsuperscript{35}. Additionally, FABP4 can quench reactive oxidized lipids such as 4-hydroxynonenal through protein carbonylation at its Cys117 site thus regulating the redox status of cells \textsuperscript{36}.

More importantly, FABP4 regulates HSL to influence lipolysis \textsuperscript{37,38} and lipid signaling. Work using fluorescence resonance energy transfer (FRET) shows that FABP4 and HSL physically interact and this interaction requires a FFA bound to FABP4 and HSL to be phosphorylated \textsuperscript{39}, shown in figure 2. Indeed, a small molecular inhibitor HTS01037 that blocks NEFA binding to FABP4 antagonizes HSL interaction and decrease the lipolytic activity of HSL \textsuperscript{40}. The HSL interaction with FABP4 is through the D17, D18, K21 and R30 on the helix together termed the charged quartet. ATGL activation during \( \beta- \)

There is a complex interaction of FABP4 and nuclear hormone receptor PPARγ activity. FABP4 expression can be controlled by PPARγ activity, but PPARγ activity can in turn be regulated by FABP4. FABP4 can translocate to the nucleus carrying an agonist to enhance the transcriptional activity of PPARγ. Alternatively, FABP4 in non-ligand bound (apo-FABP4) state can also extract ligand from the nuclear hormone receptor thus negatively regulating it, rendering this interaction very context dependent. Consequently, by regulating NEFA levels and localization, and by interaction with other proteins such as HSL, Cgi58 and PPARγ, FABP4 regulates lipid metabolism and insulin sensitivity. Ligand bound FABP4 also interacts with the unphosphorylated tyrosine kinase (Janus Kinase 2) JAK2 protein and attenuates the downstream JAK2 mediated signaling. This is another example of protein-protein interaction of FABP4 affecting cellular metabolism. Phosphatase and tensin homolog on chromosome 10 (PTEN), which negatively regulates the phosphoinositide 3-kinase pathway (PI3K), has also been reported to interact with FABP4, possibly regulating adipocyte differentiation and lipid metabolism.
Figure 2: FABP4 facilitates lipolysis
FABP4 physically binds with ATGL (Adipose triglyceride lipase) co-activator CGI-58 (Comparative gene identification-58) without the requirement of FFA binding and Hormone Sensitive Lipase (HSL) with a requirement of FFA binding on the lipid droplet. Upon activation of lipolysis, FABP4 facilitates lipolysis by shuttling FFA (represented in orange) to the plasma membrane for their release extracellularly.

2.2 Intracellular function of FABP4:
FABP4 has multiple roles intracellularly. When FABP4 deficient mice (AKO) were challenged with high fat diet, the mice became obese, but were protected from insulin resistance and hyperinsulinemia\(^ {49} \). This was true also true for the genetically induced obese (GIO) mouse model with a leptin deficient background (ob/ob mice)\(^ {50} \). In FABP4 and FABP5 double knockouts, the mice were protected from diet induced obesity (DIO) and insulin resistance and type 2 diabetes mellitus\(^ {51} \). The same metabolic benefit was observed for mice lacking FABP4, FABP5 and leptin (ob/ob FABP4\(^ {/-}\) FABP5\(^ {/-}\)) despite increased obesity\(^ {52} \). Also, FABP4 inhibitor (BMS309403) administration to
DIO and ob/ob mice decreases plasma triglycerides. FABP4 deficiency in atherogenic ApoE−/− mouse model decreases plaque formation and macrophage accumulation. In human studies, polymorphism in the promoter region of FABP4 reduced the protein transcription and the subjects have improved metabolic and cardiovascular health.

Adipose tissue macrophage (ATM) infiltration during obesity causes inflammation linked diseases. Therefore, mechanistic role of FABP4 signaling in macrophages has gained a lot of interest due to its clinical application. FABP4 loss results in increased levels of intracellular FFA in macrophages, which upregulates uncoupling protein 2 (UCP2) expression. Increased UCP2 has been shown to reduce intracellular reactive oxygen species (ROS) which can reduce ER stress and oxidative damage. Therefore, negative effects of lipotoxicity including inflammation are blunted upon FABP4 loss. Furthermore, when macrophage FABP4 expression was ablated, it diminishes the cysteine oxidation of proteins in mitochondria, decreasing the accumulation of oxidized proteins thereby regulating the redox state of the cell. As a result, there is decreased cellular stress and inflammation as observed by decrease in interleukin 1β (IL-1β) secretion, inflammasome activation and an upregulation of antioxidants.

3. Extracellular FABP4:

FABP4 was primarily studied as an intercellular protein since it discovery for over 20 years. Later it was identified that FABP4 and its isoforms were found in the serum however, it was primarily attributed to adipose tissue damage and cell lysis. For example,
FABP1 and FABP3 were found in the bloodstream due to liver damage during transplant and after myocardial infarct in men respectively \(^{64,65}\). Since, multiple studies have shown that FABP4 is secreted from adipocytes in the absence of cellular damage beginning with the first observation made by Xu and colleagues. In this study, they established that FABP4 secretion positively correlated with body weight in human subjects \(^{66,67}\). FABP4 is now being appreciated as a secreted protein found in circulation of mammals, that can signal in peripheral tissue and is therefore reclassified as an adipokine.

FABP4 lacks a leader sequence which is a 16-30 residue N-terminal secretory signal sequence that is positively charged, hydrophobic, with no specific sequence consensus with a cleavage site for signal peptidases. These leader sequences usually play a role in targeting the newly made proteins to the ER \(^{68}\). The nascent proteins are transported from ER to golgi by getting packaged into COPII vesicles, these transport or secretory vesicles then release their contents extracellularly by exocytosis \(^{69,70}\). This kind of protein secretion is classified as the classical ER-Golgi secretory pathway. Proteins that don’t follow this pathway for secretion are classified as unconventionally secreted proteins \(^{71}\). FABP4 is indeed one of the unconventionally secreted proteins \(^{66,72}\).

### 3.1 Regulation of FABP4 secretion:

The mechanism of secretion of FABP4 is a field of active research due to the multiple effects the secreted FABP4 has systemically. One of the major observations regarding secreted FABP4 was that the primary contributors to the circulating fraction of FABP4 are from adipocytes and only a small fraction is from cells of hematopoietic origin such
as macrophages. This secretion from adipocytes is stimulated in response to lipolysis. Lipolysis can be activated by β-adrenergic receptor mediated cAMP increase activating protein kinase A (PKA), or natriuretic peptide receptor-A (NPR-A)-mediated guanylyl cyclase (GC)-protein kinase G (PKG) pathways. FABP4 has been found to be secreted by both PKA and PKG activation.

Further research by both chemical and genetic models established that the secretion requires activation of downstream proteins of PKA pathway, such as ATGL and HSL. Additionally, when an excess of FFA are made available to adipocytes by exogenous FA treatment or by Plin1 genetic knock out, it potentiated FABP4 secretion. All these data indicate that the secretion is dependent on activation of proteins involved in releasing FFA rather than effects of different functions of β-adrenergic signaling or PKA activation. FABP4 is a lipid binding protein and the secretion of the protein is driven by FFA availability, therefore the most evident question is if this secretion is dependent on the ability of FABP4 to bind FA. When a lipid binding deficient mutant of FABP4 was analyzed for its secretion, there was a partial requirement for lipid binding but it wasn’t entirely dependent on its ability to bind lipids.

In addition to lipolysis, FABP4 secretion is regulated by intracellular calcium levels, increasing Ca2+ levels by using ionomycin significantly increased secretion. Under conditions of expanding tissue mass such as obesity there is a lag in vascular capacity to provide for the growing tissue thus making the environment hypoxic. Results from Wu et al., indicates that hypoxia also potentiates FABP4 release from adipocytes.
3.2 FABP4 and glucose homeostasis:

There is a complex interplay between secreted FABP4 and its effect on insulin secretion, glucose homeostasis and insulin’s effect of FABP4 secretion. Initially, it was observed that FABP4 KO mice had a reduction in insulin secretion, further studies indicated that glucose stimulated insulin secretion (GSIS) was improved in ob/ob FABP4 KO mice when compared to controls. When a similar study was performed using a chemical inhibitor instead of a genetically obese mice model lacking FABP4, insulin and glucose tolerance were not improved, indicating that there is a complex interplay between FABP4 and insulin regulation and it was proposed that this was based on how the mice got obese.

Intriguingly, a study was published wherein FABP4 increased insulin secretion from β cells in vitro with improved GSIS and glucose tolerance when FABP4 was delivered with the fatty acid linoleate. This contrasts with earlier published results which show improved GSIS in FABP4KO mice. It is still unclear, if this potentiated secretion is due to FABP4 treatment or the fatty acid availability, in this case linoleate, as FFA can regulate insulin secretion or if this contrast can be attributed to acute vs chronic effects and time dependence. Studies have also shown that insulin which is an anti-lipolytic signal can block FABP4 secretion. In addition to β cells, FABP4 acts as a paracrine adipokine and has systemic effects on glucose metabolism by regulating liver gluconeogenesis. Work done by Cao et al., shows that FABP4 potentiates gluconeogenesis from primary hepatocytes and stimulates glucose production.
3.3 Secretory pathway of FABP4:

Another important pathway that still isn’t completely understood is the trafficking of FABP4 extracellularly. There are multiple ways as to how a protein can be transported unconventionally to a cell’s exterior. Initially, it was proposed that FABP4 was secreted via their packaging into multi-vesicular bodies (MVB) and microvesicles as they can cargo unconventionally secreted proteins \(^{71}\). MVB are formed by maturation of early endosomes, which are structures that invaginate and internalizes molecules from plasma membrane along with some intercellular fluid. The MVB is characterized by the presence of many intact luminal vesicles that are formed by invagination of the endosomal membrane and incorporating some of the luminal contents of the endosomes. These rounded vesicular structures thus formed are called intraluminal vesicles (ILV). The ILV thus formed are released as exosomes \(^{82}\) as illustrated in figure 3. On the other hand, the microvesicles are formed by budding of the plasma membrane with intracellular contents. The microvesicles are heterogenous in their size and composition \(^{83}\) as shown in figure 3. Studies have shown FABP4 to be present in MVB, exosomes and microvesicles \(^{72,75,76,84}\). However, further studies by our lab and other have clarified that they only contribute to a small fraction of the secreted material and the majority of the protein is found in the fraction of extracellular material that is depleted of vesicles \(^{75,84}\), albeit the vesicular FABP4 secretion is also regulated by insulin or calcium \(^{72,75}\).
Figure 3: Vesicular biogenesis, release and uptake by recipient cell

(1) Shows endocytosis wherein plasma membrane (PM) surface receptors are internalized along with some cellular material to form early endosome (EE). (2) The formation of EE is followed by its maturation into late endosome (LE) or multi vesicular body (MVB). This maturation process involves invagination of the EE at multiple sites to form intraluminal vesicle (ILV). (3) The LE/MVB can either fuse with lysosome and be degraded or (4) fuse with the PM to release its contents into extracellular space. (5) The released ILVs are now called exosomes. (6) shows the exosomes being taken up by a recipient cell through various mechanisms like adsorption, endocytosis or membrane fusion to PM. Microvesicles are formed by the budding of plasma membrane. Both these vesicles are shown to cargo FABP4 albeit in small amounts.

3.3.1. Secretory lysosomes and FABP4 secretion:

Secretory lysosomes are modified lysosomes that can secrete proteins in response to stimuli and appears to be a regulated process. Most of what we understand about these lysosomes comes from Cytotoxic T Lymphocytes (CYL) and natural killer cells (NK)’s secretion of pore forming protein. Several genetic diseases wherein immune function is...
compromised such as Hermansky–Pudlak, Chediak–Higashi and Griscelli's syndromes have deficient secretory lysosomes\textsuperscript{86}. Conventional and secretory lysosomes are both terminal points of the endocytic pathway, therefore there are a lot of commonalities between them and further research is warranted to identify the unique features of secretory lysosomes. It is also unclear how the proteins that are packed in these lysosomes are protected from the acidic environment, however there are evidences of protein protection by carbohydrate modifications\textsuperscript{87}. 

Currently, it is accepted that secretory lysosomes utilize unique sorting and secretion pathways when compared to degradative lysosomes and a few markers such as Rab27a\textsuperscript{88} and Lyst\textsuperscript{89} are pointed out to be specific for secretory lysosome exocytosis. Membrane protein Synaptotagmins (Syt), whose activity is in a Ca\textsuperscript{2+} dependent manner, is also used as a marker for secretory lysosomes\textsuperscript{90}. Recent published work by Villeneuve et al. indicate that FABP4 is enclosed in endosomes and secretory lysosomes by using surface markers\textsuperscript{91}. Herein, they show that FABP4 secretion is independent of Golgi reassembly stacking protein (GRASP) proteins, which are implicated in mediating unconventional secretion of both transmembrane and cytoplasmic proteins\textsuperscript{92} and MVB\textsuperscript{71}. They also utilize ATG5 deficient adipocytes (with defective classical autophagy)\textsuperscript{93} to show that FABP4 secretion was not attenuated and concluded that FABP4 secretion is independent of autophagy.
4. Cross-sectional studies of effects of FABP4 in circulation:

4.1 FABP4 and metabolic syndrome:

The first study that lead to a flurry of research in the area of circulatory FABP4 was published in 2006 that indicated that FABP4 was released from adipocytes and the levels positively correlated with obesity, waist circumference, blood pressure, dyslipidemia, fasting insulin, and the homeostasis model assessment of insulin resistance index (HOMA-IR). The FABP4 levels also correlated with increasing number of metabolic diseases shown in figure 4. This observation has been supported by other studies across ages and sex differences, for example in childhood obesity and cardiovascular diseases CVD. 5-year and 10-year follow-up studies in a Chinese population measuring FABP4 in baseline levels predicted the development of T2D, which led the authors to conclude that increased FABP4 levels corresponded with the risk of developing diabetes and can be used as a predictor of development of T2D.

Additionally, treatment of T2D with PPARγ agonists such as thiazolidinedione increases plasma FABP4 concentration.

Studies also report correlation of FABP4 with other metabolic diseases like hypertension, NAFLD, ER stress in liver cells, hyperlipidemia, vascular inflammation, ischemic stroke. Recently study on 737 patients with acute ischemic stroke reports that FABP4 is a novel independent prognostic marker to improve the currently used risk measurement of ischemic stroke patients. As a part of a Tanno-Sobetsu study of 296 subjects on no medication, FABP4 levels were measured and found to be a predictor of insulin resistance and serum FABP4 was therefore proposed as a biomarker for pre-
clinical stage of metabolic syndrome (MetS)\textsuperscript{110,111}. Similar to FABP4, FABP5 is also found to be circulating and its levels are also increased with metabolic syndromes\textsuperscript{112,110,113,114}.

\textbf{Figure 4: Circulating FABP4 and its pathophysiological effects}

FABP4 secreted from adipocytes during lipolysis can contribute to the development of metabolic diseases. As the lipolysis increases during disease pathologies there is an increase in FABP4 secretion resulting in a feed-forward loop. Figure adapted from Furuhashi et al\textsuperscript{115}.

\textbf{4.2 FABP4 and Cardiovascular diseases:}

FABP4 levels also correlate with progression of atherosclerosis\textsuperscript{116–120}, coronary artery disease (CAD)\textsuperscript{121} and CVD\textsuperscript{122,123}. One of the earlier studies also showed that FABP4 suppresses cardiomyocyte contraction leading to cardiomyocyte contractile dysfunction.
in obese subjects \(^{84}\). Indeed, several reports in human subjects show that FABP4 levels can be used to predict the development of CVD \(^{122,124,125}\).

### 4.3 FABP4 and Cancer:

#### 4.3.1 FABP4 and ovarian cancer:

Increased fatty acid uptake by cancerous cells can lead to increased metastasis and epithelia to mesenchymal transition (EMT) \(^{126}\). Within the last decade multiple studies have implicated FABP4 in tumor progression. In the case of ovarian cancer, FABP4 was thought to provide the FAs that aided in ovarian cancer progression \(^{127}\). Additionally, FABP4 expression was increased in ovarian cancer cells and FABP4 suppression impairs metastasis in mice \(^{127,128}\). Since, FABP4 is considered as a biomarker for residual disease in high-grade serous ovarian cancer \(^{129}\). Further research was done by Gharpure KM et al., to understand the functional implication of FABP4 overexpression and how that drives the metastatic potential of ovarian cells. Their results show that the hypoxic environment of the cancer decreases the microRNA miR-409-3p levels. The function of miR-409-3p is to negatively regulate FABP4 expression. Treatment with miR-409-3p mimic or \(FABP4\) siRNA inhibited tumor progression in mouse models \(^{126}\). The poor prognosis of this cancer it attributed to FABP4 and is extensively researched for its therapeutic potential.

#### 4.3.2 FABP4 and breast cancer:

Breast cancer patients that have high serum levels of FABP4 have worse prognosis \(^{130,131}\) this is further exacerbated with diabetes \(^{132}\). Another study shows that a single-nucleotide
polymorphism in the 3'-UTR region of FABP4 gene in the adipose tissue is associated with triple-negative breast cancer (TNBC) occurrence and prognosis. FABP4 is not overexpressed in breast cancer cells as seen in ovarian cells but rather exogenous FABP4 treatment seems to increase FABP4 expression in breast cancer cells. Studies done in MCF-7 breast cancer cell lines treated with exogenous FABP4 suggests that FABP4 induces the PI3K-AKT and MAPK-ERK signaling pathways, and induces the expression of proto-oncogene FOXM1 and fatty acid transporter CD36 and FABP5. Thereby possibly increasing FA availability and a lipotoxic environment (with increased ROS and angiogenic properties) that can help facilitate tumor progression.

4.3.3 FABP4 and other cancers:
Elevated expression of FABP4 is correlated with multiple other cancer types including oral, cervical squamous cell carcinoma, bladder cancer, non-small cell lung cancer, and glioblastoma. FABP4 is also linked to prostate cancer progression and may be useful as a therapeutic target, herein FABP4 is overexpressed and secreted by the prostate cancer cells which activates prostate stromal cells (PSc) present in the tumor microenvironment. The PSc in turn increases the invasiveness by secretion of IL-6 and IL-8. A recent report has also shown that FABP4 is linked to obesity and aggressive myeloid leukemia (AML). Investigations revealed that FABP4 increased expression of IL-6 and signal transducer and activator of transcription factor 3 (STAT3) phosphorylation. These changes led to overexpression of DNA methyltransferase 1 (DNMT1) and further silencing of the p15 tumor-suppressor gene. This FABP4/DNMT1 axis is a potential target for improving prognosis of AML.
some indications of FABP4’s role in hepatocellular carcinoma (HCC), however, there is conflicting data and needs further investigations.\textsuperscript{145,146}.

### 4.4 FABP4 inhibitors and interventions:

There is a need for reducing the circulating levels of FABP4 given its role in causing metabolic dysfunctions and disease progression. FABP4 levels are increased in obese subjects, therefore interventional therapies to reduce obesogenic conditions have been performed in human subjects through weight loss programs\textsuperscript{147} or bariatric surgery\textsuperscript{148–150} to reduce FABP4 levels. Additionally, medical interventions\textsuperscript{151} such as Atorvastatin, a HMG-CoA reductase inhibitor, and olmesartan, an angiotensin II receptor blocker, are medicated to reduce circulating FABP4 levels\textsuperscript{152,153}. Similarly, treatment with dyslipidemic agents such as omega-3 fatty acid ethyl esters containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) also decrease circulating levels of FABP4\textsuperscript{154}. Majority of studies show that decreased FABP4 can be attributed to beneficial effects of these interventions. More importantly, Burak et al., developed a monoclonal antibody, CA33 that is specific for FABP4. This antibody was injected into dietary or genetic obesogenic mice to neutralize circulating FABP4. They found that the treatment with this antibody improved systemic insulin sensitivity, glucose metabolism, decreased fat mass and steatosis of liver\textsuperscript{155}. All these evidences point to the importance of therapeutic targeting FABP4 for metabolic diseases.
5. Goals and objectives:

Adipose tissue function and the adipokine secretion are susceptible to changes in adipose tissue morphology and environmental stimuli caused during obesity. Obesity is a global epidemic and is a financial burden for both developed and developing nations. In the United States, a study conducted by Center for Disease Control (CDC) ’s National Health and Nutrition Examination Survey reported that obesity was prevalent by 40% in adult population from 2015-2016. This shows significantly increasing trend over the last two decades with a reported 30% prevalence of obese population during 1999-2000 study.156 Another study conducted in 2009, estimated the cost associated with obesity and its related pathologies to be around $147 billion per year in US dollars157. This clearly shows that there is a need for better understanding of the molecular alterations that lead to obesity related metabolic diseases. This thesis work will provide evidence and summarize the findings of how the endocrine function of the adipose tissue plays a major role in disease progression with a special focus on the unconventionally secreted protein, FABP4. Chapter 2 will focus on the metabolic benefit of Roux-en-Y gastric bypass (RYGB) in severely obese human subjects to reduce the risk of obesity related co-morbidities.158 RYGB falls under the category of bariatric surgical procedure wherein a small stomach pouch is made which is connected directly to the ileum therefore bypassing most of the stomach and duodenum to reduce the area of intestinal gut absorption.159 The benefits of bariatric surgery has been attributed to multiple physiological changes including altered concentration of gut and pancreatic hormones or bile acids.159,160 In Chapter 2, we provide data indicating that RYGB’s metabolic benefit is due to reduction in circulating levels of FABP4. Chapter 3 focuses on unraveling the
mechanism of unconventional FABP4 secretion and regulation of its secretion from adipocytes. The secretory mechanism of this protein has been an area of intense research, and this chapter will shed light on some of the components involved in the secretory pathway. Other key findings include the regulation of this secretion, which will be vital in understanding how the pathological changes in the adipose tissue will affect secretion and how to better target this protein for metabolic benefit. While chapter 3 characterizes the secretion of FABP4 from adipocytes, chapter 4 shows evidence that indicates that a variety of other proteins including FABP4 are secreted from adipocytes. Therefore, it is possible that the bulk protein secretion will change the serum concentration of adipocyte proteins and how they influence peripheral tissues. The cumulative results presented here provide a better understanding of endocrine secretion from adipocytes during obesity, which can ultimately be utilized for development of new targets for therapeutic interventions.
6. References:

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CHAPTER 2

SERUM FABP4 CONCENTRATIONS DECREASE FOLLOWING ROUX-EN-Y GASTRIC BYPASS BUT NOT AFTER INTENSIVE MEDICAL MANAGEMENT

Cyrus Jahansouz¹, Hongliang Xu², Scott Kizy¹, Avis J. Thomas³, Ajeetha Josephrajan², Ann V. Hertzel², Rocio Foncea², John C. Connett³, Charles J. Billington⁴, Michael Jensen⁵, Judith Korner⁶, David A. Bernlohr², Sayeed Ikramuddin¹

Affiliations:
¹ Department of Surgery, University of Minnesota, Minneapolis, MN
² Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN
³ Division of Biostatistics, School of Public Health, University of Minnesota, Minneapolis, MN
⁴ Department of Medicine, Division of Endocrinology & Diabetes, University of Minnesota, Minneapolis, MN
⁵ Department of Medicine, Division of Endocrinology & Diabetes, Mayo Clinic, Rochester, MN
⁶ Department of Medicine, Division of Endocrinology, Columbia University Medical Center, New York, NY

This chapter is a reprint of the accepted manuscript for submission to the journal Surgery with minor alterations.

Ajeetha Josephrajan’s contribution to the chapter was development of figure 4 and in preparation of parts of text.
PREFACE

**Background:** Serum concentrations of fatty acid binding proteins 4 (FABP4), an adipose tissue fatty acid chaperone, have been correlated with insulin resistance and cardiovascular risk factors. The objective of this study is to: (1) To assess relationships between Roux-en-Y gastric bypass (RYGB), intensive lifestyle modification and medical management protocol (LS/IMM), FABP4, and metabolic parameters in obese patients with severe type 2 diabetes mellitus (T2DM). (2) To evaluate the relative contribution of abdominal subcutaneous adipose and visceral adipose to the secretion of FABP4.

**Methods:** Subjects were randomized to LS/IMM (n = 29) or to LS/IMM augmented with RYGB (n = 34). Relationships between FABP4 versus demographics, metabolic parameters, and 12-month changes in these values were examined. Visceral and subcutaneous adipose tissue explants from obese non-diabetic patients (n=5) were obtained and treated with forskolin to evaluate relative secretion of FABP4 in the different adipose tissue depots.

**Results:** The LS/IMM and RYGB cohorts had similar fasting serum FABP4 concentrations at baseline. At one year, mean serum FABP4 decreased by 42% in RYGB subjects (P = 0.002), but did not change significantly in LS/IMM. Percent weight change was not a significant predictor of 12-month FABP4 within treatment arm or in multivariate models adjusted for treatment arm. In adipose tissue explants, FABP4 was secreted similarly between visceral and subcutaneous adipose tissue.

**Conclusions:** Following RYGB, FABP4 is reduced 12 months after surgery but not after LS/IMM in patients with T2DM. FABP4 was secreted similarly between
subcutaneous and visceral adipose tissue explants.

INTRODUCTION

Obesity and Type 2 diabetes mellitus (T2DM) are significant sources of morbidity and mortality in the United States, and are increasing at epidemic rates\(^1\). For decades, bariatric surgery has been the most effective treatment for obesity, and more recently has been recognized for its value in the treatment of T2DM. Previously published results from the Diabetes Surgery Study (DSS) have shown that augmenting lifestyle modification and intensive medical management (LS/IMM) with RYGB increases weight loss and improvements in hemoglobin A1c (HbA1c) in patients with severe T2DM and BMI 30.0-39.9\(^2\). Other studies have corroborated these observations\(^3\).

Currently, less than 1% of the population with obesity and T2DM that meet Centers for Medicare and Medicaid Services (CMS) criteria for surgical intervention undergo surgery given its cost, invasiveness and patient suitability requirements\(^4,5\). Identifying mechanisms responsible for the efficacy of surgery may allow us to improve non-surgical care for patients with T2DM.

Proposed mechanistic explanations underlying the effectiveness of bariatric surgery include changes in bile acids, increases in glucagon-like peptide-1 (GLP-1) and fibroblast growth factor (FGF) 19 secretion, and shifts toward more metabolically favorable intestinal microbiota, among others\(^6-9\). However, adipose tissue dysfunction, a central feature of insulin resistance, remains inadequately characterized.
Fatty acid binding protein 4 (FABP4, also known as aP2) is a 15 kDa lipid carrier that is abundantly expressed in adipose tissue, and plays a major role as a fatty acid chaperone facilitating lipolysis and additionally is secreted into circulation. In mice fed a high saturated fat diet, deletion of FABP4 reduces endoplasmic reticulum stress and inflammation and improves insulin sensitivity without altering weight, thus uncoupling insulin resistance from obesity. FABP4 has primarily been investigated as a cytosolic protein, we have previously observed a rapid reduction in the mRNA and protein levels of FABP4 in subcutaneous adipose tissue following bariatric surgery.

Although primarily investigated as a cytosolic protein, several studies have shown associations between higher serum FABP4 and a number of diseases. The Framingham Heart Study found that higher circulating FABP4 is positively correlated with insulin resistance and multiple cardiovascular risk factors, including total cholesterol, lower HDL, and hypertension.

Circulating FABP4 is known to be elevated in patients with obesity and has been identified as a predictor of the development of metabolic syndrome independent from adiposity and insulin resistance. Furthermore, a polymorphism in the promoter region of FABP4 that results in decreased expression is associated with a reduced risk of heart disease and development of T2DM in subjects with obesity. Even cancer outcomes, including morbidity and mortality in breast, ovarian, and prostate cancers, have been associated with higher serum FABP4 concentrations. However, the secretion of FABP4 remains poorly understood. It remains unclear which adipose
depot, i.e. visceral versus subcutaneous, contributes to its secretion. While both depots have been shown to be independent contributors to cardiovascular disease and metabolic syndrome and play active endocrine roles, there are important physiologic differences including adipokine secretion, and rates of lipolysis and triglyceride synthesis\textsuperscript{15–17}. In fact, many diseases that affect fat, i.e. glucocorticoid excess and congenital lipodystrophy, do so in a depot specific manner\textsuperscript{18}. Although FABP4 appears to be linked to a number of diseases, studying identifying a direct relationship between FABP4 and weight change are limited, but it appears that it may also serve as a prognostic marker for weight loss\textsuperscript{28}. Given its known biology, it would be expected that FABP4 concentrations increase with rapid weight loss due to an increase in lipolysis and fatty acid transport, but long-term reductions in weight would result in its reduction\textsuperscript{29}.

While causality has not been established, the association between more favorable metabolic parameters and lower FABP4 concentrations suggests that achieving reductions in FABP4 may be of value in treating metabolic syndrome. One intervention to accomplish this may be bariatric surgery. While serum FABP4 concentrations have been observed to decrease following RYGB, it is unclear how this decrease is related to weight loss and glucose homeostasis. It is also unclear how current medical management of obesity and T2DM affects serum FABP4 concentrations\textsuperscript{30,31}. In this study, we measure serum FABP4 in a pilot cohort of subjects with T2DM, HbA1c > 8.0\% and BMI 30.0-39.9 at baseline. Subjects were randomized to LS/IMM with or without RYGB. FABP4, BMI and metabolic parameters were measured at baseline and at one year post-intervention; in this ancillary study, we explore potential relationships...
between FABP4 and the other values, both within and between treatment arms. To identify the major adipose depot contributing to serum concentrations of FABP4, we then utilized visceral and subcutaneous adipose tissue explants from obese patients at the time of bariatric surgery and assessed the relative contribution of each depot to circulating FABP4 level.

METHODS:

**Subjects.** The Diabetes Surgery Study was a randomized trial comparing the effectiveness of RYGB and LS/IMM to achieve established therapeutic targets for the treatment of T2DM. Details of subject recruitment and randomization have been previously reported. All institutions involved had Institutional Review Board approval. Between 2008 and 2011, 60 of the 120 obese subjects participating in an intensive lifestyle and medically managed weight control program were randomized to undergo RYGB while continuing a lifestyle/medical management protocol. Inclusion criteria included age >30, BMI of 30.0-39.9 kg/m², undergoing treatment for T2DM for at least 6 months, HbA1c of 8.0% or higher, and C-peptide > 1.0 ng/ml 90 minutes after a liquid mixed meal. Subjects were excluded from the study if they had serious conditions precluding surgery. The lifestyle intervention was modeled after the Diabetes Prevention Program and the Look AHEAD protocol. Subjects met regularly with a trained bariatric registered dietician. Lifestyle interventions were augmented with intensive medical therapy to obtain treatment goals established by American Diabetes Association: HbA1c < 7.0%; serum LDL cholesterol < 100 mg/dL; and systolic blood pressure < 130 mmHg. Techniques for the RYGB have been...
previously published\textsuperscript{2}.

Candidates for the current analysis were selected from among the 88 DSS subjects randomized at three clinics in the United States (University of Minnesota, Columbia University in New York, and Mayo Clinic in Rochester, MN) (Figure 1). Subjects were excluded if they lacked follow-up data or available sera at 12 months, if they were type 1 diabetics, if they used thiazolidinediones (TZDs) at baseline or during the first year of the clinical trial, or if they crossed over after the study was underway. Subjects on TZDs were excluded from the current analysis because TZDs are known to increase the activity of the nuclear receptor responsible for FABP4 expression\textsuperscript{20}. Subjects recruited for the evaluation of visceral and subcutaneous adipose tissue provided informed consent approved by the University of Minnesota Institutional Review Board. Tissue biopsies were obtained from these subjects at the time of bariatric surgery.

**Measurement and data collection.** Data were collected at baseline and at scheduled medical visits. Collected data included height, weight, medications used, and laboratory measurements including HbA1c, fasting and 90-minutes post meal glucose, and C-peptide concentrations.

Serum FABP4 was measured with human FABP4 Quantikine ELISA kit from R&D systems (Minneapolis, MN; #DFBP40) according to the manufacturer’s instructions.

**Statistical design and analysis.** Baseline characteristics and one-year outcomes for this cohort were reported using means (95% CI or ± standard deviation) for continuous data.
and percentages for categorical data. Comparisons were made using Student’s T-test, chi-squared statistics, and exact binomial models where appropriate. Univariate linear regressions were used to analyze variables potentially associated with serum FABP4, including age, gender, race/ethnicity, time since diagnosis of T2DM, BMI, % weight change, HbA1c, post-challenge C-peptide, fasting and post-challenge glucose, fasting insulin, HOMA-IR, Matsuda Index [calculated as $10,000/(\text{glucose}_0 \times \text{insulin}_0 \times \text{mean glucose} \times \text{mean insulin})^{0.5}$], and use of exogenous insulin, other anti-hyperglycemics, or statins. Variables with p values < 0.15 in univariate regression analyses were considered in developing optimal multivariate models. As this is an exploratory analysis, many hypothesis tests were carried out without prior hypotheses specified, thus no adjustments for multiple comparisons were imposed. Univariate models were used to identify determinants of 12-month levels of FABP4, and then used to construct a multivariate model for prediction of FABP4 at 12 months. All statistical analyses were completed using SAS software, version 9.3 (SAS Institute, Cary, NC).

**Non-esterified fatty acid (NEFA) Assay.** Visceral and subcutaneous adipose tissue explants were collected from human subjects. 50 mg of tissue samples were used to measure free fatty acid (FFA) release. The explants were treated with either dimethyl sulfoxide (DMSO) vehicle control or 20 uM forskolin (Fsk) for 1 and 4 hours. NEFA assay of tissue explants were performed as described and measured using NEFA-HR kit (Wako, Richmond, VA).

**Immunoblot.** The tissues were incubated in Krebs-Ringer-HEPES buffer with 1% BSA. The incubating media/extracellular material was collected for further analysis. The tissue explants were homogenized on ice in a homogenization buffer (50mM Tris 42
pH 7.4, 50 mM NaCl and 1mM EDTA, 1mM EGTA, 1mM NaP₂O₇, 50mM NaF supplemented with protease inhibitors (Calbiochem, Billerica, MA)). Homogenates were centrifuged at 1000g at 4°C for 10 min to separate the lipid cake, the infranatant was removed and sodium dodecyl sulfate was added to a final concentration of 1%. The lysate was then centrifuged at 10,000g for 20 mins at 4 °C to remove insoluble residue and the supernatant recovered. Western blot analyses were performed on equal volumes of incubating media from all samples and 5% of tissue lysate and incubating media to detect FABP4 and intracellular protein beta actin. FABP4 was assayed by running calculated amounts of purified FABP4 protein samples on respective gels. The immunoblots were imaged using Odyssey infrared imaging (Li-Cor Biosciences; Lincoln, NE). The antibodies used were anti-FABP4 and anti-actin (Sigma-Aldrich, St. Louis, MO).
RESULTS:

Of the 88 possible candidates for review, 25 were excluded: 13 for absent serum samples, 10 due to TZD use at baseline, one who was later diagnosed with Type 1 diabetes, and one patient randomized to LS/IMM patient who later obtained surgery outside of the study. Two subjects who declined surgery after randomization but participated in the LS/IMM intervention were grouped with the LS/IMM cohort for the current as-treated analysis. The resulting 29 LS/IMM and 34 surgery subjects are included in the study (Figure 1). Baseline characteristics are summarized in Table 1. There were no statistically significant differences between groups. Mean age was 51; mean BMI was 36 kg/m²; mean HbA1c was 9.5%; and mean circulating FABP4 was 38 ng/ml. Years since diabetes diagnosis and use of insulin were not different between groups.

One-year outcomes are consistent with previous DSS analyses. Compared to LS/IMM, RYGB subjects had significantly greater weight loss and improved glycemic control despite less medication. (Table 2). At one year, circulating FABP4 was 19 ng/ml (95% CI 15 to 23) in RYGB subjects versus 33 ng/ml (26 to 40) in LS/IMM subjects (42% lower; p=0.002). Mean FABP4 dropped 50% in the surgery group, with a proportionate reduction in variability. FABP4 decreased in all but two RYGB subjects, whose concentration did not change. In contrast, in the LS/IMM group, mean FABP4 decreased 13% but was accompanied by an 11% increase in variability; 10 of 29 (34%) of LS/IMM subjects exhibited either increased concentrations or no change (Figure 2). In the LS/IMM cohort, no factors (BMI,
HbA1c, fasting glucose, fasting insulin) differed significantly between subjects who had increased serum FABP4 concentrations compared to those who had decreased concentrations.

Within treatment arm, percent weight change and 12-month FABP4 were weakly associated in LS/IMM (correlation 0.35; p=0.07), but not in RYGB (correlation 0.11; p=0.52) (Figure 3). After adjustment for baseline FABP4, percent weight change was not a significant predictor of 12-month FABP4 in either arm. In the IMM arm, a 10% greater weight loss corresponded to 5.20 ng/ml lower FABP4 in the IMM group (95% CI -3.96 to 14.36; p-value = 0.25; R² = 0.34). In the surgical arm, a 10% greater weight loss was associated with 2.12 ng/ml lower FAPB4 (95% CI -1.12 to 5.37; p=0.19; R²=0.44). Both cohorts include patients with 0-20% weight loss; while everyone lost weight following RYGB, not everyone lost weight in the IMM cohort. FABP4 is much more variable for the IMM patients in this range than for the RYGB patients.
Figure 1. Study flow diagram
<table>
<thead>
<tr>
<th>Demographic/Measure</th>
<th>LS/IMM (n=29)</th>
<th>RYGB (n=34)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>50 ± 8</td>
<td>51 ± 9</td>
<td>0.60</td>
</tr>
<tr>
<td>Female</td>
<td>13 (45%)</td>
<td>22 (65%)</td>
<td>0.13</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>Black or African American</td>
<td>4 (14%)</td>
<td>5 (15%)</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>1 (3%)</td>
<td>2 (6%)</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>2 (7%)</td>
<td>2 (6%)</td>
<td></td>
</tr>
<tr>
<td><strong>General Medical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>35.7 ± 3.2</td>
<td>36.1 ± 2.7</td>
<td>0.60</td>
</tr>
<tr>
<td>Years Since Diabetes Diagnosis</td>
<td>8.9 ± 5.7</td>
<td>11.1 ± 6.4</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>Laboratory Values</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>9.5 ± 1.2</td>
<td>9.5 ± 0.9</td>
<td>0.97</td>
</tr>
<tr>
<td>Circulating FABP4 (ng/ml)</td>
<td>38 ± 17</td>
<td>38 ± 22</td>
<td><strong>0.92</strong></td>
</tr>
<tr>
<td>C peptide, 90-minute (ng/ml)</td>
<td>5.1 ± 2.6</td>
<td>4.2 ± 1.8</td>
<td>0.13</td>
</tr>
<tr>
<td>Glucose, fasting (mg/dL)</td>
<td>215 ± 58</td>
<td>224 ± 65</td>
<td>0.48</td>
</tr>
<tr>
<td>Glucose, 90-minute (mg/dL)</td>
<td>273 ± 64</td>
<td>284 ± 55</td>
<td>0.47</td>
</tr>
<tr>
<td>Insulin, fasting (mU/L)</td>
<td>22 ± 19</td>
<td>27 ± 30</td>
<td>0.46</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>11.5 ± 12.1</td>
<td>13.7 ± 13.5</td>
<td>0.50</td>
</tr>
<tr>
<td>Matsuda Index</td>
<td>2.2 ± 1.6</td>
<td>2.5 ± 2.5</td>
<td>0.63</td>
</tr>
<tr>
<td><strong>Taking Medication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Yes/No)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>15 (52%)</td>
<td>26 (74%)</td>
<td>0.11</td>
</tr>
<tr>
<td>Oral Anti-hyperglycemic</td>
<td>28 (97%)</td>
<td>28 (80%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Any Anti-hyperglycemic</td>
<td>29 (100%)</td>
<td>34 (100%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Statin</td>
<td>19 (66%)</td>
<td>24 (69%)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Table 1.** Baseline characteristics of subjects randomized to LS/IMM or RYGB. Values are reported as mean ± Standard Deviation or n (%).
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>LS/IMM (n=29)</th>
<th>RYGB (n=34)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>32.2 (30.6,33.7)</td>
<td>25.8 (24.6,26.9)</td>
<td>&lt;.00001</td>
</tr>
<tr>
<td>Weight Change (Percent of baseline)</td>
<td>-10 (-13, -7%)</td>
<td>-29 (-31, -26)</td>
<td>&lt;.00001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.1 (6.7, 7.6)</td>
<td>6.3 (6.0, 6.7)</td>
<td>0.01</td>
</tr>
<tr>
<td>HbA1c &lt; 7.0%</td>
<td>14 (48%)</td>
<td>25 (74%)</td>
<td>0.07</td>
</tr>
<tr>
<td>HbA1c &lt; 7.0% with no medication</td>
<td>0 (0%)</td>
<td>19 (56%)</td>
<td>&lt;.00001</td>
</tr>
<tr>
<td>Circulating FABP4 (ng/mL)</td>
<td><strong>33 (26, 40)</strong></td>
<td><strong>19 (15, 23)</strong></td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>C peptide, 90-minute (ng/ml)</td>
<td>5.9 (4.8, 7.0)</td>
<td>3.9 (3.3, 4.4)</td>
<td>0.002</td>
</tr>
<tr>
<td>Glucose, fasting (mg/dL)</td>
<td>139 (123, 156)</td>
<td>108 (99, 118)</td>
<td>0.003</td>
</tr>
<tr>
<td>Glucose, 90-minute (mg/dL)</td>
<td>180 (156, 205)</td>
<td>129 (116, 142)</td>
<td>0.001</td>
</tr>
<tr>
<td>Insulin, fasting (mU/L)</td>
<td>16 (10, 21)</td>
<td>7 (3, 10)</td>
<td>0.01</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>5.8 (3.2, 8.4)</td>
<td>1.9 (0.8, 3.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Matsuda Index</td>
<td>6.0 (3.8, 8.1)</td>
<td>9.3 (7.1, 11.4)</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>TAKING MEDICATION (YES/NO)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>13 (45%)</td>
<td>8 (24%)</td>
<td>0.11</td>
</tr>
<tr>
<td>Oral Antihyperglycemic</td>
<td>29 (100%)</td>
<td>13 (38%)</td>
<td>&lt;.00001</td>
</tr>
<tr>
<td>Any Antihyperglycemic</td>
<td>29 (100%)</td>
<td>14 (41%)</td>
<td>&lt;.00001</td>
</tr>
<tr>
<td>Statin</td>
<td>21 (72%)</td>
<td>17 (50%)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

**Table 2.** Characteristics of subjects one year after the intervention began. Values are reported as mean (95% CI) or n (%).
Figure 2. One year percent change in serum FABP4 concentrations in subjects following intervention.
In further exploratory analyses, we examined 15 baseline characteristics as potential univariate predictors of baseline FABP4 (Table 3). Significant associations included age (7.4 ng/ml higher FABP4 per 10-year greater age; 95% CI 1.5 to 13.2), female sex (13.4 ng/ml higher FABP4; 95% CI 3.9 to 22.8), fasting glucose (1.1 lower ng/ml FABP4 per 10 mg/dl higher fasting glucose; 95% CI 0.3 to 1.9), and 90-minute post-challenge glucose (0.8 ng/ml lower FABP4 per 10 mg/ml higher glucose; 95% CI 0 to 1.6; p = 0.05). Similarly, individual patient characteristics (including baseline, 12-month, and 12-month change, where appropriate) were examined individually as predictors of 12-month FABP4 (Table 4).

Regressions were conducted separately by treatment arm and adjusted for baseline FABP4. At 12 months, percent weight change dominated treatment arm as a predictor of HbA1c, as previously reported. However, percent weight change was not a significant predictor of 12-month FABP4 within either treatment arm, either after adjustment for baseline FABP4 or in potential multivariate models. Similarly, no other baseline value was predictive of 12-month FABP4 after adjustment for baseline FABP4.
<table>
<thead>
<tr>
<th>DEMOGRAPHICS</th>
<th>Estimate (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (per 10 years)</td>
<td>7.4 (1.5, 13.2)</td>
<td>0.01</td>
</tr>
<tr>
<td>Female</td>
<td>13.36 (3.91, 22.80)</td>
<td>0.01</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td>--</td>
<td>0.18</td>
</tr>
<tr>
<td>GENERAL MEDICAL</td>
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<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-0.41 (-2.14, 1.31)</td>
<td>0.63</td>
</tr>
<tr>
<td>Years Since Diabetes Diagnosis</td>
<td>0.00 (-0.82, 0.82)</td>
<td>1.00</td>
</tr>
<tr>
<td>LABORATORY VALUES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>-1.68 (-6.59, 3.24)</td>
<td>0.50</td>
</tr>
<tr>
<td>C peptide, 90-minute (ng/ml)</td>
<td>-0.79 (-3.04, 1.46)</td>
<td>0.48</td>
</tr>
<tr>
<td>Glucose, fasting (per 10 mg/dL)</td>
<td>-1.1 (-1.9, -0.3)</td>
<td>0.01</td>
</tr>
<tr>
<td>Glucose, 90-minute (per 10 mg/dL)</td>
<td>-0.8 (-1.6, 0.00)</td>
<td>0.05</td>
</tr>
<tr>
<td>Insulin, fasting (mU/L)</td>
<td>-0.08 (-0.28, 0.12)</td>
<td>0.42</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>-0.27 (-0.66, 0.12)</td>
<td>0.17</td>
</tr>
<tr>
<td>Matsuda Index</td>
<td>0.78 (-1.54, 3.09)</td>
<td>0.50</td>
</tr>
<tr>
<td>TAKING MEDICATION (YES/NO)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>2.56 (-7.79, 12.90)</td>
<td>0.62</td>
</tr>
<tr>
<td>Oral Anti-hyperglycemic</td>
<td>-0.16 (-15.2, 14.84)</td>
<td>0.98</td>
</tr>
<tr>
<td>Any Anti-hyperglycemic</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Statin</td>
<td>2.91 (-7.65, 13.47)</td>
<td>0.58</td>
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</tbody>
</table>

Table 3. Baseline variables as univariate predictors of a 1ng/ml increase baseline FAPB4. (Analysis based on all study subjects)
Figure 3. Predictive ellipses of serum FABP4 versus percent weight change. (A) Cross-sectional 12-month FABP4. (B) 12-month percent change in FABP4.
Table 4. Predictors of 1 ng/ml greater FABP4 at 12 months by treatment arm.

*The following potential predictors were also considered: age, gender, years since diabetes diagnosis, BMI, use of insulin, other anti-hypoglycemic medications or statins, and BMI. Only percent weight change and predictors with p<0.15 after adjustment for baseline FABP4 are shown above.

**R^2 for the optimal multivariate models was 0.50 (IMM) and 0.41 (RYGB).
In the LS/IMM group, the optimal multivariate model for 12-month FABP4 included baseline FABP4, change in fasting insulin, and change in post-challenge C-peptide. In the RYGB group, the optimal model included only baseline FABP4. $R^2$, the proportion of variability explained by the model, was 0.50 in the optimal LS/IMM model and 0.41 in the optimal RYGB model.

Next, we utilized visceral and abdominal subcutaneous adipose tissue explants obtained from obese human subjects undergoing bariatric surgery to identify the relative contributions of these depots to the secretion of FABP4 (Supplemental Table 1). The ex-vivo tissue explants effluxed non-esterified free fatty acids and responded to lipolytic stimuli (Figure 4A), indicating that the tissues were metabolically active. To analyze the secretory capacity of visceral and abdominal subcutaneous depots, we measured the FABP4 secreted from excised samples. The two types of adipose samples secreted similar amounts of FABP4 ($p=0.47$ and $p=0.17$, 1 and 4 hours following DMSO treatment, respectively; Figure 4B, C). Intriguingly, the secretion of FABP4 was not stimulated in response to lipolytic signal from human tissue ($p=0.19$ and $p=0.17$, 1 hour and 4 hours following FSK treatment, respectively). To confirm that the FABP4 in the supernatant was secreted and not present in media due to tissue cellular lysis, we evaluated the expression of $\beta$-actin in both intracellular and extracellular material. The presence of actin in the cellular lysate but not in the cell culture supernatant fraction is an indicator of intact cells and implies that FABP4 found is the secreted fraction resulting from regulated release rather than lytic release.
Figure 4. FABP4 is secreted from both visceral and subcutaneous adipose tissues. (A) Adipose tissue explants from either the visceral (Vis) or subcutaneous (Sc) depot were treated with DMSO or forskolin (FSK) for 1 to 4 hours and non-esterified free fatty acids (FFA) released into the incubation medium determined. (B) Immunoblot of FABP4 secreted from tissue explants from human subjects (S1-S5) in response to DMSO or FSK treatment. (C) Quantitation of FABP4 secreted into the incubating media. (D) Immunoblot of β-actin from tissue lysates and incubation media of DMSO and FSK-treated tissue explants.
DISCUSSION:

This study assessed serum FABP4 concentrations in a diabetic population one year after randomization to LS/IMM with or without RYGB. Mean serum FAPB4 decreased 50% in the year following surgery, versus a one-year decrease of just 13% in LS/IMM. Furthermore, no statistically significant correlation was identified between weight loss and 12-month FABP4 levels in either arm. In evaluating the relative contribution of the secretion of FABP4 from visceral and abdominal subcutaneous adipose tissue, both depots secrete approximately similar amounts of FABP4/mg tissue suggesting that majority of serum FABP4 is attributable to subcutaneous adipose tissue given its larger volume.

Our observations are consistent with findings from others showing reductions in serum FABP4 concentrations at 6 and 12 months following RYGB. However, this current study describes subjects with severe T2DM, whereas the previous cohorts were predominantly non-diabetic. It thus appears that RYGB is associated with significant one-year reductions in serum FABP4, regardless of diabetic status. The reduction of serum concentration of FABP4 may contribute to improved metabolism following RYGB. Numerous animal studies and evaluations of human polymorphisms link FABP4 to insulin resistance, increased production of pro-inflammatory cytokines, and increased reactive oxygen species. Higher serum FABP4 is associated with higher BMI and worsened dyslipidemia. Elevated FABP4 is also linked to hepatic insulin resistance and increased hepatic gluconeogenesis. Furthermore, a genetic polymorphism that decreases FABP4 expression appears to have a protective effect
against T2DM and atherosclerosis when controlled for BMI \textsuperscript{24}. While causal pathways have yet to be determined, clinical treatments that reduce serum FABP4 might be beneficial for patients with metabolic syndrome or type 2 diabetes. Animal studies targeting the reduction of FABP4 using monoclonal antibodies have been promising \textsuperscript{40}. RYGB is currently the only known treatment that reliably significantly decreases this adipokine, although angiotensin II receptor blockers, atorvastatin and omega-3 fatty acids have shown modest reductions \textsuperscript{41–43}.

While others have observed a positive correlation between FABP4 concentrations and HOMA-IR following RYGB \textsuperscript{30}, in our exploratory analysis we did not observe the same association. We did, however, observe a weak negative association between fasting glucose levels and serum FABP4 concentrations at baseline. In the LS/IMM cohort, change in fasting insulin was included in the optimal multivariate model predicting 12-month FABP4. Reasons for these differences are unclear, but may be related to medication use, extent of weight loss, and diabetic status. Further in-depth metabolic studies are needed to elucidate these relationships. Greater age and female gender were both also associated with higher baseline FABP4. Factors accounting for gender differences include androgen hormone production and body composition\textsuperscript{44}.

It is not clear how FABP4 concentrations decline following RYGB. A gene target of the nuclear receptor peroxisome proliferator-activated receptor gamma, FABP4 is the major lipid chaperone in adipocytes, facilitates lipolysis, and is secreted into serum by unconventional means \textsuperscript{45,46}. Sustained reductions in serum FABP4 at one year following RYGB may be due to alterations in adipose tissue metabolism that are specific to RYGB. This reduction may be mediated by the effect of RYGB on other
serum factors, like GLP-1 which is increased after RYGB\textsuperscript{8,9}. Sitagliptin, a dipeptidyl peptidase 4 inhibitor that increases GLP-1, results in mild reductions of FABP4 concentrations in patients with T2DM\textsuperscript{47}. It is possible that RYGB with significant weight loss itself contributes to reductions in FABP4 in the long-term, but how this may occur is also unclear. Long-term studies of FABP4 in medically managed weight loss cohorts after two years of treatment have shown reductions in FABP4\textsuperscript{48}. Baseline FABP4 has even been proposed as a prognostic factor for maintenance of weight loss after a calorically restricted medical intervention\textsuperscript{28}. However, these assessments were made in insulin sensitive populations.

FABP4 lacks a conventional secretion signal sequence, and thus utilizes an unconventional secretion mechanism. Molecular details describing this secretion have been limited. Importantly, the underlying concept of how different adipose depots contribute to circulating FABP4 is yet unexplored. Analyzing the relative contribution of FABP4 secretion from visceral and abdominal subcutaneous depots provides an insight to the relationship of body composition to whole-body FABP4 secretion. Our findings here indicate that both visceral and subcutaneous adipose tissues secrete FABP4. An intriguing finding however, is that FABP4 secretion was not potentiated in response to lipolytic stimuli, in contrast with studies in mice wherein beta adrenergic signaling stimulated FABP4 secretion\textsuperscript{37}. This observation suggests that the subcutaneous depot may be the major contributor to serum concentrations of FABP4 because of its significantly larger volume. Likewise, a substantial loss of subcutaneous adipose tissue following surgery is likely to contribute to reductions in FABP4 concentrations. However, more studies are needed to confirm our initial observations.
There are limitations of this study that should be highlighted. RYGB subjects had greater weight loss than medical management (mean=29% versus 10% in LS/IMM); the study would be strengthened by weight loss-matched cohorts. Effects of recent weight loss may confound the relationship between adipose tissue and serum FABP4. If the apparent differences in FABP4 metabolism are related to RYGB per se, RYGB subjects may be compared with another form of bariatric surgery (for instance banding or sleeve gastrectomy). Other potential confounders include medication use at baseline and differences in medication use at 12 months as well possible changes in dietary fat intake, which has been shown to alter the expression of FABP4 in adipose tissue. This study is also limited by the variability in the changes in FABP4 concentrations following each intervention, and could be strengthened by a larger sample size.

In contrast to the DSS patients providing longitudinal lab data, patients providing adipose tissue samples were insulin sensitive and were not taking any antidiabetic medications. The contrast weakens the study, but on the other hand lack of medication use in the patients providing tissue samples may limit confounding. This study would also be strengthened by the evaluation of visceral and subcutaneous adipose depots at additional sites, as well as in a larger study population. Future studies would include evaluating changes in patients who are prediabetic or diabetic off medications, as well as measurements of FABP4 in shorter time intervals following surgical or medical intervention to elucidate potential relationships with weight change. Another cohort of patients to study would be those undergoing abdominoplasty, which would provide further insight into the relative contribution of subcutaneous adipose tissue to serum...
concentrations of FABP4. An important strength of this study is its reliance on data from a randomized clinical trial, eliminating confounding due to self-selection.

In conclusion, in a randomized cohort of subjects with BMI 30.0-39.9 and severe T2DM, FABP4 levels decreased markedly 12 months after RYGB. No statistically significant reduction in FABP4 was observed 12 months exposure to an intensive lifestyle and medical management protocol. This study also failed to demonstrate a relationship between weight loss and reductions in FABP4. Further in-depth metabolic studies are required to characterize the relationship between serum FABP4 concentrations and tissue specific insulin resistance, and to understand how RYGB contributes to the marked decline in FABP4.
Acknowledgments.

We would like to thank study coordinators Joyce Schone, RD and Nyra Wimmergren, RN, at the University of Minnesota (Minneapolis, MN), and Heather Bainbridge, RD, CDN, at Columbia University Medical Center, New York, NY. We would also like to thank members of the Bernlohr lab for their technical support and contributions to the manuscript. This work was funded in part by Covidien/Medtronic and the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant Number UL1TR001873.
Supplemental Table:

<table>
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<th>Total Patients</th>
<th>5</th>
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<tr>
<td>Gender (M/F)</td>
<td>1/4</td>
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<tr>
<td>Age (years)</td>
<td>41.0 +/- 6.6</td>
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<tr>
<td>Weight at Intervention (kg)</td>
<td>123.4 +/- 11.1</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>43.7 +/- 2.9</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.7 +/- 0.4</td>
</tr>
<tr>
<td>Plasma Glucose (mg/dL)</td>
<td>83.4 +/- 6.8</td>
</tr>
</tbody>
</table>

**Supplemental Table 1.** Baseline demographics of obese subjects from which subcutaneous and visceral adipose tissue biopsies were obtained.
References:


CHAPTER 3

UNCONVENTIONAL SECRETION OF ADIPOCYTE FATTY ACID BINDING PROTEIN (FABP4) IS MEDIATED BY AUTOPHAGIC PROTEINS IN A SIRT1 DEPENDENT MANNER

Ajeetha Josephrajan¹, Ann V. Hertzel¹, Ellie K. Bohm¹, Michael W. McBurney², Shin Ichiro-Imai³, Douglas Mashek¹, Do-Hyung Kim¹ and David A. Bernlohr¹

From the ¹Department of Biochemistry, Molecular Biology and Biophysics, The University of Minnesota, Minneapolis, MN USA, ²Department of Biochemistry, Microbiology and Immunology, The University of Ottawa, Ottawa, Canada, and ³Department of Developmental Biology, Washington University St. Louis, St. Louis MO, USA

This chapter contains unpublished work that is currently being prepared for publication. Ajeetha Josephrajan’s contribution was writing the text and generating the data (except for 1D and running western blots for 2G, 3F and 4D)
Preface:

Fatty Acid Binding Protein 4 (FABP4) is a leaderless lipid carrier protein primarily expressed by adipocytes and macrophages that not only functions intracellularly, but is also secreted. The secretion is unconventional and is currently under exploration. In a variety of species, metabolic dysfunction is associated with elevated circulating FABP4 levels and neutralizing antibodies targeting serum FABP4 increase insulin sensitivity in diabetic animals. Herein, we show that in 3T3-L1 adipocytes, FABP4 is secreted in response to lipolytic stimulation in a Sirtuin-1 (SIRT1) dependent manner via a mechanism that requires some, but not all, autophagic components. Silencing of early autophagic genes such as FIP200, ULK1/2 or BECLIN-1 or chemical inhibition of ULK1/2 or VPS34, attenuated secretion while Atg5 knockdown potentiated FABP4 release. In addition, blocking SIRT1 by EX527 or activating SIRT1 by resveratrol reduced or induced secretion, respectively. Genetic knockout of SIRT1 also attenuated secretion. Similarly, serum levels of FABP4 were undetectable in SIRT1 knock out mice. Our studies suggest that FABP4 secretion from adipocytes is regulated by SIRT1 and requires early autophagic components for secretion. As such, this study not only unravels new players in this unconventional secretion mechanism of FABP4, but also identifies other secreted proteins such as FABP5 and NAMPT that utilize this pathway.
**Introduction:**

System wide pathogenesis of obesity is linked to a chronic low-grade inflammatory state due to nutrient excess, unbalanced immune system and dysfunctional cellular processes such as fatty acid metabolism and adipokine release\(^1\)\(^{-3}\). Under such conditions there is also alteration in lipid flux to and from adipose tissue\(^2\). Fatty acid binding proteins belong to a super family of lipid binding proteins performing the function of fatty acid storage, transport and lipid signaling there by directly playing a major role in mobilizing fatty acids. Adipocytes express FABP4 (major form) and FABP5 (minor form) which serve to facilitate lipolysis and FFA release from the cell in response to adrenergic signaling\(^4\). In mouse models, deletion of FABP4 provides protection from insulin resistance, asthma, atherosclerosis and inflammation\(^5\) under nutrient excess. Indeed, our lab has shown that FABP4 ablation in macrophages improves mitochondrial function\(^6,7\). Pathogenicity linked to FABP4 was originally attributed to the intracellular function of the protein and control of specific transcription factors such as PPARs\(^8,9\). However, recent literature has shown FABP4 to be secreted extracellularly from adipocytes and to a smaller extent by macrophages. Serum FABP4 levels are elevated in patients with obesity and metabolic syndrome\(^10\) and is shown to be a driver of disease progression in some cancers\(^11,12\). Circulating FABP4 has been shown by others to regulate metabolic activities systemically such as increase in production of hepatic glucose\(^13\) or production of insulin from pancreatic beta cells\(^14\). As a result, circulating FABP4 is currently being evaluated as a potential clinical biomarker for metabolic and cardiovascular diseases\(^15\)\(^{-19}\).
FABP4 is secreted unconventionally in response to lipolytic stimuli, increased intracellular Ca\textsuperscript{2+} levels and hypoxia\textsuperscript{14,20,21}. As such, FABP4 lies at the apex of energy homeostasis and this has sparked an intense research around secretory FABP4. Multiple channels of FABP4 secretion has been explored, indicating that the extracellular FABP4 is mostly non-vesicular\textsuperscript{20,22} and secreted through lysosomal exocytosis\textsuperscript{23}, and only a very small fraction of this non-canonical secretion is via multi vesicular bodies/exosomes\textsuperscript{24}. Given all the extracellular functions of FABP4, it is important that we understand the underlying regulation of secretion and the components that drive this process.

In this paper, we provide evidence for FABP4 secretion utilizing selective autophagic proteins while forgoing the use of other proteins such as ATG5 involved in canonical macro autophagy. This unconventional secretion is also dependent on the transcriptional regulator and deacetylase enzyme Sirtuin-1, which is also an autophagy regulator. The secretion pathway is also utilized by FABP5 (epidermal fatty acid binding protein) which has affinity and selectivity for long chain fatty acids as FABP4\textsuperscript{25}. Serum FABP5 is also shown to be a biomarker for atherosclerosis disease progression independently of FABP4\textsuperscript{26}. Similarly, another unconventionally secreted protein, nicotinamide phosphoribosyl transferase (NAMPT), which has been shown by Imai lab\textsuperscript{27} to require the SIRT1 activity also follows this pathway. Extracellular NAMPT has been implicated in upregulating SIRT1 activity and p53 deacetylation thereby aiding breast cancer progression\textsuperscript{28}.
Materials and Methods:

Reagents and chemicals:
Forskolin (FSK), ATGListatin (Astat), TNFα, 8-br-cAMP, Isoproterenol (ISO), 3-Methyl Adenine (3MA), N-Acetyl D- sphingosine (NAS) From Sigma-Aldrich, St. Louis, MO; LAListat from Tocris, Minneapolis, MN. Vacuolin-1 from Calbiochem, Burlington, MA; PIK-III (VPS34 Inhibitor) was gifted by Dr. Murphy’s lab, Novartis Institutes for Biomedical Research, Massachusetts, USA. MRT68921(ULK 1/2 inhibitor) from Apex Bio, Houston, TX.

Cell culture and cell lines:
3T3-L1 fibroblasts were grown to confluence and differentiated to mature adipocytes as described previously 29. Differentiated adipocytes are maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% Fetal Bovine Serum (FBS) and used for experimentation from days 10-12. Atg5 and Fip200 knockdown cells were previously made as described 30. Lentiviral knockdown of Sirt-1:
Pre-adipocytes were transduced with a short hairpin RNA (shRNA) lentivirus as described previously 31. The shRNA clones directed towards Sirt-1 were TRCN0000039294 and TRCN0000039296 from University of Minnesota Genomic Center. The full hairpin sequences used are as follows
1) 5’CCGGGCCATGGTGATATTGAGTATCTCGAGATACTCAAATCAACATG

GCTTTTTG- 3’ and
2) 5’CCGGGAGGGTAATCAATACCTGTTTTCTCGAGAACAGGTATTTGATTACCC

TCTTTTTG-3’.
2 rounds of infection were performed using the above 2 lentiviruses consecutively. Empty vector was used as a control.

**Primary cells:**

Primary adipocytes were isolated from high fat fed C57BL/6J mice on high fat diet for 12 weeks (BioServ 3282, Flemington, NJ). Epididymal and inguinal fat pads were dissected, minced, and digested with Type I collagenase (1 mg/mL CLS-1; Worthington Biochemical Corp., Lakewood, NJ) for one hour at 37°C in Krebs–Ringer–HEPES (KRH) buffer supplemented with bovine serum albumin (0.5% fatty acid free BSA) and 5 mM glucose. Adipocytes were filtered through a 100-micron membrane, followed by three washes with the BSA supplemented KRH buffer (centrifuged at 4,000xg for 10 min). The floating adipocytes were recovered and treated with 1 µg/ml bovine insulin (with 100nM (−)-N 6-(2-phenyl-isopropyl)-adenosine (PIA) and 1U/ml adenosine deaminase) or 10 µM forskolin (plus 1 U/ml adenosine deaminase) 

32. The cells were incubated at 37°C for 2 hours with gently shaking (100 rpm), followed by centrifugation at 4,000xg for 10 min allowing separation of the supernatant from the cells.

**MEFs differentiation to adipocytes:**

Immortalized mouse embryonic fibroblasts (MEFs) cells are grown in DMEM (0.1% Pen/Strep) with 10% FBS. Cells are grown to confluence and differentiated 2 days post-confluence with media containing insulin, dexamethasone, and 3-isobutyl-1-methylxanthine, troglitazone and Indomethacin (200 µM). At 48 hours, the media was replaced with maintenance DMEM medium containing 10% FBS and insulin, followed by a replenishment of the media every 2 days 33.
Sample Collection and preparation:
Differentiated adipocytes were incubated with KRH - pH 7.4 with 5 mM glucose plus 0.5% fatty acid free BSA for the treatment duration, after washing the cells twice with pre-warmed phosphate buffered saline (PBS). Lipolytic inducers or chemical inhibitors were added to KRH during the incubation period. The KRH media was collected and centrifuged at 10,000xg to remove cell debris. For chemical treatments, the cells were pre-incubated with inhibitors for 2 hrs in DMEM with 2% FBS. Cell lysis was performed with buffer containing 50 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 1% deoxycholate, 1% triton X-100, 0.1% SDS. The lysis buffer was supplemented with protease inhibitor (Calbiochem, Darmstadt, Germany) and phosphatase inhibitors (Sigma-Aldrich). Proteins were quantified using bicinchoninic acid (BCA) assay.

Oleate loading:
400 µM oleate was coupled to 100 µM fatty acid free BSA and incubated on differentiated 3T3L1s for 24 hours in full media. After 24 hours, cells were incubated with KRH with or without FSK for 4 hours and media supernatant collected for analyses.

Immunoblotting:
Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Li-Cor Biosciences, Lincoln, NE). The membrane was blocked in Odyssey blocking buffer (Li-Cor Biosciences) and incubated with primary antibodies O/N. Membranes were then washed and incubated with secondary antibody conjugated to Li-Cor IRDye for 1 hr. Visualization was performed on Odyssey infrared imaging (Li-Cor Biosciences). The primary antibodies used were polyclonal antibody to FABP4 purified in the lab, anti-β-actin (A5361), anti-ULK1 (A7481) from Sigma-
Aldrich, anti-LC3B, (2775), anti-histone H4 (2935), anti-galectin-3 (87985), anti-acetylated lysine (9441), anti-ATG14, (96752), anti-pATG14 (13155), anti-ATG14 (96752), anti-pATG14 (13155) from Cell signaling technology, Danvars, MA. Anti-PBEF/Visfatin (MAB4044), anti-caspase-3 (AF-605), anti-cleaved caspase-3 (MAB835), anti-RBP4 (AF3476) from R&D systems, Minneapolis, MN. Anti-ATG5 (110-53818) from Novus biologicals, Littleton, CO. Anti-p62 (5114) MBL, Des Plaines, IL. Anti-tubulin (sc-12462), anti-beclin-1 (sc-11427) Santa Cruz Biotechnology, Dallas, TX. Anti-sirtuin-1 (07-131) Millipore sigma, Burlington, MA and anti-FIP200 purified by DHK lab.

Non-esterified fatty acid efflux (NEFA) Assay:

Fatty acid efflux from adipocytes were measured under basal, lipolytic and various treatment conditions. KRH buffer supplemented with 0.5% fatty acid free BSA was incubated on pre-washed cells for 4 hrs and the buffer was collected to measure the released free fatty acids (FFA). Replicate measurements of FFA were measured using HR series NEFA HR (2) (Wako Chemicals, Richmond, VA) as per manufacturer’s protocol.

Expression analysis by quantitative RT-PCR (qRT-PCR):

TRIzol reagent (Invitrogen, Carlsbad, CA) was used to collect total RNA from cells and iScript (Bio-Rad, Hercules, CA) was used to make cDNA as per the manufacturer's protocol. qRT-PCR amplification was performed on Bio-Rad CFX 96 real-time system with SYBR green Supermix. Transcription factor II E (TFIIE) were used as the internal control to normalize expression. The primers used were

1) TFIIE: Fwd 5’- CAAGGCTTTAGGGGACCAGATAC-3’;

Rev: 5’-CATCCATTGACTCCACAGTGACAC-3’
2) SIRT1: Fwd 5'– GGCTACCGAGACACCTCCTG-3’;
Rev 5’– AGTCCAGTCACAGCTGGC -3’

Hypoxia:
For hypoxia experiments, hypoxia chamber (Biospherix Ltd, Parish, NY, USA) containing 1% oxygen and 5% CO2 was utilized.

ELISA analysis:
FABP4 in circulation was detected in the serum samples of C57BL/6J (WT) and whole body SIRT deficient (SIRT1 KO) mice using a sandwich enzyme-linked immunosorbent assay (ELISA) kit from LifeSpan Biosciences Inc, Seattle, WA. Product number - LS-F11412-1.

Statistical analysis:
All the results are expressed with standard errors of the mean (SEM). For all experiments performed with 3T3L1 cells, the data presented has sample size of three and individual experiments were repeated. Li-Cor software was used to quantify protein levels in western blots and graphs representing protein levels, FFA levels were made using Prism 6 (GraphPad Software). The results were summarized as standard errors of mean in the figures (SEM). Statistical significance was determined using an unpaired two-tailed Student t-test.
Results

To understand the mechanism of FABP4 secretion, we have utilized adipocyte 3T3L1 cell line to study its efflux. FABP4 is secreted in response to lipolytic agonists (Fig. 1A), also shown by others\textsuperscript{13,20,24}. Time course experiments performed to quantitate FABP4 secretion in response to lipolysis revealed that free fatty acid (FFA) efflux plateaus after 4 hrs, but FABP4 is consistently secreted until a 6 hr time point (Fig. 1B and C). Primary white adipocytes collected from epididymal and inguinal depots of mice on high fat diet showed increased FABP4 secretion in response to forskolin (FSK) when compared to insulin treated cells corroborating cell culture experiments (Fig. 1D). It’s been shown that FABP4 is secreted in an unconventional manner in response to lipolysis\textsuperscript{23,24}. This extracellular FABP4 fraction is not contributed by cell lysis, apoptosis or necrosis as shown by the absence of actin, tubulin in secreted fraction, absence of cleaved caspase 3, which is an apoptotic marker and, lack of histone H4, an indicator of necrotic cells under basal or lipolytic stimuli respectively (Fig. 1E and F). To show that the adipocytes are still functional after the lipolytic stimuli, the cells were treated with insulin after the initial FSK treatment. The cells responded to insulin stimuli as shown by decreased NEFA levels (Sup Fig. 1A). Interestingly, we found that other proteins such as FABP5, NAMPT, Galectin-3 are also secreted from adipocytes in response to lipolysis (Fig. 1F).
Figure 1: **FABP4 is secreted in response to lipolytic stimuli.**

(A) FABP4 secretion from Day 11 differentiated 3T3L1 adipocytes in response to 20uM FSK, 10 µM ISO, 1 mM 8-Br-cAMP and 500 nM INS for 4 hrs. (B) NEFA levels measured over 2, 4 and 6 hrs with or without FSK treatment of L1s. (C) Secreted FABP4 protein levels quantified over 2, 4 and 6 hrs with or without FSK treatment of L1s. (D) FABP4 secretion from primary adipocytes derived from eWAT and IWAT of 12 wks HFD fed C57BL/6J mice in response to 10 µM FSK or 1 µg/ml INS for 2 hrs (n=4) (E) 3T3L1s were treated +/- FSK and +/- TNFα for 4 hrs and intracellular and extracellular protein samples were probed for indicated proteins. (F) Secreted material from 3T3L1s treated +/- FSK for 4 hrs was probed for the indicated proteins. (G) Cells were pretreated with or without 10 µM Astat for 2 hrs, washed and then treated with or without FSK for 4hrs. Extracellular material was run on SDS-PAGE and blotted for FABP4. (H) NEFA levels were measured from cells that were treated as indicated in panel 1G.
More importantly, when the release of classically secreted protein like the retinol carrier of the lipocalin family of proteins, RBP4 was analyzed, it is unaltered by lipolysis. The results point to a possibility of specificity in this protein secretion (Fig. 1G).

Substantiating the results shown by others, we also observed the FABP4 and eNAMPT secretion is increased under hypoxic conditions (Sup Fig. 1B). There is a strong requirement of lipolytic conditions for FABP4 secretion so we tested the requirement of the enzymatic lipid hydrolase, adipose triglyceride lipase (ATGL). When ATGL activity is diminished with ATGListatin, there is a decrease in FABP4 secretion corresponding to FFA efflux (Fig. 1G and 1H)\(^{24}\).

**FABP4 secretion is independent of lipophagy but requires components of autophagic proteins**

FABP4 secretion followed FFA efflux as shown in Fig.1B, C and H. One of the mechanisms that contributes to the FFA efflux is lipophagy. Lipophagy refers to autophagic breakdown of lipid droplet, wherein lipid hydrolysis happens in the lysosomes by the enzyme, lysosomal acid lipase (LAL). We investigated if FABP4 was also secreted through lipophagy, similarly to that of FFA, by first inhibiting LAL. Impeding LAL activity by Lalistat did not affect secretion of FABP4 (Fig. 2A). Lalistat however decreased FFA efflux as expected\(^{34}\) (Fig. 2B). Next, the cells were treated with a chemical inhibitor vacuolin-1 (Vac-1), which alkalinizes the lysosomal pH and blocks lysosomal exocytosis\(^{35}\). Vac-1 decreases lysosome mediated FFA efflux and FABP4 secretion albeit not drastically.
Figure 2
Figure 2: FABP4 secretion is independent of lipophagy but requires components of autophagic proteins.

(A, B) 3T3L1 cells were pretreated with or without chemical treatment for 2 hrs, washed and then treated with or without FSK for 4 hrs. Secreted FABP4 and FFA were quantified and graphed for figures A-H. Following are the treatment conditions (A, B) 10 µM Astat (C, D) 25 µM Vac-1 (E, F) 5 mM 3MA (G, H) 30 µM NAS. (I, J) Ctrl or Atg5 knockdown adipocytes were treated with or without FSK and FABP4 and FFA were quantified from secreted material and graphed.
Our data indicates that FABP4 secretion is lipophagy independent but requires lysosomal exocytosis. This agrees with the results published by others. Multiple different pathways such as endocytosis, phagocytosis and autophagy feed materials into the lysosomes which can then exit the cell by lysosomal exocytosis. As autophagy is one of the pathways that cargoes materials into lysosomes, we explored if FABP4 secretion required the autophagy process. To test this, adipocytes were treated with classical autophagic inhibitor 3-Methyl Adenine (3MA) or autophagic inducer D-acetyl N-sphingosine (NAS). 3MA blocks autophagy as shown by the decrease in de novo lipidation of LC3, one of the commonly used marker for measuring autophagy flux. FABP4 secretion was blunted under FSK stimulated conditions (Fig. 2E), with no differences in FFA efflux levels between FSK and 3MA+FSK (Fig. 2F). Same results were observed when cells were treated with chloroquine (CQ) (Sup Fig. 2A). Concurrently, treatment with NAS induced autophagy as shown by decreased levels of autophagic substrate p62. Upon autophagy activation, FABP4 secretion was potentiated without affecting FFA efflux in response to FSK in both control and treated cells (Fig. 2G, H). Both 3MA and NAS are broad spectrum inhibitors. Hence, we conclude that secretion requires the autophagic pathway.

We then explored targeted autophagic components, published data shows that late stage autophagic proteins such as ATG5, involved in vesicle elongation of autophagosome maturation process was not required for FABP4 release. We also observed that Atg5kd
cells didn’t block secretion but rather increased both FABP4 and FFA efflux (Fig. 2I, J). The data designates that secretion is not via secretory autophagy, but instead via an unconventional secretion mechanism that packages proteins into matured autophagosomes and releases it by direct fusion with the plasma membrane in case of proteins like IL-1β. However, it is apparent that there is specificity to some parts of the autophagy pathway for this secretion process.

**FABP4 secretion requires early components of autophagy:**

Next, we tested if earlier autophagic proteins are involved in this process. First, we targeted VPS34, a class III phosphatidylinositol 3-kinase (PI3KC3), responsible for phosphorylating phosphatidylinositol (PI) to phosphotidylinositol-3-phosphate (PI3P), this protein forms a part of Vps34-Beclin-1-Vps15-Atg14L complex involved in phagophore nucleation. The PI3P pools contribute to isolation membrane formation and increases the conversion of LC3-I to LC3-II. Inhibiting VPS34 by a chemical inhibitor, PIK-III abrogated FABP4 release from adipocytes. Loss of VPS34 activity is shown by an increase in ratio of LC3B I/II when compared to WT (Fig. 3A). FFA release during lipolysis with the inhibitor treatment was comparable to WT L1 cells (Fig. 3B). Similarly, when Beclin-1, a core component of a PI3KC3 complex was deficient in MEFs that were differentiated to mature adipocytes, it blocked FABP4 secretion (Fig. 3C). However, the differentiated MEFs didn’t respond robustly to FSK also shown by others.

Next, we targeted an upstream protein, FIP200, a subunit of the ULK1-ATG13-ATG101-FIP200 complex, crucial for autophagy initiation and works by activating the VPS34-
BECLIN-1 complex \(^{40}\). The results indicate that FIP200 is essential for FABP4 secretion as shown by loss of FABP4 secretion after depleting \(Fip200\) in cells (Fig. 3D). Again, FFA efflux is not affected in these cells (Fig. 3E). The requirement of FIP200 is shown for FABP5 and NAMPT release (Sup Fig. 3A). When the requirement of another component of this complex ULK, which is a serine/threonine protein kinase was tested using \(Ulk1/2\) deficient differentiated MEF cells, the FABP4 release was undetectable (Fig. 3F). Corroborating this, ULK1/2 kinase specific inhibitor MRT68921 \(^{41}\) showed the requirement of an active ULK1/2 for FABP4 secretion. ULK1 activity loss was shown by a loss of phosphorylation of ATG14, which is an ULK1 kinase target (Fig. 3G). There was no change in FFA efflux compared to WT cells (Fig. 3H) in response to MRT68921.

It should be noted that the FFA levels are unchanged when the upstream autophagic components were targeted under basal/lipolytic conditions demonstrating that even in the presence of FFA, FABP4 secretion can be inhibited. This implies the necessity for these early autophagic proteins to possibly form some organelle structure (like an intermediate vesicle), to aid secretion.
Figure 3
Figure 3: FABP4 secretion requires early components of autophagy.

3T3L1 cells were pretreated with or without chemical inhibitors for 2 hrs, washed and then treated with or without FSK for 4 hrs. Secreted FABP4 and FFA were quantified and graphed for figures A, B and G, H. (A, B) 7.5 µM PIK-III inhibitor. (C) Secreted FABP4 was blotted using whole cell extracts from Beclin-1 deficient MEFs. (D, E) Ctrl or Fip200 knockdown 3T3L1s were treated with or without FSK and FABP4 and FFA were quantified from secreted material and plotted. (F) Ulk1/2 deficient MEFs differentiated to adipocytes treated with or without FSK for 4 hrs. (G, H) 2 µM MRT68921.
**Sirtuin-1 is required for FABP4 secretion:**

The cAMP/PKA pathway phosphorylates SIRT1 at S434 and activates the deacetylase activity of the metabolic sensor. The activated SIRT1 then promotes fatty acid oxidation and energy expenditure thereby regulating metabolic health. A growing body of evidence clearly shows that SIRT1 positively regulates autophagy and work by Sathyanarayan et al, exhibits the role of ATGL in promoting autophagy via SIRT1 activity. Moreover, Imai lab has shown that extracellular secretion of Nicotinamide phosphoribosyl transferase (NAMPT) was SIRT1 dependent. Due to these reasons, we investigated the role of sirtuin-1 in FABP4 secretion.

When SIRT1 activity was inhibited in adipocytes by a specific inhibitor EX527, there was a decrease in FABP4 secretion, when SIRT1 was activated by resveratrol, there was a corresponding increase in FABP4 release under basal conditions. eNAMPT secretion also required SIRT1 activity analogous to FABP4 (Fig. 4A) and FABP5 (Sup Fig. 4A). Total protein deacetylation by SIRT1 was used as a marker to quantitate the action of EX527 or resveratrol on SIRT1 (Fig. 4A). *Sirt1* /- MEF cells differentiated to mature adipocytes, did not secrete FABP4 (Fig. 4B).

It is possible that ATGL expression can be mediated by SIRT1 via deacetylation of FOXO-1 and the loss of FABP4 secretion here is due to loss of ATGL activity. To test this, we measured lipolytic activity of the cells and there was no defect in lipolysis in the SIRT1 deficient adipocytes (Fig. 4C). Moreover, we oleate loaded the adipocytes to
Figure 4: Sirtuin-1 is required for FABP4 secretion.

(A) Cells were treated with 10 µM EX527 or 10 µM Resveratrol for 16 hrs and the effluxed material was probed for FABP4 and NAMPT. Whole cell lysate was blotted using Acetyl Lysine antibody. (B, C) Sirtuin-1 deficient MEFs differentiated to adipocytes were treated with or without FSK for 4 hrs. FABP4 and FFA levels were measured and plotted. Oleate loaded (D) 3T3L1s or (E) Sirt1 knockdown 3T3L1s were subjected to +/- FSK treatment for 4 hrs and FABP4 efflux quantified. (F) Serum FABP4 was measured from WT vs Sirt1 deficient mice using ELISA.
Moreover, we oleate loaded the adipocytes to bypass the upstream lipolytic signals that activate the triglyceride breakdown. Upon lipid loading of L1 cells, there was a corresponding increase in FABP4 secretion basally and a modest increase with FSK (Fig. 4D). We hypothesized that this lipid availability by oleate loading might activate SIRT1 activation, indeed SIRT-6, another family member of sirtuins, was shown to activated by binding to FAs. To assess this, control and SIRT1 knockdown adipocytes were loaded with oleate to increase fatty acid availability and mimic lipolysis. FABP4 and FABP5 were found secreted from control cells and not from sirtuin-1 knockdown cells (Fig. 4E, Sup Fig. 4A, B). In conclusion, these results indicate that lipolysis mediated activation of SIRT1 can potentiate FABP4 secretion. In mice devoid of SIRT1, the circulating levels of FABP4 in the serum as measured by ELISA was undetectable (Fig. 4F).

Discussion

Secreted FABP4 has been shown to be correlative and causative of multiple metabolic dysfunctions. However, the unconventional secretion of this leaderless protein is an area of intense research. In our study, we demonstrate that the adipocytes secrete FABP4 in response to lipolysis. Upon following the route of FABP4 we found that its secretion required early stage autophagy proteins and is regulated by SIRT1.

One of our major observations, is showing the importance of canonical autophagy initiation proteins ULK1/2-FIP200 of the ULK complex and VPS34-BECLIN1 of the PI3KC3 complex for FABP4 secretion. Herein, the protein efflux is mediated via lysosomal exocytosis. Indeed, work by Goodwin JM et al, illustrate an alternative
lysosomal targeting pathway that requires ULK1/2-FIP200 complex, ATG9A and VPS34, but none of the other classical autophagy genes\textsuperscript{50}. Taken together, these observations fit well with our model for lipolytic FABP4 secretion. Villenueve et al., clearly showed FABP4 to localize in structures that are positive for late endosomal markers M6PR and Rab7. The major question, nevertheless, that was unanswered was how FABP4 got into endosomes. It is possible that there is a yet unknown mechanism of protein translocation into endosomes for their eventual fate for lysosomal exocytosis.

However, we propose another possibility of how FABP4 is present in structures positive for endosome markers. As illustrated in a model figure (Fig 5A), we speculate that FABP4 is initially recruited to the double layered isolation membrane which utilizes the activity of protein complexes ULK1/2-FIP200 and BECLIN-1-VPS34 (formed from a still unknown membrane source). This isolation membrane later extends and matures into an intermediate vesicle, due to membrane contribution from endosomes, without utilizing classical autophagy proteins such as ATG5 that will otherwise result in the formation of double membrane autophagosome. As the membrane source is endosomes in this case, we reason that this could be why FABP4 was found co-localized with endosomal markers while still requiring autophagy initiation proteins. Additionally, supporting evidence from Nishida Y et al., shows that, for ATG5 independent non-canonical autophagy, the phagophores received membranes from trans golgi or late endosomes and this autophagy requires ULK1 and Beclin\textsuperscript{1} \textsuperscript{51}. Finally, the intermediate vesicles thus formed with composite membranes from isolation membrane and endosomes can eventually fuse with lysosome and its cargo released via lysosomal exocytosis\textsuperscript{52}. Therefore, it is possible to
categorize this FABP4 secretion as an organelle based secretory mechanism. Further experiments are necessary to identify if there is co-localization of FABP4 with early autophagy proteins leading to specificity in recruitment.

Figure 5: Proposed model depicting unconventional protein secretion from adipocytes.

Schematic representation of FABP4 packaging and efflux in response to lipolysis. β-adrenergic activation signals the ULK kinase complex which in turn can activate class III PI3K complex. The PI3K complex functions to form isolation membrane, at some point during the isolation membrane formation, proteins to be secreted such as FABP4 are recruited to this structure. Isolation membrane receives membrane lipids and proteins from endosomes and matures into an intermediate vesicle without the requirement of the ATG16-ATG5-ATG12 complex. We propose that this is the reason why endosomal membrane markers were found associated with FABP4 by another study. The mature intermediate vesicle thus formed releases its contents into extracellular milieu by lysosomal exocytosis.
ATGL catalyzed lipolysis can activate SIRT1\textsuperscript{54}. Herein, we have also observed lipolysis dependent SIRT1 activation can stimulate FABP4 secretion. Furthermore SIRT1 mediated deacetylation of BECLIN1 at lysine residues 430 and 437 activates BECLIN-1 and decetylated state of VPS34 promotes autophagy initiation\textsuperscript{55,56}. Our results show that VPS34-BECLIN-1 activity is crucial for FABP4 release. Therefore, it is possible to hypothesize that lipolysis license SIRT1 activation which in turn deacetylates downstream early stage autophagy initiation proteins thereby promoting FABP4 secretion. It is intriguing yet as to how SIRT1, a protein that acts as a quality control agent of organelles, with antioxidant and antiaging properties\textsuperscript{57–59} is required for when FABP4 secretion, which in higher levels is associated with metabolic dysfunction.

Circulating FABP4 is strongly associated with progressive metabolic syndrome. However, there are still many unidentified players that regulate the secretion of FABP4. In this paper, we have identified components of a pathway that control not just FABP4 but other proteins including FABP5, NAMPT.
Acknowledgements

PIKIII (VPS34 Inhibitor) was gifted by Dr. Murphy’s lab, Novartis Institutes for Biomedical Research, Massachusetts, USA. *Ulk1/2 −/−* MEFs were provided by Dr. Kundu M, St. Jude Children's Research Hospital, Memphis, TN to DHK. *Becn1 −/−* MEFs were provided by Dr. Yue Z, Icahn School of Medicine at Mount Sinai, Newyork, NY to DHK.

**Funding:** Supported by NIH DK053189 to DAB and the Minnesota Agricultural Experiment Station (MN 70-015)

**Duality of Interests:** The authors declare that they have no conflicts with respect to any findings or conclusions from this manuscript.

**Author Contributions:** AJ developed hypotheses, carried out experiments, interpreted results and wrote manuscript. AVH developed hypotheses, carried out experiments, interpreted results and wrote manuscript. EB carried out experiments and interpreted results. MWM developed hypotheses, interpreted results and edited manuscript. SI-I developed hypotheses, interpreted results and edited manuscript. DM developed hypotheses, interpreted results and edited manuscript. DHK developed hypotheses, interpreted results and edited manuscript. DAB developed hypotheses, carried out experiments, interpreted results and wrote manuscript.
Abbreviations

- ATG5 – Autophagy related protein 5
- VPS34 - Vacuolar protein sorting 34
- FIP200 - Focal adhesion kinase family interacting protein of 200 kD
- ULK1/2 - Unc-51 like autophagy activating kinase
- M6PR – Mannose -6-phosphate receptor
- RAB7 – Ras related protein 7
- ATG9A – Autophagy related protein 9A
Supplementary figures: 1A) 3T3L1s were pre-treated with or without FSK for 4 hrs, washed and treated with INS for 30 mins and NEFA levels measured.

1B) After incubation for 18 hrs in normoxic or hypoxic chambers, the extracellular materials from the cells were blotted for NAMPT and FABP4.
2A) L1 cells were pretreated with or without 50 μM chloroquine (CQ) for 2 hrs, washed and then treated with or without FSK for 4 hrs.

3A) Ctrl or Fip200 knockdown L1s were treated with or without FSK and FABP5, eNAMPT was blotted on effluxed material.

4A) Cells were treated with 10 μM EX527 or 10 μM Resveratrol for 16 hrs and the effluxed material was probed for FABP5.

4B) mRNA expression levels were graphed to show stable lentiviral ctrl and Sirt1 knockdown in adipocytes.

4C) Oleate loaded Sirt1 knockdown 3T3L1s were subjected to +/- FSK treatment for 4 hrs and FABP5 efflux shown.
References:

CHAPTER 4:

CHARACTERIZING THE UNCONVENTIONAL PROTEIN SECRETION (UPS) FROM ADIPOCYTES

Ajeetha Josephrajan, Ann V. Hertzel, Ellie K. Bohm, Douglas Mashek, Do-Hyung Kim and David A. Bernlohr

From the Department of Biochemistry, Molecular Biology and Biophysics, The University of Minnesota, Minneapolis, MN USA,

This chapter contains unpublished work, Ajeetha Josephrajan’s contribution was writing the text and generating the data (except for figures 1F and 2C)
Preface:

Adipocyte tissue is a well-appreciated endocrine organ which secretes different factors that control metabolic, neuroendocrine and immune functions in response to various environmental stimuli, these factors are collectively called adipokines. Dysregulation of the endocrine function of the adipose tissue is one of the primary reasons for metabolic syndromes (MetS) and its pathophysiological effects in humans. Adipose tissue secretes adipokines such as leptin, adiponectin, resistin that are hormones, and tumor necrosis factor alpha (TNF), monocyte chemoattractant protein-1 (MCP-1), and plasminogen activator protein (PAI) that are inflammatory cytokines. Many of the proteins secreted follow the classical ER-golgi secretion pathway while other adipokines such as FABP4, FABP5 and NAMPT are secreted unconventionally. Herein, for the first time, we demonstrate that during lipolysis, adipocytes secrete a variety of other proteins in addition to the previously reported FABP4, FABP5 and NAMPT via an unconventional protein secretion (UPS) mechanism. We observed that the lipolytic-UPS is regulated similarly to that of FABP4 secretion which utilizes early stage autophagic proteins such as FIP200, ULK1/2 or BECLIN-1 and VPS34 while forgoing the use of later stage autophagic proteins such as ATG5 involved in canonical macro autophagy. The UPS is also dependent on the master transcriptional and autophagic regulator Sirtuin-1. Furthermore, the lipolytic-UPS also depends strongly on the nutrient status of the cells wherein presence of nutrients such as insulin or amino acids diminishes secretion.
Introduction:

Unconventional protein secretion collectively refers to the release of proteins that lack the N-terminal leader sequence involved in the classical ER-Golgi secretory pathway. This UPS has been defined to be unique as it depends heavily on the cell-type and environmental cues of the secreting cell \(^1,^2\). Proteins such as Fibroblast Growth Factor 2 (FGF2), Interleukin -1β (IL-1β) or CFTR that are revealed to follow UPS exhibit diverse ways of exiting cells. Rabouille et al., have classified these different exit pathways into 4 classes \(^3\). Type I pathway of UPS refers to proteins transported through plasma membrane pore formation such as FGF-2 \(^4\). Lipidated proteins cross the plasma membrane by utilizing the ABC transporters \(^5\) called Type II. Type III category includes IL-1β or yeast protein acyl-CoA binding protein (Acb1) secretion through organelle-based translocation of leaderless proteins via secretory autophagosomes or organelles, these are repurposed membrane organelles such as autophagosomes or endosomes to become secretory \(^6,^7\). Additionally, misfolding-associated protein secretion (MAPS) \(^8\) which is a quality-control mechanism also seem to make use of Type III. CFTR release is by Type IV golgi by-pass pathway \(^9\). These unique modes of protein export from the cell, its function after its release into the extracellular space and the requirement for these pathways are being explored intensely.

White adipose tissue (WAT) is not only a store house of excess triglycerides but it’s also one of the largest endocrine tissues of the human body \(^10\) that secretes an array of bioactive peptides called adipokines that can in an endocrine, paracrine or autocrine manner thereby causing effects to other organs via circulation. During conditions such as
obesity, characterized by dyslipidemia, hyperglycemia, hyperinsulinemia and insulin resistance, the pathophysiology is driven by many factors such as inflammatory macrophage infiltration due to chemokines secreted by AT or changes in the regulation of secretion of hormones such as decreased adiponectin or increased leptin levels in the bloodstream. Obesity also increases secretion of fibrinogen, PAI and various coagulation factors as a result of which there is condition of hyper coagulation leading to cardiovascular events.

More importantly, after the discovery of FABP4 and FABP5 as adipocyte secreted proteins, it is now apparent that these adipokines play a major role in promoting metabolic syndromes and cancers. Therefore, targeting serum FABP4 has valuable therapeutic potential. FABP4 and FABP5 are both fall under the category of lipolytic–UPS as they lack the secretory leader sequence and are secreted in response to lipolysis. Their secretion is dependent on early autophagic proteins and the deacetylase enzyme SIRT1 (data in chapter 3). Another protein that follows the same pathway as FABP4 is nicotinamide phosphoribosyl transferase (NAMPT) (data in Chapter 3). Published results indicate that NAMPT was released from adipocytes also in response to lipolysis and the secreted fraction promotes cancer progression and inhibiting the protein has beneficial effects.

This chapter shows data that suggests that a variety of proteins are secreted from mature 3T3L1 adipocytes in response to lipolysis and this process is highly regulated. The free fatty acids produced during lipolysis licenses the SIRT1 dependent activation of early
stage autophagic proteins, which in turn facilitate the secretion. Interestingly, this lipolytic-secretion is specific to adipocytes as metabolic cell do not secrete proteins in response to lipolysis. Nutrient availability is another one of the factors that regulate this secretion indicating that this secretion is receptive to physiological cues. Herein, we show that lipolytic-UPS is dependent on the presence of nutrients such as amino acids and insulin.

**Materials and Methods:**

**Reagents and chemicals:**
Forskolin (FSK), 8-br-cAMP, Isoproterenol (ISO), Amino acids from Sigma-Aldrich, St. Louis, MO. Gibco MEM Amino acids Catalog No -11130051, Thermofisher Scientific, Waltham, MA.

**Cell culture and cell lines:**
3T3-L1 fibroblasts were grown to confluence and differentiated to mature adipocytes as described previously 21. Differentiated adipocytes are maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% Fetal Bovine Serum (FBS) and used for experimentation from days 10-12. *Fip200* knockdown pre-adipocytes were previously made as described 22. Stable cell lines immortalized from bone marrow-derived macrophages of chow-fed WT C57BL/6J mice, as previously reported 23, are maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS). Primary hepatocytes derived from liver of chow-fed WT C57BL/6J mice and maintained as described previously 24.
Treatment methods:
Differentiated adipocytes were incubated with KRH - pH 7.4 with 5 mM glucose +/- 0.5% fatty acid free BSA for the treatment duration, after washing the cells twice with pre-warmed phosphate buffered saline (PBS). Lipolytic inducers or chemical inhibitors were added to KRH during the incubation period. For chemical treatments, the cells were pre-incubated with inhibitors for 2 hrs in DMEM with 2% FBS. For amino acid treatment or insulin treatment, KRH was supplemented with 1mM of each amino acid to make custom cocktails or 1X of GIBCO MEM amino acid or 500 nM of insulin and cells were pretreated for 2 hrs. The cells were then washed with twice with 1XPBS and then incubated with fresh KRH +/- treatment condition for 4-18 hrs.

Sample Collection and preparation:
KRH incubated on cells were collected and spun at 10,000xg to remove cell debris and then stored at -20º C until experimentation. Cell lysis was performed with buffer containing 50 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 1% deoxycholate, 1% triton X-100 and 0.1% SDS. The lysis buffer was supplemented with protease inhibitor (Calbiochem, Darmstadt, Germany) and phosphatase inhibitors (Sigma-Aldrich). Proteins were quantified using bicinchoninic acid (BCA) assay.

Exosomes/ microvesicle preparation:
The KRH media was collected and centrifuged at 10,000xg to remove cell debris. Microvesicle/exosome fractions were prepared as described\(^25\).
Oleate loading:

400 µM Oleate was coupled to 100 µM fatty acid free BSA and incubated on differentiated 3T3L1s for 24 hours in full media. After 24 hours, cells were incubated with KRH with or without FSK for 4 hours and media supernatant collected for analyses.

Non-esterified fatty acid efflux (NEFA) Assay:

Fatty acid efflux from adipocytes were measured under basal, lipolytic and various treatment conditions. KRH buffer supplemented with 0.5% fatty acid free BSA was incubated on pre-washed cells for 4 hrs and the buffer was collected to measure the released free fatty acids (FFA). Replicate measurements of FFA were measured using HR series NEFA kit (2) (Wako Chemicals, Richmond, VA) as per manufacturer’s protocol.

Hypoxia:

For hypoxia experiments, hypoxia chamber (Biospherix Ltd, Parish, NY, USA) containing 1% oxygen and 5% CO2 was utilized. The cells were placed in hypoxic chambers for 18 hrs.

Statistical analysis:

All the results are expressed with standard errors of the mean (SEM). For all experiments performed with 3T3L1 cells, the data presented has sample size of three and individual experiments were repeated. Li-Cor software was used to quantify protein levels in western blots and graphs representing protein levels, FFA levels were made using Prism 6 (GraphPad Software). The results were summarized as standard errors of mean in the figures (SEM). Statistical significance was determined using an unpaired two-tailed Student t-test.
Results:

Unconventional Protein Secretion (UPS) from adipocytes during lipolysis.

As a model to understand protein secretion from adipocytes, 3T3L1 adipocyte cell line was utilized. We observed that, in addition to few proteins such as FABP4, FABP5, Galectin-3 and NAMPT (Chapter 3, Fig. 1F) several other proteins are secreted in response to lipolysis from adipocytes, collectively called the lipolytic-UPS (Fig. 1A, B). It is also worth noting that proteins such as classically secreted protein, RBP4 is not stimulated in response to a lipolytic signal (Chapter 3, Fig. 1F). Therefore, there is some level of specificity to UPS. We further characterized this secreted protein fraction and concluded that most of these secreted proteins are not packaged in microvesicles or exosomes, as most proteins were present in the fraction that was depleted of small vesicles (Fig. 1C). We then wanted to test if these proteins are unconventionally secreted, by treating cells with Brefeldin-A, which is an inhibitor of the classical ER-Golgi secretory pathway. Our data indicates that the secretion is unconventional without the requirement of classical secretion pathway (Fig. 1D). This is also supported by the preliminary results from our mass spectrometry data (data not shown) as there was a clear enrichment of proteins that lacked the leader sequence. When adipocytes were subjected to hypoxic conditions, known to stimulate basal lipolysis \(^{26}\), there was an overall increase in protein secretion (Fig. 1E, F).
Figure 1: Unconventional Protein Secretion (UPS) from adipocytes during lipolysis.

A-D) Extracellular material from 3T3L1 adipocytes were collected for 4 hrs and all samples were run on SDS-PAGE gel stained with coomassie. (A) 2% of intracellular lysate and extracellular material from basal and 20 µM FSK stimulated condition (B) Extracellular material from 3T3L1 adipocytes treated with vehicle, 20 µM FSK, 10 µM ISO, 1 mM 8-Br-cAMP and 500 nM INS for 4 hrs. (C) Total protein stain of intracellular, extracellular and non-vesicular fraction, vesicular fraction of secreted material from basal and FSK treatments. (D) Treatment of 3T3L1s with Brefeldin A (10 µg/ml) for 4 hrs under basal or FSK treatment. (E) NEFA levels were measured after subjecting 3T3L1s to hypoxia or normoxia for 18 hrs. (F) Secreted proteins levels from normoxic and hypoxic conditions after 18 hrs were run on SDS-PAGE gel.
**Lipolytic – UPS is SIRT1 dependent and requires early autophagic proteins.**

FABP4, the primarily abundant protein in the adipocytes, is one of the proteins that is secreted through the lipolytic-UPS. Our earlier results indicated that FABP4 secretion required early stage autophagic proteins such as ULK1/2, FIP200 of the ULK1/2-FIP200-ATG13-ATG101 complex and BECLIN-1 and VPS34 of VPS34-ATG14-VPS15-BECLIN-1 complex (Chapter 3, Fig. 3). Therefore, we tested the requirement of early autophagic protein FIP200 for secretion. The results imply that indeed FIP200 is required for secretion (Fig. 2A). FIP200 knockdown expression is shown in Chapter 3, Fig 3D. Analogous to FABP4 the lipolytic-UPS secretion requires the activity of SIRT1. When SIRT1 activity is blocked with EX527 or activated with resveratrol there was a corresponding stimulated or attenuated secretion (Fig. 2B). Total protein deacetylation by SIRT1 was used as a marker to quantitate the action of EX527 or resveratrol on SIRT1 (Chapter 3, Fig. 4A). We hypothesized that this lipid availability might be responsible for SIRT1 activation. To assess this, adipocytes were loaded with oleate, which increased the secretion in both basal and forskolin stimulated condition (Fig. 2C). These results indicate the possibility that fatty acid dependent activation of Sirtuin-1 can potentiate FABP4 secretion.

More importantly, this secretion is unique for adipocytes under lipolytic conditions, as other cells such as hepatocytes derived from another metabolic tissue, liver didn’t show UPS in response to lipolysis (Fig. 2D) the same is true for macrophages (Fig. 2E).
Figure 2: Lipolytic – UPS is SIRT1 dependent and requires early autophagic proteins

A-D) Extracellular material from 3T3L1 adipocytes were collected after 4 hrs and all samples were run on SDS-PAGE gel stained with coomassie. (A) Ctrl or Fip200 knockdown L1s were treated with or without FSK (B) Cells were treated with 10μM EX527 or 10μM Resveratrol for 16 hrs. (C) Oleate loaded L1s were subjected to +/- FSK treatment for 4hrs. (D) Secreted material from (D) Primary hepatocytes derived from WT C57BL/6J mice liver (E) Stable macrophage cell line derived from wild-type C57BL/6J mice (WTMϕ).
**UPS is regulated by nutrient availability:**

Next, we tested if UPS is a regulated process by controlling the nutrient availability. Amino acid starvation attenuated UPS in both basal and FSK stimulated conditions (Fig. 3A). Insulin treatment and branched chain amino acid treatment also attenuated UPS. However, the FSK dependent total secretion was unchanged in insulin treatment but showed attenuation of UPS under amino acid treatment (Fig. 3B). To identify which of these amino acids are crucial in regulating the secretion process, different amino acid cocktails were made and the adipocytes were treated. Our preliminary results indicate that tyrosine and glutamate if present in the media can attenuate the lipolytic –UPS (Fig. 3C-F).
Figure 3: UPS is regulated by nutrient availability.

A-F) Secreted material from 3T3L1 adipocytes were collected after 4 hrs from +/- FSK treated conditions; +/- amino acid or insulin supplement and all samples were run on SDS-PAGE gel stained with coomassie. A) GIBCO MEM Amino acid (1X) (B) Branched chain amino acids, LIV (Leucine, Isoleucine, Valine) 1 mM each or insulin (500 nM) was supplemented into KRH buffer with 5% glucose for 4 hrs.
Figure 3: UPS is regulated by nutrient availability.

A-F) Secreted material from 3T3L1 adipocytes were collected after 4 hrs from +/- FSK treated conditions; +/- amino acid or insulin supplement and all samples were run on SDS-PAGE gel stained with coomassie. (C, D, E and F) Amino acid cocktail of different amino acids +/- FSK treatment.
Discussion:

The various mechanisms and the components of unconventional protein secretion which doesn’t utilize the classical ER/Golgi secretory pathway are being continually discovered for different proteins \(^{27-29,3}\). It is intriguing yet, as to how different secretory proteins employ different pathways, or different pathways are utilized by the same protein depending upon the stimuli \(^{29}\). This process of secretion, wherein a multitude of protein secretion is due to some form of cellular, inflammatory, nutrient, endoplasmic reticulum (ER) or mechanical stress \(^{30}\) response is collectively called UPS. In our study, we demonstrate that the adipocytes secrete a variety of proteins via UPS in response to lipolysis, including FABP4.

Upon following the route of FABP4, we found that its secretion required early stage autophagy proteins and is regulated by global metabolic regulator SIRT1. To unravel the lipolytic-UPS pathway, we tested if the bulk protein secretion also required early stage autophagic protein FIP200 and deacetylase SIRT1 in a manner similar to FABP4 (Fig. 2A-C and Chapter 3 Fig. 3,4). We found that lipolytic-UPS/FABP4 required the same components as illustrated in model figures (Chapter 3, Fig. 5A and Fig. 4). However, we haven’t yet tested if the efflux of all these proteins is via lysosomal exocytosis as was the case for FABP4 (Chapter 3, Fig. 2C). Other proteins of different complexes of the early stage autophagy pathway will also be tested to understand their requirement.

The regulated process of lipolytic-UPS from adipocytes is a very important observation that hasn’t been reported before. As a result, two major questions that arise are: 1) Why
is there a potentiated secretion of multiple proteins from adipocytes under lipolytic condition and how are the proteins utilized after getting into the extracellular milieu? 2) Is this UPS process beneficial for the cell or the organism as a whole? The deacetylase SIRT1 regulates broad metabolic functions by acting as a quality control agent of organelles, promoting antioxidant and antiaging properties. Therefore, it is tempting to speculate that this SIRT1 dependent UPS will be overall beneficial for the organism by replenishing amino acid supply in recipient cells of the metabolic tissues during starvation. On the other hand, it is also possible that this a quality control for adipocytes to get rid of damaged proteins, because in obesity and its related pathologies, there is an increase in damaged proteins in the cell due to increased ROS and inflammation. However, when the in-situ oxidized cysteine labelled proteins were examined, there was no change in the oxidation states of the secreted vs. intracellular under basal or forskolin stimulated conditions (data not shown).

More importantly, we have shown that the secretion process is tightly regulated by the presence of nutrients, in this case amino acids and insulin (Fig. 2A, B). The results imply that amino acids tyrosine and glutamine when supplemented to the cell culture media blocked the secretion. Further experiments must be performed to analyze the dose dependence of individual amino acids, to unravel the contributions of individual amino acids more clearly, as the experiments shown here might have synergistic effects due to amino acid combinations. Evidence from multiple studies also indicate nutrient starvation dependent protein secretion in yeast for proteins such as Acb1 and SOD1. This is interesting in a physiological aspect as it leads to the speculation that the secreted
proteins might act as energy source elsewhere. Amino acids and insulin starvation both activate AMP-activated protein kinase (AMPK) dependent ULK1 phosphorylation and activation and therefore, subsequently inhibit mammalian target of rapamycin complex 1 (mTORC1) \(^{35}\). We have shown the requirement of ULK complex for secretion (Chapter 3, Fig. 3F, G). It is possible that the lack of secretion after supplementation with amino acid/insulin is due to ULK1 inactivation and thus mTORC1 activation (Fig. 4). Insulin dependent mTORC1 activation is clearly established \(^{36}\).

In the case of amino acids, it has been shown that amino acids can activate mTOR and discovery of amino acid sensors such as SLC38A9 \(^{37}\), Sestrin1/2 \(^{38,39}\) and CASTOR1 \(^{40}\) explain in part as to how leucine and arginine are sensed. Also, glutamine supplements can reactive mTOR during amino acid starvation \(^{41}\). It is unclear if there are other sensors for other amino acids such as tyrosine across various subcellular compartments. As such, the nutrient sensors of the cells seem to play vital role in UPS. Further experiments to test mTOR dependence is currently underway in the lab. This will be crucial in understanding as mTOR is a master regulator and this pathway might still have multiple unexplored components.
Figure 4: Schematic representation of UPS in response to lipolysis from adipocytes.

β-adrenergic activation signals lipolysis, resulting in enzymatic hydrolysis of lipid droplet to release free fatty acids (FFA), the FFA released license the deacetylase enzyme Sirtuin-1 and activates it. Sirt-1 can activate autophagic proteins such as BECLIN-1 and VPS34 and result in the formation of an isolation membrane. However, during times of nutrient availability, mTORC1 activation will suppress the isolation membrane formation by inactivatingULK1 and VPS34. This mTORC1 activation will be antagonistic to Sirtuin1 mediated isolation membrane formation. The isolation membrane thus formed will mature with membranes from other sources (depicted in Chapter 3, Fig. 5A) and cargo proteins that are leaderless proteins packaged for secretion. We named this structure intermediate vesicle that will later release its contents extracellularly by lysosomal exocytosis.
Overall, our data reveals that a variety of proteins including FABP4, FABP5, eNAMPT and Galectin-3 are secreted from adipocytes in response to a lipolytic stimuli. Importantly, this suggests that there could be an increase in levels of adipocyte cytoplasmic proteins in the circulation. Clearly, FABP4 has multiple effects after getting into the blood stream, potentially, this is true for other proteins. It would be important to explore how the protein homeostasis is maintained in the extracellular milieu. These circulating proteins could also have an impact in the development of metabolic disorders.
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Conclusions and future perspectives:

It is now clearly established that FABP4 has function both intra and extracellularly. In chapter 3, FABP4 is shown to be exported while requiring the functions of ULK and VPS34 complex proteins. We therefore hypothesized that these proteins, whose primary function is to initiate the synthesis of isolation membrane is a required step for secretion. In that case, it is possible to speculate that FABP4 would be associated with isolation membrane components, some protein candidates of interest would be ULK1 and FIP200\(^1\).

Using confocal microscopy colocalization of FABP4 with isolation membrane components can be analyzed. This would indicate that the requirement of early autophagic components is their function to make isolation membranes bringing up another step closer to identifying the exact mechanism of FABP4 release. In the case of no colocalization, we would speculate that the requirement for these autophagic protein complexes for FABP4 efflux is due to their yet undiscovered function.

Two important unanswered questions that warrant further research and are under current pursuit following the chapter 4 UPS studies are: 1) What signals these individual proteins to get targeted for secretion from adipocytes? 2) Is there any specificity to this protein secretion? It is possible that the proteins secreted have a similar kind of post-translational modification (PTM) on them. A recent meta-analysis of the RNA-seq derived transcriptome data from various human tissues revealed that there is a fine tuning of expression of certain genes involved in the secretory pathway upon secretion demand. The expression levels of these candidate genes positively correlated with increase in nature and number of PTMs on the proteins secreted from the specific tissue \(^2\). This
indicates that when there is secretion demand, intracellular changes occur that will facilitate PTM on proteins for their targeted export. This particular study, however, was exclusively performed for classically secreted proteins. It is possible to apply this concept for UPS, wherein an abundance of a substrate can post-translationally modify proteins targeting them for secretion. For example, during the bulk protein secretion occurring after lipolytic stimuli, there is an increase in the intracellular levels of free fatty acids (FFA). These FFA can act as substrates for fatty-acylation, which can be PTM on proteins targeted for unconventional secretion. Indeed, fatty acylation is one of the mechanisms of UPS categorized under type II.

Another PTM that can focused on is acetylation, we hypothesize that the proteins need to be deacetylated to be secreted. The rationale for this hypothesis are as follows; 1) Multiple proteins that are listed as secreted from adipocytes during lipolysis from our preliminary mass-spectrometry of secreted proteins indicate that the secreted proteins comprises intermediary metabolic enzymes (data not shown), and a large fraction of these enzymes, localized in cytoplasm or mitochondria are acetylated proteins. It is possible that these proteins get deacetylated for secretion. 2) One of the proteins NAMPT which is secreted via UPS needs to be decetylated for secretion. 3) More importantly, we have shown that SIRT1 is required for UPS. Therefore, it is highly likely that the requirement of SIRT1 for secretion is for its deacetylating function.

Additionally, it’s also within the realm of possibility that the UPS proteins are recruited specifically by some chaperone proteins for export through a consensus domain or the
secreted proteins have a common feature in their sequence or structure. For example, conserved di-acidic motif like in the case of superoxide dismutase 1 (SOD1). As such it is an important area that needs to be explored, to better understand this pathway. Other important areas that need exploration includes understanding the role of these secreted proteins in the circulation, as discussed briefly in Chapter 4, and the cost of these secreted proteins.

While white adipose tissue (WAT) comprises of the predominant fraction of fat tissue in the human body, the other fat depot that is also a metabolically active is the brown adipose tissue (BAT). The BAT has properties that are antagonistic to the WAT as they are highly oxidative tissue with abundantly present mitochondria. The oxidizes the fatty acids and dissipates heat via the uncoupling protein 1 (UCP1) thereby playing an important role in thermogenesis and maintaining whole body energy balance.

Literature indicates that percentage of BAT negatively correlates with body weight and percentage of WAT. During a mild cold exposure challenge, BAT activity measured by energy expenditure was higher in lean male subjects than their obese counterparts. Therefore, BAT tissue is generally thought to be a metabolically beneficial that might be employed to combat the pathological effects of obesity. Given its contrasting functions when compared to WAT, it would be crucial to analyze the secretory protein profile of BAT. We will test the unconventional vs conventional secretion pathway, in response to lipolytic or catecholamine activation (cold challenge) from BAT. Earlier we had tested the secretory profile of other metabolic cells such as hepatocytes and macrophages and saw no change in regulated secretion in response to β-adrenergic signaling. It is possible
that the BAT might mirror similar results or more of significant interest that the proteins will get secreted in a regulated manner and the secreted proteins might have functions opposing that of WAT –UPS proteins.

The field of unconventional protein secretion is expanding rapidly due to the development of new technologies and therefore characterizing and unraveling the role of adipocyte/adipose tissue secretome in regulating whole body metabolism is pivotal to understanding disease progression under conditions of obesity.
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