

**The Effects of Leucine and Dairy Products on  
Adipose Tissue Inflammation:  
The Role of  
Adipocyte Derived Microvesicles**

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## ABSTRACT

Obesity is characterized by chronic oxidative and inflammatory stress, and adipose tissue is a significant source of inflammatory cytokines. Previous studies demonstrated that dairy products (rich in calcium and leucine) can alleviate obesity-associated inflammatory stress through suppression of 1, 25-dihydroxycholecalciferol (calcitriol) with calcium and the high leucine content in dairy. We have also shown leucine treatment increases anti-inflammatory adiponectin expression and decreases pro-inflammatory cytokines TNF- $\alpha$  [alpha], MCP-1, and IL-6 expression in adipocytes. Therefore, we sought to determine if these alterations in inflammatory cytokine production could have a functional effect on the inflammatory process, specifically monocyte – endothelial cell adhesion as this is one of the initial events of the inflammatory process. We demonstrate that leucine treatment of adipocytes reduces monocyte CD11b expression, endothelial cell ICAM-1 expression and, consequently, monocyte – endothelial cell adhesion *in vitro* while calcitriol exerted the opposite effects. Furthermore, plasma samples from obese individuals consuming high dairy diets (> [greater than] 3.5 servings/day) over a 12-week period reduced monocyte – endothelial cell adhesion, *ex vivo*. Recently, adipocyte derived microvesicles (ADMs) have been suggested to play a role in communication between adipose tissue and systemic circulation, so we sought to determine if adiponectin present on ADMs was responsible for the anti-inflammatory effects we have observed when treating adipocytes with leucine. Therefore, after adipocytes were treated for 48 hrs with leucine, the whole conditioned media (CM), purified ADMs, and remaining supernatant were applied to human peripheral blood to measure monocyte CD11b expression. Compared to control,

leucine CM and the isolated ADMs both reduced monocyte CD11b expression while the supernatant fraction did not. Knocking down adiponectin with siRNA attenuated these effects, suggesting adiponectin associated with ADMs plays a role in mediating the anti-inflammatory effects we have observed. Collectively, these data suggest that dairy products can provide beneficial effects at reducing obesity-associated inflammation, and ADMs, in part, mediate some of these effects.

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## LIST OF ABBREVIATIONS AND SYMBOLS

|                  |                                                                           |
|------------------|---------------------------------------------------------------------------|
| ADM              | adipocyte derived microvesicles                                           |
| Ca <sup>2+</sup> | Calcium                                                                   |
| Calcitriol       | 1,25-Dihydroxycholecalciferol                                             |
| AT               | Adipose tissue                                                            |
| CM               | Conditioned Medium                                                        |
| M:E              | monocyte to endothelial cell ratio                                        |
| ROS              | reactive oxygen species                                                   |
| TNF- $\alpha$    | tumor necrosis factor alpha                                               |
| IL-6             | interleukin 6                                                             |
| UCP1             | uncoupling protein 1                                                      |
| UCP2             | uncoupling protein 2                                                      |
| MCP-1            | monocyte chemotactic protein 1                                            |
| MIF              | macrophage migration inhibitory factor                                    |
| CD11b            | cluster of differentiation molecule 11b                                   |
| CD54             | cluster of differentiation molecule 54                                    |
| IL-8             | interleukin 8                                                             |
| VEGF             | vascular endothelial growth factor                                        |
| DiI              | 1,1'-Dioctadecyl-3, 3, 3', 3'- tetramethylindocarbocyanine<br>perchlorate |
| PBS              | phosphate buffered saline                                                 |
| HAT              | Hypoxanthine - Aminopterin – Thymidine                                    |
| DMEM             | Dulbecco's modified Eagle's medium                                        |
| FBS              | fetal bovine serum                                                        |
| SIRT1            | sirtuin 1                                                                 |
| SEM              | standard error mean                                                       |
| ADM              | adipocyte derived microvesicles                                           |

**CHAPTER I:**  
**INTRODUCTION**

Overweight and obesity, defined as having a body mass index  $>25 \text{ kg/m}^2$  and  $>30 \text{ kg/m}^2$ , respectively, is a growing problem in both the United States and worldwide. According to the World Health Organization in 2008, 1.4 billion or 35% of the world's population age 20 and older were overweight (1). Approximately 500 million or 11% of the world's population are obese, and in the United States in 2012, nearly 35% of adults age 20 and older are obese (1, 2). Obesity is associated with increased risk factors for multiple chronic diseases including cardiovascular disease, cancer, and type 2 diabetes (3), and the cost to treat obesity related diseases was estimated to be \$147 billion in the United States in 2008 (4).

Adipose tissue is a significant source of both reactive oxygen species (ROS) and inflammatory cytokines (5, 6). In obesity, enlarged adipocytes produce more pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF  $\alpha$ ) and interleukin 6 (IL-6), and less anti-inflammatory factors such as adiponectin (7, 8). The regulation of ROS production and interactions among ROS, cytosolic  $\text{Ca}^{2+}$  signaling and mitochondrial uncoupling have been intensively investigated, and we have shown that calcitriol (1,25-Dihydroxycholecalciferol) increases ROS production in both murine and human adipocytes by inhibiting adipocyte UCP2 expression, thereby increasing mitochondrial potential, and increasing cytosolic  $\text{Ca}^{2+}$  signaling (9). Accordingly, since dietary calcium suppresses calcitriol, high calcium diets may correspondingly decrease adipose tissue ROS production and systemic oxidative stress. Consistent with this concept, increasing dietary calcium decreases pro-inflammatory cytokine production and increases adiponectin production in mice (10, 11).

These data provide a framework to explain a protective effect of dietary calcium against oxidative and inflammatory stress. However, milk also contains high concentrations of leucine and bioactive peptides that may further protect against oxidative and inflammatory stress. Leucine has been shown to increase mitochondrial UCP2 expression and adiponectin production in differentiated 3T3-L1 adipocytes, and promote fatty acid oxidation in C2C12 muscle cells, while calcitriol exerted the opposite effects (12). Furthermore, we have recently confirmed that dairy exerts a significantly greater effect on suppressing both local adipose tissue and systemic oxidative and inflammatory stress compared to supplemental calcium in mice (11). Additionally, our human data demonstrate that dairy-rich diets result in suppression of oxidative and inflammatory biomarkers (11). In a recent follow-up randomized crossover study in overweight and obese subjects, we found that dairy supplementation suppressed both oxidative and inflammatory stress within seven days of initiation of supplementation, and that these effects increased in magnitude with increased duration of supplementation (13). Similar results were found in a 12-week study of metabolic syndrome patients (14).

The adipose tissue cytokines we have demonstrated to be regulated by calcitriol and dairy components include several which affect vascular endothelial and smooth muscle cells (e.g. MCP-1, MIF, CD14, TNF- $\alpha$ , IL-6, IL-8, VEGF) and vascular infiltration by monocytes (15). Accordingly, the purpose of this work was to: a) determine if the modulation of adipocyte cytokine production observed with leucine and dairy treatment has a functional effect on the inflammatory process, b) determine which cytokine plays the most prominent role in this process, and c) determine if adipocyte derived microparticles can play a role in this process.

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**CHAPTER II:**  
**LITERATURE REVIEW**

## **2.1.0 Adipose Tissue Overview:**

The term adipose tissue (AT) refers to loose connective tissue in the body where fat is stored (1). AT is interspersed throughout the body where it can serve a variety of functions and play multiple roles. Various forms of AT are present throughout the body. These forms include white adipose tissue (WAT), brown adipose tissue (BAT), and beige/brite adipose tissue (1). Each of these forms are critically important for energy metabolism and their functions are tightly regulated (1). The sections below will discuss the various aspects of adipose tissue including the specific cellular components and the role those components play in regulating energy metabolism.

### **2.1.1 Adipocytes:**

The two primary forms of adipose tissue are white adipose tissue (WAT) and brown adipose tissue (BAT), whose primary functions are energy storage and thermogenesis, respectively (2). These two distinct forms of AT are comprised of different cell types that serve specific roles in each of their respective tissues. In humans, WAT can typically make up 15-20% of the total body mass of a healthy, young adult (1). WAT can be found dispersed throughout the body including under the skin (subcutaneous), in the abdominal cavity, and around bodily organs (visceral) (1). The most abundant cell type present in WAT is the white adipocyte, or more commonly known as the fat cell. White adipocytes are large (typically 30-70  $\mu\text{m}$  in diameter), spherical cells filled with a single, large lipid droplet that constitutes ~65% of the cell mass (3). The lipid droplet, due to its size, causes the mitochondria and nucleus to be forced up against the cell wall of the plasma membrane (3).

The primary function of the white adipocyte is to store energy in the body as fat until a demand for energy increases, where the stored fat is mobilized and used throughout the body as a source of fuel (1, 3). Like many other cell types, adipocytes have an active glycolytic metabolism, are capable of oxidizing pyruvate and fatty acids via the citric acid cycle, and also carry out oxidative phosphorylation (3). During periods of high carbohydrate intake, white adipocytes can convert glucose to fatty acids, which are stored in the adipocyte's lipid droplet as triacylglycerol (TAG) (3). White adipocytes can also store lipid exported from the liver (1, 3). When fuel sources become scarce, lipase enzymes in the adipocyte hydrolyze the stored TAGs and release them as free fatty acids into the blood stream to be used as fuel (1, 3).

BAT is less abundant in adults and more abundant in newborn infants, where it is involved in thermogenesis (4). In adult humans, BAT can be found deep in the neck and close to muscle tissues (5, 6). The predominant cell type present in BAT is the brown adipocyte, which is structurally and functionally distinct from white adipocytes (3). Compared to white adipocytes, brown adipocytes are typically smaller (20-40  $\mu\text{m}$  in diameter), polygonal shaped cells that store triglycerides (TAGs) in several, smaller lipid droplets per cell (3). Furthermore, brown adipocytes have more mitochondria and the surrounding tissue has a richer supply of capillaries, thus causing the cells to appear brown in color (3). One key genetic difference between white and brown adipocytes is the expression of mitochondrial uncoupling protein 1 (UCP1) by brown adipocytes (7). UCP1 is a 32 kDa transmembrane protein located on the inner mitochondrial membrane that promotes the uncoupling of the mitochondrial electron transport chain from ATP synthesis during oxidative phosphorylation and  $\beta$ -oxidation of fatty acids, resulting in the

dissipation of the mitochondrial membrane potential and allowing the energy to be dissipated as heat (4). This controlled exothermic release of stored energy occurs during periods of cold exposure in order to increase the core body temperature, a process known as non-shivering thermogenesis (3). Upon exposure to a cold environment, the sympathetic nervous system activates  $\beta_3$ -adrenergic receptors in brown adipose tissue to initiate lipolysis and activation of UCP1, which will use the mitochondrial membrane potential generated during electron transport from lipolysis to bypass ATP synthase, thus allowing the stored energy to be dissipated as heat to warm the body (8). Consequently, BAT is highly metabolically active and can be detected as hot spots of radiolabeled glucose uptake by positron emission tomography – computed tomography (PET-CT) (2).

Both white and brown adipocytes are believed to be from a mesodermal origin, but different mesenchymal stem cell (MSC) lineages (2). Although the number of steps and cell types remain unclear, it is believed that MSCs give rise to a common early precursor, known as the adipoblast, which in turn develops into the committed white and brown pre-adipocyte and under specific stimulatory conditions will differentiate into mature white and brown adipocytes (2, 9). The process of pre-adipocytes differentiating into mature adipocytes is collectively known as adipogenesis (9). The transition from pre-adipocyte to mature adipocyte is thought to occur in four stages: growth arrest, clonal expansion, early differentiation, and terminal differentiation (9). These stages are organized by a cascade of transcriptional regulators involving the nuclear receptor peroxisome proliferator-activated receptor gamma ( $PPAR\gamma$ ) and members of the CCAAT-enhancer-binding-protein (C/EBP) family (9).  $PPAR\gamma$  activates a variety of genes that regulate fatty acid storage and glucose metabolism, and thus is required for

adipogenesis, as PPAR $\gamma$  knockout mice fail to generate adipose tissue when fed a high fat diet (10, 11). PPAR $\gamma$  has two isoforms, namely PPAR $\gamma$ 1 and PPAR $\gamma$ 2, in which PPAR $\gamma$ 2 seems to be more dominant for adipocyte differentiation (8, 9). The C/EBP family consists of five different members: C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$ , C/EBP $\gamma$ , and CHOP (2). During differentiation, these various members are sequentially expressed and help drive the differentiation process. For example, early expression of C/EBP $\beta$  and C/EBP $\delta$  promote the expression of C/EBP $\alpha$  and PPAR $\gamma$ 2 later on during the adipogenic program (12). Although both white and brown adipocytes appear to be of a common lineage, studies have shown that traditional brown adipocytes are derived from myoblasts and are positive for the Myf5 protein while white pre-adipocytes do not express this protein (13). Furthermore, two key transcriptional regulatory factors involved in the brown adipogenic program are C/EBP $\beta$  and the PR domain-containing protein-16 (PRDM16) (5, 8, 13). PRDM16 binds and co-regulates C/EBP $\beta$ , PPAR $\gamma$ , PPAR $\alpha$ , and PGC-1 $\alpha$  to promote brown fat-specific gene induction (5, 13). PGC-1 $\alpha$ , or peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$ , is involved in regulating mitochondrial biogenesis, oxidative metabolism, and thermogenesis (14, 15). The primary physical characteristic of adipocyte differentiation is lipid droplet accumulation. Although many cell types can accumulate lipids, adipocytes collect the lipids into either a unilocular lipid droplet (WAT) or in smaller multilocular droplets (BAT) surrounded by the protein, perilipin (16).

Although white and brown adipocytes have distinct structural features, characteristics and functions, recent evidence suggests a third type of adipocyte known as the beige adipocyte (also called inducible brown, brown-in-white, or brite) (17, 18). The

accumulation of beige adipocytes in WAT is often referred to as “browning” of WAT, and occurs after extended cold exposure, from  $\beta_3$ -adrennergic receptor agonists, or from exercise-induced myokines, including irisin and meteorin-like. (7, 19). Recent evidence has shown that these beige adipocytes that arise in WAT are not of the traditional Myf5 positive precursor lineage that give rise to traditional brown adipocytes present in BAT depots (20, 21). Although the exact mechanism of “browning” of white adipose tissue remains unclear, some groups claim that the “browning” process arises via the transdifferentiation of white adipocytes into beige/brite adipocytes (20, 21).

Interestingly, “paucilocular” adipocytes (UCP1-positive cells containing lipid droplet distribution that is intermediate between brown and white adipocytes) have been reported and further suggest an intermediate form of adipocyte that is transitioning from white to beige/brite adipocytes (22, 23). Furthermore, recent data based on genetic labeling of adipose cells support the existence of bidirectional interconversion between beige/brite and white adipocytes (24). The “browning” process has become a popular area of research due to its therapeutic potential of expending energy without the production of ATP and warrants further investigation (7).

### **2.1.2 Stromal/Vascular Fraction of Adipose Tissue:**

In addition to adipocytes, adipose tissue is comprised of other cell types commonly referred to as the stromal/vascular fraction, which consists of blood vessels containing endothelial cells, leukocytes such as monocytes/macrophages, and cells that exhibit characteristics of progenitor cells (25). The stromal/vascular fraction supplies the tissue with blood, nutrients and energy to support growth and for energy storage (25).

Endothelial cells line the blood vessels wall and play a role in the extravasation of leukocytes from blood circulation into the adipose tissue (25). Specifically, monocytes and macrophages are the most prominent leukocyte present in adipose tissue, and can secrete a variety of cytokines and chemokines that generate cross-talk between adipocytes and the stromal/vascular fraction (26).

### **2.1.3 Endocrine Function of Adipose Tissue:**

In addition to its role in energy storage, adipose tissue is an active endocrine organ that expresses and secretes a variety of peptides and hormones into circulation (27, 28). The peptides and hormones produced in adipose tissue are collectively referred to as adipokines (29). Adipokines can act locally, via an autocrine or paracrine action, or systemically, via an endocrine action to carry information about the adequacy of the energy reserves (TAGs) stored in adipose tissue to other tissues and to the brain (3). Under normal conditions, adipokines cause changes in fuel metabolism and feeding behavior that will reestablish adequate fuel reserves and, thus, maintain body mass (3). If adipokines are over or under produced, it can result in life-threatening alterations that increase the risk for a variety of diseases (3). The three classic adipose tissue derived hormones are leptin, resistin and adiponectin (3).

The first adipokine, discovered in 1994 by Zhang *et al.*, was the peptide hormone leptin, which is a 167 amino acid peptide that, upon reaching the brain, acts on receptors in the hypothalamus to change appetite (30). Leptin essentially messages to the body that fat reserves are sufficient, and it promotes a reduction in food intake and increases energy expenditure (31). The leptin receptor is expressed in regions of the brain that are known

to regulate feeding behavior called the arcuate nucleus of the hypothalamus (3). The two types of neurons in the arcuate nucleus control fuel intake and metabolism (3). The first type known as orexigenic neurons, stimulate eating by producing and releasing neuropeptide Y, which is suppressed by leptin (3). Furthermore, leptin stimulates the second type of neuron called anorexigenic neurons to produce  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH; also called melanocortin), which suppresses appetite (3). Therefore, as adipose tissue mass decreases, leptin levels also decrease (3).

A second adipose tissue specific adipokine is resistin, which plays a role in obesity related insulin resistance, as depletion of circulating resistin by neutralizing antibodies in obese mice resulted in improved insulin action (32) and resistin knockout mice fed a high fat diet show signs of improved glucose metabolism (33). The third classic adipokine is adiponectin, and will be discussed in detail in subsequent sections.

#### **2.1.4 Adipose Tissue and Inflammation:**

Adipose tissue can produce multiple factors that can directly interact with the immune system, both locally and systemically, to alter the inflammatory response (27). Inflammation is a complex biological process by which an organism's immune system responds to an infectious stimuli, pathogen or tissue damage through the recruitment of activated immune cells to repair or heal the tissue (34). Inflammation can be classified as either acute or chronic inflammation, where acute inflammation typically refers to an initial immune response to a site of infection or damage while chronic inflammation is a prolonged version and typically occurs systemically in the organism (1, 3). Classic signs of inflammation are pain, redness, swelling and heat and are typically present at a

localized site of tissue damage or infection (35). Upon stimulation, resident immune cells in the tissue will recognize pathogens or tissue damage through expression of pattern recognition receptors (PRRs) that recognize molecular patterns of invading pathogens or tissue damage (35). These patterns are distinguishable from host patterns and are collectively referred to as pathogen-associated recognition patterns (PAMPs) (35). The activation of these receptors initiates the release of inflammatory mediators by the immune cells, which result in vasodilation and subsequent increased blood flow to the sites of inflammation (35). The inflammatory mediators also cause increased adhesion molecule expression on the surface of endothelial cells lining the blood vessel to recruit additional immune cells to the site, resulting in migration of immune cells through the endothelial layer into the tissue to begin repairing the damaged tissue or eliminating the pathogen (35). The following sections will describe the role of the various adipose tissue inflammatory mediators, how they are involved in altering the inflammatory response, and how obesity alters this process.

Obesity is a condition characterized by an excess of adipose tissue that results in adverse effects on health by increasing risk for a type 2 diabetes mellitus, cardiovascular disease, cancer, sleep apnea, and many others (36). Obesity, defined as a body mass index greater than or equal to  $30 \text{ kg/m}^2$ , is caused primarily by excess energy intake, lack of physical activity, and genetic susceptibility, which ultimately leads to an alteration of energy balance resulting in excessive weight gain (36).

Adipose tissue can produce multiple components, such as inflammatory cytokines, that can alter inflammation (27), and enlarged or obese adipocytes produce more pro-inflammatory cytokines and less anti-inflammatory cytokines compared to non-

obese adipocytes (27, 36). This excess production of pro-inflammatory cytokines can result in a feed forward cycle that ultimately leads to chronic, systemic inflammation, which contribute to obesity related diseases (27, 36). Furthermore, the transition from normal to obese adipose tissue is accompanied by an increase in macrophage infiltration in response to the increased production of pro-inflammatory cytokines (37).

Additionally, obesity also causes a shift in polarization of adipose tissue macrophages from an anti-inflammatory state (M2) to a pro-inflammatory state (M1) (38, 39). It is believed that the link between obesity, inflammation, and metabolic complications is directly related to the failure of adipose tissue to expand and meet storage demands, causing lipid spilling from adipocytes (40, 41). The free lipids are taken up by resident macrophages leading to formation of foam cells, which are then inhibited from scavenging the apoptotic adipocytes and can further increase insulin resistance (40, 41).

The first inflammatory cytokine demonstrated to be released by adipose tissue is tumor necrosis factor alpha (TNF- $\alpha$ ) (42). TNF- $\alpha$  is a potent pro-inflammatory cytokine that is primarily produced by macrophages present in adipose tissue (42). TNF- $\alpha$  is involved in recruitment of additional immune cells through activation of various cell signaling pathways, such as NF- $\kappa$ B and MAPK, that lead to transcriptional activation of various proteins and cytokines involved in inflammation (29). TNF- $\alpha$  interferes with insulin signaling, leading to obesity-induced insulin resistance (43). This is believed to occur through activation of JNK1, which phosphorylates and inactivates insulin receptor substrate-1 (IRS1) and, thus, blocks the signal from the insulin receptor to the intracellular signaling pathway PI3K/Akt (43). Conversely, mice lacking TNF- $\alpha$  or its receptors are protected from obesity-induced insulin resistance and hyperglycemia (43).

Free fatty acids can promote TNF- $\alpha$  production in macrophages, which can in turn promote lipolysis to increase free fatty acid release from adipocytes, causing a feed-forward cycle of further insulin resistance (44).

Additionally, adipose tissue secretes monocyte chemotactic protein 1 (MCP-1), a pro-inflammatory chemokine involved in the recruitment of monocytes, memory T cells, and dendritic cells to the site of inflammation (45). MCP-1 production and secretion by adipose tissue resident macrophages and adipocytes is positively correlated with the expansion of adipose tissue, and increased MCP-1 expression in adipose tissue results in increased macrophage infiltration, insulin resistance, and hepatic steatosis associated with obesity in mice (45).

Interleukin 6 (IL-6) is a pro-inflammatory cytokine produced by multiple cell types including immune cells, fibroblasts, endothelial cells, skeletal muscle, and adipose tissue, and it is positively associated with obesity and insulin resistance (46). IL-6 is primarily involved in fever production and the acute phase response, thus leading to production of acute phase proteins, such as C-reactive protein, that is considered a clinical marker of systemic inflammation(47). Although IL-6 seems to be involved with glucose metabolism and insulin resistance, the exact mechanism of its actions is currently unknown (47).

The interleukin 1 (IL-1) family of inflammatory cytokines include 11 different members that play a role in the immune and inflammatory response (29). IL-1 cytokines are produced primarily by monocytes, macrophages, fibroblasts, and dendritic cells, and they are involved in increasing adhesion molecule expression on the surface of endothelial cells to promote immune cell migration from blood to the inflamed tissue

(29). Like most other pro-inflammatory cytokines, IL-1 in adipose tissue is positively correlated with the development of obesity.

Macrophage inflammatory protein 1  $\alpha$  and  $\beta$  (MIF-1 $\alpha$  and -1 $\beta$ ), also known as CCL3 and 4, respectively, are pro-inflammatory chemokines produced by macrophages to initiate the immune response by activating neutrophils to fight infection or inflammation (29). As with most other pro-inflammatory factors, MIF is also positively correlated with the development of obesity. One of the only anti-inflammatory cytokines produced by adipose tissue is adiponectin and will be discussed in detail in the next section.

### **2.2.0 Adiponectin:**

Adiponectin is one of the most abundant adipokines secreted by adipocytes, with circulating plasma levels ranging from 2-20  $\mu\text{g/mL}$  in humans with a half-life of 2.5-6 hours (48). The gene encoding the adiponectin protein is located on chromosome 3q27, which is a locus associated with susceptibility to diabetes and cardiovascular disease (49). Adiponectin is a 247 amino acid protein with an NH<sub>2</sub>-terminal hyper-variable region, a conserved collagen-like domain comprising 22 Gly-X-Y repeats and a COOH-terminal C1q-like globular domain (50). Adiponectin is secreted from adipocytes as three different oligomeric complexes, which include a trimer, hexamer, and a high molecular weight (HMW) multimer consisting of at least 18 monomers (Figure 2-1) (51). The trimeric form of adiponectin is assembled through hydrophobic interactions within the globular head domain and is stabilized by the non-covalent interactions of the collagen-like domains in the triple-helix stalk (52). The subsequent assembly of the

higher ordered structure requires the formation of an intermolecular disulfide bond between highly conserved cysteine residues within the hyper-variable region (53). The cysteine residue is located at amino acid 22 (numbering starting after the signal sequence domain) and site directed mutagenesis to an alanine residue (C22A) prevents formation of the higher ordered oligomeric structures (53).

The three forms of adiponectin have been shown to possess different biological activities, and the HMW form is considered the major active form in circulation, providing the insulin-sensitizing and cardiovascular protective effects observed with adiponectin (55, 56). The synthesis and secretion of adiponectin oligomers in adipocytes are tightly regulated by multiple molecular chaperones in the endoplasmic reticulum (ER) (52), which include ER protein of 44 kDa (ERp44) (57), ER oxidoreductase 1- $L\alpha$  (Ero1- $L\alpha$ ) (58), and disulfide-bond A oxidoreductase-like protein (DsbA-L) (59). ERp44 will inhibit the secretion of adiponectin oligomers through a thiol-mediated retention (57). Conversely, Ero1- $L\alpha$  releases HMW adiponectin that becomes trapped by ERp44 (58), while DsbA-L promotes the intracellular assembly and secretion of HMW adiponectin (59).

Adiponectin exerts its signaling actions through two receptors, adiponectin receptor 1 and 2 (AdipoR1 and AdipoR2) (60). AdipoR1 and AdipoR2 are both seven-transmembrane receptors that share a 67% homology with one another but differ significantly at the amino terminus (52, 61). Compared to other traditional 7 transmembrane receptors, the adiponectin receptors have the opposite orientation with the carboxyl terminus residing outside the cell and the amino terminus located in the cytoplasm (61). AdipoR1 is ubiquitously throughout the body with the highest

expression levels in skeletal muscle, while AdipoR2 is predominantly expressed in the liver (60, 61). Activation of the adiponectin receptors leads to intracellular communication through activation of serine/threonine kinases such as AMPK and Akt2, increased phospholipase C activity, and small G-proteins such as Rab5, ultimately leading to increased magnitude of insulin signaling on target cells to facilitate glucose uptake and energy homeostasis (62).

### **2.2.1 Adiponectin and AMPK Signaling:**

One way adiponectin sensitizes target tissue to insulin actions is through increasing 5'-adenosine monophosphate kinase (AMPK) activity, which stimulates fatty-acid oxidation, increases PPAR $\alpha$  expression, and increases glucose uptake while simultaneously inhibiting glycolysis, lipogenesis and gluconeogenesis (62). AMPK is activated by factors that signal the need to shift metabolism away from biosynthesis and towards energy generation through the increased production of AMP, and is therefore considered a key nutrient sensor in the cell (1, 3). Activation of AMPK occurs during periods of nutrient deprivation and exercise, causing the upregulation of ATP-producing processes such as fatty acid oxidation (1, 3). Furthermore, AMPK can directly increase insulin sensitivity through phosphorylation of peroxisome proliferator-activated receptor  $\gamma$  co-activator 1 alpha (PGC-1 $\alpha$ ), a transcription co-activator that plays a critical role in mediating mitochondrial biogenesis and oxidative phosphorylation (63). AMPK is a heterotrimeric protein composed of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits that stabilize and activate the protein kinase upon interaction of AMP to Bateman repeats of the  $\gamma$  subunit (64). AMPK can be phosphorylated on threonine-172 by upstream targets liver kinase B1 (LKB1) and

Calcium/calmodulin kinase kinase  $\beta$  (CaMKK $\beta$ ) through action of an adaptor protein APPL1, which binds to the N-terminus of AdipoR1 (52, 63, 65). APPL1 also binds to the protein kinase Akt2 and the catalytic subunit of phosphoinositide-3-kinase (PI3K) leading to increased translocation of GLUT4 to the plasma membrane to transport glucose inside the cell for utilization (65).

Adiponectin can also exert anti-atherosclerotic effects through modulation of multiple points of the inflammatory process (66). For example, adiponectin prevented atherosclerosis by increasing cholesterol efflux from macrophages (67). Furthermore, in human monocyte-derived macrophages, which express the adiponectin receptor, adiponectin induced upregulation of IL-10 (68, 69). Adiponectin also increased expression of CD36, acetyl CoA oxidase, and uncoupling protein 2 in skeletal muscle, while causing a decrease in CD36 in the liver (70). In the endothelial cell, adiponectin was shown to inhibit the production of inflammatory cytokines and adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM) and E-selectin (71). Furthermore, adiponectin has been shown to increase endothelial nitric oxide synthase (eNOS) production leading to the production of nitric oxide (NO) in the vasculature to stimulate vasodilation (72). Additionally, adiponectin inhibited TNF- $\alpha$  induced activation of NF- $\kappa$ B (73). These results suggest that adiponectin can play a protective role against key processes associated with atherosclerosis.

### **2.3.0 Modulation of Adipose Tissue Function and Energy Balance with**

#### **Dairy Products:**

Fat mass expansion and accumulation occur through adipocyte hypertrophy, or the increase in size of the adipocyte, and adipocyte hyperplasia, or the increase in adipocyte number (74). Typically, adipocyte hyperplasia occurs by proliferation and subsequent differentiation of pre-adipocytes, while adipocyte hypertrophy results from excessive lipid storage within adipocytes. Adipocyte hypertrophy is frequently seen in obese adipose tissue and is often associated with the remodeling of adipose tissue, inflammatory and oxidative stress, all of which contribute to metabolic abnormalities at both the local and systemic levels (75). The sections below will discuss how dairy products and specific dairy bioactive components are involved in altering obesity with regards to weight management, oxidative stress, and inflammation.

#### **2.3.1 Dairy Overview:**

The term, dairy products, refers to food products that are produced from the milk of mammals. According to the current MyPlate guidelines, it is recommended that children between ages 2-3 years old consume 2 cups of dairy daily, children between 4 and 8 should consume 2.5 cups per day, while all individuals above the age of 9 should consume 3 cups per day ([choosemyplate.gov](http://choosemyplate.gov)). All fluid milk products and many foods made from milk are considered to be apart of this group, and it is recommended that food choices be made from the low-fat or fat free dairy category.

Many studies have shown that consumption of dairy products can provide multiple health benefits, specifically related to bone health (76). Dairy products are rich

in minerals such as calcium, potassium and vitamin D that are vital to overall health, as well as being rich in protein (76). Calcium is beneficial for building and maintaining bone mass and teeth; potassium is beneficial for maintaining a healthy blood pressure; vitamin D helps maintain circulating levels of calcium and phosphorous, thereby helping maintain healthy bone mass (76).

### **2.3.2 Dairy and Weight Loss:**

Although multiple studies have been performed to investigate the relationship between dairy consumption and weight loss, the findings from these studies are somewhat conflicting. According to the most recent meta-analysis of randomized controlled trials by Chen *et al.*, which focused on the effects of dairy intake on body weight and fat in 29 randomized control trials which included 2,441 participants, there is not a beneficial effect of increasing dairy consumption on body weight and fat loss in long-term studies or studies without energy restriction (77). However, the meta-analysis did find that dairy products may have a modest effect in facilitating weight loss in short-term or in energy-restricted randomized controlled trials (77). Specifically, the clinical trials discussed in this meta-analysis concluded that increasing dairy intake (1.8-3.3 servings per day versus 1-2.7 servings of dairy per day for the control group) without energy restriction does not significantly affect weight, body fat mass, lean mass, and waist circumference (77). However, the majority of clinical studies with an energy-restriction diet indicated that dairy-rich diets augment weight loss, fat loss, and reduce adiposity. Specifically, the meta-analysis showed that increased consumption of dairy with an energy-restriction diet (500 kcal less than maintenance) led to 1.29 kg greater

weight loss, 1.11 kg greater reduction in body fat mass, 0.72 kg gain in body lean mass and 2.43 cm additional reduction in waist circumference compared with control groups. Wennergren *et al.* reported no change in body weight or body fat during 6 months of 3-5 portions/day of dairy products consumption (78). However, they did observe positive effects on waist circumference in subjects with a low baseline calcium intake (78). This demonstrates the possibility of a threshold in relation to effects on body composition. Overall, these studies suggest that the inclusion of 3-5 portions/day of dairy products may have modest benefits in facilitating weight loss in energy-restricted diets (76, 77).

### **2.3.3 Dairy and Oxidative/Inflammatory Stress:**

Low-grade systemic inflammation is considered as a key etiologic factor in the development and progression of cardiovascular disease, type 2 diabetes mellitus, and metabolic syndrome (79). A cross-sectional study suggests that consumption of dairy products is inversely associated with low-grade systemic inflammation (80). It is well known that elevated plasma concentrations of C-reactive protein (CRP) and pro-inflammatory cytokines such as TNF- $\alpha$  and IL-6 are associated with an increased risk of cardiovascular disease (81). Additionally, monocyte chemoattractant protein-1 (MCP-1) is involved in recruitment of monocytes to sites of vascular inflammation, thereby promoting atherosclerosis, and MCP-1 expression is increased in obese individuals (76). Furthermore, oxidative stress is a common mediator in the pathogenicity of cardiovascular risk factors (76). Multiple studies have proposed that dairy components may play a role in down-regulating genes that encode pro-inflammatory cytokines while up-regulating those genes that regulate antioxidant enzymes (76).

Stancliffe *et al.* demonstrated reduced markers of oxidative stress in overweight and obese subjects consuming dairy foods (82). Specifically, adequate dairy consumption (3.5 servings/day) decreased malondialdehyde and oxidized LDL levels after 1 week of intervention, with further decreases after the 12 week study (82). Similarly, the adequate dairy group exhibited reduced inflammatory markers, including TNF- $\alpha$ , IL-6 and MCP-1 (82). Additional studies have shown consumption of dairy products rich in cis-9, trans-11 conjugated linoleic acid for 10 weeks showed a reduction in the inflammatory markers IL-6, IL-8 and TNF- $\alpha$  in 10 healthy subjects (83). Other clinical trials have also demonstrated that diets supplemented with dairy resulted in suppression of oxidative stress markers (plasma malondialdehyde and F<sub>2</sub>-isoprostane) and lower inflammatory markers (TNF- $\alpha$ , IL-6, and MCP-1) in overweight and obese subjects (84, 85). Conversely, Nestel *et al.* did not observe differences in the oxidative stress marker F<sub>2</sub>-isoprostane in 12 overweight and obese subjects consuming a low-fat dairy diet and two high-fat dairy diets (86). Other clinical studies also found no changes in inflammatory markers, oxidative stress markers or cytokine gene expression (78, 87-89). However, some of these studies have been performed in subjects that already consumed an adequate dairy diet, leaving little room for a slightly higher dairy diet to exert its effects. Other studies have included a 3 week “run-in” period before the trial begins. It is possible that these run-in periods in otherwise inactive individuals allows for reductions in baseline levels of oxidative and inflammatory stress markers, leaving little room for improvement from the clinical intervention. Further clinical trials are warranted and require carefully design parameters.

### 2.3.4 Calcium Homeostasis:

Plasma calcium levels are typically maintained at ~2.5 mM (9-10 mg/dL), and this level is maintained via endocrine systems that respond to increases or decreases in plasma ionized calcium ( $\text{Ca}^{2+}$ ).  $\text{Ca}^{2+}$ -sensing receptors in the parathyroid (as well as clear cells in the thyroid) detects a drop in circulating  $\text{Ca}^{2+}$ . This initiates the release of parathyroid hormone (PTH) from the parathyroid gland, and facilitates the conversion of 25-hydroxy-Vitamin D (primary form in circulation) to the active form, 1 $\alpha$ -25 dihydroxycholecalciferol, also called calcitriol. This process occurs in the renal proximal tubule of the kidneys and is mediated by 1- $\alpha$  hydroxylase activity, which is increased in response to the increase in PTH. Once calcitriol is produced, it acts at multiple sites, including the intestine to increase calcium absorption, the kidneys to increase calcium reabsorption, and on the bones to mobilize calcium stores. These actions release calcium into the blood stream to return the previously low calcium levels back to normal. Once this occurs, the calcitriol inactivating enzyme 24-hydroxylase is activated and hydroxylates calcitriol on the 24<sup>th</sup> carbon, which marks it for degradation. This is the feedback mechanism to prevent calcitriol from continuously raising plasma calcium levels (1). An additional feedback mechanism is also in place when calcium levels are elevated. Calcitonin is a 32-amino acid polypeptide hormone produced by parafollicular cells in the thyroid that, essentially, counteracts the effects of calcitriol by inhibiting calcium absorption in the small intestine, osteoclast activity in the bones, and calcium reabsorption in the kidneys to allow calcium to be excreted in the urine (1).

Suboptimal calcium intakes are common (90) and require a PTH/calcitriol response in order to maintain calcium homeostasis. Our lab (and others) have shown that

calcitriol exerts significant effects on adipocyte biology. Although a classical nuclear receptor hormone, calcitriol can also bind to a plasma membrane receptor (1, 25-MAARs) and cause rapid calcium influx, which typically has a  $\text{Ca}^{2+}$  concentration of ~100 nM. The rapid influx of calcium causes an increase in ROS production by inhibiting ROS clearing enzymes, such as superoxide dismutase and glutathione peroxidase (91), resulting in a decrease in mitochondrial function,. Furthermore,  $\text{Ca}^{2+}$  influx and signaling that occurs in the presence of calcitriol coordinately stimulates fatty acid synthase activity and inhibits lipolysis in adipocytes (92-96). Calcitriol can freely diffuse across the plasma membrane and interact with another receptor in the cytosol, the nuclear vitamin D receptor (nVDR). Once calcitriol binds to the nVDR, it is translocated to the nucleus where it dimerizes with the retinoid X receptor (RXR) and binds to the vitamin D response element (VDRE) where it can initiate the transcription of a plethora of genes. Among these is fatty acid synthase, which is a rate-limiting enzyme in *de novo* lipogenesis (97-99). It also decreases fatty acid oxidation and key mitochondrial metabolizing enzymes and transcription factors, such as PGC1- $\alpha$  (100). Another mitochondrial protein that is suppressed with calcitriol treatment of adipocytes is uncoupling protein 2 (UCP-2) (100, 101). UCP-2 helps to dissipate the mitochondrial membrane potential by uncoupling oxidative phosphorylation and ATP synthesis (102). UCP-2 allows protons to leak back across the mitochondrial inner membrane, which will allow for more efficient flow of electrons through the electron transport chain in the mitochondria (102). Since calcitriol decreases UCP-2 expression (103), this will also lead to increased ROS production (91) leading to oxidative stress and an increase in pro-inflammatory cytokine production (104).

### **2.3.5 Leucine Overview:**

Leucine is an essential amino acid that is a member of the branched-chain amino acid (BCAA) family, along with isoleucine and valine. Leucine is abundant in foods that are high in protein, as leucine is a building block for proteins. Some examples of foods rich in leucine include the whey fraction of dairy, meats, eggs and soy protein. Although leucine supplementation is primarily thought of to help with building lean muscle mass through activation of the mammalian target of rapamycin (mTOR), recent studies have shown that leucine can also play a role in energy partitioning and intracellular signaling pathways. The section below will describe leucine metabolism and its role in energy partitioning and cell signaling pathways.

### **2.3.6 Leucine Metabolism:**

Most amino acid metabolism occurs predominantly in the liver, however the liver contains relatively low expression levels of hepatic BCAA catabolic enzymes (105). As a result, circulating BCAA levels remain unchanged after passing through the liver and, therefore, the plasma and peripheral tissue concentration of BCAAs are highly dependent on dietary intake of amino acids (105, 106). Once ingested, leucine is transaminated to  $\alpha$ -ketoisocaproate (KIC) in a reversible reaction by the mitochondrial enzyme BCAA transferase (BCAT), (105); KIC is then capable of entering two metabolic pathways to generate either isovaleryl-CoA (90-95% of leucine metabolism) or  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB; 5-10% of leucine metabolism) (105). The primary route for KIC during leucine metabolism first involves oxidative decarboxylation through the mitochondrial branched-chain ketoacid dehydrogenase complex (BCKDH) (105). The

BCKDH complex consists of three enzymes including a branched-chain  $\alpha$ -keto acid decarboxylase (E1), a dihydrolipoyl transacylase (E2) and a dihydrolipoyl dehydrogenase (E3). The activity of the BCKDH is regulated by a BCKDH phosphatase and kinase through phosphorylation and dephosphorylation of the E1 subunit, thus capable of regulating BCAA metabolism (107) (106). Through a series of reactions, KIC is converted to HMG-CoA and ultimately acetyl-CoA and acetoacetate, which can enter into the citric acid cycle (105). Excess KIC can also be released into circulation, taken up by other organs such as the liver and adipose tissue, where they can be resynthesized into BCAAs or oxidized as fuels to generate ATP (25) (105). The second possible fate of KIC generated from leucine involves conversion to HMB by the enzyme KIC-dioxygenase (105). Up to 40% of HMB is excreted in the urine with the remaining HMB being converted to HMB-CoA then HMG-CoA (108). HMG-CoA has multiple fates as well: 1. interconversion to MG-CoA and back to HMB-CoA; 2. Conversion to acetoacetyl-CoA and acetyl-CoA, or 3. conversion to mevalonate by HMG-CoA reductase to eventually yield cholesterol (108). Figure 2-2 summarizes this process.

### **2.3.7 Sirtuin Signaling:**

Sirtuins are a member of the class III histone deacylases that consume one molecule of nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) with each deacylation cycle (109). The first sirtuin protein was identified in *Saccharomyces cerevisiae* as silent information regulator 2 (SIR2) (110). It was later demonstrated that SIR2 extended the replicative lifespan of yeast (111, 112), and this model was further expanded to *Caenorhabditis elegans* (113, 114) and *Drosophila melanogaster* (115) and to mediate

beneficial effects of calorie restriction (CR) on health and longevity (115-118). More recent studies in many organisms have now confirmed the original hypothesis that sirtuins are conserved, diet-sensitive, anti-aging proteins. The sections below will describe the relevant forms of the sirtuin proteins, their corresponding signaling cascade, and the role leucine plays in this process.

There are seven known mammalian sirtuins, SIRT1-7, which function to regulate metabolism in various ways in many tissues (119). SIRT1, SIRT6 and SIRT7 are localized in the nucleus, where they are involved in deacetylation of histones to influence gene expression epigenetically (120). SIRT2 was originally thought of as a cytosolic sirtuin, however recent studies show that SIRT2 is also found in the nucleus where it functions to modulate cell cycle control (121-123). SIRT3-5 are localized in the mitochondria, where they function to regulate the activities of metabolic enzymes and moderate oxidative stress by switching cells to favor mitochondrial oxidative metabolism (124). This report will focus primarily on SIRT1, the most highly studied sirtuin, as well as SIRT3 and its role in modulating oxidative stress.

During times of fasting or CR, blood glucose levels decrease causing hepatic metabolism to immediately shift to glycogen breakdown and then gluconeogenesis to ensure glucose supply and ketone body production to bridge periods of energy deficits. Additionally, fasting also activates muscle and liver oxidation of fatty acids produced by lipolysis in white adipose tissue. Multiple transcription factors are involved in this switch to adapt to the energy deprivation, and SIRT1 mediates the metabolic switch during fasting (125). In the initial phase of fasting, pancreatic alpha cells produce glucagon to activate hepatic gluconeogenesis through the cyclic AMP response-element-binding

protein (CREB) and its coactivator, CREB-regulated transcription coactivator 2 (CRTC2). However, during periods of extended fasting this effect is cancelled by SIRT1-mediated CRTC2 deacetylation and, thus, targeting the coactivator for ubiquitin/proteasome-mediated destruction (125). Next, SIRT1 deacetylates and activates PGC-1 $\alpha$ , a coactivator for forkhead box O1 (FOXO1), which is also important for stimulating mitochondrial biogenesis to assist the liver in accommodating the reduced energy status (126). In order to increase energy production, SIRT1 can stimulate fatty acid oxidation by deacetylating and activating the nuclear receptor, PPAR $\alpha$  (127). SIRT1 can also stop the production of energy from glycolysis by deacetylating and repressing glycolytic enzymes such as phosphoglycerate mutase-1 (PGAM-1) (128).

In addition to glucose homeostasis, the liver also plays important roles in regulating lipid and cholesterol homeostasis (119). During fasting, fat and cholesterol synthesis in the liver is turned off, and lipolysis in WAT is favored (119). The primary hepatic transcription factors for lipogenesis and cholesterol synthesis are proteins belonging to the sterol regulatory element binding protein (SREBP) family (129). SIRT1 deacetylates SREBP1, targeting the protein for destruction through the ubiquitin-proteasome system, and ultimately results in repression of fat and cholesterol synthesis (130).

During fasting or CR, the body switches from carbohydrate to lipid use for energy production and this effect is mediated, in part, via SIRT1 deacetylation and activation of PGC-1 $\alpha$  (131). AMPK is also activated by energy depletion, causing an increase in cellular AMP levels and, thus, can also drive the expression of the PGC-1 $\alpha$  gene under these conditions (132). In order to be fully activated, PGC-1 $\alpha$  can be phosphorylated by

AMPK and deacetylated by SIRT1 (133). This ultimately results in increased mitochondrial biogenesis and fatty acid oxidation in the muscle (134). Interestingly, the effect of SIRT1 and AMPK can also be amplified through a reciprocal positive regulatory loop. AMPK can increase NAD<sup>+</sup> levels by upregulating nicotinamide phosphoribosyltransferase (NAMPT) (135, 136), which is one of the crucial enzymes for NAD biosynthesis. On the other hand, SIRT1 can deacetylate the serine/threonine kinase liver kinase B1 (LKB1) to activate AMPK (137).

Sirtuins also play a crucial role in WAT, where they can act with secreted adipokines, specifically adiponectin (63). During exercise, the muscle adiponectin receptor is activated and induces expression of SIRT1, AMPK, and PGC-1 $\alpha$  in a Ca<sup>2+</sup>-dependent manner (63). This, in turn, stimulates fatty acid oxidation and mitochondrial biogenesis (63). Furthermore, SIRT1 can promote fat mobilization from WAT to support lipid oxidation in the liver and muscle (138). SIRT1 can also deacetylate two critical lysine residues on PPAR $\gamma$ , resulting in a white adipocyte switch into the metabolically active brown fat (139). SIRT1 is also capable of inhibiting NF- $\kappa$ B signaling, thereby reducing inflammatory stress (140-142). In summary, SIRT1 has multiple beneficial effects on skeletal muscle, liver and adipocytes during exercise or a caloric restricted state to promote fatty acid oxidation, and these events are the result of cumulative effects of adiponectin/SIRT1/AMPK signaling.

In addition to the nuclear sirtuin (SIRT1), mitochondrial SIRT3 is critical in fatty acid oxidation in the mitochondria (119). SIRT3 has been shown to be a downstream target of PGC-1 $\alpha$  and plays an important role in the suppression of ROS through PGC-1 $\alpha$ -dependent induction of ROS-detoxifying enzymes and several components of the

respiratory chain, including glutathione peroxidase-1, superoxide dismutase-2, ATP synthase 5c, and cytochrome c (133). During fasting or CR, SIRT3 protein level and activity are upregulated in the mitochondria to promote lipid catabolism and fatty acid oxidation by deacetylating long-chain-specific acyl coenzyme A dehydrogenase (LCAD) (134). Therefore, it appears that SIRT1/SIRT3/AMPK are a part of a signaling pathway that can provide beneficial effects for oxidizing fat and reducing oxidative and inflammatory stress. Multiple studies have confirmed that in addition to resveratrol (143), leucine is a stimulator of the sirtuin pathway(144-147). These studies will be discussed in detail below.

### **2.3.8 Effects of Leucine in Muscle and Fat:**

Perhaps the most well-known attribute of leucine is its potential effect on promoting muscle protein synthesis during periods of fasting, caloric restriction, or exercise (148). Supplementation with leucine alone or in combination with other BCAAs (isoleucine and valine) stimulates muscle protein synthesis in both rodent and human model systems (149-151). The leucine-induced muscle protein synthesis occurs by targeting the mammalian target of rapamycin pathway (mTOR), a Ser/Thr kinase (152). Studies have shown that leucine activates mTOR kinase and promotes the phosphorylation of eukaryotic initiation factor 4 complex causing dissociation from its binding protein and activation of eIF4E. Activated mTOR results in downstream activation of p70S6 kinase and phosphorylate S6 ribosomal proteins (153). Furthermore, leucine can also activate a second initiation factor, eukaryotic initiation factor-4G (eIF4G) in an mTOR-independent manner (153). Overall, leucine supplementation

induces the activation of multiple transcriptional factors, causing the promotion of protein translation and synthesis (152, 153).

Leucine also has beneficial effects regarding mitochondrial metabolism and inflammatory cytokine production. For example, leucine treatment (0.5 mM) increases mitochondrial UCP2 expression and adiponectin production in differentiated 3T3-L1 adipocytes (154). Leucine also promoted fatty acid oxidation in C2C12 murine muscle cells (154). The effects of leucine on fatty acid oxidation and are presumably due to direct effects of mitochondrial biogenesis, as leucine treatment increases mitochondrial mass by 30% and 53% in C2C12 myocytes and 3T3-L1 adipocytes, respectively (147). Furthermore, leucine is capable of activating SIRT1 in a cell free system (144) as well as in both adipocytes and muscle cells (146, 147). For example, leucine stimulated mitochondrial biogenesis genes SIRT1, PGC-1 $\alpha$  and NRF-1 as well as mitochondrial component genes UCP3, COX, and NADH expression by 3-5 fold in C2C12 cells (147). Knockdown of SIRT1 resulted in suppression of leucine-induced stimulation of PGC-1 $\alpha$  and NRF-1, indicating that SIRT1 mediates leucine induced mitochondrial biogenesis in muscle cells (147). Consistent with these observations, leucine has been found to markedly enhance oxidative capacity and increase mitochondrial density in skeletal muscle, partially through AMPK-mediated increases of PGC-1 $\alpha$  (155). A more recent study indicates that synergy between leucine/HMB and a six-carbon ring structure bound to a carboxylic group seem to be a necessary element for leucine/HMB synergy with other stilbenes and hydroxycinnamic acids to stimulate AMPK/SIRT1 dependent fatty acid oxidation (145). Six-carbon ring structures, such as pyridoxal phosphate, have been shown to inhibit Ca<sup>2+</sup> influx *in vitro* (156), resulting in significant decreases in fatty acid

synthase expression and activity and corresponding reductions in adipocyte triglyceride content (156), because  $\text{Ca}^{2+}$  signaling coordinately stimulates fatty acid synthase activity and inhibits lipolysis in adipocytes (92-96). In further support, recent studies have shown that leucine and its metabolite  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) activate AMPK synergistically with resveratrol and metformin, resulting in increased fatty acid oxidation, AMPK activation, SIRT1 and SIRT3 activity in murine muscle cells (146).

The beneficial effects of leucine regarding energy partitioning and oxidative metabolism can further be expanded to *in vivo* models of mice and humans (146, 156, 157). Bruckbauer *et al.* recently demonstrated that 6 week feeding of low-dose resveratrol combined with either leucine or its metabolite HMB to diet-induced obese mice resulted in increased adipose SIRT1 activity, muscle glucose and palmitate uptake (via PET/CT), insulin sensitivity (HOMA-IR), improved inflammatory stress biomarkers (CRP, IL-6, MCP-1, adiponectin), and reduced adiposity, while low-dose resveratrol exerted no independent effects, suggesting leucine or HMB can act synergistically with low-dose resveratrol to exert SIRT1-dependent outcomes (146). In two separate 24 week, placebo-controlled, randomized trials (one with weight maintenance and one hypocaloric diets) in human obese subjects consuming 2.25 g leucine combined with 30 mg vitamin B6, the supplement resulted in fat loss at both 12 and 24 week time points in the weight maintenance group study, while the hypocaloric group study lost up to twice as much weight and fat compared to the placebo group (157). In summary, dairy products are rich in both leucine and  $\text{Ca}^{2+}$ . Leucine promotes mitochondrial biogenesis, fatty acid oxidation, and adiponectin production through AMPK/SIRT1/SIRT3 mediated mechanisms, while also reducing ROS, and pro-inflammatory cytokine production. On

the other hand,  $\text{Ca}^{2+}$  inhibits the production of calcitriol. Calcitriol causes rapid  $\text{Ca}^{2+}$  influx and activation of nVDR gene expression resulting in increases in lipogenesis through increased fatty acid synthase activity, ROS production, and pro-inflammatory cytokine production with reductions in adiponectin production. Therefore, this information provides a strong framework for providing leucine and  $\text{Ca}^{2+}$  in the form of dairy to support weight loss and reduce the oxidative and inflammatory stress associated with obesity.

#### **2.4.0 Cellular Microvesicles Overview:**

Microvesicles are a collective term that refers to small, insoluble factors that are generated from most, if not all, eukaryotic cells, and they have gained a high level of interest among researchers in recent years due to their role in intracellular communication. Microvesicles are small vesicles ranging from 50 nm up to 3  $\mu\text{m}$  in diameter, which are released from the origin cell type under normal physiological conditions. In general, there are three types of vesicles: apoptotic bodies ranging from 500 nm-3  $\mu\text{m}$  in diameter (158), microvesicles (100 nm-1  $\mu\text{m}$ ) (159), which directly bud from the plasma membrane, and nanovesicles (30-100 nm), which include exosomes released via exocytosis from multivesicular bodies of the endosome (160) (Figure 2-3).

The properties of cellular vesicles have been reviewed extensively (41, 160-167). Briefly, these vesicles have been shown to carry diverse membrane and cytosolic proteins as well as messenger and microRNAs (miRNAs) (160-162). These factors can affect the physiology of their target cells in various ways. For example, they can induce intracellular signaling following binding to receptors, or they can confer new properties

after the acquisition of new receptors, enzymes, or genetic material by fusion or endocytosis (160-162). Furthermore, they participate in physiological processes including hemostasis and thrombosis, inflammation, immune interactions, and angiogenesis (162, 168). Studies have shown elevated levels of microvesicles in blood circulation in various disorders, including atherosclerosis and coronary heart disease, pre-eclampsia, hematological and inflammatory diseases, diabetes, and cancer (162). Therefore, microvesicles may be useful as prognostic and diagnostic biomarkers for early detection of a wide variety of diseases and could also have a potential role in monitoring the treatment of these diseases. The sections below will review common nomenclature, sources of these vesicles, and some standardized isolation and analysis techniques for studying microvesicles.

#### **2.4.1 Extracellular Vesicle Nomenclature:**

Extracellular vesicles (EVs), which include both exosomes and microvesicles, are heterogeneous, membranous, cell-derived vesicles approximately 40-5000 nm in diameter that are released by a variety of cells into their microenvironment (169-172). The terminologies used for addressing EVs have significantly changed over the last 10 years. Initially, isolated EVs were named based on their sample source where they were derived and based on their size (163, 172). Some of these names include oncosomes (exosomes derived from tumor cells), exosome-like vesicles, microparticles, apoptotic bodies, exosomes, prostasomes, nanoparticles, microvesicles, and shedding microvesicles (172). The confusion in the variously used terminologies has caused ambiguity in isolation methods and data reporting. For example, exosomes have classically been

defined as originating from the endosomal compartment by fusion of multivesicular bodies with plasma membrane, whereas microvesicles, ectosomes or shed vesicles/particles have been thought to originate by direct budding from the plasma membrane (163, 167). The literature describes exosomes as smaller than 100 nm in diameter, while microvesicles are considered larger than 100 nm in diameter (162, 166, 167). However, this strict definition to separate the two types of vesicles based on size and origin has not definitively been established (173-175), and there is currently no consensus on markers that distinguish the origin of these vesicles once they have left the cell (175). For example, there is little evidence that particles less than 100 nm in diameter cannot bud from the plasma membrane, and likewise, vesicles containing exosome-like markers are not always smaller than 100 nm (176). Therefore, for the purposes of this review the term “microvesicles” will be used to describe both exosome-like vesicles and vesicles that have bud from the plasma membrane.

#### **2.4.2 Microvesicle Methodology Overview:**

Microvesicles have garnered significant attention because of the specific mechanisms for their release from cells, their involvement in cell-to-cell signaling, and their utility as potential markers of disease (166, 176-187). In the midst of growing interest in microvesicles, technical standardization is of central importance due to the multitude of possible isolation and analyzing techniques available (176). The sections below will describe the various isolation techniques and analysis technologies currently used to study microvesicles.

#### **2.4.2.1 Isolation Methods – Differential Centrifugation:**

Microvesicles have been successfully isolated from cell culture conditioned media (188), and various body fluids including plasma (189, 190), serum (189), saliva (190), amniotic fluid (191, 192), breast milk (190), urine (193, 194), pleural effusions (195), ocular effluent and aqueous humor (196), ascites (197), semen (198), nasal secretions (199), cerebrospinal fluid (200), bronchoalveolar lavage (201), synovial fluid (202), and bile (203). The gold standard and most commonly used protocol for microvesicle isolation/purification is differential centrifugation (172). However, this depends on the biological fluid the microvesicles are being isolated from, as the various biological fluids have different viscosities and, therefore, require different speeds and centrifugation times to acquire the appropriate vesicles (171, 172, 188). Although various protocols are available, differential centrifugation typically begins with a low speed spin at approximately 300 x g for ten minutes to eliminate dead cells and bulky apoptotic debris (171, 172, 188). The supernatant is then centrifuged in a second step to eliminate larger vesicles and debris, typically anywhere from 1,000-20,000 x g. The slower the spin (closer to 1000 x g), the more plasma membrane budding vesicles will be present in the sample, whereas the faster spin (closer to 20,000 x g) will eliminate/pellet these vesicles, leaving the smaller exosome-like vesicles in the supernatant (176). The remaining supernatant is subjected to a final, high-speed spin step at approximately 100,000 x g for 1-8 hours (172). This step typically takes place at 4°C, but can vary depending on the specific protocol (172). Once the high-speed centrifugation step occurs, the microvesicles will be pelleted and can then be resuspended in a solution, typically phosphate buffered saline (PBS), for analysis (171, 172, 188). For a more purified

microvesicle sample and to eliminate contaminants, the pellet can be washed again in a large volume of PBS and centrifuged one last time at 100,000 x g, however this results in a relatively low recovery of microvesicles ranging from 5-25% of the starting microvesicle MHC class II concentration (171, 172, 176, 204). An additional way to use centrifugation to separate vesicles is to use a sucrose gradient, which separates vesicles based on their different flotation densities. Exosomes have a flotation density of 1.08-1.22 g/mL on sucrose gradients (205), while vesicles purified from the endoplasmic reticulum float at 1.18-1.25 g/mL and vesicles from the golgi at 1.05-1.12 g/mL (188).

#### **2.4.2.2 Isolation Methods – Microfiltration:**

A second method used to separate microvesicles based on their size is microfiltration technologies. Essentially, the biological fluid is applied to a filtration apparatus containing a membrane with a specific pore sizes that will prevent a specific sized vesicle from passing through while allowing smaller vesicles to pass. Although filtration techniques by themselves have only been recently introduced, many differential centrifugation protocols apply initial microfiltration steps rather than the initial low speed spins described above (172). For example, a biological fluid could initially be filtered through a 1  $\mu$ m filter to eliminate dead cells and larger cell debris followed by an ultracentrifugation spin at 100,000 x g. Although filtration technologies are improving, they face several challenges, such as co-purifying abundant proteins with microvesicle isolation, contamination of isolated microvesicles with components of the filtration membrane and trapping of microvesicles into nano- or micro-pores (172). Thus, isolation

conditions, such as the membrane material and pore size, must be optimized for maximal recovery of microvesicles and a more pure enrichment (170-172).

#### **2.4.2.3 Isolation Methods – Antibody-Coated Magnetic Beads:**

A third isolation method involves using magnetic beads that are coated with an antibody specific to a protein present on the microvesicle. Proteomic studies characterizing the molecular composition of microvesicles have revealed the presence of both ubiquitous and cell-specific proteins that can be used as markers for microvesicles (206). Therefore, an antibody for the protein of interest is coated on magnetic beads and the beads are incubated with the biological fluid for a period of time. Afterwards, a magnet is applied to the sample to retain the beads, which will be bound to the microvesicle via the antibody-protein complex (176). This method is quick and useful for isolating a specific sub-population of microvesicle. However, this method requires initial proteomic analysis to reveal a specific protein present on the microvesicle population of interest and, therefore, presents a problem with samples of unknown proteins. Furthermore, this method is not intended for isolation of large amounts of microvesicles (172). In this case, it is suggested that pre-concentration of samples and prior centrifugations should be considered to reduce the sample volume.

A similar technology called microfluidics has also been shown to be an effective method to isolate microvesicles containing a specific surface protein (176). This technology involves perfusing the biological fluid across an antibody-coated surface to capture exosomes or microvesicles. In a recent 2010 study by Chen *et al.*, rapid recovery of small microvesicles was demonstrated using serum and conditioned culture medium

(207). As with antibody coated magnetic beads, microfluidics will only capture microvesicles that present a specific surface protein, thus making total microvesicle isolation not applicable (172, 176, 206).

#### **2.4.2.4 Isolation Methods – Precipitation Techniques:**

Many biotechnology companies are producing kits that will quickly and easily isolate microvesicles from biological fluids using a polymeric based precipitation methods. For example, the ExoQuick™ kits (System Biosciences, CA, USA) are designed to precipitate exosomes from various fluids (urine, plasma, serum, and tissue culture media) through an overnight incubation period, followed by a simple spin to pellet the exosomes for further analysis (208). Drawbacks include the tendency of polymer-based precipitation methods to include numerous non-microvesicle contaminants such as lipoproteins (176). In a recent study, Yamada *et al.* demonstrated the highest yield of microvesicles was achieved using ultracentrifugation with ExoQuick™ precipitation, whereas higher quality microvesicle isolation with intact morphological structures was achieved through ultracentrifugation with density gradient centrifugation (209).

#### **2.4.3.0 Investigative Technologies:**

Due to the small size, heterogeneity, and unknown surface markers, microvesicle research is held back by the currently available technologies (210). Although traditional methods, such as western blotting, are often used to analyze microvesicle protein content, these methods lack the ability to investigate the microvesicles while they are in their

unaltered physiological state (211). Current research is geared towards understanding the size, concentration and proteomic/genomic profile of microvesicles as well as their application to functional aspects in biological processes (211). The various analysis methods currently available have both positive and negative aspects, and the sections below will discuss those aspects in detail.

#### **2.4.3.1 Investigative Technologies – Electron Microscopy:**

Electron microscopy (EM) techniques have been well established for many decades, and the application of this technique towards microvesicle research has been shown to be very useful by providing direct evidence for the presence of vesicular structures (205). Furthermore, the use of heavy metal stains such as osmium tetroxide and uranyl acetate in transmission electron microscopy (TEM) allows for the recognition of membrane-surrounded vesicles (212). The most common techniques involve applying concentrated microvesicle suspensions to grids and fixing them with paraformaldehyde, or fixing the pelleted microvesicles and generating ultra-thin sections of the pellet for analysis on a TEM (212). Although using a suspension of microvesicles would be simpler to work with, the concentration of microvesicles will be lower, thus producing fewer vesicles per field of view. TEM can be combined with immunoglobulins conjugated with nanogold particles to target a specific marker of interest, providing the ability to see detailed images and specific location for a protein of interest. Other types of EM, such as scanning EM (SEM) or cryo-EM are often used (213, 214). Cryo-EM allows for analyzing frozen samples and avoids the various dehydration and chemical fixatives associated with SEM that could alter the microvesicle state (215). In

conclusion, EM techniques are valuable for determining size, morphology and specificity of microvesicles, but EM is not quantitative and it lacks the ability to assess concentration.

#### **2.4.3.2 Investigative Technologies – Capture ELISA:**

Capture, or sandwich enzyme-linked immunosorbent assay (ELISA) is a common biochemical technique that involves coating a 96-well plate surface with an antibody specific to an antigen of interest. The sample is added to the plate, which will bind the antigen to the antibody. Then a detecting antibody is added to the plate to bind to the antigen, followed by an enzyme-linked secondary antibody. A substrate is then added to the plate and the enzyme will convert the substrate to a fluorescent or colormetric version that can be detected by a plate reader. Combined with a standard curve of known antigen concentration, this method allows for the indirect calculation of an antigen present in the sample. Although this method is advantageous for determining antigen concentration, capture ELISA cannot discriminate between the various sized microvesicles or between microvesicle-associated versus free antigen (211).

#### **2.4.3.3 Investigative Technologies – Dynamic Light Scattering:**

Dynamic light scattering (DLS) is a technique used to determine the average particle size in a fluid suspension by shining a laser into the sample and measuring the light scatter (213, 216, 217). This measurement is determined by the particle core size, the size of surface structures, particle concentration, and the types of ions present in the medium. Since the output data from this technique is a mean particle size, DLS is

advantageous for determining microvesicle concentration in a mono-disperse sample (176, 211). However, microvesicles are heterogeneous in size and, therefore, DLS cannot discriminate among the polydisperse sample (211).

#### **2.4.3.4 Investigative Technologies – Flow Cytometry:**

Flow cytometry is an extremely powerful method for both the qualitative and quantitative characterization of a large number of cells and smaller vesicles (176, 211). Cells or vesicles in suspension are passed through a chamber containing a laser and various detectors that can measure cell size (forward scatter), cell granularity (side scatter), or various wavelengths of fluorescent filters (fluorescent intensity). Antibodies conjugated with a fluorophore can be added to the sample to allow for the detection/measurement of a specific antigen. Furthermore, the application of Megamix bead gating, which utilizes a synthetic bead mixture of known size and concentration, can allow the investigator to calculate concentration and size of the cells or particles (218, 219). Therefore, flow cytometry is a useful technique to determine concentration and specificity. However, most conventional flow cytometers do not detect vesicles smaller than 500 nm, which will only allow for the analysis of larger microvesicles and completely excludes exosomes (176, 218, 219). Newer instruments, such as the Gallios (Beckman Coulter), BD-Influx (Becton Dickinson) and Apogee (Apogee Flow Systems) claim the ability to discriminate between particles as small as 200 nm (220). However, these claims are determined using polystyrene beads with a higher refractive index compared to the microvesicles (221), which has been the subject of multiple discussions regarding the standardization of the technology. Furthermore, typical light-scattering

methods (forward and side scattering) can be confounded by protein aggregates, and a term known as the “swarm” effect (222). Essentially, the “swarm” effect involves the detection of multiple small microvesicles as one single light scattering event and, therefore will significantly alter the calculation of microvesicle concentration (222). In conclusion, flow cytometry can be advantageous for analyzing concentration, size and specificity of microvesicles >500 nm, however it excludes the ability to analyze the exosome fraction of microvesicles.

#### **2.4.3.5 Investigative Technologies – Nanoparticle Tracking Analysis:**

Nanoparticle tracking analysis (NTA) is a recently developed technology that allows for direct, real-time visualization and analysis of nanoparticles in liquids that overcomes many of the problems associated with the previously described methods (170, 171, 211, 213, 216, 217). NTA relates the rate of Brownian motion to particle size through the use of a light microscope and video camera (211, 217). The vesicles are visualized by light scattering with the light microscope, and a video is taken for 30 seconds to one minute (depending on the concentration and heterogeneity of the sample) (211, 217). The software then tracks each individual particle’s Brownian motion and the velocity of particle movement is used to calculate particle size by applying the two-dimensional Stokes-Einstein equation to solve for particle diameter, thus generating a histogram of particle size versus concentration (211, 217). This technology has been further developed to utilize fluorescent markers to determine antigen specificity in combination with particle size and concentration, allowing for the ability to determine a percentage of the total microvesicle population expressing an antigen of interest (223).

Although NTA can analyze particles as large as 1  $\mu\text{m}$  (Brownian motion is too slow to measure above this size), the large number of small vesicles in these preparations requires that the sample be diluted for analysis, thus significantly reducing the larger particles and, therefore, underestimating their concentration (224, 225). Thus, analysis of larger vesicles (>500 nm) may be better carried out on a flow cytometer. In conclusion, NTA technology overcomes most of the issues associated with the techniques mentioned above and is considered the gold standard for exosome and microvesicle research (223, 224). However, the optimization and standardization can be extensive, depending on the preparation methods.

#### **2.4.4 Adipocyte Derived Microvesicles - Protein:**

Like other eukaryotic cell types, multiple studies have shown that adipocytes release microvesicles containing a variety of proteins and miRNAs, and these microvesicles have been termed adipocyte derived microvesicles (ADMs) (41, 164, 206, 226-230). One of the first studies that investigated ADMs was by Aoki *et al.*, where they characterized the protein composition of ADMs secreted from the adipocyte cell line 3T3-L1 (226). Aoki *et al.* found that 3T3-L1 cells secreted milk fat globule-epidermal growth factor 8 (MFG-E8) associated microvesicles, and they displayed a heterogeneous mixture of exosome-like and larger membrane vesicles as shown by electron microscopy (226). Furthermore, proteomic and biochemical analysis revealed that the microvesicles with exosomal features contained a variety of secreted, integral, cytosolic and nuclear proteins (226).

Of particular interest, Aoki *et al.* and others have demonstrated that adiponectin was associated with the ADM fraction, and the HMW form was the most abundant form present in the ADMs (226, 231, 232). Sano *et al.* isolated ADMs from differentiated 3T3-L1 cell culture supernatants and demonstrated that ADMs isolated from cells cultured under hypoxic conditions are enriched in enzymes related to lipogenesis (acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, fatty acid synthase) and promote lipid accumulation in recipient 3T3-L1 adipocytes (232). In a recent study from Poonsawat *et al.*, ADMs isolated from mouse serum contained HMW adiponectin and trace amounts of resistin, but not leptin (231), confirming not only that ADMs can reach systemic circulation, but also that adiponectin can be used as a marker for circulating ADMs.

Other groups have provided evidence that stimulus-induced lipid synthesis between differently sized adipocytes is controlled by the release of microvesicle-associated CD73 from large cells and its subsequent translocation to LD of small cells (164, 229, 230). This information transfer via microvesicles harboring the glycosylphosphatidylinositol (GPI) anchored proteins may shift the burden of TAG storage from large to small adipocytes (164, 230). Therefore, it is likely that ADMs may mediate intercellular communication between adipose, vascular tissue, and systemic circulation.

#### **2.4.5 ADM miRNA Expression:**

MicroRNAs (miRNA) are highly conserved noncoding RNA molecules of approximately 22 nucleotides long that are capable of exerting post-transcriptional effects

on gene expression (41). These miRNAs generally bind to the target sequence localized in the 3'-untranslated region of their target mRNA and regulate protein translation or mRNA stability (233, 234). Early studies in *Caenorhabditis elegans* determined that miRNA-mediated regulation was at the post-transcriptional level, because large effects were observed on protein expression and no effects on mRNA abundance (235). In other biological systems, modest effects on the amounts of the mRNA target were seen in addition to substantial degrees of regulation at the protein level (236). Therefore, miRNAs are expressed in a tissue and cell type specific manner and they have been shown to play an essential role in multiple biological processes such as proliferation, apoptosis, development, and differentiation (41).

In obese individuals, miRNAs are capable of regulating adipocyte differentiation, oxidative stress, inflammation, and angiogenesis in the adipose tissue (41, 237). Specifically, the miRNA-17-92 cluster, miRNA-21, miRNA-103, miRNA-143, miRNA-371, and miRNA-378/378\* have been shown to increase adipogenesis through the up-regulation of adipogenic markers and by increased triglycerides (238-242). The miRNA-17-92 cluster can accelerate adipocyte differentiation by negatively regulating the key cell cycle regulator and tumor suppressor Rb2/p130 (241). MiRNA-21 can inhibit the TGF- $\beta$  signaling pathway, which is known to inhibit adipogenesis (240, 243), and mi-143 increases adipogenesis through down-regulating ERK-5 (240). MiRNA-103 and miRNA-143 are also capable of inhibiting multiple targets in pathways that involve cellular acetyl-CoA and lipid metabolism, and several antiadipogenic factors, such as aryl hydrocarbon receptor nuclear translocator (ARNT), frizzled homolog 1 (FZD-1), and runt-related transcription factor 1 (RUNX1T1/ETO/MTG8) (242). MiRNA-371

stimulates expression of adiponectin and fatty acid binding protein 4 (FABP-4) (238) and miRNA378/378\* specifically increases transcriptional activity of C/EBP $\alpha$  and C/EBP $\beta$  on adipocyte gene promoters and also increases transactivation of the glucose transporter type 4 (GLUT4) promoter by C/EBP $\beta$  (239).

Conversely, multiple miRNAs can inhibit the adipogenic differentiation program, such as let-7, miRNA-27, miRNA-130, miRNA-138, miRNA-369-5p, and miRNA-448, through down-regulation of adipogenic factors and through a decrease in triglyceride uptake and storage (238, 244-248). Specifically, let-7 has been shown to inhibit adipogenesis by inhibiting high-mobility group AT-hook 2 (HMGA-2) (247); miRNA-27 and miRNA-130 by directly inhibiting PPAR $\gamma$  (245, 249, 250); miRNA-138 by inhibiting the nuclear receptor coregulator adenovirus early region 1-A (E1A)-like inhibitor of differentiation 1 (EID-1) (248); miRNA-369-5b by reducing adiponectin and FABP-4 (238); miRNA-448 by reducing the expression of Kruppel-like factor (KLF-5) (244). A list of dysregulated miRNAs in adipose and vascular tissue is available (251)

Impairment of adipocyte function is also associated with endoplasmic reticulum and mitochondrial oxidative stress, which further exacerbates adipose tissue dysfunction (251). Dysfunctional adipocytes, such as those in obese adipose tissue, exhibit an inflammatory phenotype, with increased production of pro-inflammatory cytokines and decreased production of anti-inflammatory cytokines (251-253). Of particular interest, miRNA221 and 222 correlate positively with TNF- $\alpha$  and negatively with adiponectin (254), while miRNA-132 contributes to the inflammatory phenotype and macrophage accumulation by activating the inflammatory NF- $\kappa$ B pathway in adipocytes to stimulate chemokine production (255). MiRNA-221 and -222 also inhibit endothelial cell

migration, proliferation, and angiogenesis, which contribute to hypoxia (256). Hypoxia can induce inflammatory cytokine production in adipocytes and causes increases in miRNA27 in adipose tissue of obese mice, therefore, leading to impaired adipogenesis and contributing to the development of obesity-related insulin resistance and type 2 diabetes (246, 251). Specifically, miRNA-29a/b/c expression is increased in muscle, liver, and adipose tissue of diabetic rats, leading to insulin resistance in adipocytes (257). Furthermore, miRNA-320 increases insulin sensitivity by improving insulin-PI3K signaling pathways (258).

Recent evidence indicates that microvesicles are enriched in both RNAs and miRNAs, and these RNAs can be taken up by resident macrophages, which can potentially enter circulation (259). Furthermore, cells can selectively package miRNAs into microvesicles and actively secrete them into the blood, where they can actively travel to distant tissues in the body for communication purposes (237, 259). For example, IL-1 $\beta$  has been shown to be secreted/released from monocytes/macrophages via 5 different mechanisms: exocytosis of IL-1 $\beta$ -containing secretory lysosomes, release of IL-1 $\beta$  from shed plasma membrane microvesicles, fusion of multivesicular bodies with the plasma membrane and subsequent release of IL-1 $\beta$  containing exosomes, export of IL-1 $\beta$  through the plasma membrane using specific membrane transporters, and release of IL-1 $\beta$  on cell lysis (260).

In a recent study, 71 miRNAs were found to be coexpressed in microvesicles in plasma and peripheral blood monocytes (261). The majority of these miRNAs were involved in regulating cellular differentiation of blood cells, metabolic pathways and immune function (261). Therefore, it is possible that miRNA levels in microvesicles in

plasma mirror specific pathological processes in peripheral blood monocytes, vascular and adipose tissue, which could provide insight into the metabolic disease state in a non-invasive manner (251).

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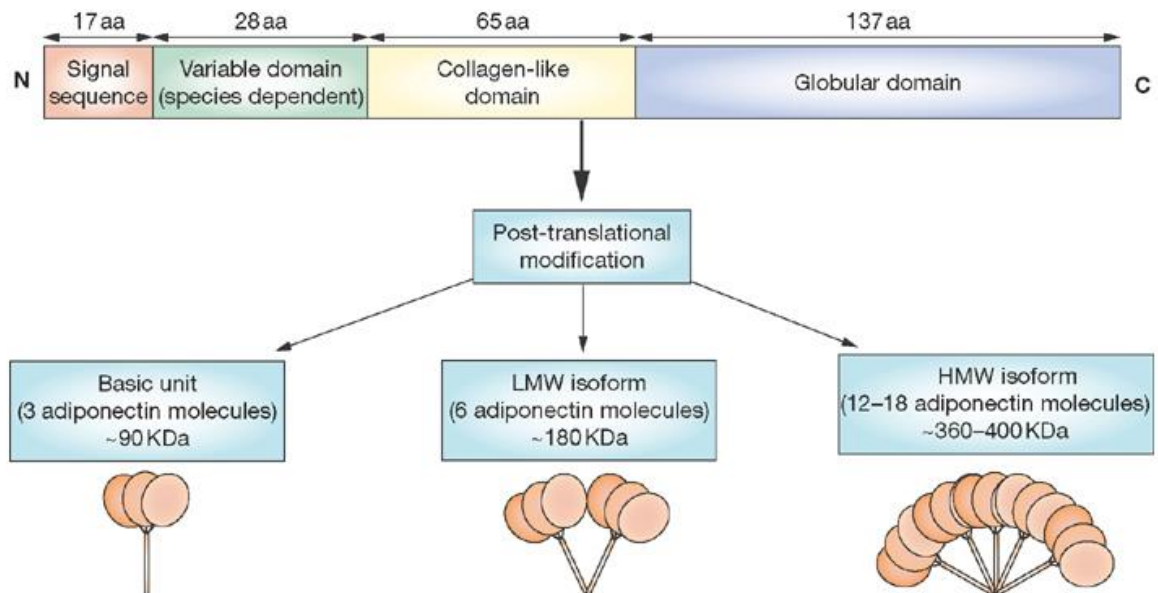
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## APPENDIX



**Figure 2-1: Adiponectin Structure (54).**

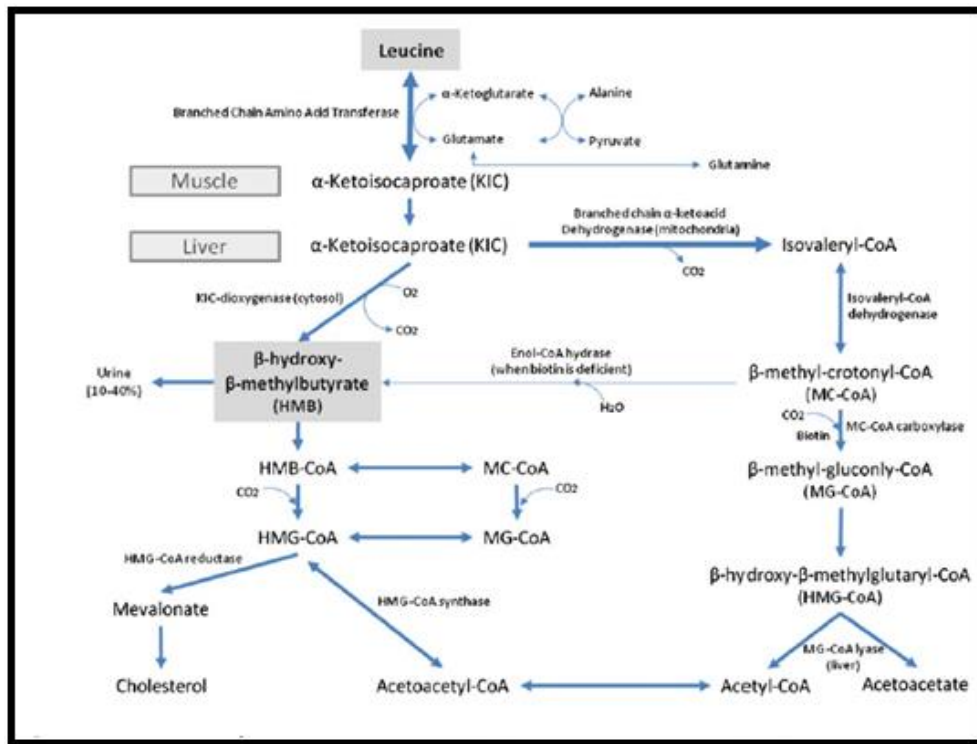
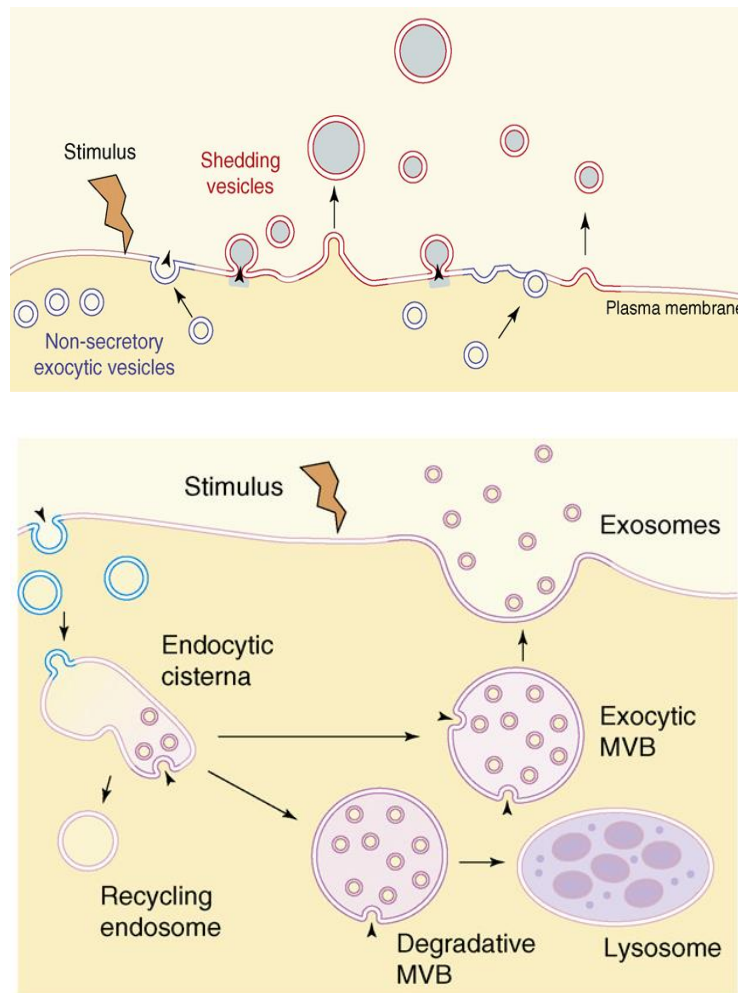


Figure 2-2: Leucine catabolism (108).



**Figure 2-3: Generation of cellular microvesicles via membrane shedding (top) and exocytosis (bottom) (159).**

**CHAPTER III:**  
**Effects of Calcitriol, Leucine and Dairy Products On Monocyte –**  
**Endothelial Cell Interactions *In vitro* and *Ex vivo***

### 3.1 Abstract:

We previously demonstrated that 1,25-Dihydroxycholecalciferol (calcitriol) and leucine have the ability to modulate adipocyte and macrophage inflammatory cytokine production. Since several of these affected cytokines are also involved in endothelial cell adhesion and infiltration by monocytes, we sought to determine if calcitriol and leucine treatment of adipocytes could cause functional effects on this inflammatory process. 3T3-L1 adipocytes were treated with vehicle, calcitriol, leucine or both for 48 hr and conditioned medium (CM) was collected and incubated with fluorescently labeled U937 monocytic cells while being perfused across a monolayer of EA hy.926 endothelial cells. Compared to vehicle, calcitriol treatment resulted in a  $3.47 \pm 0.39$  fold increase in the monocyte to endothelial cell ratio (M:E), while leucine ( $0.61 \pm 0.07$  fold decrease) and the combined treatments ( $0.81 \pm 0.02$  fold decrease) had the opposite effect. This same trend was observed for both CD11b and CD54 expression on U937 and EA hy.926 cells, respectively. The adhesion assay was also applied using an *ex vivo* design in which human plasma was taken at baseline, day 7, and day 84 from obese individuals that consumed either high ( $>3.5$  servings/d) or low ( $<0.5$  servings/d) dairy diets for 12 weeks. Plasma from the high dairy group significantly decreased U937 adhesion to the EA hy.926 monolayer, while plasma from the low dairy group had the opposite effect. Data from this study suggests dairy products, via calcitriol suppression and high leucine content, could play a beneficial role in reducing inflammation associated with obesity.

### 3.2 Introduction:

Adipose tissue is a significant source of both reactive oxygen species (ROS) and inflammatory cytokines (1, 2). In obesity, enlarged adipocytes produce more pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF  $\alpha$ ) and interleukin 6 (IL-6), and less anti-inflammatory factors such as adiponectin (3, 4). The regulation of ROS production and interactions among ROS, cytosolic  $\text{Ca}^{2+}$  signaling and mitochondrial uncoupling have been intensively investigated, and we have shown that calcitriol (1,25-Dihydroxycholecalciferol) increases ROS production in both murine and human adipocytes by inhibiting adipocyte UCP2 expression, thereby increasing mitochondrial potential, and increasing cytosolic  $\text{Ca}^{2+}$  signaling (5). Accordingly, since dietary calcium suppresses calcitriol, high calcium diets may correspondingly decrease adipose tissue ROS production and systemic oxidative stress. Consistent with this concept, increasing dietary calcium decreases pro-inflammatory cytokine production and increases adiponectin production in mice (6, 7).

These data provide a strong framework to explain a protective effect of dietary calcium against oxidative and inflammatory stress. However, milk contains high concentrations of leucine and bioactive peptides that may further protect against oxidative and inflammatory stress. Leucine has been shown to increase mitochondrial UCP2 expression and adiponectin production in differentiated 3T3-L1 adipocytes, and promote fatty acid oxidation in C2C12 muscle cells, while calcitriol exerted the opposite effects (8). Furthermore, we have recently confirmed that dairy exerts a significantly greater effect on suppressing both local adipose tissue and systemic oxidative and inflammatory stress compared to supplemental calcium in mice (7). Additionally, our

human data demonstrate that dairy-rich diets result in suppression of oxidative and inflammatory biomarkers (7). In a recent follow-up randomized crossover study in overweight and obese subjects, we found that dairy supplementation suppressed both oxidative and inflammatory stress within seven days of initiation of supplementation, and that these effects increased in magnitude with increased duration of supplementation (9). Similar results were found in a 12-week study of metabolic syndrome patients (10).

The adipose tissue cytokines we have demonstrated to be regulated by calcitriol and dairy components include several which affect vascular endothelial and smooth muscle cells (e.g. MCP-1, MIF, CD14, TNF- $\alpha$ , IL-6, IL-8, VEGF) and vascular infiltration by monocytes (11). Accordingly, we tested the hypothesis that dairy food components, through modulation of adipokine production, can affect monocyte adhesion and subsequent migration across endothelial cells.

### **3.3 Methods:**

#### *3.3.1 Chemicals*

Calcitriol, leucine, 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and penicillin/streptomycin were obtained from Sigma (St. Louis, MO, USA). Calcein AM and phosphate buffered saline (PBS) were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum for cell culture was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Hypoxanthine - Aminopterin - Thymidine (HAT) was obtained from ATCC (Manassas, VA, USA). CD11b primary antibody (IgG) was purchased from AbCam (Cambridge, MA, USA). CD54 primary antibody (IgG1) was purchased from Beckman Coulter (Brea, CA, USA). AlexaFluor 488 goat anti-

mouse secondary antibody, IgG and IgG1 specific, were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

### 3.3.2 Cell Culture

3T3-L1 preadipocytes were purchased from ATCC (Manassas, VA, USA) and incubated at a density of 8000 cells/cm<sup>2</sup> (10 cm<sup>2</sup> dish) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (adipocyte medium) at 37°C in 5% CO<sub>2</sub> in air. Confluent preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM-F10 (1:1, v/v) medium supplemented with 1% FBS, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and antibiotics (1% penicillin/streptomycin). Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium (day 0). Cultures were re-fed every 2 to 3 days to allow 90% cells to reach full differentiation before chemical treatment. After 12-14 days, differentiated adipocytes were treated with vehicle (control), 0.5 mM leucine, 10 nM calcitriol, or both for 48 hours. After treatment, the conditioned medium (CM) was collected, aliquoted and frozen at -80°C until needed for subsequent assays. For comparative purposes, all assays utilized CM (control vs. treatment group) derived from the same set of 3T3-L1 cultures (ie. the same passage and treatment period).

EA hy.926 endothelial cells (12) were a gift from Dr. Ali Amirkhosravi (Florida Hospital, Center for Thrombosis Research, Orlando, FL), and were cultured in T-75 cm<sup>2</sup> flasks (Corning) with DMEM supplemented with 10% FBS, antibiotics (1% penicillin streptomycin), and HAT according to manufacturer's instructions. U937 monocyte cells

were obtained from ATCC (Manassas, VA, USA) and cultured in T-75 cm<sup>2</sup> flasks with RPMI 1640 medium supplemented with 10% FBS and antibiotics (1% penicillin/streptomycin).

### 3.3.3 Cell Preparation

EA hy.926 cells were cultured as described above until ~90% confluent. Cells were then removed from the T-75 cm<sup>2</sup> flask with trypsin (0.25%) / EDTA (0.1μM), and centrifuged at 200 x g for 5 minutes. The cells were resuspended with 3 mL of cell culture media and counted using an improved Neubauer cell counting chamber with trypan blue staining to assess viability. The cells were seeded onto 40 mm glass coverslips at a final concentration of  $2.5 \times 10^5$  cells/mL and incubated at 37°C, 5% CO<sub>2</sub> for 12-24 hours until ~90% confluent. Cells were washed 3 times with 1X PBS and fluorescently labeled with Calcein AM (1 μg/mL) for 30 minutes at 37°C, 5% CO<sub>2</sub>. Afterwards, the coverslips were washed 3 times with 1X PBS to remove excess Calcein AM.

U937 cells were centrifuged at 200 x g for 5 minutes. The cells were resuspended in 3 mL of FBS free, phenol red free medium and counted as previously described. Viability of U937 cells was >90% for all assays. U937 cells were adjusted to a final concentration of  $1.0 \times 10^6$  cells/mL. The cells were fluorescently labeled with DiI at a final concentration of 4.16 μg/mL for 20 minutes at 37°C, 5% CO<sub>2</sub>. After labeling, the cells were topped off with FBS free, phenol red free RPMI 1640 medium and centrifuged at 200 x g for 10 minutes. The supernatant was removed and the labeled U937 cells were

resuspended with 3 mL of adipocyte CM ( $1.0 \times 10^6$  cells/mL final concentration), or 3 mL of clinical trial human plasma (diluted 1:1 in 1X PBS).

#### 3.3.4 U937 Cell Adhesion Assay

This assay was modified from the principles previously described (13). Briefly, a coverslip containing a monolayer of Calcein AM labeled EA hy.926 cells was assembled in an FCS2 microscope stage mounted perfusion system (Biopetechs Inc, Butler, PA) containing 0.5 mm gaskets. FBS free, phenol red free RPMI 1640 cell culture media was perfused across the EA hy.926 monolayer at a flow rate of 0.5 mL/min (shear rate =  $132.9 \text{ s}^{-1}$ ) for 10 minutes. Afterwards, the pump was stopped and the affluent tubing was placed in a 12 x 75 mm tube containing the prepared U937 cells in adipocyte CM. The pump was restarted and run for 5.5 minutes, which was the optimized time to allow fluid to flow through the entire system to the effluent end. The pump was then stopped, and the effluent end of the tubing was placed back into the original 12 x 75 mm tube, allowing for a continuous, recycled perfusion. The pump was then restarted and run for 1 hr. Afterwards, unbound U937 cells were washed off for 10 minutes with FBS-free, phenol red-free RPMI 1640 media. For comparative purposes, a perfusion with the control CM was run every day prior to running a perfusion with a given treatment group CM. This allowed for each treatment group perfusion (leucine, calcitriol, or both) to be normalized to the control CM each day.

### 3.3.5 Cell Migration Assay

This assay was modified from the principles previously described (13). EA hy.926 cells were seeded onto 8  $\mu\text{m}$  pore size transwell inserts (Corning) at a concentration of  $2.0 \times 10^6$  / mL and incubated at 37°C, 5% CO<sub>2</sub> for 24 hours or until cells were confluent. U937 cells were fluorescently labeled with DiI and resuspended in adipocyte CM as previously described.  $2.0 \times 10^5$  cells were added to the top of the transwell insert, and the adipocyte CM was placed in the bottom of the well to act as the chemoattractant. The plate was incubated at 37°C, 5% CO<sub>2</sub> conditions for 18 hours and cell migration was assessed using a Perkin-Elmer Victor 3 fluorescent plate reader and confirmed by microscopy.

### 3.3.6 Adhesion Molecule Expression by Flow Cytometry

U937 cells adjusted to a concentration of  $5.0 \times 10^5$  cells/mL were centrifuged at 200 x g for 5 minutes. Cell pellets were resuspended in 1 mL of adipocyte CM and incubated at 37°C, 5% CO<sub>2</sub> for 1 hour. After treatment, U937 cells were centrifuged at 200 x g for 5 minutes and resuspended in 1 mL of 2% paraformaldehyde for 10 minutes. The tubes were topped off with 1 X PBS and centrifuged as previously described. One microgram of mouse, anti-human CD11b monoclonal antibody was diluted in 1 mL of 1 X PBS and used to resuspend the cell pellets. The primary antibody was incubated at 25°C for 1 hr. The cells were washed with 1 X PBS and centrifuged as previously described. One microgram of goat, anti-mouse IgG secondary antibody conjugated to Alexaflour 488 was diluted in 1 mL of 1 X PBS and allowed to incubate with the cells for 30 minutes at 25°C. Cells were washed, centrifuged and resuspended in 1 X PBS. Flow

cytometry was performed on an Accuri C6 Cytometer (Ann Arbor, MI). Twenty thousand events were collected and a marker gate was set on the isotypic control. The same gate was applied to the CD11b stained samples generating a percent of cells that are positive for CD11b  $\pm$  SEM.

EA hy.926 cells were cultured as previously described. Cells ( $1.5 \times 10^5$ ) were seeded in each well of a 6 well plate and grown at 37°C, 5% CO<sub>2</sub> for 12-24 hours until ~90% confluent. EA hy.926 cells were washed with PBS and incubated with 2 mL of the various adipocyte CM for 8 hours at 37°C, 5% CO<sub>2</sub>. After incubation, the cells were washed 3 times with PBS and removed from the wells with trypsin (0.25%) / EDTA (0.1 $\mu$ M). Cells were centrifuged at 300 x g for 5 minutes and resuspended in 2% paraformaldehyde for 10 minutes for fixation. The tubes were topped off with PBS and centrifuged as previously described. Cell pellets were resuspended in 100  $\mu$ L of PBS and incubated with one microgram of mouse, anti-human CD54 monoclonal antibody for 1 hr. at 25°C. Cells were washed with PBS and centrifuged as previously described. Cell pellets were resuspended in 100  $\mu$ L of PBS and incubated with one microgram of goat, anti-mouse Alexa 488 (IgG1) secondary antibody for 45 minutes at 25°C. Cells were washed with PBS and centrifuged as previously described. Pellets were resuspended in 300  $\mu$ L of PBS and analyzed by flow cytometry as described above.

### *3.3.7 Microscopy and Image Analysis*

Microscopy was performed on a Nikon Eclipse Ti-E Epi-fluorescence microscope equipped with an automated stage and a 20 X objective. A 3 x 3 large image scan was taken in each of 5 random fields by multi-channel capture (channel 1: excitation/emission

= 488/517 nm, channel 2: ex/em = 550/567 nm). Image analysis was performed using Nikon Elements 3.1 software. Binary thresholding was individually applied to both cell types to exclude non-specific staining, generating a percent area occupied by fluorescence, which has been shown to be a direct correlation to the cell count (14). Results are presented as the ratio of percent area occupied by fluorescence (U937 : EA hy.926).

### *3.3.8 Clinical Samples*

Plasma samples from a recently completed clinical trial (10) were utilized to evaluate the effects of feeding a dairy-rich diet on monocyte adhesion to endothelial cells. These studies were conducted as described above, with plasma (diluted 1:1 in 1 X PBS) substituted for the adipocyte CM. Briefly, 40 overweight and obese subjects participated in a 12-week randomized clinical trial of low (<0.5 serving/day) vs. high (>3.5 servings/day) intakes of dairy foods. Blood was drawn at baseline, day 7 and day 84 for assessment of inflammatory and oxidative stress markers, and archival samples were used for this study. This study was approved from an ethical standpoint by the Institutional Review Board at the University of Tennessee-Knoxville; standardized informed consent was obtained from all subjects, and the research was conducted in accordance with the ethical standards outlined in the Helsinki Declaration. Details of this clinical trial are published elsewhere (10).

### 3.3.9 Statistical Analysis

Data from each day was normalized to control by dividing each individual treatment value by the mean control value from that days experiment. Normalized data were analyzed via analysis of variance after confirming equality of variance among groups and normality of distribution, and significantly different group means were separated by Fisher's LSD.

## 3.4 Results:

Calcitriol conditioned medium (CM) stimulated a 347% increase in monocyte adhesion when perfused across a monolayer of EA hy.926 cells compared to the control CM (representative images shown in Fig 3-1A vs. B;  $p < 0.0001$ ). In contrast, leucine CM elicited a 61% reduction in monocyte adhesion to the endothelial cell monolayer compared to the control CM (Fig 3-1A vs. C;  $p = 0.0485$ ). Interestingly, when the two treatments were combined, leucine was able to completely abolish the effects of calcitriol, resulting in an 81% reduction in monocyte adherence compared to the control (Fig 3-1A vs. D;  $p = 0.0096$ ). These quantitative comparisons are summarized in Fig 3-1E.

Parallel experiments were performed to assess the effects of the CM on monocyte CD11b expression, as CD11b is directly involved in adhesion to and migration across the endothelial layer. When monocytes were incubated for 1 hr with calcitriol CM, the percentage CD11b-positive cells increased from  $3.9\% \pm 0.12$  (control) to  $4.3\% \pm 0.06$  (Fig 3-2A;  $p = 0.0196$ ). Conversely, monocytes treated with leucine CM resulted in a decrease in CD11b-positive cells ( $2.7\% \pm 0.06$ ; Fig 3-2A;  $p = 0.0069$ ). When the leucine

and calcitriol treatments were combined, the percent of cells positive for CD11b was reduced to  $2.6\% \pm 0.13$ , comparable to the leucine treatment in the absence of calcitriol (Fig 3-2A;  $p=0.0029$ ). These results show an identical trend to the monocyte adhesion data, suggesting a positive role for leucine in modulating adhesion molecule expression via adipocyte derived compound(s).

An additional adhesion molecule required for diapedesis of monocytes across the vascular endothelial layer, ICAM-1 or CD54, which is expressed by activated endothelial cells, was also measured by flow cytometry. After 8 hrs of treatment with the control CM, the percent of EA hy.926 cells positive for CD54 was  $2.38\% \pm 0.29$  (Fig 3-2B). The calcitriol CM treatment resulted in a significant increase to  $4.74\% \pm 0.69$  percent positive cells compared to control (Fig 3-2B;  $p=0.0006$ ). Similar to the CD11b expression data, when the two treatments were combined, leucine was able to significantly overcome the effects of calcitriol resulting in  $1.58 \pm 0.17\%$  of EA hy.926 cells positive for CD54 (Fig 3-2B;  $p=0.0147$  vs. calcitriol). This CD54 expression data shows a similar trend to the CD11b expression data, suggesting that the adipocyte derived factors generated by treatment with leucine can affect adhesion molecule expression on both monocytes and endothelial cells.

The effect of the various CM on diapedesis was also performed, as monocyte migration across the endothelial layer is directly involved in the inflammatory response. Although the observed trend was similar to the adhesion data, these differences were not significant (Fig 3-3).

Because dairy foods are rich in both leucine and calcium, resulting in suppression of calcitriol and elevated postprandial leucine, an *ex vivo* approach was used to assess the

effects of low vs. high dairy diets on monocyte adhesion to endothelial cells. When human plasma samples from obese subjects on low or high dairy diets were used in place of the adipocyte CM, plasma from the high dairy group significantly reduced U937 adhesion by 68% in perfusions with plasma samples taken after the patients were on a high dairy diet for 7 days (Fig 3-4E;  $p = 0.0031$ ), and this reduction was sustained (57%) following 84 days on the high dairy diet (Fig 3-4F;  $p = 0.0129$  vs. baseline). In contrast, plasma samples from patients receiving a low dairy diet exerted the opposite effect with an 80% increase at day 7 (Fig 3-4B;  $p = 0.0006$ ) and a further 116% increase at day 84 (Fig 3-4C;  $p = 0.0001$  vs baseline). Quantitative comparisons are summarized in Fig 3-4G.

### 3.5 Discussion:

Data from this study demonstrate that treatment of adipocytes with calcitriol significantly increases pro-inflammatory events, including monocyte CD11b expression, endothelial ICAM-1 expression, and monocyte adhesion to an endothelial cell monolayer, while leucine exerts the opposite effect. Similarly, Zehnder *et. al.* reported that calcitriol treatment of endothelial cells significantly increased U937 adhesion (15). Moreover, calcitriol induces monocyte-macrophage differentiation (16, 17), further contributing to a pro-inflammatory state. Notably, the effects of leucine were sufficiently robust to fully reverse these effects of calcitriol when the two treatments were combined.

We have previously demonstrated that dietary calcium-induced suppression of calcitriol reduces oxidative and inflammatory biomarkers in both mice and humans (7, 9, 10). However, calcium from dairy exerts greater effects than supplemental calcium,

suggesting the presence of an additional bioactive component(s) (7, 18). Dairy products are rich in the branched-chain amino acid leucine; our previous data (8, 19) coupled with the data from the present study suggest that leucine is the primary contributor to this additional bioactivity. Leucine increases both mitochondrial UCP2 expression and adiponectin production in differentiated 3T3-L1 adipocytes while calcitriol exerts the opposite effects (8). Increasing mitochondrial uncoupling status in adipocytes provides a potential mechanism to alleviate the increased oxidative stress present during an obese state. Since high levels of ROS can lead to an increase in pro-inflammatory cytokine production in adipocytes (6, 7), it is possible that leucine indirectly reduces pro-inflammatory cytokines by attenuating ROS production and oxidative stress. Adiponectin, an adipocyte-derived cytokine that increases expression upon leucine treatment, has anti-inflammatory effects by suppressing TNF- $\alpha$  induced adhesion molecule expression through inhibition of I $\kappa$ B- $\alpha$ -NF- $\kappa$ B activation via a cAMP-dependent pathway in human aortic endothelial cells (20). In support of this concept, Macotela *et al.* recently demonstrated that a two-fold increase in dietary leucine prevented the macrophage infiltration of adipose tissue induced by dietary fat in mice and reduced inflammatory markers, including TNF- $\alpha$ , by 40-45% (21). Moreover, our recent data demonstrates that leucine stimulates SIRT1 activity in skeletal muscle and adipocytes, resulting in stimulation of PGC1- $\alpha$  expression and mitochondrial biogenesis and decreases in ROS production and NF- $\kappa$ B activation (22). Accordingly, using dairy to deliver both leucine and calcium may reduce obesity-induced oxidative and inflammatory stress via multiple mechanisms.

It is possible that dairy modulation of adipokine production could generate a functional effect on cell-to-cell interactions that occur during an obesity-induced inflammatory response, *in vivo*. Therefore, we have applied this concept in a novel *ex vivo* assay to assess monocyte – endothelial cell adhesion in the presence of human plasma from obese clinical trial participants consuming either an adequate dairy (>3.5 servings/d) or low dairy (<0.5 servings/d) diet for 12 weeks. Our results show adequate dairy consumption results in significant, sustained decreases in monocyte – endothelial cell adhesion, whereas low dairy consumption had the opposite effect in this model system. These findings are further supported by our *in vivo* data from the clinical trial demonstrating significant decreases in the inflammatory stress biomarkers TNF- $\alpha$ , MCP-1, IL-6, and C-reactive protein in those individuals consuming adequate dairy over the 12-week period (9, 10). Furthermore, a recent study from Thomas *et al.* showed significant reductions of white adipose tissue pro-inflammatory cytokines in diet induced obese mice fed a high-Ca<sup>2+</sup> plus non-fat dairy diet when compared to high-Ca<sup>2+</sup> alone, although these differences were not observed after controlling for body weight (18). In contrast, two other studies showed little or no effect of dairy foods on inflammatory biomarkers (23, 24). However, in both cases the levels of calcium and dairy consumption prior to intervention were close to adequate, leaving little opportunity for dairy to exert an effect. A more recent study from Van Loan *et al.* showed no effect of dairy food consumption on inflammatory biomarkers (25); the reason for the discrepancy is not clear.

Obesity is associated with sub-clinical chronic inflammation (26) that plays an important role in the initiation and progression of cardiovascular diseases such as

atherosclerosis, which is characterized by a thickening of the arterial wall with fatty deposits (plaque). An additional hallmark of atherosclerotic lesion development is a localized increase in monocyte adhesion to the endothelium and infiltration to the intima where they can differentiate into macrophages. The adipose tissue cytokines we have previously demonstrated to be regulated by calcitriol and dairy components include several which affect vascular endothelial and smooth muscle cells (e.g. MCP-1, MIF, ICAM-1, TNF- $\alpha$ , IL-6, IL-8, VEGF) and vascular infiltration by monocytes (MAC-1, MCP-1, ICAM-1, TNF- $\alpha$ ) (11). Therefore, modulation of adipocyte-derived inflammatory cytokines via dairy consumption could provide a potential mechanism to reduce the initial stages of atherosclerotic lesion development independently of its proposed anti-obesity and anti-hypertensive properties.

Strengths of this study include targeting the cumulative effects of calcitriol and leucine treatment of adipocytes through the collection of adipocyte-derived factors present in conditioned media. Since adipose tissue is capable of producing a variety of co-factors that could affect monocyte – endothelial cell interactions, it is important to account for the balance between the components rather than solely utilizing a specific compound. Additionally, we applied a novel, *ex vivo* style approach to confirm our *in vitro* model of assessing monocyte – endothelial cell adhesion by using human plasma samples from patients consuming adequate or low dairy diets. Similar to the *in vitro* assessment, this approach targets the cumulative effects of dairy consumption on a systemic level rather than solely its effects on adipose tissue. However, our model of assessing cell adhesion has some limitations. In this study design, we did not assess the mechanisms responsible for the changes in cell adhesion, and we are unable to yet

identify how leucine alters adipokine expression. Similarly, the *ex vivo* approach also lacks the mechanistic detail, and although it does not provide direct evidence, it does model what would occur in a clinical setting.

In summary, data from this study confirms our previous work and provides additional evidence suggesting that dairy components have the ability to modulate adipocyte inflammatory cytokine production that attenuates monocyte – endothelial cell adhesion, *in vitro*. Furthermore, utilization of human plasma from obese patients who increase their dairy intake significantly reduces monocyte – endothelial cell adhesion using a novel, *ex vivo* approach, suggesting a possible *in vivo* mechanism to reduce obesity-associated inflammation and risk for cardiovascular disease.

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## APPENDIX

**Figure 3-1: Effects of adipocyte CM on U937 – EA hy.926 cell adhesion under flow conditions.** DiI labeled U937 cells (red) were resuspended in the various CM and perfused across a monolayer of Calcein AM labeled EA hy.926 cells (green) for 1 hr. Adhesion was quantified by microscopy and image analysis. Representative images of control (A), calcitriol (B), leucine (C) or the two treatments combined (D) are shown. Results are presented as the normalized percent area occupied by fluorescence  $\pm$  SEM (E). Differing letters above the bars denote significant differences between groups (a = different from control  $p < 0.05$ ; b = different from calcitriol  $p < 0.0001$ ;  $n=3$ ).

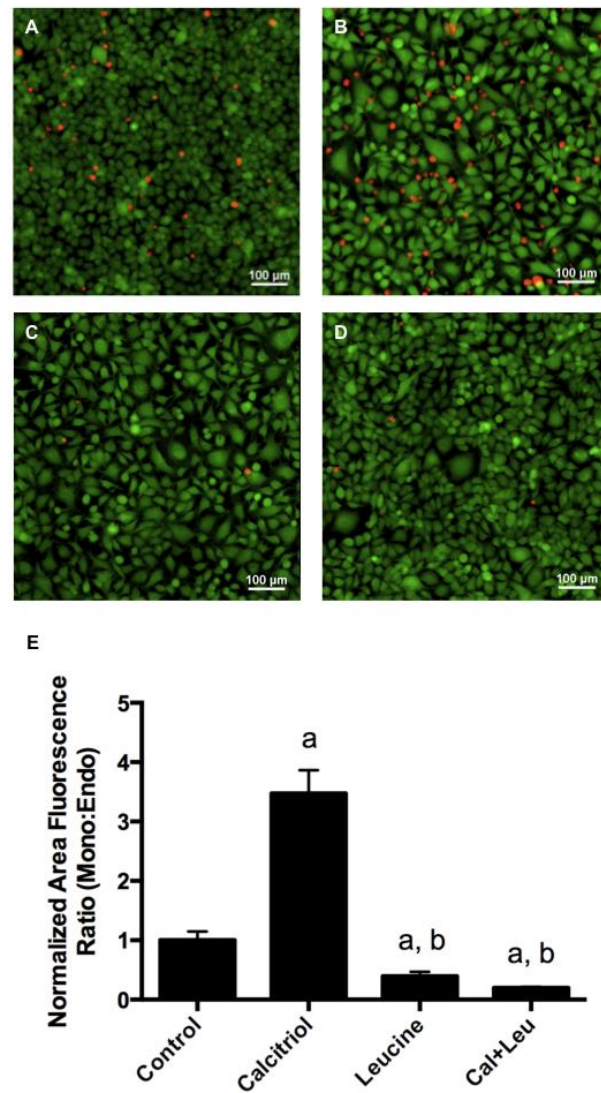
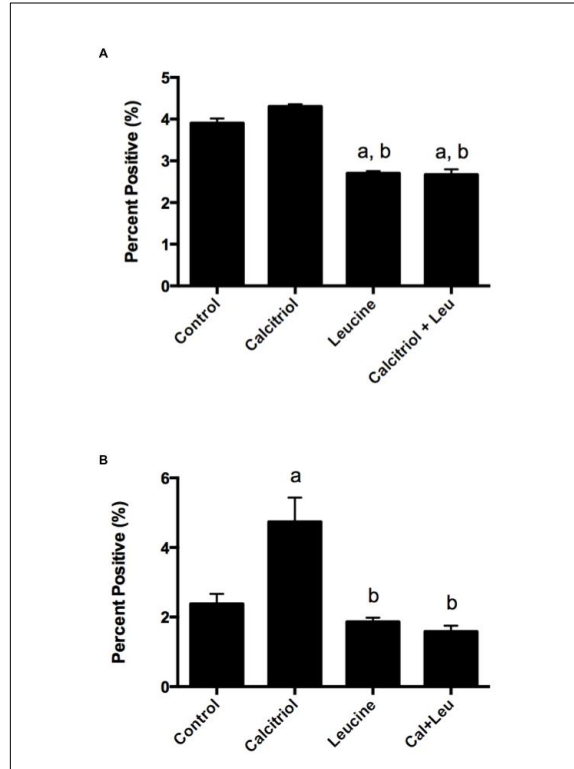
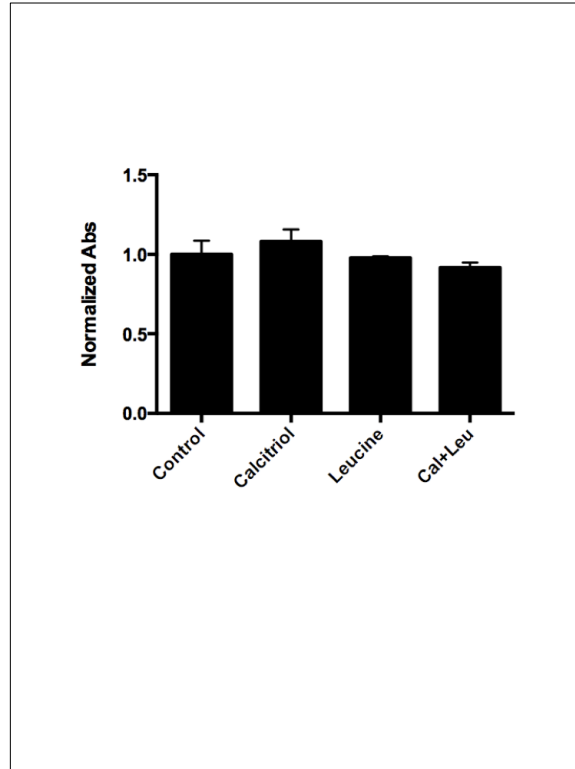


Figure 3-1. Continued



**Figure 3-2: Effects of adipocyte CM on U937 – CD11b expression (A) and EA hy.926 – ICAM-1 expression (B).** Differentiated 3T3-L1 adipocytes were treated for 48 hrs with calcitriol (10 nM), leucine (0.5 mM), or the two combined and CM was collected. U937 or EA hy.926 cells were incubated with the adipocyte CM for 1 hr and 8 hrs, respectively. Cells were labeled with specific mAbs and analyzed by flow cytometry. Results are presented as percent positive cells  $\pm$  SEM. Differing letters above the bars denote significant differences between groups (a = different from control; b = different from calcitriol  $p < 0.01$ ;  $n=3$ ).



**Figure 3-3: Effects of adipocyte CM on transmigration of U937 cells across an EA hy.926 cell monolayer.** DiI labeled U937 cells were resuspended in the previously described adipocyte CM and added to transwell inserts containing a monolayer of EA hy.926 cells. After 18 hr incubation, U937 migration was assessed using a fluorescent plate reader. Results are presented as normalized fluorescent intensity  $\pm$  SEM (n=5).

**Figure 3-4: Effects of plasma from humans consuming low and high dairy diets on monocyte – endothelial cell adhesion *ex vivo*.** Similar to the *in vitro* assay, plasma samples taken at baseline, day 7 and day 84 were prepared as described. DiI labeled U937 cells were resuspended in the prepared plasma and perfused across a monolayer of Calcein AM labeled EA hy.926 cells for 1 hr. Adhesion was quantified by microscopy and image analysis. Representative images of low dairy baseline (A), day 7 (B), day 84 (C), high dairy baseline (D), day 7 (E), and day 84 (F) are shown. Results are presented as the percent area occupied by fluorescence normalized to control  $\pm$  SEM (G). Differing letters above the bars denote significant differences between groups (a = different from baseline;  $p < 0.01$ ;  $n=3$ ).

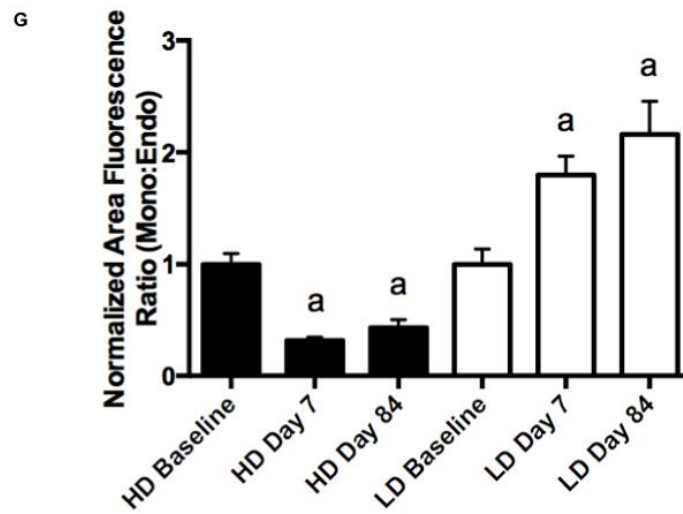
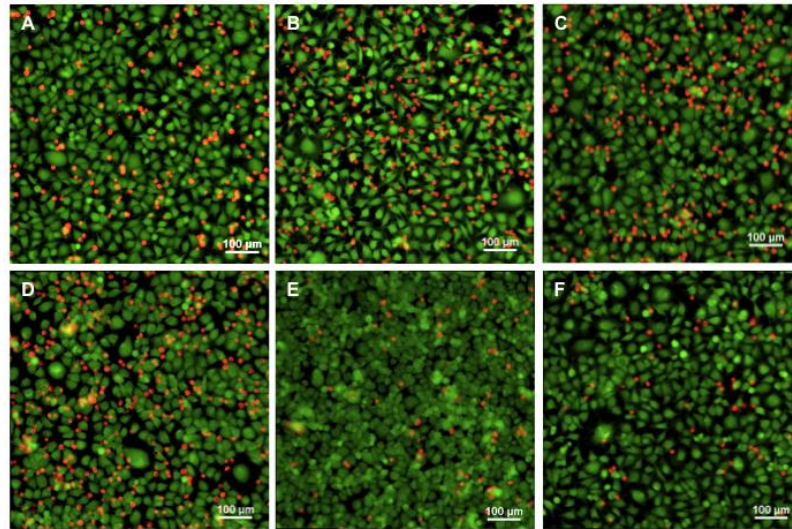


Figure 3-4. Continued

## **CHAPTER IV:**

### **Leucine Induced Adiponectin Expression on Adipocyte Derived Microvesicles Reduces Peripheral Blood Monocyte CD11b Expression**

#### 4.1 Abstract:

We previously demonstrated that calcitriol and leucine modulate adipocyte cytokine production. Specifically, calcitriol increases the production of pro-inflammatory cytokines, such as TNF- $\alpha$ , MCP-1 and IL-6, and decreases the production of anti-inflammatory cytokines (adiponectin) while leucine exerts the opposite effect. The modulation of inflammatory cytokine production also had corresponding effects on monocyte-vascular endothelial cell adhesion *in vitro* and *ex vivo* and on monocyte CD11b and endothelial cell ICAM-1 expression. Recent evidence suggests that adipocyte derived microparticles (ADMs) may serve as a messenger system between adipose tissue and peripheral blood. Therefore, we tested the hypothesis that ADMs isolated from leucine treatment of 3T3-L1 adipocytes will reduce monocyte CD11b expression in fresh human blood. 3T3-L1 cultures were treated with vehicle (control) or 0.5 mM leucine for 48 hrs and the conditioned media (CM) was collected. ADMs were isolated by ultracentrifugation and samples of CM, supernatant fraction, and ADM fraction were incubated with fresh whole blood for 2 and 18 hrs. Monocyte CD11b expression was assessed using flow cytometry. After 2 hrs, monocyte CD11b<sup>+</sup>, expressed as the percent of monocytes positive for CD11b, decreased from  $68.6 \pm 3.4\%$  (control) to  $47.7 \pm 7.6\%$  in the presence of leucine CM, and the leucine ADMs recapitulated this effect. Similarly, leucine ADMs reduced monocyte CD11b<sup>+</sup> from  $72.1 \pm 4.0\%$  (control) to  $48.4 \pm 2.7\%$  ( $p < 0.05$ ) following 18-hr incubation. These effects were reversed when cells were transfected with adiponectin siRNA. These data suggest that leucine treatment of adipocytes reduces CD11b expression on fresh human monocytes and that adiponectin present on ADMs mediate these effects.

## 4.2 Introduction:

Microvesicles are membrane vesicles that range from 50 nm to 3  $\mu$ m in diameter, and are released from the origin cell type into the microenvironment under normal physiological conditions (1-3). The term “microvesicles” is used here as a collective term referring to both membrane budding vesicles and exosomes. The properties of cellular vesicles have been reviewed extensively (3-11). Briefly, these vesicles have been shown to carry diverse membrane and cytosolic proteins as well as messenger and microRNAs (miRNAs) (3-5). These factors can affect the physiology of their target cells by transferring these proteins and genetic material. For example, they can induce intracellular signaling following binding to receptors, or they can confer new properties after the acquisition of new receptors, enzymes, or genetic material by fusion or endocytosis (3-5). Furthermore, microvesicles can participate in physiological processes including hemostasis and thrombosis, inflammation, immune interactions, and angiogenesis (5, 12). Studies have shown elevated levels of microvesicles in blood circulation in various disorders, including atherosclerosis and coronary heart disease, pre-eclampsia, hematological and inflammatory diseases, diabetes, and cancer (5).

Recent studies have confirmed that adipocytes also release microvesicles into their microenvironment (6, 8, 13-19). Aoki *et al.* characterized the protein composition of adipocyte derived microvesicles (ADMs) secreted from the adipocyte cell line 3T3-L1 (13). In another study, microvesicles isolated from the adipose tissue of obese mice were injected into wild type showed that the ADMs are taken up by monocytes, which differentiate into macrophages and secrete increased levels of TNF- $\alpha$  and IL-6 (14). Sano *et al.* isolated ADMs from differentiated 3T3-L1 cell culture supernatants and

demonstrated that exosomes isolated from cells cultured under hypoxic conditions are abundant in enzymes that promote lipogenesis, such as acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, fatty acid synthase, and these ADMs promoted lipid accumulation in recipient 3T3-L1 adipocytes (19). Studies by Muller *et al.* provided evidence that stimulus-induced lipid synthesis between differently sized adipocytes is controlled by the release of ADM-associated CD73 from large cells and its subsequent translocation to lipid droplets of small cells (8, 16, 17). This information transfer via ADMs harboring the glycosylphosphatidylinositol (GPI) anchored proteins may shift the burden of TAG storage from large to small adipocytes (8, 17). These studies suggest that adipocytes produce microvesicles that may play a role in communication between adipocytes and macrophages, and could also serve as communication vectors between adipose tissue and systemic circulation.

Adiponectin, a 247-amino acid protein that is one of the most abundant adipokines secreted by adipocytes, with circulating plasma levels ranging from 2-20 µg/mL in humans with a half-life of 2.5-6 hours (20). Adiponectin has been shown to activate AMPK and sirtuin signaling pathways, which can directly increase insulin sensitivity through phosphorylation of peroxisome proliferator-activated receptor  $\gamma$  co-activator 1 alpha (PGC-1 $\alpha$ ), a transcription co-activator that plays a critical role in mediating mitochondrial biogenesis and oxidative phosphorylation (21). In the endothelial cell, adiponectin inhibits the production of inflammatory cytokines and adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM) and E-selectin (22). Leucine, a branched-chain amino acid abundant in dairy products, can increase adiponectin secretion in 3T3-L1 adipocytes

compared to control (23). Furthermore, conditioned media collected from differentiated 3T3-L1 cells after a 48 hr leucine treatment (0.5 mM) decreased CD11b expression in U937 cells (unpublished data).

Adiponectin is one of the proteins carried by ADMs, and the HMW form is the most abundant form present in the exosomes (13, 18). Most recently, Phoonsawat *et al.* has isolated ADMs carrying HMW adiponectin from the plasma of mice (18). Since ADMs carrying adiponectin can enter systemic circulation, its possible that they can also exhibit anti-inflammatory effects, such as decreasing monocyte CD11b adhesion molecule expression. Therefore, the purpose of this study is to determine if ADMs carrying adiponectin isolated from 3T3-L1 conditioned media mediate the effects observed with leucine treatment.

### **4.3 Materials and Methods:**

#### *4.3.1 Chemicals:*

Leucine, and penicillin/streptomycin were obtained from Sigma (St. Louis, MO, USA). Phosphate buffered saline (PBS) was obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum for cell culture was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). CD11b primary antibody (IgG2b) was purchased from AbCam (Cambridge, MA, USA). CD14 primary antibody (IgG1) was purchased from R and D Systems (Minneapolis, MN, USA). AlexaFlour 488 goat anti-mouse (IgG1 specific) and AlexaFlour 647 goat anti-rat (IgG2b specific) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

#### 4.3.2 Cell Culture

3T3-L1 preadipocytes were purchased from ATCC (Manassas, VA, USA) and incubated at a density of 8000 cells/cm<sup>2</sup> (10 cm<sup>2</sup> dish) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (adipocyte medium) at 37°C in 5% CO<sub>2</sub> in air. Confluent preadipocytes were induced to differentiate (day 0) with a standard differentiation medium consisting of DMEM-F10 (1:1, v/v) medium supplemented with 1% FBS, 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and antibiotics (1% penicillin/streptomycin). Preadipocytes were maintained in this differentiation medium for 3 days and subsequently cultured in adipocyte medium. Cultures were re-fed every 2 to 3 days to allow 90% cells to reach full differentiation before chemical treatment. After 7 days, differentiated adipocytes were treated with vehicle (control) or 0.5 mM leucine for 48 hours. After treatment, the conditioned medium (CM) was collected, aliquoted and frozen at -80°C until needed for subsequent assays. For comparative purposes, all assays utilized CM (control vs. treatment group) derived from the same set of 3T3-L1 cultures (ie. the same passage and treatment period).

#### 4.3.3 siRNA Knockdown of Adiponectin:

3T3-L1 adipocytes were cultured and differentiated as described above. On day 6 of differentiation, 3T3-L1s were removed from the T-75 cm<sup>2</sup> flask using trypsin/EDTA 0.25% and 0.5 µg/mL type I collagenase (to prevent clumping of cells). Cells were centrifuged at 400 x g for 5 min and resuspended in 24 mL of media (10% FBS, 1%

pen/strep). One mL was placed into each well of a 24 well plate and allowed to re-adhere overnight.

Small interfering RNA for adiponectin and scrambled RNA (negative control) were obtained from Dharmacon, Inc (ThermoScientific; Waltham, MA, USA). A 5x siRNA solution was made according to the manufacture's instructions and diluted to 1x for use. The knockdown was optimized so that >60% reduction in adiponectin RNA expression was achieved. Re-seeded 3T3-L1s were treated with siAdipoQ for 24 and 48 hrs at a final concentration of 100, 200, and 300 nM. After treatment, the media was changed to complete media and left for 48 hrs to confirm the duration of the knockdown. Optimized conditions were determined to be a 24 hr treatment with 200 nM siAdipoQ followed by 48 hrs of control (no treatment) or 0.5 mM leucine treatment.

#### *4.3.4 Total RNA Isolation:*

RNA isolation was performed per the manufacturer's instructions using a total cellular RNA isolation kit (Ambion – Life Technologies; Grand Island, NY, USA). The concentration and purity of the isolated RNA was then measured using a NanoDrop (A280/A260 between 1.9 and 2.1).

#### *4.3.5 RT-PCR:*

Adipocyte 18s and adiponectin were quantitatively measured using an ABI 7300 Real-Time PCR System (Applied Biosystems, Branchburg, NJ) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems, Branchburg, NJ). Primer and probe sets were purchased from Applied Biosystems TaqMan Assays-on-Demand and utilized according

to manufacturer instructions. A standard curve was generated using pooled total RNA from each sample and were serial-diluted (50-1.5625 ng). Reactions of quantitative RT-PCR for standards and unknown samples were performed per ABI 7300 Real-Time PCR System and TaqMan Real-Time PCR Core Kit instructions. The mRNA quantitation for each sample was normalized using the corresponding 18s.

#### *4.3.6 Preparation of ADMs from Conditioned Media:*

The stored 3T3-L1 CM was thawed using a 37°C water bath for 15 min. The tubes were centrifuged at 1500 x g for 15 minutes to remove cell debris (24) and the supernatant was transferred to a 5 mL ultracentrifuge tube (Beckman Coulter; Pasadena, CA, USA). The CM samples were subjected to high-speed centrifugation for 1.25 hrs at 115,000 x g and 4°C to remove the ADM fraction from the CM. The supernatant was carefully removed and placed in a separate tube. The ADM pellet was re-suspended in 500 µL of antibiotic/serum-free DMEM.

#### *4.3.7 Phlebotomy:*

Blood draws were approved from an ethical standpoint by the Institutional Review Board at the University of Tennessee (IRB #8961 B). Subjects interested in donating blood were required to meet standard inclusion criteria guidelines that were outlined in the screening form. Blood was drawn using a 19-gauge butterfly needle equipped with a vacutainer connection and 10-30 mL of blood was collected each time into sodium heparin vacutainer tubes (BD Biosciences; San Jose, CA, USA). Blood was immediately used for all assays described below.

#### *4.3.8 Whole Blood Monocyte CD11b Expression:*

Five hundred  $\mu\text{L}$  of whole blood was added to 200  $\mu\text{L}$  of the prepared ADMs, supernatant, or whole conditioned media in a 2 mL eppendorf tube. Samples were gently mixed with a pipette and incubated at 37°C. After both 2 hrs and 18 hrs, a 100  $\mu\text{L}$  blood/ADM sample was added to 65 $\mu\text{L}$  of 10% paraformaldehyde (4% final concentration) and incubated at room temperature for 10 minutes to fix the cells. The tubes were topped off with 1X PBS and centrifuged for 10 min at 300 x g. The supernatant was removed and the pellet was gently resuspended in 100  $\mu\text{L}$  of 1X PBS. Then, 0.5  $\mu\text{g}$  of each primary antibody (AbCam; Cambridge, England, UK) specific for human CD11b (IgG2b) and CD14 (IgG1) were added to each tube and incubated for 1 hr at room temperature. The same concentration of matching isotype was added to a separate tube and used as a negative control. After primary antibody incubation, the tubes were topped off with 1X PBS and centrifuged for 10 min at 300 x g. The supernatant was removed and the pellet was gently resuspended in 100  $\mu\text{L}$  of 1X PBS. Then, 0.5  $\mu\text{g}$  of each secondary antibody (IgG1-DyLite 488 and IgG2b-APC) were added to each tube and incubated at room temperature for 45 minutes. After secondary antibody incubation, the tubes were topped off and centrifuged as before. Cell pellets were resuspended in 1 mL of red blood cell lyse solution (BD Biosciences; Franklin Lakes, NJ, USA) and incubated for 10 minutes. After incubation with the lyse solution, tubes were centrifuged as previously described and resuspended in 300  $\mu\text{L}$  of 1X PBS and analyzed by flow cytometry.

#### *4.3.9 Flow Cytometry of Whole Blood:*

The dual color staining method was designed to allow for the identification of monocytes (CD14+) also expressing the adhesion molecule CD11b. Therefore, CD14 was tagged with a green fluorescent marker (DyLite 488) and CD11b was tagged with a red fluorescent marker (APC) as previously described (25). Samples were analyzed on an Accuri C6 flow cytometer (BD Biosciences). Initially, 10,000 events were collected to generate a typical whole blood profile on the forward scatter (FSC) vs. side scatter (SSC) plot. Then, a gate was placed around the monocyte population (Gate 1). Gate 1 was then applied to two separate histogram plots of green fluorescence (CD14 expression; H1 plot) and red fluorescence (CD11b expression; H2 plot) where isotypic control gates were set to 1% positive. Therefore, anything above the fluorescence level of the isotypic control for both green and red fluorescence was considered positive for CD14 and CD11b, respectively. A test sample was allowed to run until 10,000 events in gate 1 were collected. Those events were then applied to the H1 plot and the positive events were further applied to the H2 plot. Then, the percent of CD14+ cells also positive for CD11b were recorded. Each treatment condition was run in triplicate and repeated 3 times. Results are presented as mean percent positive  $\pm$  standard error mean from the 3 independent experiments.

#### *4.3.10 Statistical Analysis:*

Unless otherwise specified, data is presented as mean  $\pm$  standard error mean (SEM). Data was analyzed via one-way analysis of variance after confirming equality of

variance among groups and normality of distribution. Significantly different means ( $p < 0.05$ ) were separated by Fisher's LSD using GraphPad Prism (GraphPad Software, Inc.).

#### **4.4 Results:**

Compared to control, leucine CM incubated with whole blood for 2 hrs. reduced monocyte CD11b expression from  $71.6 \pm 2.9\%$  to  $52.7 \pm 5.8\%$  (Fig 4-1A;  $p < 0.03$ ), and this effect was sustained throughout the 18 hr incubation (Fig 4-1B;  $p < 0.004$ ). The isolated ADM fraction from the leucine CM mimicked the effects of the whole CM at both the 2 and 18 hr time points, reducing monocyte CD11b expression from  $73.4 \pm 3.2\%$  to  $50.6 \pm 7\%$  (2 hr;  $p < 0.008$ ) and from  $75.6 \pm 3.4\%$  to  $49.6 \pm 1.9\%$  (18 hr;  $p < 0.0001$ ), respectively (Fig 4-1A and B). The supernatant fraction from the CM exhibited no significant effects at either time point (Fig 4-1A and B), suggesting the ADM fraction of the CM contains an active component capable of reducing monocyte CD11b expression.

We have previously shown that treating 3T3-L1 adipocytes with leucine (0.5 mM) increases adiponectin production (23), and others have shown that adiponectin is present on ADMs (13, 14, 18). Therefore, we knocked down adiponectin production using siRNA by ~60% compared to untransfected cells and cells treated with non-specific scrambled RNA (Fig 4-2A;  $p < 0.02$ ). After optimizing our knockdown efficiency, we applied our adipocyte treatments to determine transcriptional levels of adiponectin. In untransfected 3T3-L1 adipocytes, leucine treatment significantly increased adiponectin mRNA levels by 35% compared to control (Fig 4-2B;  $p < 0.02$ ). However, when the cells were treated with siAdipoQ, it significantly reduced adiponectin mRNA levels compared

to untransfected cells (Fig 4-2B;  $p<0.02$ ) within each treatment group, but there was no difference between leucine and control treatment groups (Fig 4-2B).

The same siAdipoQ transfection and treatments were used to generate adipocyte CM for the control (no treatment) and 0.5 mM leucine groups. The CMs were further separated using ultracentrifugation to isolate ADMs and the remaining supernatant. The whole CM, supernatant, and isolated ADMs were applied to samples of whole blood and incubated for 2 and 18 hours. Consistent with our previous data, untransfected leucine CM decreased monocyte CD11b expression from  $24.2 \pm 1.4\%$  to  $20.4 \pm 1.2\%$  (Fig 4-3A;  $p<0.04$ ) at the 2 hr time point and from  $73.9 \pm 1.1\%$  to  $68.6 \pm 1.3\%$  at 18 hrs (Fig 4-4A;  $p<0.001$ ). Adiponectin siRNA reversed this effect, resulting in a significant increase compared to the leucine untransfected group at both time points (Fig 4-3A and 4-4A;  $p<0.004$ ). The supernatant fraction had no significant effects between treatment groups or between transfection groups at the 2 hr time point (Fig 4-3B). The ADM fraction showed the same trend as the whole CM with leucine significantly reducing monocyte CD11b expression from  $24.2 \pm 1.8\%$  to  $9.1 \pm 0.3\%$  after a 2 hr incubation (Fig 4-3C;  $p<0.0001$ ) and from  $68.7 \pm 1.8\%$  to  $64.1 \pm 0.7\%$  after 18 hrs. (Fig 4-4C;  $p<0.003$ ). Again, adiponectin siRNA reversed this effect, resulting in a significant increase compared to the leucine untransfected group at both time points (Fig 4-3C and Fig 4-4C;  $p<0.0001$ ). Collectively, these results suggest that leucine treatment of adipocytes decreases monocyte CD11b expression, and adiponectin present on ADMs are directly involved in this process.

#### 4.5 Discussion:

Data from this study demonstrate that treating adipocytes with leucine reduce CD11b expression on peripheral blood monocytes and adipocyte derived microvesicles mediate this process. We have shown similar effects with CD11b expression in U937 cells and ICAM-1 expression on endothelial cells treated with control and leucine CM (unpublished data). Isolating the ADM fraction exhibited similar effects on monocyte CD11b expression compared to the whole CM, while the supernatant fraction did not, suggesting the ADM fraction mediates this process. Microvesicles appear to be involved in intercellular cross-talk in both normal and pathological conditions (26, 27). Adipocyte derived microvesicles released from obese mice (obADMs) are preferentially taken up by peripheral blood monocytes, and stimulate monocyte to macrophage differentiation (14). Furthermore, when the obADMs were injected into wild-type mice it induced an insulin-resistant phenotype (14), suggesting ADMs mediate communication from adipose tissue to cells in systemic circulation. Therefore, its possible that leucine supplementation through dairy products can alter the ADM profile that acts on circulating monocytes, leading to decreased adhesion to endothelial cells. This would decrease monocyte/macrophage infiltration into adipose tissue to subsequently reduce obesity associated inflammatory stress.

Our previous *in vitro* studies (23) and studies in mice and humans demonstrate that leucine increases adiponectin secretion (28, 29) through a Sirtuin 1 (SIRT1)/AMPK mediated mechanism. In the current study, we demonstrate leucine treatment of adipocytes also increases adiponectin mRNA levels. Therefore, adiponectin is a probable candidate mediating the reduction in monocyte CD11b expression. Indeed, after

knocking down adiponectin expression, the collected leucine CM did not reduce monocyte CD11b expression compared to control (Fig 4-3). These effects were also observed in the isolated ADM fraction, but not the supernatant fraction, suggesting adiponectin is present on the ADM fraction. Similarly, multiple other studies have also demonstrated adiponectin associated ADMs, specifically the high-molecular weight form (14, 18, 19). The three forms of adiponectin have been shown to possess different biological activities, and the HMW form is considered the major active form in circulation, providing the insulin-sensitizing and cardiovascular protective effects observed with adiponectin (30, 31).

Adiponectin exerts its signaling actions through two receptors, adiponectin receptor 1 and 2 (AdipoR1 and AdipoR2) (32). One pathway that adiponectin activates is 5'-adenosine monophosphate kinase (AMPK) activity, which stimulates fatty-acid oxidation, increases PPAR $\alpha$  expression, and increases glucose uptake while simultaneously inhibiting glycolysis, lipogenesis and gluconeogenesis (33). Adiponectin can also exert anti-atherosclerotic effects through modulation of multiple points of the inflammatory process (34). For example, adiponectin prevented atherosclerosis by increasing cholesterol efflux from macrophages (35). Furthermore, in human monocyte-derived macrophages, which express the adiponectin receptor, adiponectin induced upregulation of IL-10 (36, 37). Adiponectin also increased expression of CD36, acetyl CoA oxidase, and uncoupling protein 2 in skeletal muscle, while causing a decrease in CD36 in the liver (38). In the endothelial cell, adiponectin was shown to inhibit the production of inflammatory cytokines and adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM) and E-selectin

(22). Furthermore, adiponectin has been shown to increase endothelial nitric oxide synthase (eNOS) production leading to the production of nitric oxide (NO) in the vasculature to stimulate vasodilation (39). Additionally, adiponectin inhibited TNF- $\alpha$  induced activation of NF- $\kappa$ B (40). These studies, combined with our recent data suggest that adiponectin can play a protective role against key processes associated with atherosclerosis.

Leucine has recently been demonstrated to directly activate SIRT1 through an allosteric mechanism (41). SIRT1 increases adiponectin gene transcription by activating Foxo1, thereby enhancing Foxo1 and C/EBP $\alpha$  interaction (42), which then binds to the adiponectin promoter to increase transcription (42). Adiponectin binding to AdipoR1 increases calcium influx, causing activation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase  $\beta$  (CaMKK $\beta$ ), which phosphorylates CaMK $\beta$  resulting in increased PGC-1 $\alpha$  gene expression (21). CaMKK $\beta$  also activates AMPK leading to increased NAD<sup>+</sup> levels and subsequent activation of SIRT1, resulting in a positive feedback cycle. The connection between adiponectin and decreased CD11b expression presumably occurs through suppression of NF- $\kappa$ B. NF- $\kappa$ B is retained in the cytoplasm through interactions with inhibitory proteins from the inhibitory factor kappa B family (I $\kappa$ B) (43). Upon activation, I $\kappa$ B is phosphorylated by I $\kappa$ B kinase and becomes disassociated from NF- $\kappa$ B, allowing NF- $\kappa$ B translocation to the nucleus where it increases the transcription of pro-inflammatory cytokines such as TNF- $\alpha$  and MCP-1 (43, 44). Adiponectin increases the expression and protein levels of I $\kappa$ B causing NF- $\kappa$ B to be retained in the cytoplasm, thereby reducing pro-inflammatory cytokine production (45). The role of pro-inflammatory cytokines such as TNF- $\alpha$  and MCP-1 are to increase immune cell

infiltration to combat inflammatory stimuli. CD11b is a common monocyte activation marker that responds to inflammatory cytokines, and it is required for monocyte adhesion and migration through the endothelium (46). Therefore, decreased pro-inflammatory cytokine production at the adipocyte level and systemically can decreased CD11b expression resulting in decreased monocyte adhesion and infiltration through the endothelium.

In summary, our results indicate that leucine treatment of adipocytes reduces CD11b expression on fresh human monocytes and suggest that adiponectin present on ADMs mediate some of these effects. Translation of these effects to a clinical setting could provide a framework to reduce obesity associated inflammation and further supports the ability of ADMs to serve as a communication vector between adipose tissue and systemic circulation.

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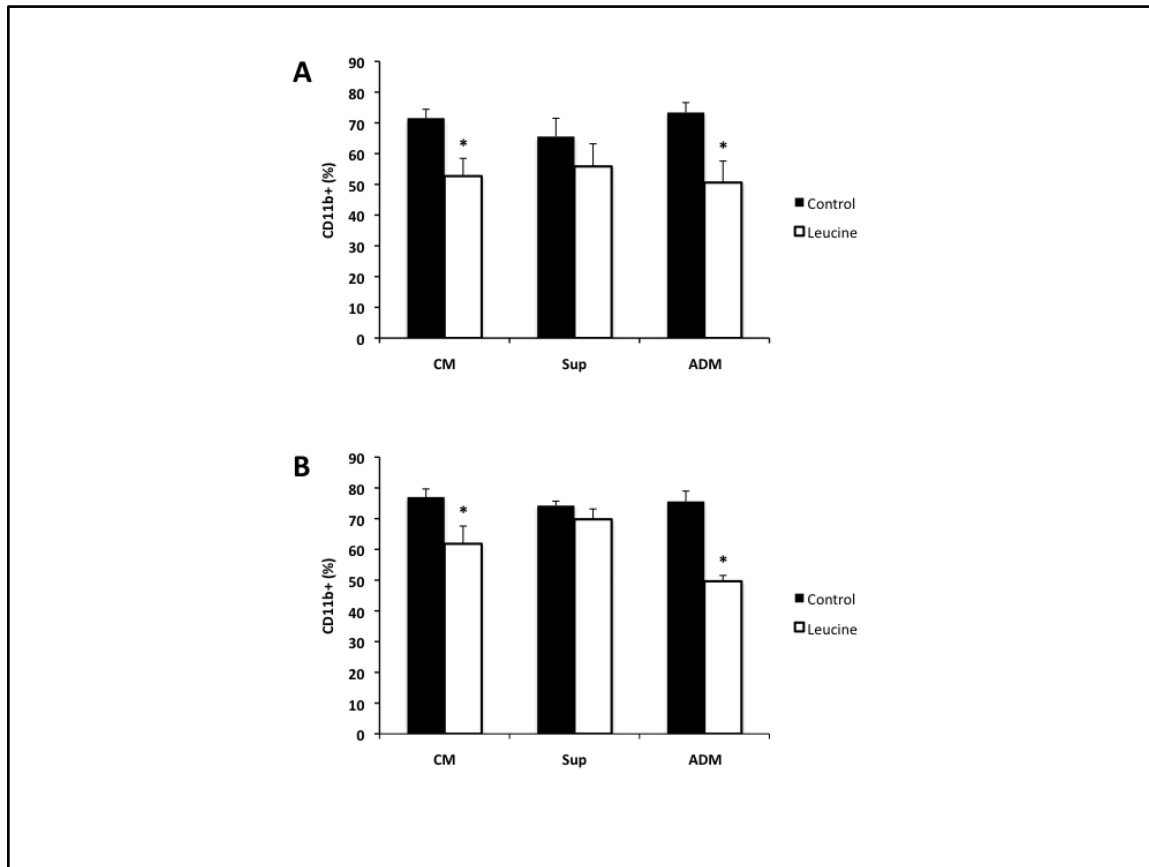
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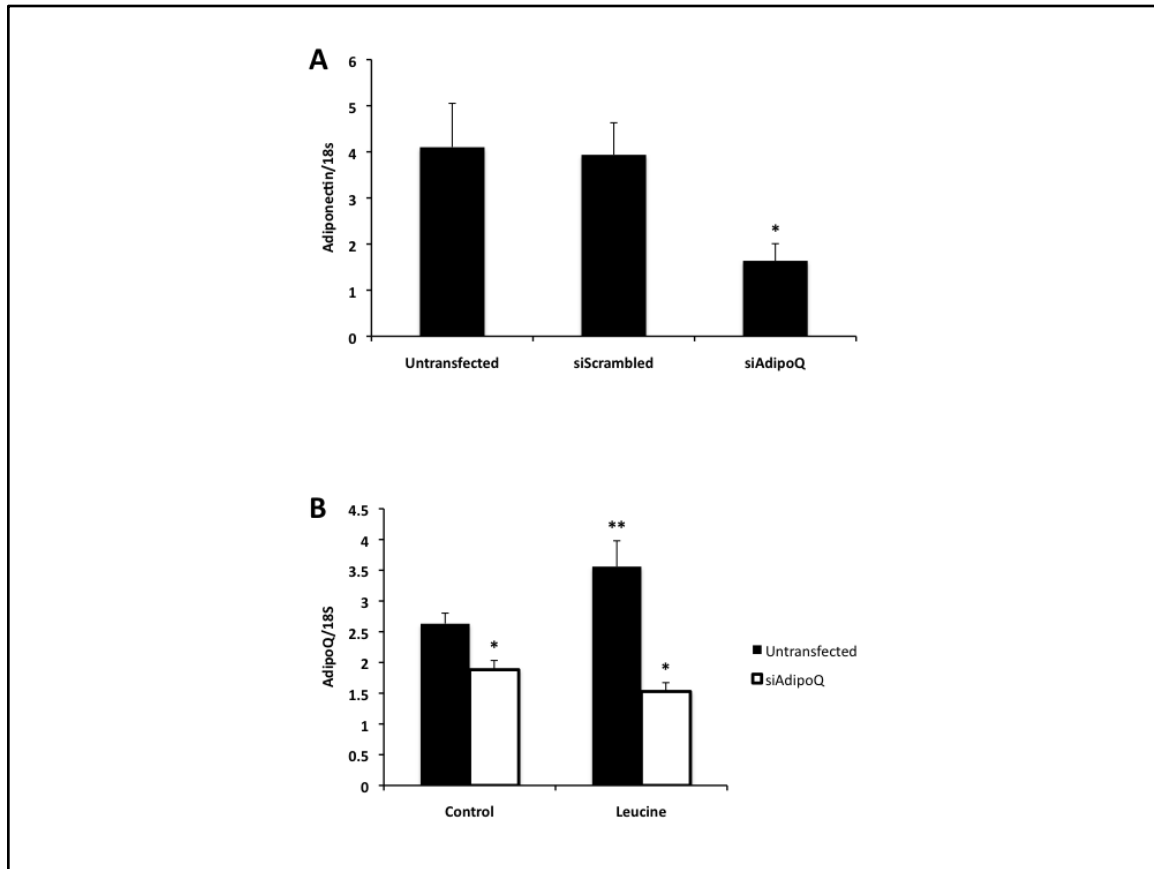
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## APPENDIX

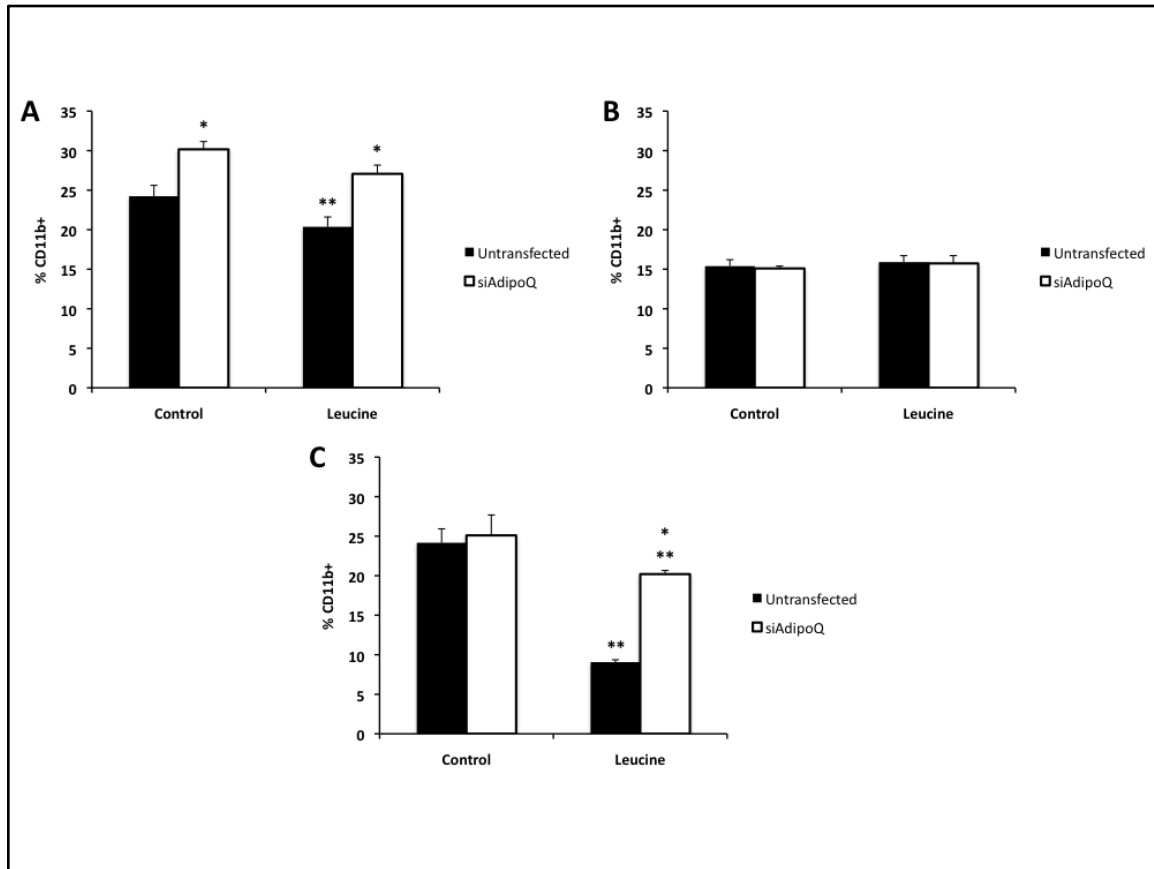


**Figure 4-1: Effects of adipocyte CM on peripheral blood monocyte CD11b expression.** Fully differentiated 3T3-L1 adipocytes were treated for 48 hrs with leucine (open bars) or control (black bars). Conditioned media (CM) was collected and the ADMs were separated from the supernatant (sup) by ultracentrifugation. Whole CM, supernatant, and ADMs were incubated with whole blood for 2 hrs (A) and 18 hrs (B) at 37°C. Results are presented as percent of monocytes (CD14+) that are CD11b+  $\pm$  SEM (n=3). \* Denotes significance compared to control (\*  $p < 0.02$ ).

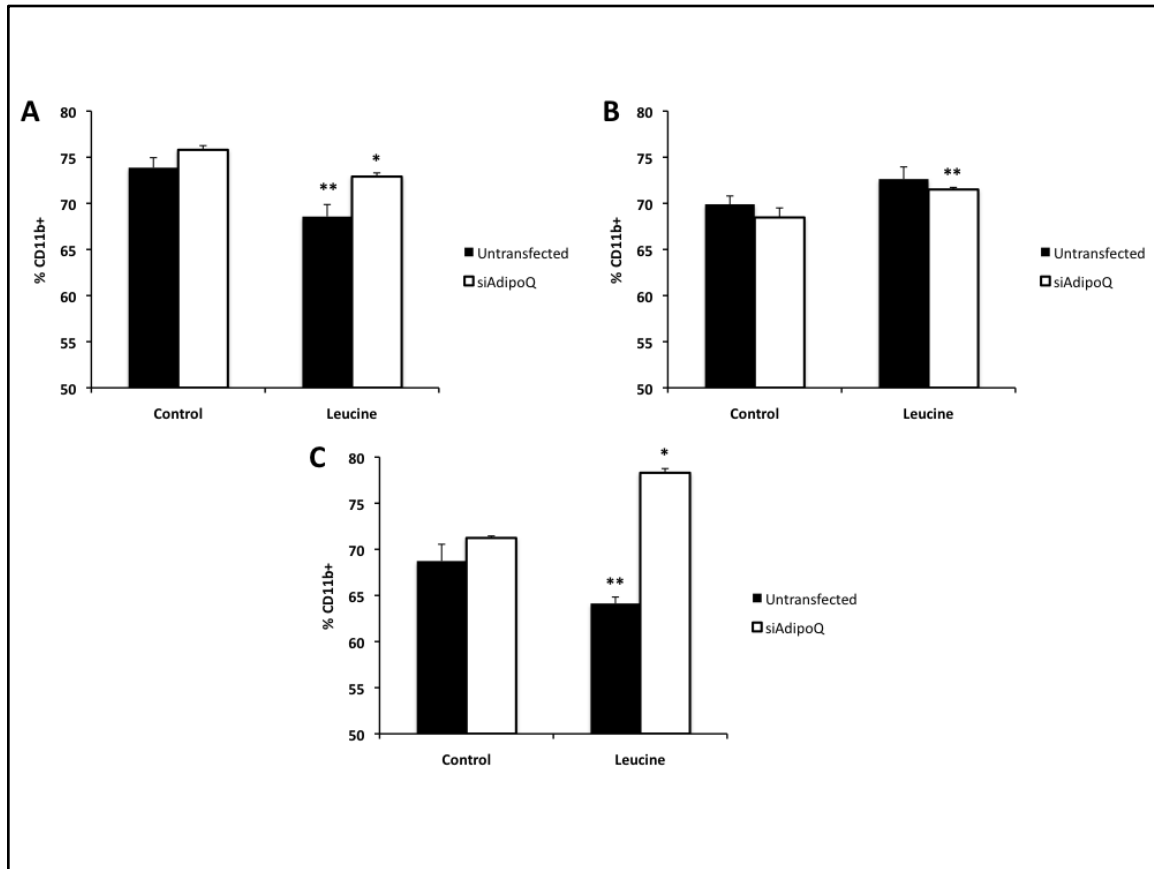


**Figure 4-2: Adiponectin knockdown efficiency and expression in 3T3-L1 cells.**

Differentiated 3T3-L1 cells (day 7) were untransfected, transfected with scrambled siRNA, or transfected with adiponectin siRNA (siAdipoQ) for 24 hrs and adiponectin mRNA levels were measured using RT-PCR (A; n=6). Differentiated 3T3-L1 cells were untransfected (black bars) or transfected with siAdipoQ (open bars) as described above. Cells received no treatment (control) or treated with 0.5 mM leucine for 48 hrs and mRNA levels were measured by RT-PCR (B; n=3). Results are presented as the ratio of adiponectin to ribosomal 18S  $\pm$  SEM. \* Denotes significance compared to untransfected ( $p < 0.02$ ), \*\* denotes significance compared to control ( $p < 0.02$ ).



**Figure 4-3: Adiponectin and ADMs mediate reduction in peripheral blood monocyte CD11b expression after 2 hours.** Differentiated 3T3-L1 adipocytes were untransfected (black bars) or transfected with siAdipoQ (open bars), and then treated for 48 hrs with 0.5 mM leucine or not treated (control). Whole conditioned media (A), isolated supernatant (B) or isolated ADMs (C) were incubated for 2 hrs. with whole blood. Results are presented as percent of monocytes (CD14+) that are CD11b+  $\pm$  SEM (n=3). \* Denotes significant compared to untransfected ( $p < 0.002$ ). \*\* Denotes significant compared to control ( $p < 0.04$ ).



**Figure 4-4: Adiponectin and ADMs mediates reduction in peripheral blood monocyte CD11b expression after 18 hours.** Differentiated 3T3-L1 adipocytes were untransfected (black bars) or transfected with siAdipoQ (open bars), and then treated for 48 hrs with 0.5 mM leucine or not treated (control). Whole conditioned media (A), isolated supernatant (B) or isolated ADMs (C) were incubated for 18 hrs. with whole blood. Results are presented as percent of monocytes (CD14+) that are CD11b+  $\pm$  SEM (n=3). \* Denotes significant compared to untransfected and control ( $p < 0.002$ ). \*\* Denotes significant compared to control treatment ( $p < 0.002$ ).

**CHAPTER V:**  
**CONCLUSIONS**

## 5.1 Conclusions:

The data presented in this dissertation suggests that leucine, reduces monocyte – endothelial cell adhesion, and adipocyte derived microvesicles carrying adiponectin partially mediate these effects. Treatment of adipocytes with leucine produces factors that decrease monocyte – endothelial cell adhesion, while treatment with calcitriol has the opposite effect. The reduction in monocyte – endothelial cell adhesion observed with leucine treatment of adipocytes was further expanded using an *ex vivo* model using plasma samples from a recent clinical trial in which overweight and obese subjects were given either high (>3.5 servings/day) or low (<0.5 servings/day) dairy diets over a period of 12 weeks. Compared to baseline plasma samples, the high dairy group decreased monocyte – endothelial cell adhesion after just 7 days and these effects were sustained throughout the 12 week study, while the low dairy group exhibited the opposite effect. These effects are due, in part, to an alteration in adipokine production, causing a decrease in pro-inflammatory cytokines (TNF- $\alpha$ , MCP-1, IL-6, CRP) and an increase in anti-inflammatory cytokines (adiponectin) (1, 2), resulting in a decrease in adhesion molecule expression on both monocyte (CD11b) and endothelial cells (CD54). Our data shows that leucine treatment of adipocytes causes a decrease in peripheral blood monocyte CD11b expression, and the adipocyte derived microvesicles (ADMs) isolated from the conditioned media mimic the effects observed with whole conditioned medium, suggesting ADMs carry an active component(s), such as adiponectin, that mediates these effects. We confirmed this concept by knocking down adiponectin production using siRNA, causing the previously observed reduction in CD11b expression to be blocked. Collectively, this data suggest that dairy products can be beneficial in reducing

inflammatory stress associated with obesity and adipocyte derived microvesicles play a role in this process.

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## VITA

Benjamin Jones Curry, son of Ken and Cindy Curry, was born on March 11, 1985 in Kansas City, Kansas, USA. After graduating from high school in May 2003, Ben attended the University of Tennessee – Knoxville to pursue a Bachelor of Science in Biological Sciences with a concentration in Microbiology. In May 2008, Ben graduated with a Bachelor's degree and began conducting research for Dr. John Biggerstaff. Ben started graduate school at the University of Tennessee – Knoxville in August 2008, pursuing a Master's degree in the department of Biochemistry, Cellular and Molecular Biology. In January 2010, Ben transferred to the Nutrition Department to pursue a Ph.D in Cellular and Molecular Nutrition with a cognate in Biochemistry, Cellular and Molecular Biology under supervision of Dr. Michael B. Zemel. Ben defended his dissertation, titled “The Effects of Leucine and Dairy Products on Adipose Tissue Inflammation: The Role of Adipocyte Derived Microvesicles” on July 10, 2014.