Sex-related Differences in Microvascular Adaptations to Obesity

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

Graduate Program in

Kinesiology and Health Science

York University

Toronto, Ontario

August 2017

Abstract

Capillary networks within skeletal muscle and adipose tissue have a critical role in the delivery of energy sources and the maintenance of proper tissue function. A reduction in capillary density is correlated with impairments in whole-body metabolic functions, as occurs in obesity. The susceptibility to develop obesity-related metabolic disturbances differs between males and females. However, studies investigating these sex-related differences have overlooked the potential contribution of capillary content. The goal of this study was to determine whether there are sex-related differences in skeletal muscle and adipose tissue microvascular content and to relate these to the development of metabolic dysregulations in mice fed either a normal chow (NC) diet or a high-fat (HF) diet.

Following 16 weeks of HF diet, whole-body, as well as skeletal muscle and adipose tissue, response to insulin was maintained in HF female but not HF male mice. In the muscle, this was accompanied by higher levels of endothelial cell marker PECAM-1 in HF females. However, no significant difference in capillary and arteriole distribution was established. Microvascular content in the visceral adipose was reduced in HF males, but maintained in HF females which also had a healthier adipokine profile. This was further supported by higher levels of PECAM-1 and pro-angiogenic factor VEGF-A in HF females vs. HF male mice.

These results provide evidence that sex-related differences exist in the angiogenic responses to a HF diet. The maintained capillary content within visceral adipose of HF female mice may in turn contribute to the better metabolic profile in HF females. This highlights the possible associations that may exist between metabolic and vascular parameters and provides the opportunity to explore sex-dependent signaling pathways that regulate capillary content within the context of diet-induced obesity.

Special Acknowledgements:

I would like to thank Tara for her guidance and encouragement during my Master's program. Thank you for creating a work environment where we can learn from each other and expand our knowledge.

Thank you Martina and Emmanuel for all the help and advice you have given me in the past two years.

Thank you Arya for all of your love and support.

A special thanks to my family for helping me be where I am today. I could not have done this without your encouragement, love, and support.

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AKT	Protein kinase B
ATP	Adenosine triphosphate
C:F	Capillary-to-fiber ratio
CAM	Chick chorioallantoic membrane
cAMP	Cyclic adenosine monophosphate
CD	Capillary density
CD36	Cluster of differentiation 36
cGMP	Cyclic guanylate monophosphate
EDL	Extensor digitorum longus
EDN-1	Endothelin-1
eNOS (NOS3)	Endothelial nitric oxide synthase
ETA	Endothelin receptor A
FFA	Free fatty acids
FGF-2	Fibroblast growth factor 2
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
GTT	Glucose tolerance test
HF	High-fat
HIF-1	Hypoxia-inducible factor -1
HIF-1α	Hypoxia-inducible factor -1α
HIF-1β	Hypoxia-inducible factor -1β
Hprt-1	Hypoxanthine-guanine phosphoribosyltransferase-1
HSL	Hormone-sensitive lipase
IP	Intraperitoneal
IR	Insulin receptor
IRS1/2	Insulin receptor substrate 1/2
ISO	Isoproterenol
ITT	Insulin tolerance test
KLF2	Kruppel-like factor 2
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinases
MYH11	Myosin heavy chain 11
NC	Normal chow
NO	Nitric oxide
OB-Ra	Leptin receptor a
OB-Rb	Leptin receptor b
PDGFB	Platelet derived growth factor subunit B
PECAM-1	Platelet endothelial cell adhesion molecule-1

PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
ROS	Reactive Oxygen Species
SMC	Smooth muscle cell
TA	Tibialis anterior
TGF-β	Transforming growth factor-β1
THBS-1	Thrombospondin-1
VEGF-A	Vascular endothelial growth factor -A
VEGFR	Vascular endothelial growth factor receptor

Chapter 1: Literature Review

1.1 Microvasculature

The microvasculature, comprised of capillaries, arterioles and venules, is the primary site for the delivery and exchange of nutrients, gases and removal of metabolic waste. Therefore, microvessel structure and function is critical for maintenance of metabolic health and function of the surrounding tissues. Capillaries are the smallest (5-8μm in dimeter) and most abundant unit of the vasculature (Wiedman 1963). Capillaries are lined by a single layer of endothelial cells and surrounded by the extracellular matrix. Endothelial cells lining capillaries are estimated to be 0.5-1 μm in thickness and can be distinguished by cell surface antigens such as platelet endothelial cell adhesion molecule-1 (PECAM-1) (Pries et al. 2000). The structural composition of capillaries enables efficient trans-capillary diffusion (Bruns and Palade 1968; Krogh 1919). The capacity of capillaries for nutrient and gas exchange is dependent on blood flow and total delivery of molecules. This is controlled by upstream tissue arterioles which in addition to the endothelium, are lined by layers of smooth muscle cells (SMCs) (Ellsworth and Pittman 1990; Segal 2005). The Presence of SMCs gives arterioles the ability to constrict or dilate and alter vessel lumen size. This permits the control of blood flow and in turn influences capillary recruitment and perfusion of the target tissue. Capillary number is not static and it can change depending on the requirements of the surrounding tissue and in response to molecular and mechanical stimuli (Haas and Nwadozi 2015; Olfert et al. 2016). In adults, adaptations in capillary number can be in the form of capillary rarefaction, stabilization, or growth through the process of angiogenesis.

1.1.1 Angiogenesis

Angiogenesis is the formation of new capillaries from a pre-existing capillary network (Flier et al. 1995). *In vivo*, this process was first explored by Clark & Clark, in transparent tails of larva and by insertion of a transparent chamber within the ear of rabbits, where growth of capillary networks was observed by microscopy (1939). Angiogenesis occurs in embryonic development and in adults in the female reproductive system, during wound healing, tumour growth, and in response to exercise (Gustafsson 2011). Physiological angiogenesis occurs to maintain metabolic homeostasis within growing tissues (e.g. muscle) to ensure appropriate delivery of oxygen and nutrients (Haas 2002). An increase in capillary number increases surface area for exchange and reduces the diffusion distance of molecules in times of increased metabolic demand (Krogh 1919).

1.1.2 Abluminal Sprouting and Intussusceptive Angiogenesis

There are two forms of angiogenesis termed abluminal sprouting and intussusceptive, both of which result in the formation of endothelium-lined capillaries. Abluminal sprouting angiogenesis requires lateral growth of endothelial cells from the abluminal side of one capillary to connect to another existing capillary (Haas 2002). This type of angiogenesis requires activation of endothelial cells and loosening of endothelial cell junctions by extracellular stimuli. Once activated, matrix metalloproteinase (MMP) enzymes degrade the extracellular matrix (ECM) and pave the way for the newly proliferated endothelial cells to migrate (Haas 2005). Endothelial cells at the stalk of the sprout continue to proliferate while endothelial cells at the tip of the sprout migrate to form a connection with an adjacent pre-existing capillary. This is followed by formation of a lumen and stabilization of the new sprout by attachment of pericytes and reconstitution of the basement membrane (Flier et al. 1995; Pepper 1997). In intussusceptive angiogenesis, endothelial

cells activate and send pillar-like projections into the lumen of the existing vessel which eventually splits the vessel into two parallel capillaries (Caduff et al. 1986; Mentzer and Konerding 2014). In this process, compared to abluminal sprouting, there is minimal endothelial cell proliferation and no need for the MMP degradation of ECM (Zhou et al. 1998). In adults, both of these processes are co-ordinated through the integration of chemical signals such as hypoxia, metabolites, growth factors and inflammatory factors, and mechanical signals including hemodynamic forces of blood and mechanical stretch of muscle. In response to these chemical and mechanical stimuli, an angiogenic response can be regulated by the release of pro-angiogenic and anti-angiogenic factors. Studies have established that for angiogenesis to occur, pro-angiogenic stimuli must up-regulate and overcome the influence of anti-angiogenic factors (Olfert and Birot 2011).

1.2 Angiogenic Regulators

1.2.1 Vascular Endothelial Growth Factor-A (VEGF-A)

VEGF-A is one of the most dominant pro-angiogenic factors and is able to stimulate endothelial cells in multiple steps of the angiogenic process (Egginton 2011; Ferrara and Henzel 1989). VEGF-A is required for the formation of vascular system as lack of a single VEGF allele results in embryonic lethality in mice (Carmeliet et al. 1996). Within skeletal muscle and adipose tissue, VEGF-A is secreted from multiple cell types, including endothelial cells, myocytes, macrophages and fibroblasts (Duffy, Bouchier-Hayes, and Harmey 2013; Maharaj et al. 2006). Multiple splice variants of VEGF-A exist, which differ by their ability to bind proteoglycans within the extracellular matrix (Neufeld et al. 1996). Secreted VEGF-A is retained within the ECM surrounding capillaries and can stimulate endothelial cells upon activation. VEGF-A can be expressed by shear stress (Milkiewicz et al. 2001; Williams et al. 2006), hypoxia (Shweiki et al.

1992), and muscle stretch (Milkiewicz et al. 2007). There are 3 VEGF receptors: VEGFR-1, VEGFR-2 and VEGFR-3 (Ferrara et al. 2003b). VEGF-A binds two receptors on the surface of endothelial cells, VEGFR-1 (membrane bound and soluble) and VEGFR2 which is considered to be the major mediator of endothelial cell activation and angiogenesis (Terman et al. 1992; de Vries et al. 1992). VEGF-A promotes activation, proliferation and migration of endothelial cells through mitogen activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) signaling pathways (Bernatchez, Soker, and Sirois 1999; Gerhardt et al. 2003). In a murine model of muscle specific VEGF deletion, absence of this pro-angiogenic factor induces muscle capillarity rarefaction and is associated with a blunted glucose disposal in response to insulin (Bonner et al. 2013).

1.2.2 Leptin

Leptin, a product of the *ob* gene, is an adipocyte-secreted cytokine (adipokine) traditionally known for its involvement in the regulation of food intake, metabolism, and inflammatory responses (Margetic et al. 2002; Scarpace and Zhang 2007). To date, six isoforms of the leptin receptors which differ by their cytoplasmic domain length (short or long) have been identified (Margetic et al. 2002). The presence of leptin receptors OB-Ra and OB-Rb on the surface of endothelial cells and the ability of leptin to stimulate tyrosine kinase-dependent signaling pathways suggests that the endothelium is a target for leptin signaling (Sierra-Honigmann et al. 1998). Leptin also has been recognized as a modulator of angiogenesis due to its pro-angiogenic influence on endothelial cells (Anne Bouloumié et al. 1998; Sierra-Honigmann et al. 1998). Leptin's angiogenic activity has been tested in both *in vitro* and *in vivo* angiogenesis models (Anne Bouloumié et al. 1998). Treatment of HUVECS with a physiological concentration of leptin promotes endothelial

cell proliferation and survival (Anne Bouloumié et al. 1998; Sierra-Honigmann et al. 1998). This effect is associated with formation of capillary-like structures in *in vitro* angiogenesis assay. Also, leptin has been shown to enhance formation of capillaries *in vivo* in chick embryos and rat corneas. In addition to its direct pro-angiogenic activity, leptin can also promote angiogenesis by upregulating VEGF expression and facilitating expression and activity of matrix metalloproteinanses (MMP-2 and MMP-9), which are key regulators of sprouting angiogenesis (Park et al. 2001; Suganami et al. 2004). Leptin-dependant modulation of angiogenesis is dose-dependent. When present in high concentrations, leptin can enhance capillary permeability and generation of reactive oxygen species, which can promote endothelial cell dysfunction and have pathophysiological effects on the tissue vasculature (Bouloumie et al. 1999; Cao et al. 2001).

1.2.3 Thrombospondin-1 (Thbs-1)

Thbs-1 is a potent anti-angiogenic factor synthesized and released into the extracellular matrix by a variety of cell types including endothelial cells, smooth muscle cells, platelets and fibroblasts (Mosher, Doyle, and Jaffe 1982; Raugi et al. 1982). Thbs-1 can inhibit multiple steps of the angiogenic process. At low concentrations, Thbs-1 inhibits corneal neovascularization as well as proliferation and migration of endothelial cells *in vitro* (Good et al. 1990; Taraboletti et al. 1990; Tolsma et al. 1993a). Also, exogenous addition of Thbs-1 blocks the ability of endothelial cells to organize into tube-like structures and prevents lumen formation (Tolsma et al. 1997). The anti-angiogenic domain of Thbs-1 has been mapped to the second and third type-1 repeats in its structure (Iruela-Arispe et al. 1999; Tolsma et al. 1993b). The inhibitory action of Thbs-1 is through multiple signaling pathways and through interaction with multiple growth factors and proteases (Bornstein 2009). Thbs-1 binds Fibroblast growth factor 2 (FGF2), Transforming

growth factor-beta (TGF-β), VEGF-A and MMP-2 and prevents their pro-angiogenic influence on endothelial cells (Iruela-Arispe et al. 1999; Margosio et al. 2008; Rodriguez-Manzaneque et al. 2001; Schultz-Cherry et al. 1995). Furthermore, binding of Thbs-1 to Cluster of Differentiation 36 (CD36) promotes EC apoptosis (Jiménez et al. 2000). Compared to wild type mice, Thbs-1 knock-out mice have increased vascularization documented in several tissues (cardiac and skeletal muscle, retina, pancreas), which confirms the role of Thbs-1 as an important inhibitor of angiogenesis (Crawford et al. 1998; Malek and Olfert 2009; Wang et al. 2003).

1.3 Arteriole Tone and Remodeling

1.3.1 Endothelial Nitric Oxide Synthase (eNOS)

Within endothelial cells, eNOS catalyzes conversion of L-arginine to L-citrulline and nitric oxide (NO) as a by-product (Epstein, Moncada, and Higgs 1993). Expression of eNOS can be regulated by multiple stimuli. To name a few, shear-stress (the force of blood flow on the surface of endothelial cells), estrogen, and hypoxia have been shown to increase eNOS expression within endothelial cells (Arnet et al. 1996; Davis et al. 2001; Hishikawa et al. 1995). Activity of eNOS in endothelial cells is regulated by multi-site phosphorylation. One of the main activators of eNOS is shear-stress. Shear-stress leads to the activation of Akt which phosphorylates eNOS on ser ^{1177}, a main regulatory site for eNOS activation and production of NO (Fulton et al. 1999; Zeiher et al. 1999). Also, several factors such as VEGF, estrogen, insulin and bradykinin result in activation of the PI3-Akt pathway with consequent phosphorylation of eNOS and generation of NO (Harris et al. 2001; Liao et al. 2000; Papapetropoulos et al. 1997). Production of NO is required for dilation of blood vessels and regulation of blood flow to the surrounding tissue. In arterioles, NO diffuses into SMCs, where it activates guanylate cyclase which mediates the production of cGMP. This

promotes calcium uptake into the sarcoplasmic reticulum and induces relaxation of SMCs, thereby increasing blood flow via vasodilation. Evidence for the critical role of NO in the regulation of vessel tone is based on experiments that show inhibitors of eNOS (e.g. nitro-L-arginine) increase vessel resistance and reduce blood flow (Sakuma et al. 1992). Furthermore, eNOS knockout mice exhibit deficits in vascular relaxation and develop hypertension and microvascular rarefaction (Dai and Faber 2010; Kubis et al. 2002; Liu and Huang 2008). This shows that while vasodilation is a major function of NO, endothelial production of NO is also critical for adaptive vascular remodeling within the alterations blood flow.

1.3.2 Endothelin-1 (Edn-1)

Edn-1 is a dominant endogenous vasoconstrictor mainly produced by endothelial cells under physiological conditions (Yanagisawa et al. 1988). Multiple stimuli induce expression of Edn-1 within endothelial cells including, hypoxia, hormones (e.g. androgens) and inflammatory mediators (Hu et al. 1998; Pearson et al. 2008; Taddei and Vanhoutte 1993). The product of Edn-1 gene transcription is a pre-pro-edn-1 mRNA which undergoes post-transcriptional cleavage to produce big Edn-1. Mature and biologically active Edn-1 is then produced by endothelial converting enzymes (Bohm and Pernow 2007; Maguire et al. 1997). The biological effects of edn-1 are mediated by two receptors subtypes: ETA and ETB. In arterioles, binding of Edn-1 to the ETA receptor promotes the outflow of calcium and contraction of SMCs, thereby inducing vasoconstriction and reduction of blood flow to the surrounding tissue (Yanagisawa et al. 1988). Administration of Edn-1 in humans impairs endothelium-dependant vasodilation. This highlights the important interaction between Edn-1 and endothelium-derived factors such as NO in the regulation of vessel tone and vascular remodeling (Bourque, Davidge, and Adams 2011).

1.3.3 Shear stress and Arteriole Remodeling

Vascular endothelial cells are highly sensitive to shear stress. Mechanotransduction of endothelial cells, induced by laminar shear stress, is essential for the proper distribution of blood, regulation of blood pressure, and maintenance of vessel structure (Chien 2007; Mack et al. 2009). In arterioles, an increase in shear stress can alter vascular tone or induce structural remodeling depending on the duration of the shear stimulus. Acute stimulation of endothelial cells by shear stress, due to a transient increase in blood flow, promotes release of shear responsive stimuli such as NO and prostaglandins. This induces vasodilation of arterioles and adequate delivery of nutrients to the surrounding tissue (Takahashi et al. 1997). Sustained shear-stress promotes structural remodeling of the arterioles, which under physiological conditions, serves as a homeostatic response to accommodate increases in flow demand and facilitates delivery of nutrients within a growing tissue (Mack et al. 2009). This could result in adaptive outward remodeling of arterioles (arterioles with wider lumen), or arteriolarization of tissue capillaries (increase in arteriole content). Both of these processes require higher endothelial and smooth muscle cell content. In endothelial cells, prolonged shear stress can induce expression of a number of signaling molecules such as platelet-derived growth factor B (PDGFB), kruppel-like factor 2 (KLF-2), and Transforming growth factor-β (TGF-β) (Dekker et al. 2002; Mitsumata et al. 1993; Walshe, dela Paz, and D'Amore 2013). The role of these endothelial-derived factors in the control of SMC and/or endothelial cell proliferation and integrity of vessel structure have been characterized by others. Models of arterial injury have identified TGF-β as a critical regulator of smooth muscle cell proliferation and migration (Song et al. 2000). Furthermore, studies have shown that deletion of KLF2 or PDGFB genes results in embryonic lethality of mice, due to defects in vessel formation and vascular wall stability (Lindahl et al. 1998). Overall, shear-stress induced

remodeling of arteriole structure constitutes a control mechanism to maintain vascular homeostasis and delivery of substrates to the tissue.

1.4 Skeletal Muscle Angiogenesis

Skeletal muscle is a highly plastic tissue and can adapt to changes in contractile activity (e.g. exercise and denervation), nutrient supply, and environmental stimuli (e.g. hypoxia) (Flück and Hoppeler 2003). These adaptations entail alterations in muscle capillary content which can significantly impact local tissue and whole-body metabolism given that skeletal muscle makes up ~40% of body mass in humans (Olfert et al. 2016). Within skeletal muscle, capillaries run in parallel with the longitudinal axis of muscle fibers. Thus, an increase in muscle capillary content (angiogenesis) increases the surface area for nutrient and gas exchange, thereby facilitating function of individual muscle fibers. Physiological angiogenesis in skeletal muscle has been mainly studied in response to repeated exercise since it serves as one of the main angiogenic stimuli *in vivo* (Egginton 2011).

Capillary growth in skeletal muscle is initiated when oxygen and nutrient delivery is inadequate to meet the metabolic demand of myocytes. This activates a complex array of mechanical and metabolic stimuli which can simultaneously activate endothelial cells and promote angiogenesis. The mechanical stimuli include muscle stretch and flow-mediated shear stress (Egginton et al. 1998; Milkiewicz et al. 2001). Muscle stretch can alter endothelial cell shape. This leads to the activation of integrins and production of growth factors such as VEGF-A, as well increases in the activity of MMPs required for extracellular matrix remodeling during angiogenesis (Haas et al. 2000; Milkiewicz et al. 2007). Shear stress in capillaries results from increases in blood flow by vascular conductance and flow-meditated dilation. Elevated shear stress by administration

of vasodilator drugs (α1 adrenergic receptor antagonists) can increase skeletal muscle capillary content (Gee, Milkiewicz, and Haas 2010). Actions of shear stress are in part mediated by increases in VEGF-A and eNOS expression necessary for endothelial cell proliferation and survival (Baum et al. 2004; Wragg et al. 2014). Hypoxia can stimulate angiogenesis via activation of HIF-1 pathway. HIF-1 is a transcription factor consisting of the subunits HIF-1α and HIF-1β. In normoxia, HIF-1α is targeted for degradation. However, hypoxia supresses the degradation of HIF-1α, which then stabilizes and gets translocated to the nucleus to induce VEGF-A expression. *In vitro*, hypoxia can promote formation of capillary like structures via VEGF induced proliferation and migration of endothelial cells (Shweiki et al. 1992). Furthermore, secretion of local metabolites can also exert angiogenic influences within skeletal muscle. For example, adenosine and lactic acid can induce proliferation and migration of endothelial cells (Langberg et al. 2002; Murray and Wilson 2001). The homeostatic balance between metabolic demand and capillary content can be disrupted with inactivity and in various disease conditions such as obesity, diabetes and peripheral artery disease. This may be as a result of microvascular dysfunction or a decrease in capillary content which will be discussed in further detail in the context of obesity.

1.5 Adipose Tissue Angiogenesis

Of all tissues in the body, adipose tissue may be the most plastic and has the greatest capacity to expand (Corvera and Gealekman 2014). Healthy expansion of adipose tissue requires a concomitant increase in the adipose vascular network for a maintained substrate delivery and metabolic homeostasis of the tissue. Regulation of adipose tissue angiogenesis relies on reciprocal local cues from adipocytes and endothelial cells (Cao 2007). Experimental evidence has shown that adipose tissue homogenates and pre-adipocyte condition media can induce angiogenesis *in*

vivo, within cornea and chick chorioallantoic membranes (CAM) (Ledoux et al. 2008; Silverman et al. 1988). Adipocyte release of numerous pro-angiogenic factors and pro-angiogenic adipokines can stimulate an angiogenic process. Adipocytes are a source of VEGF-A and leptin which can stimulate endothelial cell proliferation and migration (Cao et al. 2001). Experiments with adipocyte specific deletion of VEGF show decreased vascularization within adipose tissue. Conversely, overexpression of VEGF seems to rescue this phenotype by maintaining the angiogenic capacity of the adipose tissue (Sung et al. 2013). Furthermore, VEGFR-2 blockade in implanted pre-adipocytes reduces tissue angiogenesis, as observed by intravital microscopy (Fukumura et al. 2003). This highlights the dominant role of VEGF-A in adipose tissue angiogenesis. Adiponectin is another pro-angiogenic adipokine that can enhance the angiogenic potential within adipose tissue. Adiponectin can activate Akt signaling within endothelial cells and indirectly induce endothelial cell migration and survival (via eNOS phosphorylation and VEGF expression) (Ouchi et al. 2004). Furthermore, over-expression of adiponectin within adipose tissue of mice increases tissue vascular density and perfusion, as assessed by microsphere injection (Aprahamian 2013). Studies have also demonstrated that mature adipocytes secret matrix metalloproteinases (e.g. MMP-2 and MMP-9) (Brown et al. 1997), which can facilitate sprouting angiogenesis by promotion of ECM degradation. Tissue hypoxia may also have a role in adipose tissue angiogenesis.

Under hypoxic conditions, expression of angiogenic factors VEGF-A and leptin may be increased because both of these genes are hypoxia inducible (Grosfeld et al. 2002; Shweiki et al. 1992). Consistent with this idea, it has been shown that under low oxygen conditions, cultured pre-adipocytes release leptin, VEGF and MMPs, which can potentially induce an angiogenic response (Lolmède et al. 2003). An alternate approach in understanding the influence hypoxia on adipose

angiogenesis has been through over-expression or HIF-1 subunits. Overexpression of adipose HIF-1α fails to induce a pro-angiogenic response and increases adipose tissue inflammation and fibrosis instead (Halberg et al. 2009). To date, limited data exist regarding physiological regulation of angiogenesis within adipose tissue. The majority of studies documenting adipose tissue angiogenesis have been conducted in obese individuals or models of HF-diet obesity, which will be discussed in detail in an upcoming section.

1.6 Skeletal Muscle and Adipose Tissue Metabolism

Skeletal muscle and adipose tissue are key insulin targets involved in the regulation of energy storage and utilization. Insulin is synthesized by β-pancreatic cells and released into the vasculature in response to post-prandial glucose influx. Insulin signaling initiates by binding of insulin to its receptor (IR) located on the cell membrane (Kahn and White 1988). This leads to the phosphorylation and activation of insulin receptor substrates 1/2 (IRS1/2). IRS1/2 can activate two main downstream signaling pathways, the phosphatidylinositol 3-kinase (PI3K) pathway and the Ras-mitogen-activated protein kinase (MAPK) pathway (Rask-Madsen and Kahn 2012). Activation of the PI3K pathway is required for insulin-stimulated glucose transport into myocytes and adipocytes (Cheatham et al. 1994). Akt is one of the main downstream targets of PI3K which gains full enzymatic activity upon phosphorylation. Phosphorylated Akt stimulates the translocation of Glucose Transporter 4 (GLUT4) to the plasma, leading to the uptake of glucose (Kupriyanova and Kandror 1999). Within skeletal muscle and adipose tissue, basal uptake of glucose can also be facilitated via Glucose trasporter 1 (GLUT1), which does not rely on the presence of insulin (Kraegen et al. 1993). However, GLUT4 is the predominant controller of glucose influx into myocytes and adipocytes (Ishiki and Klip 2005).

Approximately 80% of insulin-stimulated glucose disposal occurs within skeletal muscle (Rask-Madsen and Kahn 2012). Insulin regulates the rate of glucose transport and glycolysis mainly through the PI3K-AKT pathway. Healthy skeletal muscle alternates between carbohydrate or lipid use (Stump et al. 2006a). In times of glucose abundance, skeletal muscle uses glucose as the main source of ATP production (via oxidative phosphorylation), or stores it in the form of glycogen. In times of energy deprivation such as low glucose availability or high metabolic demand of exercise, skeletal muscle utilizes free fatty acids (FFAs) for energy production. This requires break down of muscle triglyceride stores or uptake FFAs from the circulation.

The primary function of adipose tissue is synthesis and storage of triglycerides in periods of energy excess, and their hydrolysis and release of free fatty acids in periods of energy deprivation. <10% of insulin-mediated glucose uptake occurs within adipose tissue (Dimitriadis et al. 2011) Similar to skeletal muscle, interaction of insulin with its receptor stimulates the activation of the PI3K-AKT pathway and uptake of glucose, where it can be used to synthesize glycerol. Within adipose tissue, circulating free fatty acids released from the liver are taken up by fatty acid transporters (Schwenk et al. 2010). In a fed state, insulin promotes adipose tissue lipogenesis, where glycerol and FFA are used to synthesize triglycerides. This serves as the main reservoir of energy for the body and prevents accumulation of lipids within plasma and peripheral organs (Newsholme and Dimitriadis 2001). During periods of energy demand, triglycerides are rapidly mobilized by the activity of hydrolytic lipases. In adipose tissue, hydrolysis of triglycerides depends in large part on the activity of Hormone-sensitive lipase (HSL) (Ahmadian et al. 2007). HSL can be phosphorylated and activated via beta-adrenergic stimulation. In adipocytes, catecholamines bind to Gs- coupled receptors which in turn activate adenylyl cyclase to generate cyclic AMP. Rising levels of cAMP activates protein kinase A (PKA) which can phosphorylates

HSL at three serine residues (Jocken and Blaak 2008). Phosphorylated HSL catalyzes triglyceride breakdown into FFAs in a process called lipolysis. FFAs can be be utilized by adipocytes for ATP production (lipid oxidation) and released as a source of energy and membrane components for other organs. In the post-prandial state, insulin inhibits lipolysis which limits FFA release into the plasma. Anti-lipolytic actions of insulin are mostly mediated through activation of the phosphodiesterase enzymes which lower cAMP levels and activity of PKA as a consequence. Overall, skeletal muscle and adipose tissue metabolism is coordinated through integration of processes (glycolysis, lipolysis and lipogenesis) that are regulated by the metabolic actions of insulin. To carry out such metabolic actions, in addition to a need for a competent insulin signaling pathway, insulin must first arrive and have access to the target tissue via the microvasculature.

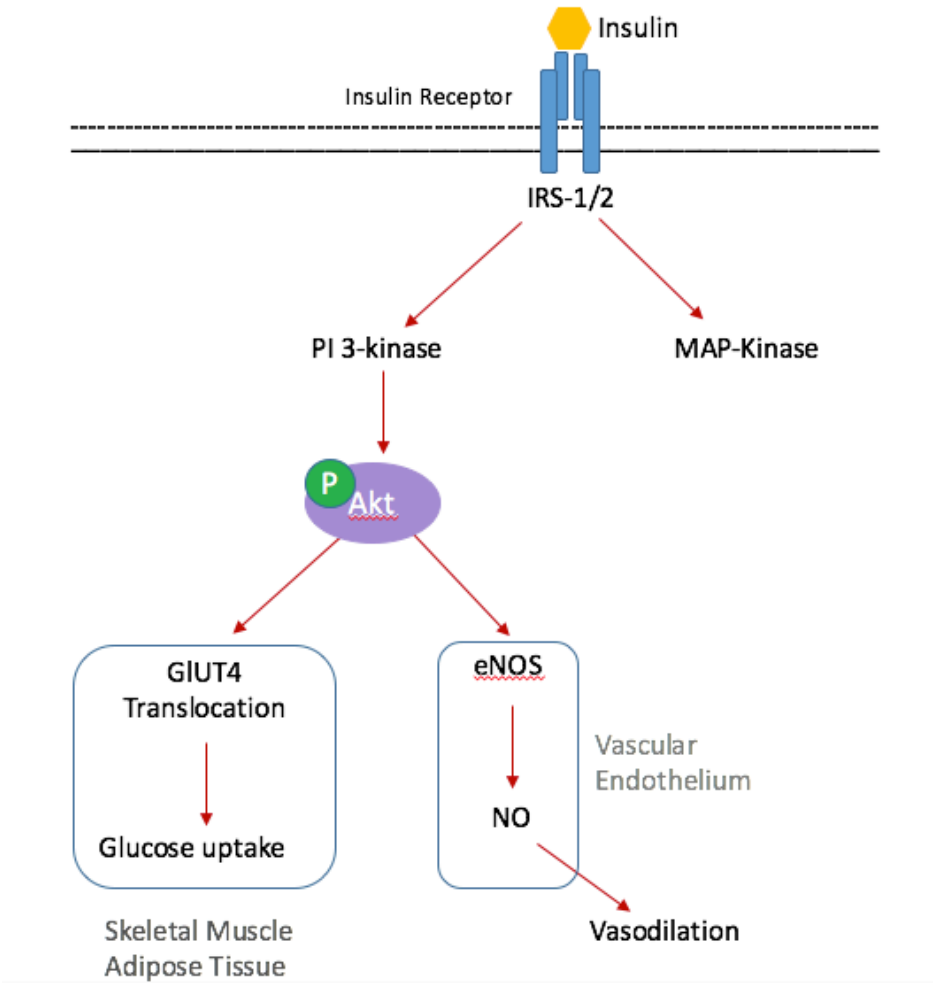

Figure 1.1: Insulin signaling
Insulin binds to its receptor on the cell membrane. This activates insulin receptor substrates 1/2 (IRS1/2). IRS1/2 can activate two main downstream signaling pathways, the PI3K pathway and the MAPK pathway. Akt is one of the main downstream targets of PI3K pathway. Within skeletal myocytes and adipocytes, phosphorylated Akt stimulates the translocation of Glucose Transporter 4 (GLUT4) to the plasma membrane and uptake of glucose. Within the vascular endothelium, Akt promotes production of NO via activation of eNOS. NO induces vasodilation and increases perfusion of capillaries and delivery of insulin to the tissue.

1.6.1 Vascular Influence on Insulin Signaling and Metabolism

Insulin secreted by beta pancreatic cells is released into the circulation. In both skeletal muscle and adipose tissue, delivery of insulin to carry out metabolic functions relies on the tissue vasculature. This is regulated by the vasculature via hemodynamic changes in blood flow as well as microvascular content and function. The first component of insulin delivery is that blood carrying the hormone must perfuse the target tissue via arterioles and capillaries. In principal, if tissue capillary and arteriole number is higher, there is more blood flow and a higher capacity for insulin delivery and insulin signaling within the tissue. Importantly, insulin itself increases blood flow by inducing vasodilation of arterioles (Raitakari et al. 1995). Similar to myocytes and adipocytes, insulin signaling in the endothelium activates the PI3K-AKT pathway. In endothelial cells, this leads to the activation of eNOS and production of NO which then inhibits calcium influx into SMCs and induces vasodilation. This has been observed in animal studies using euglycemic clamps, showing that increases in plasma insulin concentration increases hind limb blood volume, which precedes the activation of the insulin signaling pathway (Raitakari et al. 1995; Vincent et al. 2004). However, insulin-stimulated changes in blood flow can be partially reduced by inhibitors of eNOS (Vincent et al. 2004). The net increase in blood flow increases perfusion of capillaries and enhances insulin arrival to the tissue.

The second component of insulin delivery to the tissue is that insulin must cross the capillaries to reach the interstitium. In muscle and adipose tissue where capillaries have a continuous arrangement of endothelial cells joined by cell-cell junctions, insulin can diffuse between adjacent endothelial cells or by endothelial transcytosis (Lee and Klip 2016). The role of endothelial cells in this step of insulin delivery is less understood and currently under investigation. Regardless of the means of insulin transfer across the endothelium, total capillary content can

influence the net amount of insulin available to myocytes and adipocytes. A higher capillary number within the tissue increases the surface area for transfer of insulin across the endothelium. This can facilitate binding of insulin to its receptor and activation of downstream pathways for glucose disposal. On the other hand, if there is a reduction in capillary number, total supply of insulin to the tissue decreases. This may in turn result in a decrease in tissue responsiveness to insulin, thereby compromising systemic insulin sensitivity and glucose tolerance. Insulin's influence on tissue uptake of glucose and metabolism is closely linked to the vascular delivery of insulin. Therefore, deficits in skeletal muscle and adipose tissue vascular niche may contribute to impairments in the metabolic action of insulin in obesity-induced metabolic dysfunctions.

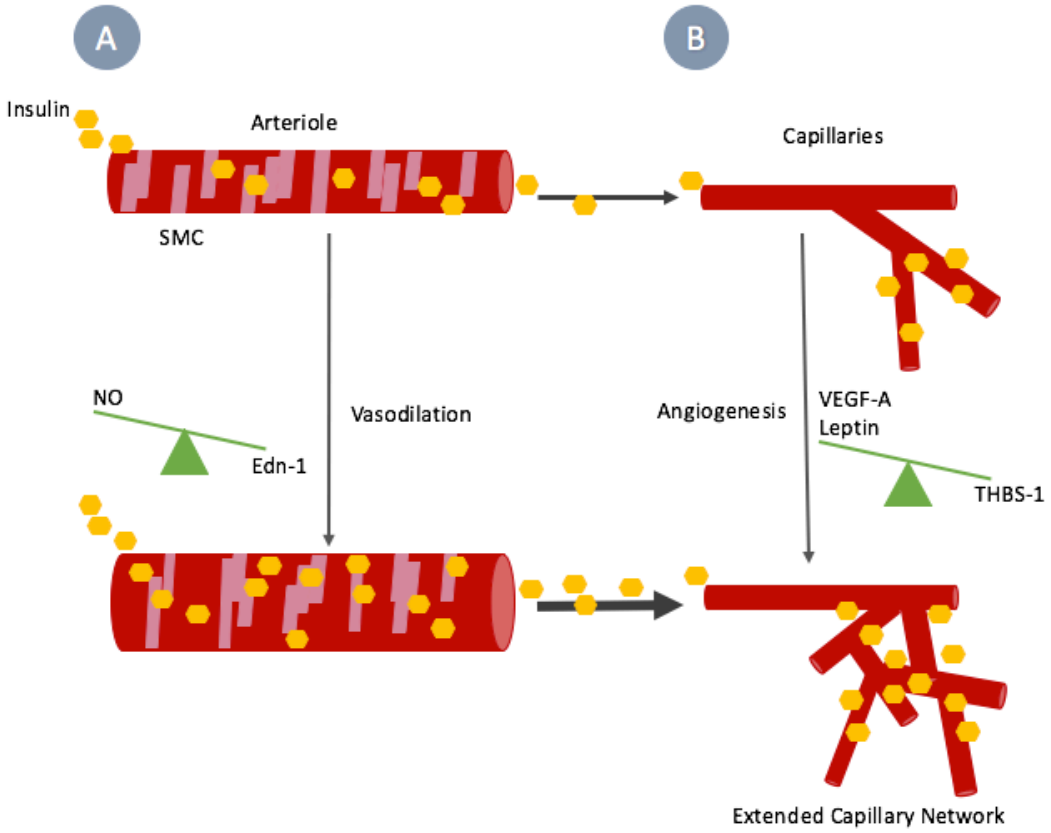

Figure 1.2: Vascular dependent delivery of insulin: influence of blood flow and capillary number

A) Insulin is released from the pancreas and is delivered via tissue arterioles. The net delivery of insulin depends on arteriole tone, which is regulated by smooth muscle cells (SMC). Increased levels of vasodilators (e.g. NO) paralleled by a decreased release of vasoconstrictors (e.g. edn-1) induces vasodilation of arterioles. As a result, blood flow increases, which enhances total insulin arrival to the tissue. **B)** At the level of capillaries insulin must exit the endothelium to reach the tissue interstitium. Angiogenesis, induced by an increase in the level of pro-angiogenic factors (VEGF-A, Leptin) relative to anti-angiogenic factors (e.g. THBS-1) increases capillary number within the tissue. A higher capillary number increases the surface area for transfer of insulin across the endothelium, which can facilitate binding of insulin to its receptor and activation of downstream pathways for glucose disposal.

1.7 Obesity-induced Metabolic Dysregulation: The Vascular Side

Obesity is a multifactorial condition associated with the development of ectopic lipid accumulation, resistance to metabolic stimuli, and chronic systemic inflammation. Central adiposity is closely associated with an increased risk in the development of metabolic disorders such as insulin resistance and type 2 diabetes (Martyn, Kaneki, and Yasuhara 2008). Insulin resistance refers to impairments in the tissue response to insulin. In both skeletal muscle and adipose tissue, this leads to deficits in insulin's regulation of glucose and fatty acid metabolism and dysregulation of metabolic processes mentioned earlier. The majority of experimental studies documenting the deleterious effects of a high-fat diet have mainly focused on tissue metabolism and overlooked the critical role that tissue capillaries and arterioles play the maintenance of insulin delivery and tissue metabolic function. Over the past few decades, a link between the tissue microvascular content and function, exchange of metabolic stimuli and insulin sensitivity has been recognized (Barrett et al. 2009; Frisbee et al. 2006; Goodwill and Frisbee 2012). Also, a growing body of evidence has shown that vascular dysfunction occurs prior to, or in parallel to, conditions such as insulin resistance and type II diabetes (Kim et al. 2008; Natali et al. 2006). In insulin resistant states, endothelial dysfunction at the levels of tissue arterioles has been shown to reduce blood flow to the muscle. This involves reduced vasodilation due to increases in the production of reactive oxygen species (ROS) paralleled with impairments in endothelial NO production (Goodwill and Frisbee 2012; Xiang et al. 2008). Furthermore, increases in the release of fatty acids, as occurs in obesity, can impair endothelial production of NO and further reduce delivery of insulin to the tissue (Steinberg and Baron 2002). Although impairments in the delivery of insulin via tissue arterioles has gained attention (Muniyappa and Quon 2007), only few studies have

examined the impact of HF diet-induced obesity on tissue capillary content, which is the focus of the next two sections.

1.7.1 Skeletal Muscle Capillaries: Influence of Obesity

Capillary number and distribution within a muscle must be sufficient to maintain a balance between nutrient supply and metabolic demands of the myocytes**.** In some pathological conditions, this balance gets disrupted, in which case the capillary network fails to maintain perfusion of myocytes. Several studies have shown that skeletal muscle capillary content is reduced (capillary rarefaction) in conditions of HF diet-induced obesity, insulin resistance or diabetes. In rodent studies, capillary density and capillary-to-fiber ratio is significantly decreased within skeletal muscle of diet-induced obese diabetic male animals (Frisbee 2003; Frisbee et al. 2006; Kivela et al. 2006; Kondo et al. 2011). This pattern is also evident in humans as capillary density has been shown to be inversely correlated with waist circumference and decreased in the muscle of obese and diabetic males (Gavin et al. 2004; Lillioja et al. 1987). Such reductions in microvascular content in turn are closely linked to impairments in skeletal muscle insulin responsiveness (Solomon et al. 2011). Insulin-stimulated blood flow and glucose uptake have been shown to be proportional to capillary density, meanwhile, fasting glucose and insulin levels are inversely related to capillary density (Hedman et al. 2001; Krotkiewski et al. 1983; Lillioja et al. 1987). A reduction in muscle capillary content may be due to capillary rarefaction, or restraint in the growth of new capillaries. A high-fat diet may reduce muscle capillary content by promoting an angiostatic microenvironment, which in turn contributes to disturbances in the metabolic pathways of skeletal muscle. For example, levels of Thbs-1 have been shown to increase in the plasma and muscle of male mice fed a high-fat diet (Inoue et al. 2013). Importantly, the majority of studies that have

reported a reduction in muscle capillary content have used models of obesity associated with diabetes or insulin resistance (Frisbee et al. 2006). In this case the skeletal muscle response may be more severe because diabetes can repress an angiogenic response by inducing tissue inflammation and production of reactive oxygen species which can promote endothelial cell dysfunction and apoptosis (Fridlyand and Philipson 2006; van den Oever et al. 2010).

Conversely, some studies have reported a lack of change or an increase in capillary content within skeletal muscle of rodents fed a HF diet. Roudier et al. have shown that capillary density and capillary-to-fiber ratio does not change within plantaris muscle of female rats fed a HF diet (Roudier et al. 2009). However, in a mouse model of chronic HF feeding, Silvennoinen et al. reported that HF diet induces angiogenesis and increases capillary content within skeletal muscle of male mice. This was paralleled with an increase in VEGF-A and HIF-1 mRNA expression within endothelial cells of HF fed animals. However, within the muscle homogenate, only VEGF-A protein levels were increased (Silvennoinen et al. 2013). Thus currently, there is mixed evidence regarding the influence of obesity on skeletal muscle angiogenesis. The majority of present data suggests a reduction in capillary content when obesity is associated with metabolic dysfunctions such as type 2 diabetes. However cellular mechanisms underlying this reduction remain to be identified. Therefore, more research is needed to build a comprehensive knowledge regarding influence of HF diet-induce obesity on capillary adaptations within skeletal muscle.

1.7.2 Adipose Tissue Capillaries: Influence of Obesity

Expansion of adipose tissue requires an increase in tissue vascularization for the maintenance of metabolic homeostasis. However, in obesity, adipose capillary content may not keep pace with the rapid growth of visceral white adipose tissue (Goossens and Blaak 2015). In

obese humans and rodents, adipose tissue blood flow is significantly reduced, which correlates with a decrease in adipose tissue capillary density (Gealekman et al. 2011; Goossens et al. 2011; Hosogai et al. 2007; Pasarica et al. 2008). A reduction in blood flow may be accompanied by adipose tissue hypoxia in obese individuals (Pasarica et al. 2009). Low oxygen tension within adipose tissue of genetically obese mice (e.g. ob/ob mice) and mouse models of diet induced obesity has also been observed using needle-type O2 sensors or by measurement of hypoxia marker pimonidazole (Hosogai et al. 2007; Rausch et al. 2008; Trayhurn and Alomar 2015). Under physiological conditions, this should lead to the stabilization of HIF-1, upregulation of VEGF-A protein, and a concomitant increase in tissue angiogenesis. However, VEGF-A level does not increase within adipose tissue of obese male mice (Voros et al. 2005). In obesity, hypoxia seems to have a primary role in triggering a pro- inflammatory and fibrotic environment within the adipose tissue (Corvera and Gealekman 2014; Halberg et al. 2009). For example, an increase in the production of pro-inflammatory adipokines (e.g. leptin) and activation of tissue macrophages can induce production of tumor necrosis factor-α which can induce endothelial cell apoptosis, resulting in capillary rarefaction (van der Heijden et al. 2015). Studies have shown an up-regulation in gene expression of anti-angiogenic factors such as THBS-1 within adipose tissue of animals on a high-fat diet (Moraes et al. 2003; Voros et al. 2005). Upregulation of anti-angiogenic along with a lack of hypoxia-induced VEGF expression may suggest that in obesity, anti-angiogenic pathways may overcome pro-angiogenic pathways, further contributing to capillary rarefaction. To date, mechanisms underlying the failure of an angiogenic response within adipose tissue of obese and insulin resistant individuals have not been established.

1.8 Sex Differences in Obesity-Related Metabolic Disorders

In most industrialized countries, the increased prevalence of obesity and comorbidities associated with it is a major health concern. Although both males and females are affected, the susceptibility to develop obesity-related metabolic disorders is higher in males (Fuente-Martín et al. 2013; Kanter and Caballero 2012; Power and Schulkin 2008; Shi et al. 2012). In developed countries, more males are obese versus pre-menopausal females (Kanter and Caballero 2012). This coincides with a higher prevalence of insulin resistance, hypertension, and dyslipidemia in males compared to females (Beigh and Jain 2012; Eliza B. Geer and Shen 2009). In a report released by Statistics Canada, 62% of men versus 46 % of women have an increased health risk due to excess weight gain (http://www.statcan.gc.ca/pub/82-625-x/2015001/article/14185-eng.htm). In experimental animal models, the metabolic challenge of a HF diet induces more weight gain in males compared to females (Benz et al. 2012; Hong et al. 2009a). Moreover, with chronic consumption of a HF diet, male rodents are more often to develop severe hypertension, systemic glucose intolerance and insulin resistance (Benz et al. 2012; Renee E. Stubbins et al. 2012). Interestingly, even in a study of weight matched male and female mice, HF-fed females maintain a better glucose tolerance compared to HF-fed male mice (Nickelson et al. 2012).

1.8.1 Mechanisms Involved in Sex-specific Influence of Obesity

To date, established molecular mechanisms responsible for the sex-specific responses to a high-fat diet and obesity are limited. Differences in patterns of fat distribution and metabolism may be one of the underlying reasons for sex-dependent differences in the metabolic risk of obesity. Pre-menopausal females have a lower waist-to-hip ratio and predominantly accumulate subcutaneous adipose tissue, whereas males store more fat in the visceral region (Kotani et al.

1994; Lönnqvist et al. 1997). Studies have shown that higher visceral adiposity substantially increases the risk of insulin resistance and is correlated with mortality from cardiovascular and metabolic disorders (Björntorp et al. 1997; Kaplan 1989). Therefore, accumulation of visceral adipose tissue may predispose males to the development of the aforementioned disorders. Furthermore, visceral adipose tissue of female rats has been shown to have a higher lipolytic capacity compared to males (Lladó et al. 2002). This is accompanied by a lower expression of hormones involved in lipid hydrolysis (i.e. HSL) in the adipose tissue of males (Stubbins et al. 2012). Thus, in females, fat accumulated within visceral adipose tissue may be more easily oxidized or mobilized as a source of energy for other organs. In addition, FFA uptake is higher in subcutaneous adipose tissue of pre-menopausal females than in males (Shadid et al. 2007). This may in turn contribute to lower levels of circulating lipids, lower ectopic fat accumulation and an improved metabolic status in females compared to males during weight gain.

HF diet-induced adipose tissue expansion is also accompanied by sex differences in inflammatory responses. Production of pro-inflammatory cytokines is higher in the skeletal muscle and adipose tissue of male mice compared to female mice (Estrany et al. 2013). A HF diet results in greater increases in TNF- and IL 6 in male compared to female mice (Estrany et al. 2013; Taylor and Sullivan 2016). Also, the presence of crown–like structures (macrophages surrounding apoptotic cells) is significantly higher within adipose of HF-fed male compared to HF-fed female mice (Nickelson et al. 2012). Females are reported to have higher levels of adiponectin, which is an anti-inflammatory cytokine (Cnop et al. 2003). In mice, a HF diet induces a decrease in adiponectin levels in males, whereas, in females adiponectin levels are maintained (Nickelson et al. 2012).

Notably, following menopause and the decline in estrogen levels, females gain more visceral adiposity and have an increased risk to develop insulin resistance (Catalano et al. 2008; Toth et al. 2000). In experimental models, ovariectomized female mice mimic male mice in their propensity to develop weight gain and insulin resistance (Hong et al. 2009a). This brings forth the protective role of estrogen in the development of metabolic dysfunctions (Riant et al. 2009). Estrogens act through two nuclear receptors ERα and ERβ. Loss of ERα signaling has been shown to exacerbate the adverse effects of a HF diet and insulin resistance in both skeletal muscle and adipose tissue of female rats (Gorres et al. 2011). The beneficial effects of estrogen in the maintenance of insulin sensitivity is mediated by different mechanisms. Estrogen has direct effects on insulin-mediated uptake of glucose by increasing expression of GLUT4 (Rüegg et al. 2011). Furthermore estrogen may have anti-inflammatory properties, since expression of inflammatory cytokines (e.g. TNF and IL6) is significantly reduced with estrogen treatment in ovariectomized rats compared to their untreated counterparts (Arenas et al. 2005). However, effects of estrogen therapy in post-menopausal women is controversial and estrogen therapy does not significantly improve the metabolic status of obese woman (Ryan et al. 2002; Saglam 2004). This suggests that a higher level of estrogen alone is not the only contributor to a maintained metabolic profile in females.

Overall, sex-related differences in HF diet-induced weight gain and metabolic function likely are multifaceted. Capillaries within skeletal muscle and adipose tissue are critical regulators of metabolic homeostasis, enabling the appropriate exchange of gasses, hormones and energy sources. Thus, their content and phenotype can influence the overall health of the tissue. To date, most of what we know about the influence of a high-fat diet on tissue capillary beds is from studies that have investigated only male animals. Furthermore, the majority of experimental studies

investigating sex-associated differences in obesity have predominantly focused on tissue metabolism and fat distribution, while the microvascular contribution to these differences has been overlooked. In this context, the goal of this project is to examine if a high-fat diet influences the vascular niche of male and female skeletal muscle and adipose tissue in a sex-dependent way and whether this can be linked to differences in obesity-related metabolic complications.

1.9 Study Objectives

1. To examine if exposure to a HF diet alters muscle and adipose tissue responsiveness to metabolic stimuli in a sex-specific manner.

2. To examine if sex-related differences exist in microvascular content within the muscle and adipose tissue of male and female mice on a normal diet or under the metabolic stress of a HF diet.

1.10 Hypotheses:

1. Sex-dependent differences in angiogenic responsiveness exist in skeletal muscle and adipose tissue of male and female mice on a high-fat diet.

2. This contributes to a worsened metabolic profile in males compared to females.

Chapter 2: Methods

2.1 Ethical Approval

Animal studies were approved by York University Committee on Animal Care, and performed in accordance with the Canadian Council for Animal Care Guidelines.

2.2 Mouse Model of High-fat Diet-induced Obesity

FVB/B6 Mouse colonies were initiated and maintained in York University vivarium on a 12-hour light-dark cycle. Male and Female mice at 6-8 weeks of age were placed on a High-fat (HF) or a calorie matched normal chow (NC) diet for 16 weeks (n=8 per group). The HF diet consisted of 58 kcal% fat, 25.5% carbohydrate and 16.4% protein and the NC Diet consisted of 10.5 kcal% fat, 73.1% carbohydrate and 16.4% protein (D12331 and D12329 respectively; Research Diets Inc., NJ, USA). Water and food was provided *ad libitum*. Body weight and food consumption were recorded weekly. Body weight gain was calculated over 14 weeks (week 14 -week 0). Total Kcal consumed calculated by multiplying grams of food consumed by kcal/g (NC: 4.07 kcal/g, HF 5.56 kcal/g) averaged over 14 weeks. Feed efficiency, which is the amount of kcal needed to gain 1 g of weight, was calculated by dividing cumulative weight gain by cumulative kcal consumed over 14 weeks. Systemic glucose handling characteristics of the mice were assessed by insulin and glucose tolerance tests on weeks 8, 14 and 15. Blood pressure and heart rate were recorded using a non-invasive tail-cuff pressure system (CODA surgical monitor, Kent Scientific, CT, USA) on week 13, while mice were lightly sedated by isoflurane inhalation and body temperature was maintained by placing mice on a heat pad. Animals were allowed to recover at least 72 hours after which hind-limb skeletal muscles and visceral adipose tissue were removed under isoflurane

anesthesia. Tissues were weighed and snap frozen in liquid nitrogen for biochemical analyses, or frozen in cooled isopentane for histology and kept at -80°C. Mice were euthanized by exsanguination.

2.3 Systemic Metabolic Profiling

Insulin tolerance tests (ITT) were conducted during diet weeks 8 and 14 after 4 hours without food. Fasted blood glucose levels were measured before mice receive intraperitoneal (IP) injections of insulin (0.75U/kg body weight, Humalog, Lilly, ON, Canada). Blood glucose levels were measured 20, 40 and 60 minutes post insulin injection. Glucose tolerance test (GTT) was taken on week 15 after 16 hours without food. Fasted blood glucose levels were measured before IP injections of glucose (1.75g/kg body weight, D-(+)-Glucose dissolved in ddH_2O, Sigma Aldrich Co., MO, USA). Blood glucose levels were measured 30, 60, 90, 120, and 180 minutes post glucose injection. For both tests, blood was collected by saphenous vein puncture and blood glucose levels were measured with a glucometer (Freestyle Lite, Abbott Diabetes Care, ON, Canada). IP-ITT: Blood glucose levels relative to baseline (time 0) were graphed over time and glucose response curve was calculated using baseline glucose levels as reference for each animal (Area over curve-AOC). IP-GTT: blood glucose values were graphed over time and the area of the glucose response curve was calculated using baseline glucose levels as reference for each animal (Area under curve-AUC).

2.4 *In vivo* and *ex vivo* Functional Analysis

Insulin stimulation of skeletal muscle was conducted *in-vivo* while mice were maintained under isoflurane anesthesia. One extensor digitorum longus (EDL) muscle was collected and snap frozen. The mice were then injected intraperitoneally with 0.12 U of insulin (0.25U insulin/ ml of sterile HEPES-saline). After 15 minutes, EDL from the contralateral leg was removed and snap frozen in liquid nitrogen for protein analysis.

Ex-vivo stimulation of visceral white adipose was conducted by surgically removing a portion of perigonadal adipose prior to insulin injection. This adipose was divided into 3 portions (~80 mg each) and pre-incubated in low-glucose DMEM containing 1% fatty-acid free Bovine serum albumin (BSA; Sigma Aldrich, #A8806-5G, ON, Canada) for 30 minutes at 37°C, followed by addition of insulin (25mU/ml), isoproterenol (0.1mM, #1747 Tocris Bioscience, Bristol, UK) or vehicle and incubation at 37°C for 30 minutes. At the end of the incubation, depots were snap frozen in liquid nitrogen for protein analysis.

2.5 Skeletal Muscle Immunohistochemistry

10 μm cryo-sections of EDL muscle were fixed in 3.7% formaldehyde in 1x PBS for 20 minutes. Sections were blocked using 5% goat serum (#318773, Thermo Fisher Scientific, MA, USA) and 0.01% triton in 1x PBS for 30 minutes. Sections were stained for 1 hour with fluorescein isothiocyanate-conjugated *Griffonia Simplicifolia* Lectin-1 (1:100; Vector Labratories, ON, Canada) to detect capillaries and Rhodamine Wheat Germ Agglutinin (1:1000; Vector Labratories, ON, Canada) to detect muscle fibers. Sections were washed with 1xPBS, mounted on coverslips and placed in a moist dark area over night. Slides were viewed using an Axiovert 200M Zeiss microscope with 20x objective. Images were captured using a cooled CCD camera and Metamorph

imaging software. The numbers of capillaries and fibers in 3-4 independent fields of view were counted for each animal. Capillary-to- fiber ratio was calculated as a measure of skeletal muscle capillary content. Capillary number was quantified as capillary density (#capillaries/mm^2) and myocyte cross-sectional area was calculated by image thresholding on the ImageJ software

2.6 Adipose Tissue Histology

Visceral white adipose tissue from the perigonadal region was sectioned into small pieces (~1 mm^3) and incubated with Bodipy® 493/503 (0.25ug/ml; Molecular Probes, Thermo Fisher Scientific, MA, USA) as a lipid droplet marker and Rhodamine *Griffonia Simplicifolia* Lectin-1 (1:100 dilution; Vector Laboratories, ON, Canada) as an endothelial cell marker for 20 minutes and shielded from light. Samples were washed 3 times with 1x PBS (10 minutes per wash). Whole-mount adipose tissue was visualized using a Zeiss LSM 700 confocal microscope with 10x objective. Setting one channel for lectin and another for Bodipy®, an average of 4 scans were taken from the same focal plane using the speed of 3 to ensure optimal quality of images. 3-4 independent fields of view were selected randomly for each animal. Percent area of field of view (% FOV) covered by lectin (as an indicator of tissue capillary content) was quantified by image thresholding, and Vessel diameter and number of branch points were quantified on the ImageJ software.

2.7 Protein Extraction

Protein was extracted using 20-30 mg of frozen skeletal muscle (EDL and EHP) or visceral white adipose tissue (perigonadal adipose region) in Radio immunoprecipitation assay (RIPA) lysis buffer containing 1X protease inhibitor cocktail (Complete mini; Roche Diagnostics, QC, Canada)

and 1X phosphatase inhibitor cocktail (PhosSTOP; Roche Diagnostics, QC, Canada). Tissue Lysis was performed using a Retsch MM400 tissue lyser (GmbH, Haan, Germany) and metal beads, followed by one hour of agitation at 4°C. Samples were then centrifuged at 12,000g (Heraeus Biofuge Stratos, Thermoscientific, ON, Canada) for 20 minutes at 4°C to remove cell debris. Supernatant was collected and stored at -80°C.

2.8 Protein Quantification

Protein extracts were quantified by bicinchoninic acid assay (BCA) (Pierce, Fisher Thermoscientific, ON, Canada). Lysates were loaded in duplicates on 96 well plates with working reagent and compared to a standard curve using serial dilutions of bovine serum albumin (BSA: 0-2000ug/ml, Pierce, Thermo Fischer scientific, ON, Canada). Sample absorbance at 562nm was measured using the Cytation^3 microplate reader (BioTek, Vermont, USA).

2.9 Western Blot

20-30 ug of total protein per sample, as determined by BCA, was prepared in 4xDTT leading buffer containing 0.72M β-mercaptoethanol, 69.4mM sodium dodecyl sulfate, 2.74M glycerol, 14.29 μM bromophenol blue, 62.4 mM Tris pH 6.8, and RIPA lysis buffer as a diluent. Samples were boiled for 5 minutes, centrifuged, and placed on ice. The prepared lysates were loaded (20-25μg per lane) and separated through 8% or 10% SDS-Polyacrylamide gel electrophoresis under reducing conditions. Proteins were transferred on a polyvinylidene difluoride (PVDF) membrane (Immobilon P, EMD Millipore, ON, Canada) using wet transfer at 100V for varying durations, depending on the molecular weight of the protein of interest. Membranes were blocked for one hour with 5% milk in a 0.05% Tween Tris buffered saline solution (TTBS). After Blocking,

membranes were incubated with primary antibodies [Phospho-Akt-ser 473 (1:1000 dilution; #4058, Cell Signaling, ON, Canada), Akt (1:1000; #9272, Cell Signaling), Phospho-HSL-Ser563 (1:1000; #4139, Cell signaling), HSL (1:1000; #4107, Cell Signaling), αβ-Tubulin (1:1000; #2148, Cell Signaling) and β-Actin (1:1000; #Sc-47778, Santa Cruz Biotechnology, CA, USA)] in 5% BSA (Bioshop, Canada Inc, ON, Canada) in 0.05% TTBS overnight at 4°C. The following day, membranes were washed with TTBS and incubated with secondary antibodies [goat anti-rabbit IgG-horseradish peroxidase (1:10000 dilution, #111-035-003, Jackson ImmunoResearch Laboratories Inc., USA) and goat anti-mouse (1:10000; 115-165-003, Jackson ImmunoResearch Laboratories Inc., USA) in 5% BSA for 1 hour at room temperature. The antibodies were detected by enhanced chemiluminescence (Pierce, Thermo Fischer Scientific, ON, Canada) using Microchemi DNr Bio-imaging system (Neve Yamin, Israel). Blots were quantified and analyzed using Image J software which determines density values based on the net intensity of each band. For each sample, the raw value obtained from the protein of interest was divided by the raw value obtained from the loading control. Relative intensity values were used for statistical analyses.

2.10 RNA Extraction

10-15 mg of frozen tibialis anterior (TA), EDL muscle, and visceral white adipose tissue were homogenized in RLT (muscle) or Qiazol buffer (adipose) (Qiagen, ON, Canada) using the Retsch MM400 tissue lyser. Total RNA was purified using the RNeasy Fibrous Tissue Mini Kit for muscle and RNeasy Mini Kit for Adipose (74704 and 74104, Qiagen, ON, Canada) as per the manufacturer's instructions.

2.11 q-RT PCR

For each sample, 190 ng of RNA was reversed transcribed to cDNA in a two-step PCR reaction on a Thermal cycler (2720 cycler, Applied Biosystems, CA, USA) using [dNTP (#N0447S, New England Bioabs, ON, Canada), Oligo DT (#100002344 Invitrogen, CA, USA), Ribolock RNase inhibitor (#EO038, Thermo Fisher Scientific, MA, USA), Random hexamers (#100026484, Invitrogen, CA, USA), M-MuLV reverse transcriptase and buffer (New England BioLabs, ON, Canada)]. The cDNA was diluted in 40 µl of RNase free water. 2 µl of cDNA was combined with Taqman® Fast Advanced Master Mix (#4444557, Applied Biosystems, Thermo Fisher Scientific, CA, USA) and TaqMan® FAM-Labelled probe sets for murine *Hprt1* (Mm00446968-m1), β*actin*(Mm004394036-g1), *Pecam1* (Mm00476712-m1), *Vegfa* (Mm00437306-m1), *NOS3* (Mm00435217-m1), *Leptin* (Mm00434759-m1), *Slc2a1* (Mm00441480-m1), *Edn1* (Mm00438656-m1), *Thbs1* (Mm001335418-m1), *adipoq* (Mm00456425-m1), *Klf2 (*Mm00500486-m1*),* Pdgfb (Mm00440677-m1), *Tgfβ1* (Mm01178820-m1). q-RT PCR was performed using the PCR cycler Rotor-Gene Q system (Qiagen, ON Canada) in the following thermal conditions: 60 °C for 30 minutes, 95 °C for 10 minutes for 40 cycles. Samples were assessed in duplicates. To determine the amount of target sample mRNA, the average cycle threshold (C_T) was calculated and compared to the average C_T of *Hprt1* or β*actin* (housekeeping genes) for the same samples with the formula ΔC_T= Average C_T (gene of interest)- Average C_T (housekeeping gene). The amount of target gene amplification relative to the control was calculated using the formula $2^{\wedge -_{\Delta} CT}$.

2.12 Statistical Analysis

Results were presented as Mean ± S.E.M. Analyses was done by unpaired student's t-test and two-way ANOVA followed by Bonferroni *post hoc* tests as appropriate (Prism5; Graphpad software Inc; La Jolla, CA, USA). In all cases, $p < 0.05$ was considered statistically significant.

Chapter 3: Results

3.1 Sex Differences in Body Weight and Energy Intake Following HF Diet-induced Obesity

Male and Female FVB/B6 mice were placed on a 16week high-fat diet to induce weight gain and metabolic disturbances **(Fig. 3.1A)**. Weight gain was significantly higher in HF male vs. NC male mice **(Fig. 3.1B)**. Meanwhile, there was no significant difference in weight gain between NC- and HF-fed females (~7g weight gain in both groups). Consistent with the existing literature (Hong et al. 2009b), weight gain was significantly lower in HF female compared to HF male mice. This was in accordance with lower total calorie (kcal) consumption of females compared to males in both NC and HF diet groups, although, HF-fed males and females consumed more calories relative to their NC-fed counterparts **(Fig. 3.1C)**. Furthermore, cumulative feed efficiency, which is the amount of calories needed to gain one gram of body weight, was significantly lower in HF female vs. HF male mice **(Fig. 3.1D)**.

There was no significant effect of diet on the absolute weights (mg) of hindlimb muscles of male or female mice **(Table 3.1)**. As expected, females had smaller EDL, gastrocnemius, and plantaris muscles compared to males. When taking body weight into consideration, all hindlimb muscles, except soleus, weighed less in HF male vs. NC males **(Table 3.2)**. However, no significant difference was found in the relative hindlimb muscle weights (mg/ g body weight) of NC- and HF-fed females. The absolute weights of visceral white adipose tissue from both perigonadal and retroperitoneal regions were significantly higher in HF males compared to NC males. In females however, there was no significant change in the weight of these regions following HF feeding. Absolute adipose tissue weight (perigonadal, retroperitoneal and BAT) was significantly lower in HF females compared to HF males, meanwhile, relative adipose tissue

weight was not significantly different between groups. HF feeding also increased the mass of heart and liver in males but not females. HF females had significantly smaller heart and liver compared to HF males. This difference in sex was absent in animals fed a NC diet, although, the relative weights of HF female hearts were significantly higher compared to HF male mice, which is accounted for by a lower overall body weight in females.

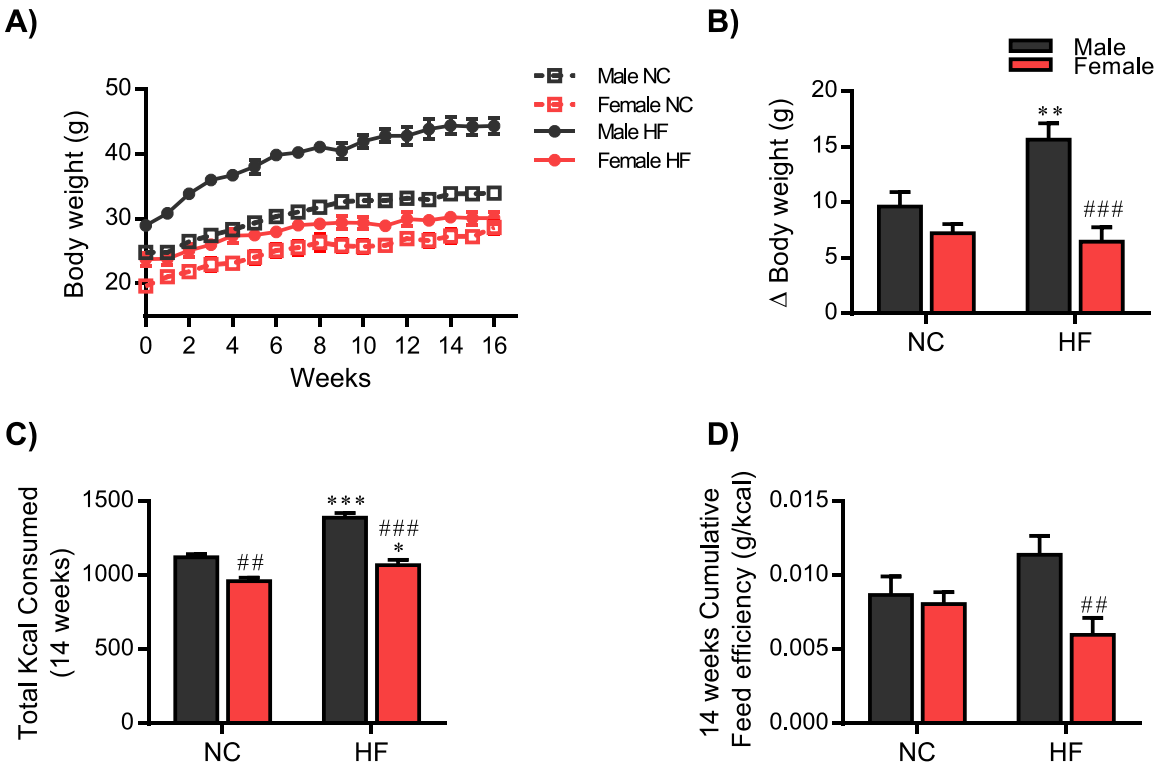

Figure 3.1: Weight gain is lower in female mice following 16 weeks of HF diet.
FVB/B6 male and female mice fed a NC or HF diet for 16 weeks (n=6-8). **A)** Body weights for the duration of the diet. **B)** Change in body weight on week 14 compared to week 0. **C)** Total Kcal of food consumed from week 1 to week 14. Weight gain and food consumption calculations utilized weeks 0-14, before glucose tolerance and insulin tolerance tests (GTT and ITT) were conducted. **D)** Cumulative feed efficiency (g body weight gained / Kcal food consumed) over 14 weeks. Data analyzed using two-way ANOVA followed by Bonferroni *post-hoc*. Mean +/- S.E.M. *represents post hoc significant difference in diet. # represents post hoc significant difference in sex. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ HF vs. corresponding NC, ##$p<0.01$ and ###$p<0.001$ Female vs. corresponding Male (n=6-8).

Table 1: Absolute tissue weights of male and female mice after 16 weeks of NC or HF diet.

	Normal Chow		High Fat Chow	
Tissue mass (mg)	***Male***	***Female***	***Male***	***Female***
Tibialis Anterior	39.2±1.4	34.3±1.3	42.8±2.2	37.8±2.4
EDL	9.9±0.5	8.3±0.2#	10.4±0.5	8.8±0.3
Gastrocnemius	118.1±2.5	97.8±5.3##	130.9±4.3	100± 0.7###
Plantaris	14.3±0.7	11.9±0.6#	15.6±0.7	11.8±0.4###
Soleus	7.1±0.3	7.3±0.8	8.2±0.5	6.6± 0.4
Perigonadal AT	1177.4±104.6	937.9±127.4	1839.1±197.7*	1176.9±203.8#
Retroperitoneal AT	561.2±43.1	552.9±84.6	881.71±86.1*	450.52±119.4#
Brown AT	169.1±19.9	124.3± 4.1	189.2±21.2	93.7±7.7#
Heart	103.4±2.4	100.8±3.0	134.9±4.2***	109.4±4.1###
Liver	1177.6±85.8	982.9±67.0	1517.9±93.3*	908.2±69.0###

Tissues weighed following extraction. Data expressed as mean ± S.E.M (n=6-8). Significance established using two-way ANOVA followed by Bonferroni post hoc. * $p< 0.05$, *** $p< 0.001$ HF *vs.* corresponding NC. # $p< 0.05$, ## $p<0.01$, ### $p<0.001$ Female vs. Male.

Table 2: Tissue weights of male and female mice after 16 weeks of NC or HF diet relative to body weight.

	Normal Chow		High Fat Chow	
Tissue mass (mg/g BW)	***Male***	***Female***	***Male***	***Female***
Tibialis Anterior	1.2±0.03	1.2±0.05	1.0±0.05*	1.3±0.07##
EDL	0.3±0.03	0.3±0.02#	0.2±0.01*	0.3±0.02#
Gastrocnemius	3.6±0.2	3.3±0.1	3.0±0.1*	3.3±0.1
Plantaris	0.4±0.03	0.4±0.01	0.4±0.01*	0.4±0.01
Soleus	0.2±0.02	0.3±0.02	0.2±0.01	0.2±0.01
Perigonadal AT	36.2±2.6	35.8±5.5	41.3±4.0	38.5±6.9
Retroperitoneal AT	17.4±1.4	18.3±2.3	20.01±2.0	15.8±3.0
Brown AT	5.2±0.7	4.2±0.1	4.3±0.5	3.1±0.3
Heart	3.2 ± 0.1	3.4±0.2	3.0±0.1	3.7±0.2#
Liver	33.6±1.9	33.05±1.6	34.1±1.3	30.5±2.9

Tissues weighed after extraction and normalized to body mass. Data expressed as mean± S.E.M (n=6-8). Significance established using two-way ANOVA followed by Bonferroni post hoc. * $p< 0.05$ HF *vs.* corresponding NC. # $p< 0.05$, ## $p<0.01$ Female vs. Male.

3.2 Glucose Handling and Blood Pressure Maintained in HF Female but not HF Male Mice

Next, we examined whether this model of HF diet-induced obesity induces systemic cardiovascular and metabolic disturbances in male and female mice. There was no influence of diet on heart rate in either sex **(Fig. 3.2A)**. However, systolic and diastolic blood pressures were significantly increased in HF males but not HF females when compared to their NC-fed counterparts **(Fig. 3.2B, C)**. Fasted plasma glucose level was significantly lower in HF female compared to HF male mice, in whom fasting plasma glucose was significantly increased by the HF diet **(Fig. 3.2D)**. Following an IP-GTT test, HF male mice exhibited impaired glucose tolerance compared to HF female mice who maintained a response similar to the NC-fed groups. GTT-AUC was significantly higher in HF male vs. HF female mice due to a higher peak in plasma glucose, and glucose levels remaining higher during the following 3 hours after glucose injection **(Fig. 3.2E, F)**. Skeletal muscle and adipose tissue are both metabolically active tissues involved in the regulation of whole-body metabolism and glucose disposal. In both of these tissues, glucose uptake may occur by both insulin dependent and insulin independent mechanisms. Basal uptake of glucose is facilitated by the insulin independent Glucose Transporter 1 (GLUT1) (Kraegen et al. 1993). To examine whether differences in glucose tolerance were associated with GLUT1 levels, I measured GLUT1 mRNA in both EDL muscle and perigonadal adipose tissue. In the EDL muscle, there was a trend for higher GLUT1 mRNA levels in HF females compared to HF males (overall sex effect $p=0.06$) **(Fig. 3.2G)**. In adipose tissue, GLUT1 mRNA level was significantly higher in HF vs. NC males and there was a trend for lower GLUT1 expression in female compared to male mice **(Fig. 3.2H)**.

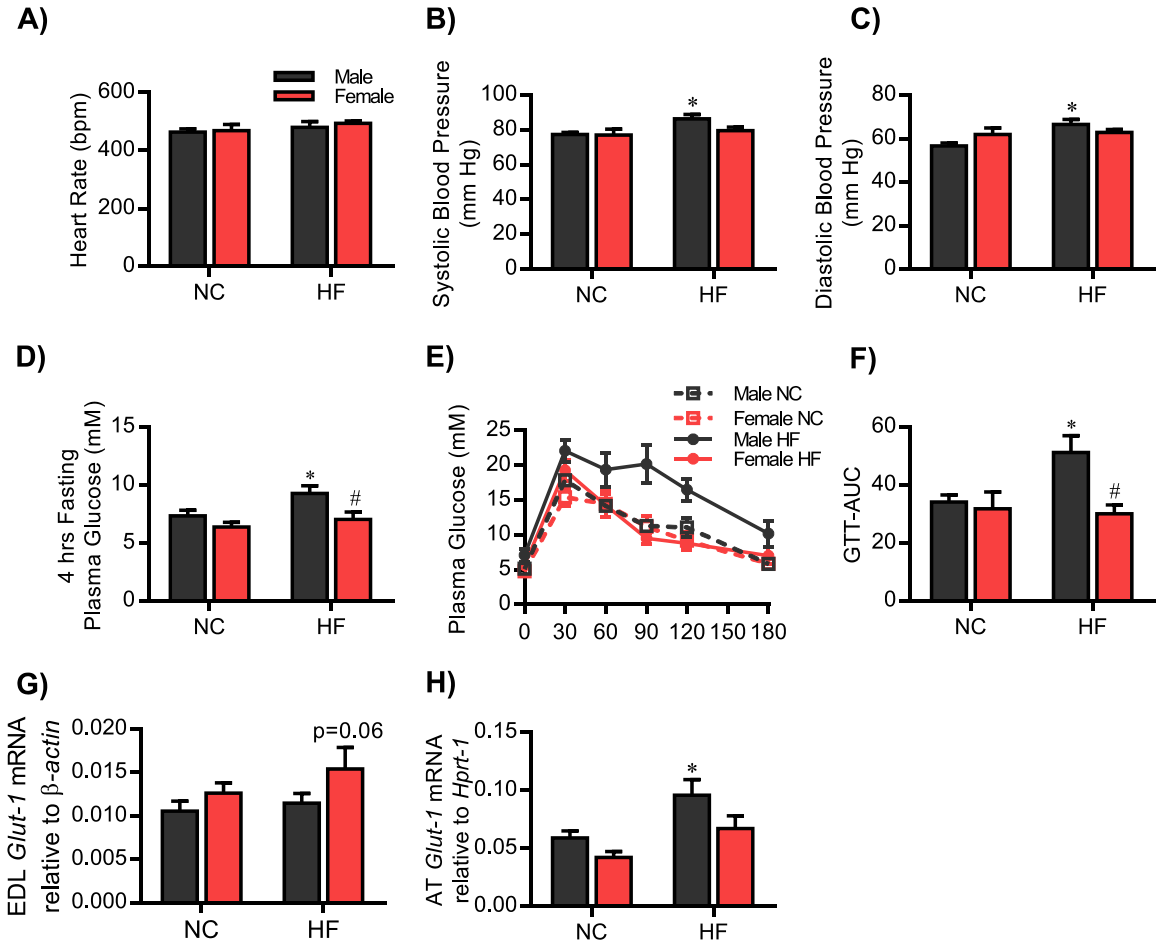

Figure 3.2: Blood pressure and glucose tolerance are maintained in HF-fed female mice.
A) Heart rate, **B)** Systolic blood pressure and **C)** Diastolic blood pressure of NC- and HF-fed male and female mice recorded using a non-invasive tail-cuff pressure system on diet week 13. **D)** Plasma glucose (mM) after 4 hrs of fasting, **E)** line graph of plasma glucose and **F)** the corresponding AUC graph of IP-GTT after 16 hours of overnight fasting on diet week 15. **G)** EDL muscle quantification of *glut1* mRNA normalized to β-*actin* and H) Perigonadal white adipose tissue quantification of *glut1* mRNA normalized to *hprt1*. Expression of mRNA assessed by real-time quantitative PCR and values expressed as $2^{-\Delta CT}$. Data analyzed using two-way ANOVA followed by Bonferroni *post-hoc*. Mean +/- S.E.M. *p<0.05 HF vs. corresponding NC, #p<0.05 Female vs. corresponding Male (n=6-8).

3.3 Systemic and Skeletal Muscle Sensitivity to Insulin Maintained in HF Female Mice

Changes in glucose tolerance may in large part depend on differences in the insulin-dependent uptake of glucose. Following an IP-ITT test, HF male mice demonstrated an impaired capacity to lower their plasma glucose levels in response to the injected insulin, as characterized by a lower ITT-AOC in HF vs. NC male mice. In contrast, HF females maintained a response similar to their NC counterparts and the ITT-AOC was significantly higher in HF female compared to HF male mice **(Fig. 3.3A, B)**. The majority of insulin-induced glucose uptake occurs within skeletal muscle (Leto and Saltiel 2012). However, to have this metabolic effect, insulin in the circulating blood must first be delivered via capillaries, then exit the vessel wall to reach the tissue (Lee and Klip 2016). Thus the insulin-dependent activation of downstream pathways (i.e. Akt pathway) relies on the vascular delivery of insulin to the target tissue. Consequently, sex differences in systemic insulin sensitivity may involve differences in skeletal muscle responsiveness to insulin via altered delivery of insulin by the muscle microvasculature. In animals fed a NC diet, both males and females exhibited a similar level of phosphorylated Akt in response to *in-vivo* delivery of insulin **(Fig. 3.3C)**. In contrast, insulin stimulation resulted in significantly greater Akt phosphorylation within the muscle of HF females compared to HF males **(Fig. 3.3D)**. Together, these findings indicate that systemic and skeletal muscle response to insulin is preserved in females but impaired in males in response to a HF diet.

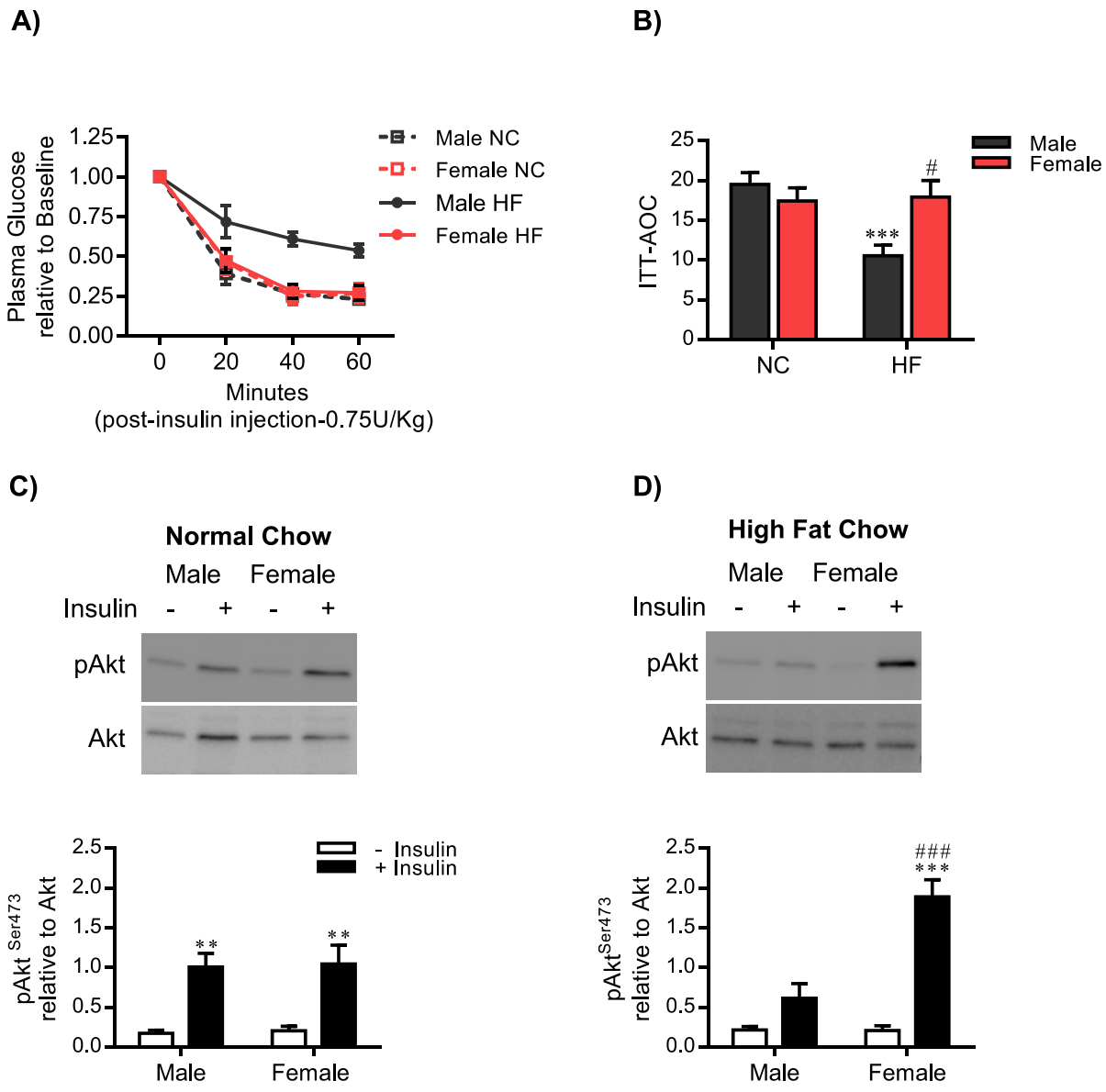

Figure 3.3: Systemic and skeletal muscle sensitivity to insulin is impaired in HF male but is maintained in HF female mice.

A) Line graph of blood glucose relative-to-baseline and **B)** the corresponding AOC graph of IP-ITT after 4 hours of no food on diet week 14. Data analyzed using two-way ANOVA followed by Bonferroni *post-hoc*. Mean +/- S.E.M. ***p<0.001 HF male vs. NC male. #p<0.05 HF female vs. HF male (n=5-8). **C)** Representative western blots and protein quantification of p-Ser473-Akt and total Akt assessed in control and *in vivo* insulin stimulated EDL muscle of male and female mice on a NC diet and **D)** male and female mice on a HF diet. Results are expressed as p-Akt relative to total Akt. Data analyzed using two-way ANOVA followed by Bonferroni *post hoc*. Mean +/- S.E.M. **p<0.01 and ***p<0.001 +Insulin vs. corresponding –Insulin. ###p<0.001 Female +Insulin vs. Male +Insulin (n=5-7).

3.4 Adipose Tissue Sensitivity to Insulin and Isoproterenol Maintained in HF Female Mice

To examine if the male and female response to stimuli involved in metabolism is also different in visceral white adipose tissue, we stimulated fat from the perigonadal region with insulin or isoproterenol. Within adipose tissue, insulin induces the storage of lipids (Rosen and Spiegelman 2006), meanwhile, isoproterenol, as a β-adrenergic agonist, stimulates the breakdown of lipids through the process of lipolysis (Lafontan et al. 1997). Insulin-dependent phosphorylation of Akt in the adipose tissue was similar between males and females on a NC diet **(Fig. 3.4A)**. In contrast, in animals fed a HF diet, phosphorylated Akt levels were significantly increased only in female mice. Moreover, HF female mice had significantly higher pAkt levels compared to HF male mice, suggesting that on a HF diet, visceral white adipose tissue of females is more sensitive to insulin compared to males **(Fig. 3.4B)**.

Hormone-sensitive lipase (HSL) has been established as a downstream effector of β-adrenergic receptor signaling (Lafontan et al. 1997). In response to *ex-vivo* isoproterenol, phosphorylation of HSL was significantly increased in the adipose tissue of NC male and NC female mice **(Fig. 3.4C)**. Phosphorylated HSL was also significantly increased in male and female animals fed a HF diet, however, this response was significantly higher in HF females compared to HF males **(Fig. 3.4D)**. Together, these results confirm that HF feeding influences the white adipose tissue of male and female mice differently. In HF male mice adipose tissue response to insulin and isoproterenol was compromised, whereas HF female adipose tissue response to these stimuli was maintained.

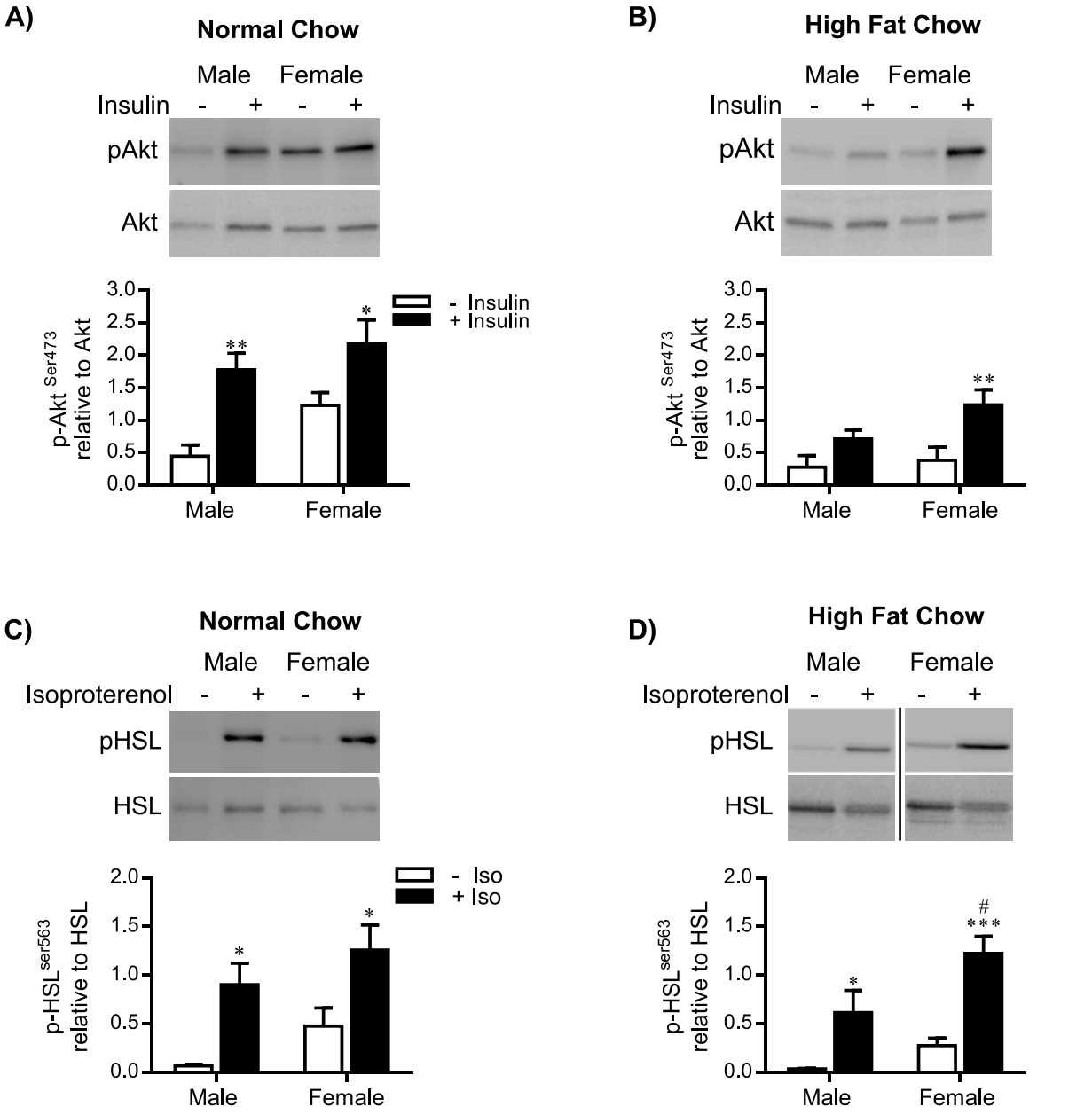

Figure 3.4: White adipose tissue response to insulin and isoproterenol is maintained in HF female mice.

Perigonadal white adipose tissue from NC and HF-fed male and female mice was incubated for 30 minutes *ex vivo* in the presence of insulin (25 mU/ml), isoproterenol (0.1mM) or low- glucose medium as a control. **A)** Representative western blots and protein quantification of p-Ser473-Akt and total Akt in male and female mice on a NC diet (n=5-7) or **B)** a HF diet (n=6). **C)** Representative western blots and protein quantification of p-Ser563-HSL and total HSL in male and female mice on a NC diet (n=6) and **D)** on a HF diet (n=4-5). Samples originated from the same blot; vertical line indicates lanes are not continuous. Data analyzed using two-way ANOVA followed by Bonferroni *post hoc*. Mean +/- S.E.M. *p<0.05, **p<0.01,***p<0.001 stimulated vs. corresponding control. #p<0.05 Female +Iso vs. Male +Iso.

3.5 Adipokine Profile of Visceral White Adipose Tissue is Different in Male vs. Female Mice

Male and female adipose response to metabolic stimuli may be influenced by HF diet induced alterations in the release of adipokines. It has been previously reported that mRNA levels of leptin are significantly lower in the inguinal and mesenteric white adipose tissue of females compared to males on a NC or a HF diet (Priego et al. 2009). Our results confirm that on a HF diet, mRNA expression of leptin is significantly lower in the prigonadal white adipose tissue of HF female vs. HF male mice. However, in the NC-fed mice, leptin mRNA levels were not significantly different between males and females. Moreover, a significant increase in leptin mRNA expression was only observed in the adipose tissue of HF males when compared to their NC counterparts **(Fig. 3.5A)**. Visceral white adipose tissue expression of adiponectin has been established to be higher in female compared to male rodents (Estrany, Proenza, Gianotti, and Lladó 2013). We observed that adiponectin mRNA level was significantly higher in the perigonadal adipose tissue of HF female compared to HF male mice. However, no significant difference was detected in adiponectin mRNA levels of NC fed animals **(Fig. 3.5B)**. Overall, the adipokine profile and the maintained response to metabolic stimuli in HF females may contribute to the preserved health of the local tissue.

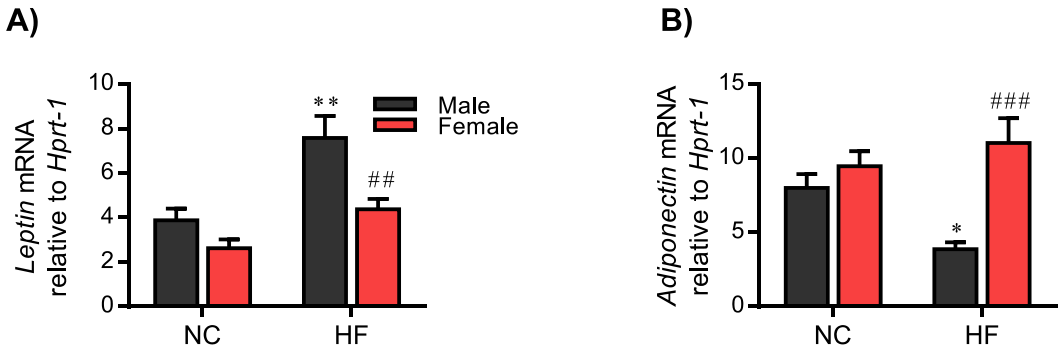

Figure 3.5: Protective adipokine profile maintained in white adipose tissue of HF female mice. **A)** *Leptin* (n=4-6) and **B)** *Adiponectin* (n=6-8) mRNA levels in the perigonadal white adipose tissue of NC- and HF-fed male and female mice as assessed by real-time quantitative PCR. Values were normalized to *Hprt1* and expressed as $2^{-\Delta CT}$. Data analyzed using two-way ANOVA followed by Bonferroni *post-hoc*. Mean +/- S.E.M. *p<0.05 and **p<0.01 Male HF vs. Male NC, ##p<0.01 and ###p<0.001 Female HF vs. Male HF.

3.6 Skeletal Muscle Vascular Content Maintained in HF-fed Male and Female Mice

Differences in skeletal muscle sensitivity to *in-vivo* insulin may reflect changes in vascular content or function. In principle, a higher capillary content within skeletal muscle, through the process of angiogenesis, will increase the surface area for the flux of insulin and may enhance muscle perfusion of insulin. Conversely, vasodilation or outward remodeling of existing vessels, which both increase vessel circumference, can increase blood flow and total delivery of insulin to skeletal muscle. I first did a histological assessment of muscle fibers and capillaries to see whether sex differences exist in skeletal muscle capillary content **(Fig. 3.6A)**. Capillary-to-fiber ratio (C:F) was used as an indicator of angiogenesis. C:F was not significantly different within the EDL muscle of NC- or HF-fed male and female mice **(Fig. 3.6B)**. Also, no significant difference was detected in EDL muscle capillary density **(Fig. 3.6C)**. Capillary density is influenced by the size of individual muscle fibers. Therefore, I measured muscle fiber cross-sectional area (um^2) in the

EDL muscle and found no significant difference between NC- and HF-fed male and female mice **(Fig. 3.6D)**.

Next, I measured PECAM-1 mRNA level as a marker of endothelial cells within the EDL and TA muscles with the assumption that endothelial cell content would confirm the histological assessment of capillary number. HF diet significantly increased PECAM-1 mRNA level in the EDL muscle of HF females but not males, Furthermore, PECAM-1 expression was significantly higher in HF females vs. HF males **(Fig. 3.6E).** However, within the TA muscle, no significant differences were observed in PECAM-1 mRNA levels **(Fig. 3.6F)**.

Vascular Endothelial Growth Factor A (VEGF-A) and Thrombospondin-1 (THBS-1) are critical regulators of endothelial cell content and angiogenesis within skeletal muscle. VEGF-A promotes endothelial cell proliferation and capillary growth whereas THBS-1 has anti-angiogenic effects via promotion of endothelial cell apoptosis (Bornstein 2009; Ferrara, Gerber, and LeCouter 2003a). VEGF-A mRNA expression was significantly higher in the TA muscle of NC female vs. NC male mice **(Fig. 3.6G**). However, no significant influence of sex was observed in VEGF-A mRNA levels of HF male and HF female mice. Also, no significant difference was found in THBS-1 mRNA levels between NC- or HF-fed males and females **(Fig. 3.6H)**. Leptin has been reported to have pro-angiogenic influences on endothelial cells (A Bouloumié et al. 1998; Sierra-Honigmann et al. 1998)Leptin mRNA level was significantly higher within the TA muscle of HF male vs. NC male mice. Furthermore, there was a strong trend for HF females to have lower leptin expression compared to HF males ($p=0.06$) **(Fig. 6I).** Together, the expression pattern of angiogenic factors agreed with lack of change in capillary content within skeletal muscle. However, this would not explain why PECAM-1 mRNA level was different within the EDL muscle of HF-fed males and Females.

Endothelial cells are present in all vessels, including arterioles which contain smooth muscle cells. We hypothesized that increased PECAM1 mRNA may be associated with arteriolarization of capillaries or outward remodeling of existing arterioles. Both of these adaptations require the presence of more endothelial cells, however, this would not be reflected in muscle capillary number. We measured Myosin heavy chain 11 mRNA level as a marker of smooth muscle cells and indicator of arteriole content. However, no significant difference was found in MYH11 mRNA expression within the EDL muscle of HF-fed male and female mice **(Fig. 3.6J)**.

No conclusive results were found to indicate that the enhanced insulin responsiveness of skeletal muscle in females is related to adaptations in vessel content. Thus, we assessed the expression of factors that regulate vessel tone and may influence the delivery of insulin to skeletal muscle. Among them, eNOS (via production of NO) and endothelin (Edn-1) are two endothelium-specific factors that induce vasodilation and vasoconstriction respectively. It has been previously shown that eNOS levels decrease and Edn-1 levels increase within the skeletal muscle of male rats fed a HF diet(Bourgoin et al. 2008). No significant influence of diet or sex was observed in the expression of eNOS mRNA within the TA muscles of the male and female mice **(Fig. 3.7A)**. Measurement of phosphorylated eNOS (p-Ser473-eNOS) is a more accurate estimation of its enzymatic activity. I attempted to measure protein levels of p-eNOS relative to total eNOS by western blotting. The expected molecular weight for p-eNOS is 140 kDa. However, I was unable to detect bands at the correct molecular weight with antibodies currently available. No significant influence of diet was detected in Edn-1 mRNA levels, although there was a trend for higher Edn-1expression in females compared to males ($p=0.2$ in NC, $p=0.06$ in HF groups) **(Fig. 3.7B)**.

We could not measure vasodilation or muscle blood flow directly, but we postulated that higher blood flow would increase the expression of genes activated by shear stress, the frictional

force of blood moving past the endothelial cell surface. We assessed the expression of shear-stress induced factors TGFβ, PDGFB, and KLF2 and found no significant differences in the expression of these factors, although a trend for higher KLF2 expression was present within the EDL muscle of HF female compared to HF male mice **(Fig. 3.7C-E)**. Thus far, assessment of vessel content and function within the skeletal muscle of male and female mice shows inconclusive results and the role of vascular system in the maintained skeletal muscle response to insulin in HF females but not HF males remains to to be clarified.

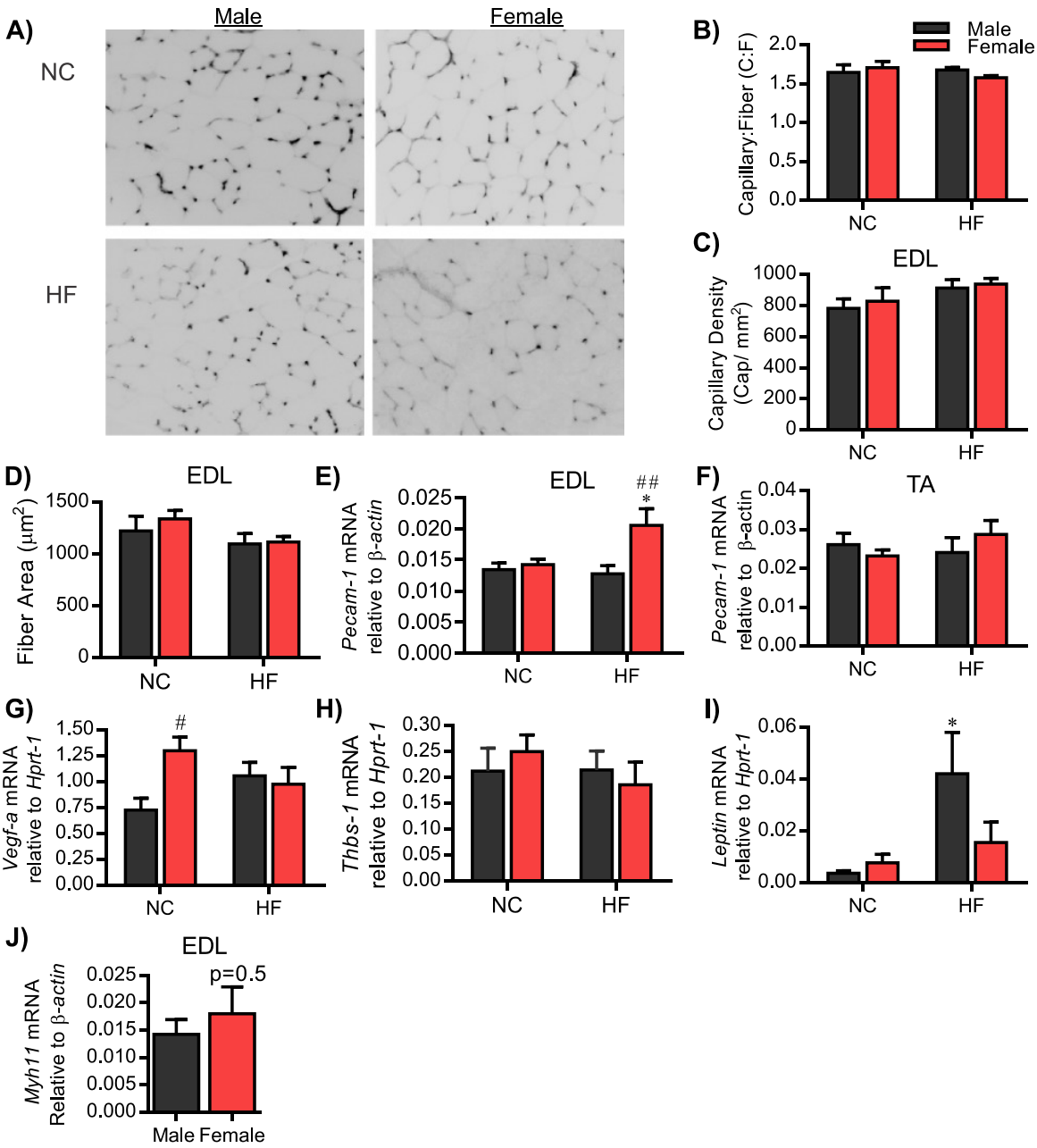

Figure 3.6: 16 weeks of HF diet does not affect skeletal muscle capillary or arteriole content.
A) Representative images of EDL muscle sections stained with Lectin-FITC to visualize capillaries in NC- and HF-fed male and female mice. Greyscale images were inverted to enhance visualization of capillaries. **B)** Capillary-to-fiber (C:F) ratio **C)** Capillary density and **D)** Muscle fiber cross-section areas were calculated from 3-4 fields of view per mouse (n=5-7). **E)** TA muscle and **F)** EDL muscle *pecam1* mRNA normalized to β-*actin*. **G)** *vegfa* **H)** *thbs1* and **I)** *leptin* mRNA normalized to *hprt1* in the TA muscle of NC and HF fed male and female mice. **J)** *myh11* mRNA normalized to β-*actin* in the EDL muscle of HF-fed male and female mice. Expression of mRNA assessed by real-time quantitative PCR and results expressed as $2^{-\Delta Ct}$. Data analyzed using two-way ANOVA followed by Bonferroni post-hoc. Mean +/- S.E.M. *p<0.05 HF vs. corresponding NC, #p<0.05 and ##p<0.01 Female vs. corresponding Male (n=5-7).

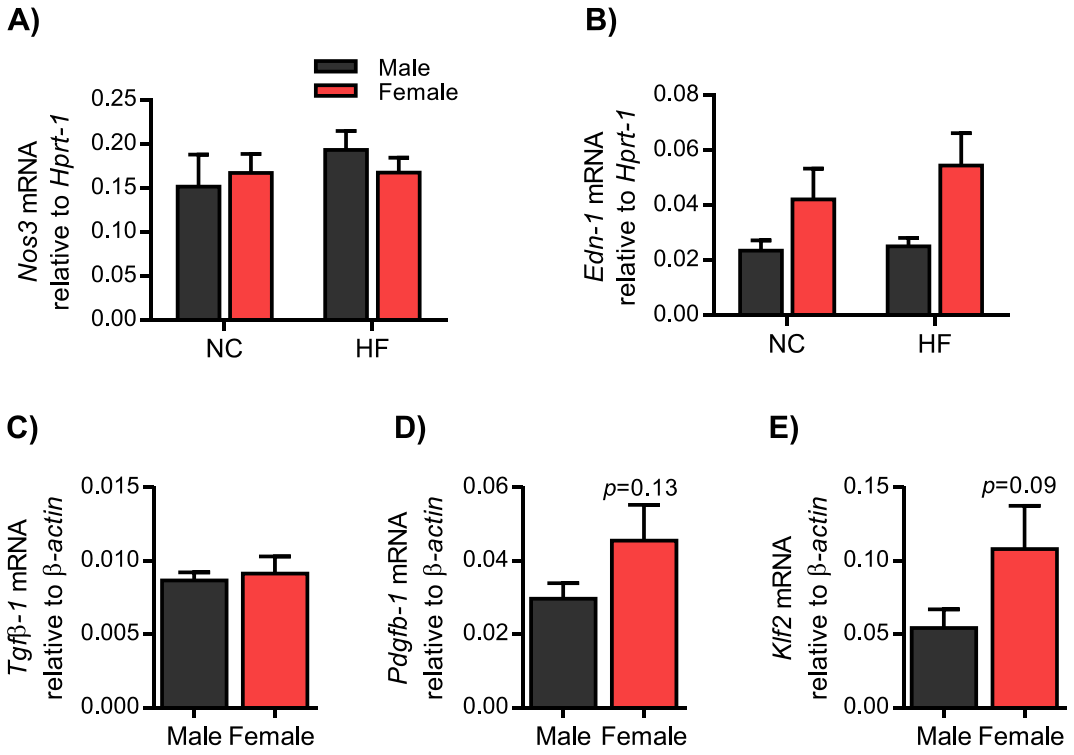

Figure 3.7: Factors associated with vessel tone and shear-stress responsiveness within skeletal muscle are unchanged by HF diet.

A) *Nos3* and **B)** *Edn1* mRNA normalized to *hprt-1* in TA muscle of NC- and HF- fed male and female mice**. C)** *Tgf*β*-1* **D)** *Pdgfb1 and* **E)** *Klf2* mRNA normalized to β-*actin* in EDL muscle of HF male and HF female mice, as assessed by real-time quantitative PCR and results expressed as $2^{-\Delta Ct}$. Data analyzed using two-way ANOVA followed by Bonferroni *post-hoc*. Mean +/- S.E.M. (n=5-7).

3.7 Adipose Tissue Microvascular Content Maintained in HF Female Mice

I also hypothesized that sex differences in adipose tissue sensitivity to metabolic stimuli may be related to differences in the vascular niche of male and female adipose tissue. To examine this, I first established whether male and female adipose vascularization was different in response to HF feeding. Perigonadal white adipose tissue quantification of lectin staining as a percentage of field-of-view showed no significant difference in the adipose vascularization of NC male and NC female mice. In contrast, HF males had a significantly reduced, but HF females had a maintained, adipose vascular content **(Fig. 3.8A, B)**. We observed that vessel branch point number per field-of-view, which serves as an indicator of vessel sprout formation, was significantly higher in HF female vs. HF male mice. Furthermore, branch point number was significantly reduced only in male mice in response to the HF diet **(Fig. 3.8C)**. This was further supported by higher mRNA levels of vascular marker PECAM-1 in HF females vs. HF males **(Fig. 3.8D)**. However, the mean diameter of capillaries within the adipose tissue of HF females was smaller compared to HF males **(Fig. 3.8E, F)**.

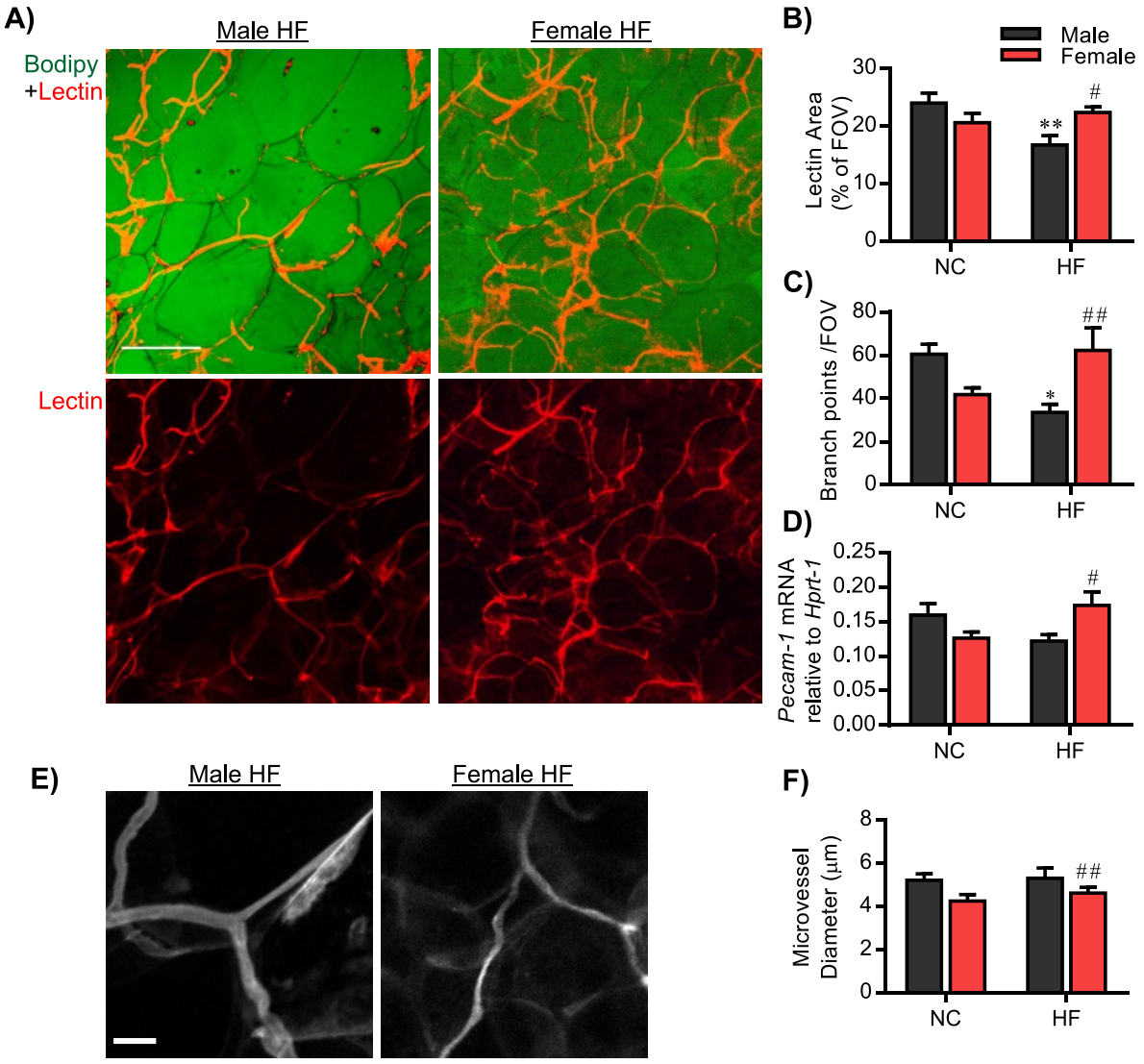

Figure 3.8: Visceral adipose tissue vascular content maintained in HF female but impaired in HF male mice.

A) Adipocyte and capillary staining (*Green: bodipy, Red: lectin*). Representative confocal images of whole-mount adipose of HF male and HF female mice are shown (10x, scale bar 100µm). **B)** Capillary content in NC- and HF-fed male and female mice was represented as the % surface area of lectin within the field-of-view, and **C)** The number of branch points measured from 3-4 independent fields of view per mouse (n=5-7). **D)** *Pecam1* mRNA normalized to *hprt-1* in perigonadal adipose tissue, as assessed by real-time quantitative qPCR and expressed as $2^{-\Delta Ct}$ (n=6-8). **E)** Representative confocal images of whole-mount adipose of HF male and HF female mice are shown in black and white (20x, scale bar 25µm). **F)** Average microvessel diameter was calculated from 3-4 images per mouse. Data analyzed using two-way ANOVA followed by Bonferroni post-hoc. Mean +/- S.E.M. *p<0.05 and **p<0.01 HF male vs. NC male. #p<0.05 and ##p<0.01 HF female vs. HF male.

3.8 Adipose Tissue Expression of Vascular Regulatory Factors

To identify contributing factors in the sex-dependent regulation of visceral white adipose vascularization in response to a HF diet, I measured the expression of stimuli that influence vascular content and vascular tone. The mRNA expression of anti-angiogenic factor THBS-1 was not significantly different between NC male and NC female mice. However, THBS-1 mRNA level was significantly higher in HF female as compared to NC female and HF male mice **(Fig. 3.9A)**. In response to a HF diet, VEGF-A mRNA level was significantly reduced in the perigonadal adipose of male but not female mice. Interestingly, under the normal condition of a NC diet, female mice had significantly lower VEGF-A mRNA level compared to males. However, this observation was reversed on a HF diet, where female mice had significantly higher VEGF-A expression vs. HF male mice **(Fig. 3.9B)**. A trend similar to VEGF-A expression was also observed in the expression of eNOS and in the adipose tissue of female mice, eNOS mRNA level was significantly increased in response to the HF diet **(Fig. 3.9C).** I also measured expression of the vasoconstrictor factor EDN-1. However, no significant influence of sex or diet was detected in EDN-1 mRNA levels **(Fig. 3.9D)**.

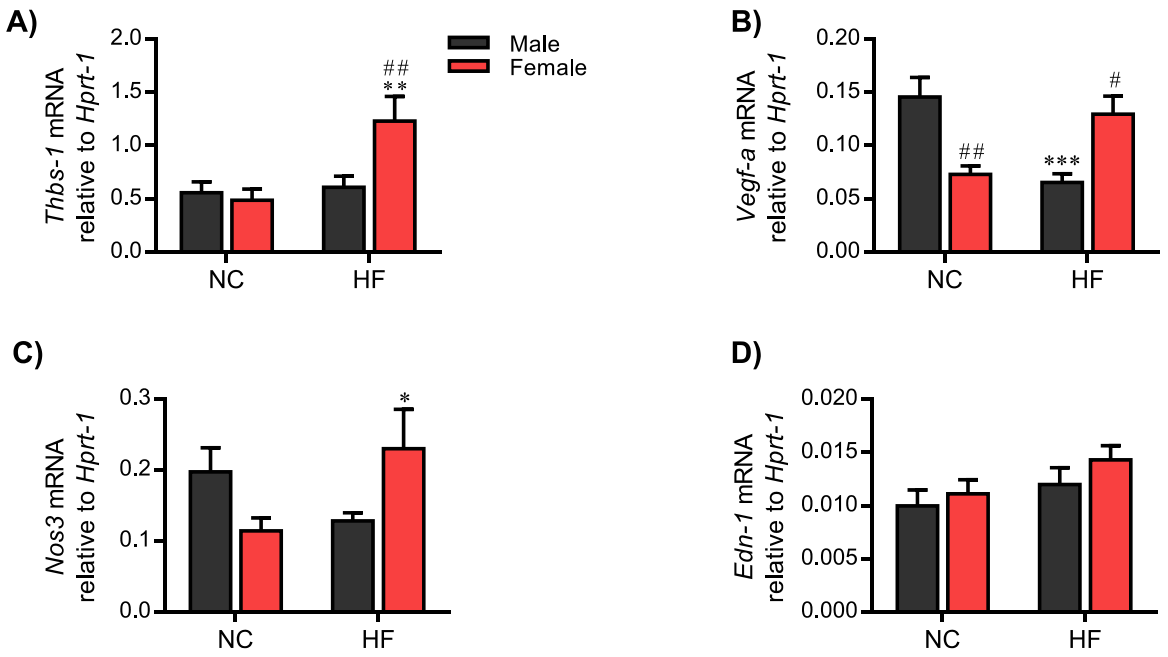

Figure 3.9: Regulators of angiogenesis and vessel tone in visceral adipose tissue of NC- and HF-fed male and female mice.

A) *Thbs-1*, **B)** *Vegfa,* **C)** *Nos3,* and **D)** *Edn1* mRNA normalized to *Hprt-1* in perigonadal adipose tissue of NC-and HF- fed male and female mice, as assessed by real-time quantitative qPCR and expressed as $2^{-\Delta Ct}$. Data analyzed using two-way ANOVA followed by Bonferroni *post-hoc*. Mean +/- S.E.M. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ HF vs. corresponding NC, #$p<0.05$ and ##$p<0.01$ Female vs. corresponding Male (n=6-8).

Chapter 4: Discussion

In this project, I have established that sex-related differences exist in microvascular adaptations in response to a HF diet. Following 16 weeks of HF feeding, I have shown that skeletal muscle and adipose tissue responsiveness to insulin is maintained in female mice but is severely impaired in male mice. Despite the indication of increased endothelial cell content within the skeletal muscle of HF females, no difference in capillary content was observed in either male and female mice. I report the novel finding that capillary content was significantly higher within the adipose tissue of HF female mice compared to HF male mice. Furthermore, HF female adipose tissue was characterized by an increase in the gene expression of adiponectin and pro-angiogenic factors. Altogether, these findings suggest that maintenance of tissue vasculature in HF female mice may be a contributing factor to a healthy tissue microenvironment and a maintained metabolic response.

4.1 Sex Differences in Systemic Metabolic Functions

Marked sex differences have been established in the progression of metabolic disturbances in animals following HF feeding (Medrikova et al. 2012). When faced with the metabolic challenge of a HF diet, previous studies have shown males to be more prone to develop insulin resistance and weight gain compared to pre-menopausal females (El Akoum et al. 2011; Palmer and Clegg 2015; Shi et al. 2012). My findings confirmed that, following 16 weeks of HF feeding, female mice exhibit less weight gain and maintain systemic insulin sensitivity and glucose tolerance versus male mice, which develop impairments in both. To date, established mechanisms responsible for the sex-specific effects of a HF diet are unsettled. In both humans and animals, evidence of a strong link between visceral adiposity and insulin resistance suggests that the higher

distribution of visceral adipose tissue in males may predispose them to the development of metabolic dysfunction (Després and Lemieux 2006; Eliza B Geer and Shen 2009). Other studies examine the influence of sex hormones and speculate on the protective role of estrogen in insulin-induced glucose uptake and cardiovascular function (Baker et al. 2003; Mauvais-Jarvis, Clegg, and Hevener 2013; Paranjape et al. 2005; Riant et al. 2109; Renee E Stubbins et al. 2012). Existing data also show that the lower production of pro-inflammatory cytokines and pro-inflammatory adipokines in females compared to males may protect against or delay the development of metabolic disturbances (Estrany et al. 2013; Mauvais-Jarvis et al. 2013). The majority of the aforementioned studies have examined a single tissue or systemic metabolism in general. This limits the conclusions that can be drawn considering that obesity-induced metabolic dysfunction is a systemic condition stemming from damage to multiple organs (Dimitriadis et al. 2011; Neto-Ferreira et al. 2014). In this project, the combined analysis of skeletal muscle and adipose tissue aimed to better capture the underlying causes of sex differences in susceptibility to metabolic disruptions.

4.2 Sex Differences in Tissue-specific Responses to Metabolic Stimuli and Adipokines

HF diet-driven sex differences in systemic insulin sensitivity may be due to tissue-specific responses to insulin. Unlike their male counterparts, HF female mice exhibited a maintained skeletal muscle insulin responsiveness. This was evident as *in vivo* injection of insulin, which relies on the vascular delivery of insulin to muscle, induced a significant increase in phosphorylation of Akt within the skeletal muscle of HF females only. Conversely, HF males exhibited impaired impaired Akt phosphorylation in response to insulin, which confirms findings from other studies (van Haare et al. 2015; Senthil Kumar et al. 2014; White et al. 2014). HF diet-induced insulin

resistance could also involve impairments in adipose tissue insulin signaling and catecholamine-induced lipolysis (Dimitriadis, Mitrou, Lambadiari, Maratou, and Sotirios A. Raptis 2011; Jocken et al. 2007; Mulder et al. 2003; Nagy, Levy, and Grunberger 1990; O'Carroll et al. 2013). I found that visceral adipose tissue responsiveness to insulin is maintained in female mice but impaired in male mice following HF feeding. This was evident as *ex vivo* insulin stimulation of adipose tissue induced a significant increase in phosphorylation of Akt in HF female mice but not HF male mice. This also is consistent with published data showing higher *in vivo* insulin-stimulated Akt activation in the adipose tissue of female mice compared to male mice on a HF diet (Senthil Kumar et al. 2014).

A blunted catecholamine-induced lipolysis response has previously been linked to a reduction in adipose tissue HSL mRNA and protein in insulin resistant obese individuals (Jocken et al. 2007). Furthermore, HSL- deficient mice develop impaired insulin sensitivity, which reveals the important role of this hormone in glucose handling (Fortier et al. 2005; Mulder et al. 2003). Building on these data, I established that sex differences exist in adipose tissue response to *ex vivo* isoproterenol stimulation, with significantly higher HSL phosphorylation in the visceral adipose of HF females compared to HF males (but no apparent differences in total HSL levels were observed). This raises the possibility that higher HSL activity in females may contribute to enhanced lipid mobilization and breakdown, whereas triglyceride accumulation and adipocyte expansion, associated with reduced HSL activity may subsequently impair insulin signaling in HF male mice.

In HF female mice, maintained adipose tissue responsiveness to hormonal stimuli may be promoted by a healthier adipokine profile within the adipose microenvironment. It has previously been established that compared to males, females have higher circulating plasma levels of leptin

and adiponectin (Cnop et al. 2003; Hickey et al. 1996). I found no baseline differences in mRNA expression of leptin and adiponectin within the visceral adipose tissue of NC males and NC females. I measured mRNA expression of these adipokines within a section of visceral adipose as an indicator of the local environment of the tissue. Therefore, it may not match plasma levels of leptin and adiponectin which is the accumulation of adipokines released by total adipose tissue (both visceral and subcutaneous adipose depots). Multiple studies have linked higher leptin but lower adiponectin plasma levels to a state of reduced insulin sensitivity in obese rodents and humans, similar to the adipokine profile of our HF male (Kwon and Pessin 2013; Lihn et al. 2005; Weyer et al. 2001; Yadav et al. 2013). In contrast to this "obesogenic" adipokine profile, HF female mice had lower leptin but higher adiponectin mRNA expression within the visceral white adipose.

Higher adiponectin levels in the visceral adipose tissue of HF female mice confirms previous data showing higher plasma adiponectin levels in HF-female mice associated with reduced tissue inflammation and a later onset of insulin resistance (Cnop et al. 2003; Medrikova et al. 2012). Montague et al. have reported that in female mice, leptin is predominantly released from subcutaneous adipose tissue (Montague et al. 1997). This may in turn account for the discrepancy between plasma and visceral adipose tissue leptin levels in females. Within the visceral adipose tissue of HF female mice, lower leptin (pro-inflammatory) but higher adiponectin (anti-inflammatory) levels may be associated with reduced inflammation and accumulation of immune cells, further contributing to a healthier tissue microenvironment (Kwon and Pessin 2013; Mancuso 2016; Ouchi et al. 2011). One key determinant of this phenotype is the tissue microvasculature, which controls the exchange of nutrients, oxygen and hormones (e.g. adipokines) as well as immune cells between the circulatory system and the surrounding tissue.

4.3 Capillary Content and Angiogenic factors in Skeletal Muscle

Within the microenvironment of skeletal muscle and adipose tissue, capillaries can be thought of as gate keepers that control the flux of hormones and nutrients from the circulatory system. Thus, capillaries serve as a key determinant of health and metabolic function of the local tissue. Skeletal muscle insulin sensitivity is strongly influenced by the microvasculature, which controls two critical factors: 1. insulin distribution through the capillary network (number of vessels/surface area); 2. regulation of insulin delivery to the muscle, which depends on blood flow and is modified by vascular tone (vasodilation or vasoconstriction of arterioles). Therefore, an increase in skeletal muscle vascularization or blood flow can facilitate insulin delivery and enhance insulin sensitivity of the tissue(Akerstrom et al. 2014; Kusters and Barrett 2016; Prior et al. 2015). I postulated that the maintained insulin sensitivity within skeletal muscle of HF female mice is a consequence of better tissue vascularization. The cellular actions of estrogen may enhance this phenotype in females since estrogen can promote VEGF-A expression, a dominant stimulator of angiogenesis, and induce proliferation and migration of mature progenitor endothelial cells (Bausero et al. 2000; Liang, Han, and Chen 2013; Minokoshi et al. 2002; Strehlow et al. 2003; Zhao et al. 2008). However, I did not detect a significant difference in capillary content of male and female EDL muscle. This indicates that vasculoprotective effects of estrogen on endothelial cells may not affect capillary number within skeletal muscle of female mice. Furthermore, I confirmed that HF feeding does not alter capillary number within skeletal muscle of either male or female mice. To my knowledge, HF diet-induced alterations in skeletal muscle angiogenesis have been studied in only a few experiments, which investigated only male rodents. These studies show conflicting results ranging from improvements to impairments in capillary content. Silvennoinen et al. reported that 19 weeks of HF diet induces angiogenesis and increases capillary

content within skeletal muscle of male mice (2013). On the contrary, HF diet has been reported to impair skeletal muscle angiogenesis after ischemia-induced capillary rarefaction (Li et al. 2007). Also, Roudier et al. found no significant difference in capillary content between NC and HF rats (2009). In a recent study, our lab also observed no significant difference in capillary content of male mice fed a NC- or HF- diet for 16 weeks (Nwadozi).

Regulation of capillary number is mediated by the expression of pro-angiogenenic and anti-angiogenic factors (Gustafsson 2011). VEGF-A is a predominant stimulator of angiogenesis and THBS-1 is a potent inhibitor of angiogenesis (Bornstein 2009; Gustafsson 2011; Haas 2002). I found that neither sex nor diet influenced VEGF-A and THBS-1 mRNA levels within the EDL muscle. This closely matched with the unchanged capillary content within the EDL muscle of male and and female mice. Maintained expression of angiogenic stimulators and inhibitors may be attributed to lack of a strong stimulation by the HF diet (either due to diet duration or fat content). Silvennoinen et al. reported increased VEGF-A protein levels in quadriceps femoris muscle of male mice following 19 weeks of HF feeding, using a HF diet (60% fat) as we have used but for a longer duration (2013). On the other hand, the long-term duration of a diet may mask the initial anti- angiogenic responses such as changes in the expression of THBS-1. In male mice, THBS-1 levels has been shown to increase in early stages of HF feeding (first 3 weeks) (Inoue et al. 2013). Furthermore, I looked at leptin expression because it is elevated in obesity and has a pro-angiogenic influence by promoting endothelial cell activation, proliferation and survival (Anne Bouloumié et al. 1998; Kurtovic et al. 2015; Scarpace and Zhang 2007; Sierra-Honigmann et al. 1998). Thus, leptin may be a relevant angiogenic regulator in the context of HF-diet induced obesity. No significant influence of sex was observed in muscle mRNA expression of leptin. However, HF feeding significantly increased leptin mRNA expression within the skeletal muscle

of male mice. Since adipocytes are the main source of leptin, this may be due to higher intramuscular adipose within skeletal muscle of HF males as others have observed increased intramuscular adipose and lipid accumulation in obese and insulin resistant individuals (Pan et al. 1997; Stump et al. 2006b).

PECAM-1 is localized on the endothelial cell junctions and its expression in skeletal muscle has been used as indicator of endothelial cell number and capillary content (DeLisser et al. 1994; Roudier et al. 2009). The higher PECAM-1 mRNA expression in HF female mice suggests that more endothelial cells are present within skeletal muscle of HF female compared to HF male mice. Although this is consistent with the reported proliferative effects of estrogen on endothelial cells (Oviedo et al. 2011), it is inconsistent with the lack of change in capillary number in female mice. Endothelial cells also are a major component of arterioles and changes in PECAM-1 could reflect altered arteriole structure or number. I then turned my attention to structural adaptations that may occur in the arterioles of female mice, resulting in higher number of endothelial cells.

4.4 Arteriole Remodeling and Vasodilator Stimuli in Skeletal Muscle

Estrogen can bind to receptors on the surface of endothelial cells and, via PI3k-Akt activation of eNOS, induce NO-dependent vasodilation (Haynes et al. 2000). This increases blood flow which serves as a stimulus for outward remodeling of arterioles or arteriolarization of existing capillaries (Korshunov et al. 2007; Renna et al. 2013). These adaptations may in turn enhance delivery of insulin (due to a wider vessel lumen) and contribute to the maintained insulin responsiveness in HF females compared to HF males. Outward remodeling of arterioles or arteriolarization both require higher endothelial cell content, as observed in HF females, and higher smooth muscle cell content (Baum et al. 2005; Renna et al. 2013). However, MYH11 (a

marker of smooth muscle cell content) mRNA expression was not significantly different between HF male and HF female mice, which does not support the assumption that structural modifications in muscle arterioles may account for the observed sex differences in muscle sensitivity to *in-vivo* insulin.

I then hypothesized that maintained insulin sensitivity in HF female muscle may occur as a result of estrogen induced vasodilation and enhanced delivery of insulin, which could occur independent of changes in capillary or arteriole content. I could not measure blood flow and vascular tone directly in the current experiment. Therefore, mRNA expression of eNOS and Edn-1, which are dominant regulators of vessel tone, was measured. However, no significant influence of sex or diet was observed, which suggests that a vasodilation-vasocontraction balance may be maintained within skeletal muscle of NC- and HF-fed male and female mice. Measurement of eNOS and Edn-1 at the mRNA level may not capture sex or diet-induced differences in the expression of these factors. In contrast to my results, which assessed mRNA levels, previous studies have shown that both diet and sex hormones can influence protein expression and activity of eNOS and Edn-1. It has been reported that HF diet decreases eNOS activity associated with impairments in endothelium dependent dilation of arterioles within skeletal muscle of male rats (Erdei et al. 2006). Furthermore, administration of estrogen has been shown to decrease endothelial cell production of Edn-1 while testosterone can increase edn-1 levels (Akishita et al. 1998; Polderman et al. 1993). Edn-1 is transcribed as a prepro-endothelin1 peptide which must get cleaved at multiple sites for an active end product, therefore its final protein levels may be substantially different from mRNA levels.

Vascular endothelial cells are constantly exposed to shear stress (mechanical force of blood flow on the endothelium) which stimulates expression of genes involved in capillary remodeling.

In this study, expression of three shear-stress induced genes (TGFβ, PDGFβ, and KLF2) was also used as an indirect measurement of blood flow (Walshe et al. 2013). Considering that an increase in the expression of these genes would be associated with higher flow, we can infer that delivery of insulin would also be higher which may account for a higher muscle insulin response in HF females compared to HF males. However, the lack of a significant difference in the expression of TGFβ, PDGFβ, and KLF2 mRNA between HF male and HF female mice did not provide evidence that flow and delivery of insulin is sexually dimorphic. Although, the possibility that shear stress may influence regulation of other genes in a sex-dependent manner cannot be excluded.

To gain a better understanding if sex differences exist in the regulation of other shear stress induced genes, *in vitro* endothelial cell culture experiments examining the direct influence of shear stress on male and female vasculature can be conducted using a parallel plate flow system (Milkiewicz et al. 2011). Altogether, data obtained from the skeletal muscle of male and female mice does not provide conclusive evidence of the influence of sex or diet on structural adaptations in muscle capillaries or arterioles. Moreover, indirect measurements of blood flow and stimuli involved in the regulation of vessel tone does not support the assumption that insulin delivery to muscle may be different between male and female mice as a result of vasculoprotective effects of estrogen. Therefore, the vascular influence on the maintained insulin response of HF female skeletal muscle, but the impaired response in HF male mice, remains to be established.

4.5 Capillary Content and Angiogenic Factors in Visceral Adipose Tissue

Maintained capillary content in the visceral adipose tissue of HF females but not HF male mice provides novel evidence that sex differences exist in adipose tissue capillary adaptations to a high-fat diet. This was detected by lectin staining and confirmed by adipose PECAM-1 mRNA

levels. Accordingly, a lower number of vessel sprouts (branch points) is indicative of capillary rarefaction within adipose tissue of HF male mice. This is consistent with previous data showing lower capillary content within adipose tissue of obese individuals and HF fed male rodents (Michailidou et al. 2012; Pasarica et al. 2009; Spencer et al. 2011). The process of angiogenesis is an integral component of adipose tissue expansion since growth of adipocytes requires a concomitant increase in gas and substrate exchange (Cao 2013; Rupnick et al. 2002; Sun, Kusminski, and Scherer 2011). A healthy adipose tissue expansion requires coordinated paracrine signaling between adipocytes and endothelial cells to maintain adequate vascularization. I postulate that a maintained capillary content within adipose tissue of HF females preserves this cross-talk, which can contribute to a maintained insulin response. In contrast, capillary regression in the adipose of HF males limits distribution of oxygen, hormones, and nutrients. Consecutively, this can be associated with profound changes within adipose tissue microenvironment including local hypoxia, inflammatory cell infiltration, oxidative damage, and fibrosis, all of which can compromise tissue function and response to metabolic stimuli such as insulin (Michailidou et al. 2012; Sun et al. 2011).

A maintained capillary content within adipose tissue of HF females compared to HF males may in part be due to the pro-angiogenic influence of estrogen on VEGF-A and leptin expression. I found that in response to the HF diet, VEGF-A and leptin mRNA levels were maintained within the adipose tissue of female mice. As mentioned earlier, estrogen can stimulate VEGF-A gene expression and induce proliferation of endothelial cells (Bausero et al. 2000). Furthermore, studies show that following ovariectomy, serum leptin levels and expression of the leptin (ob) gene in white adipose tissue is significantly decreased; meanwhile, this can be reversed by estrogen hormone therapy in both women and rodent models (Messinis et al. 1999; Shimizu et al. 1997;

Yoneda et al. 1998). Therefore, estrogen can maintain leptin levels and preserve its pro-angiogenic role in endothelial cell proliferation and survival (Anne Bouloumié et al. 1998; Sierra-Honigmann et al. 1998). In HF male mice, lower capillary density is paralleled by a lower mRNA expression of the dominant pro-angiogenic factor VEGF-A. This is consistent with data showing that the induced deletion of VEGF-A decreases adipose tissue vascularization, whereas its over-expression in HF-fed male mice enhances vascular density (Sun et al. 2011; Sung et al. 2013). Increases in pro-angiogenic factor leptin mRNA did not rescue HF male adipose from capillary regression. This may be due to HF- diet induced upregulation of anti-angiogeneic factors which may negate the pro-angiogenic influence of leptin. However, I found that THBS-1 expression within the HF male adipose was not higher. There is a possibility that endothelial cells may have become resistant to leptin (Hsuchou et al. 2013; Mark et al. 2002), which is another indication of the loss of cross-talk between endothelial cells and adipocytes in HF male mice.

Higher adiponectin expression may also contribute to the maintenance of adipose capillary content in HF female mice, considering that adiponectin has a pro-angiogenic role in differentiation and migration of endothelial cells into capillary-like structures (Maeda et al. 2002). Studies have shown that adiponectin-deficient mice develop insulin resistance in response to both NC and HF diets (Kubota et al. 2002; Maeda et al. 2002). Furthermore, Adiponectin-KO mice exhibit impaired endothelium-dependent vasodilation associated with endothelial cell dysfunction (Ouchi et al. 2003). Collectively, this suggests that a significant reduction in adiponectin expression, as occurred in the adipose of our HF males, may participate in loss of tissue capillaries and induce deficits in adipose tissue response to insulin. Finally, higher expression of eNOS within the adipose tissue of HF female mice may be indicative of higher NO bioavailability and NO-induced vasodilation. As a result, increased blood flow and oxygen delivery helps preserve

vascularization and maintain a healthy adipose tissue environment in HF female mice. Altogether, data obtained from analysis of the adipose tissue capillary content imply that tissue release of adiponectin and pro- angiogenic factors help maintain the vascular niche within the adipose of HF female mice, whereas the metabolic stress of a HF diet induces capillary regression in adipose tissue of males. This established difference may in turn contribute to sex differences in the adipose tissue response to metabolic stimuli and ultimately influence whole body metabolic functions such as glucose homeostasis.

4.6 Summary

In this project I confirmed that whole-body, as well as skeletal muscle and adipose tissue, response to insulin is maintained in HF females but not HF male mice. In skeletal muscle, this was accompanied by higher PECAM-1 expression in HF female mice (EC content), although no significant difference in capillary and arteriole distribution or function was established. This leaves the underlying signaling mechanisms responsible for sex differences in skeletal muscle response to insulin open to be explored. Maintenance of adipose tissue capillary content in HF females was paralleled by a maintained adipose response to metabolic stimuli and a healthy adipokine profile. This highlights the important role of the capillaries in the regulation and maintenance of a healthy microenvironment within adipose tissue, which may contribute to sex-differences in the development of metabolic disorders.

Chapter 5: Conclusion

5.1 Significance

My master's thesis provides novel evidence that sex differences in vascular content exist in response to the metabolic stress of a HF diet. This study, along with others in humans and animal models, demonstrates lower susceptibility of females to the development of diet-induced metabolic dysfunctions (insulin resistance and glucose intolerance) as compared to male mice (Frias et al. 2001; Krotkiewski et al. 1983; Medrikova et al. 2012; Priego et al. 2008). To date, studies investigating sex-associated differences in obesity have predominantly focused on tissue metabolism and fat distribution, while the microvascular contribution to these differences has been overlooked. To fill this knowledge gap, I had the unique opportunity to examine vascular adaptations in two metabolically active tissues (skeletal muscle and adipose tissue), as a possible underlying cause of sex differences in HF-diet induced metabolic dysregulations. This project is a starting point to establish that sex differences in angiogenic responses to a HF diet exist. This provides a glimpse into the identification of novel sex-hormone-dependent pathways that regulate angiogenesis, in addition to providing significant insight into the possible link between capillary content and development of insulin resistance. Collectively, findings from this project highlight the possible associations that may exist between metabolic and vascular parameters and provides the opportunity to explore essential signaling pathways that regulate capillary content within the context of diet-induced obesity.

5.2 Limitations

A limitation of this study is that none of the angiogenic factors or adipokines were measured at protein level. Level of mRNA could be altered by post-transcriptional modifications and differ from final protein levels. To gain a better representation of the angiogenic and adipokine

profile of male and female mice, measurement of these factors at protein level is necessary. However, available antibodies for some of these factors, particularly VEGF-A and eNOS are not accurate. The use of an *in-vivo* insulin stimulation model enabled me to examine the influence of vascular delivery of insulin to skeletal muscle. A recognized limitation of this analysis is that it does not exclude myocyte response to insulin which may contribute to the observed differences. However, our lab has recently observed that in HF male mice, Akt phosphorylation in muscle incubated with insulin *ex-vivo* (where insulin delivery is not dependent on the vasculature) is not impaired in HF male mice when compared to their NC-fed counterparts (unpublished data). Female tissue was collected irrespective of the ovarian cycle. Considering the role estrogen has in transcription of angiogenic factors such as VEGF-A and insulin signaling pathways, differences in estrous cycle of mice at the time of tissue collection may have introduced variability in the results and masked the true extent of statistical significance when compared to male mice (Bausero et al. 2000; Fuente-Martín et al. 2013; Liang et al. 2013). Furthermore, I did not have a direct measurement of blood flow to examine vessel tone and insulin delivery within adipose tissue and skeletal muscle. This can be addressed in future studies by injection of microspheres into the circulation and measurement of blood flow in tissue relative to tissue mass.

5.3 Future Directions

Two mechanisms may be responsible for sex differences in adipose tissue capillary number in the context of HF diet-induced obesity: 1. Steroid sex hormones directly influence regulation of capillary number within adipose tissue (Ajayi, Ogungbade, and Okorodudu 2004; Liu et al. 2002; Losordo and Isner 2001); 2. HF-diet induced changes in sex chromosome gene expression contribute to changes in the expression of angiogenic factors and alter capillary number in a sex-

dependent manner (Link et al. 2013; Lorenz et al. 2015). While it is appreciated that estrogen influences vascular functions and has a protective role in the development of metabolic dysfunctions (Riant et al. 2109), the influence of estrogen and testosterone hormone on the angiogenic regulation of adipose tissue is not established yet. A follow-up project to my Master's thesis project can be to elucidate sex-hormone dependent regulation of capillary number within adipose tissue. This can be accomplished by surgical removal of gonads (gonadectomy) in male and female mice. Both male and female mice can then undergo hormone therapy by injection of estrogen or testosterone while fed a NC or HF diet to see if switch to the opposite sex hormone, while keeping the genetic environment constant, can alter capillary number and angiogenic regulation within the adipose tissue.

Recently, our group has reported the anti-angiogenic influence of FoxO1 transcription factors in HF diet-induced capillary regression within skeletal muscle of male mice (Nwadozi et al. 2016). Measurement of FoxO1 in male and female adipose tissue may help identify if sex-dependent differences in upregulation of FoxO1 accounts for capillary regression in adipose tissue of males, as compared to females which may be protected from this angiogenic influence. If sex differences in FoxO1 levels do exist, we can then examine if sex hormones modify the expression of FoxO1 using cultured murine endothelial cells. Collectively, these experiments can help establish if steroid sex hormones contribute to differences in adipose tissue capillary content in male and female mice faced with the metabolic challenge of a HF diet. Furthermore, they can help gain a better understanding of the microvascular contribution to metabolic dysfunction in the context of HF diet-induced obesity.

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