THE EFFECT OF DIABETES ON THE REGULATION

OF PLACENTA GROWTH FACTOR

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Abstract: Cardiovascular disease is the number one cause of death worldwide, representing 31% of all global deaths. Among them, over 14 million are due to ischemic coronary heart disease and stroke. Additionally, peripheral artery disease is the major cause of limb amputations in the US. Type 2 diabetic patients are at highest risk of mortality and morbidity due to ischemic cardiovascular disease. Arteriogenesis is the normal physiological response to occluded blood vessels by structural remodeling of preexisting collateral arteries. Although, arteriogenesis is vital for diabetic patients, diabetes inhibits this process with poorly known mechanism.

Placenta growth factor (PLGF) is a key mediator of arteriogenesis. Genetic deletion of PLGF reduced arteriogenesis while over expression resulted enlarged stable vessels making it a suitable therapeutic target for compromised arteriogenesis in diabetes. However, little is known about PLGF regulation. Our previous work suggests that PLGF is sensitive to reactive oxygen species. Since physiological oxidative balance is altered in diabetes, PLGF expression and regulation may be affected by diabetes contributing to reduced arteriogenesis in diabetes.

Here, we report that PLGF is down regulated in mouse heart, skeletal muscle and pig coronary arteries and upregulated in mouse aorta under diabetes related metabolic conditions. PLGF inhibition *in vivo* is highly correlated with hypercholesterolemia, oxidative stress (heart and coronary arteries) and hyperinsulinemia (skeletal muscle). *In vitro* finding suggests advanced glycated end product mediated inhibition of PLGF in multiple cell types. In a physiologically relevant model of hind limb ischemia, we found that PLGF is shapely elevated in skeletal muscle. However, this stimulation is severely hampered in the presence of diabetes associated metabolic conditions.

In conclusion, the research presented in this dissertation identifies key metabolic parameters that affect PLGF expression under basal level as well as in the presence of arteriogenic stimulus. Our data from large animal, small animal and multiple cell types are consistent and strongly suggest similar mechanisms of PLGF inhibition occur in diabetic patients. These findings benefit the research aiming to minimize ischemic cardiovascular disease in diabetic patients.

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REVIEW OF LITERATURE

Vascular system and homeostasis

Vascular growth creates the first functional organ system during vertebrate organogenesis (1). The functional circulatory system is a prerequisite for embryogenesis. Failure of vascular growth results in embryonic death by E10 in mice (2). During gastrulation, the vascular system develops from the mesoderm, where hemangioblast differentiation and formation of immature blood vessels occurs to form the primary vascular plexus. This process is known as vasculogenesis (1). Angiogenesis refers to capillary formation, which is also important in embryogenesis establishing blood supply to ectoderm derived organs (3). Post-natally, blood vessel growth (mainly angiogenesis) continues to be important in growth and development. In addition to angiogenesis, blood vessel growth in adults also involves a related process referred to as arteriogenesis or collateral remodeling. Rather than proliferation of capillaries, arteriogenesis mediates formation of functional arteries from preexisting anastomoses. Temporal, tissue specific and genetic variation of regulation of wide range of mediators, along with environmental cues, governs the development and maintenance of a functional circulation system. Dysfunction of the adult vasculature is implicated in the majority of leading causes of mortality.

The primary goal of the vascular system is to deliver nutrients and oxygen to tissue. The blood supply to tissue is variable, allowing it to fluctuate primarily according to the vastly divergent needs of tissue for oxygen according to their metabolism. Moreover, maintaining oxygen and nutrient homeostasis in the internal fluid environment that surrounds and exchanges materials with cells requires dramatic changes in blood flow in time scales varying from seconds to months or years. For instance, during exercise additional blood is delivered to skeletal muscles to meet their increased metabolic needs. This increase happens

in seconds by a process called active hyperemia. Acute changes in blood flow such as this are due to release of vasoactive mediators or changes in vascular tone which result in local arteriolar dilation. In contrast, chronic changes to a blood flow to an organ require long term vascular changes, both during normal growth and development and upon pathological vascular obstruction. However, both acute and chronic adaptations are complementary and are driven by similar environmental variables, which include local oxygen tension and hemodynamic forces such as shear stress. After birth, there are two such major mechanisms of vascular remodeling: angiogenesis, which generates neovessels, mainly capillaries; and arteriogenesis, growth and expansion of existing collateral arterioles leading to formation of large conductance arteries as a compensatory route when the major supply artery is occluded. Despite the high clinical versatility of arteriogenesis in ischemic cardiovascular diseases, the mechanism governing the arteriogenesis remains unknown. Stimulating arteriogenesis in ischemic cardiovascular disease is rapidly developing area of research.

Arteriogenesis is inhibited in diabetes by an unknown mechanism. Dysregulation of arteriogenic growth factors is a major area of research aiming to uncover the basis of this inhibition. The overall goal of the research presented in this thesis is to characterize the effect of hyperglycemia and hyperlipidemia on the regulation of placenta growth factor, an arteriogenic growth factor. The initial part of the chapter one covers the introduction of key concepts and up-to-date literature review. The objectives and the hypothesis will be summarized at the end of chapter one.

Arteriogenesis

First identified by Longland (4), collateral arterioles are small vessels that connect the perfusion zone of one conductance vessel with the perfusion zone of another neighboring conductance vessel. Later, Fulton (5) reported the existence of coronary collaterals in normal hearts as well as in those with heart disease. He also demonstrated a caliber increase of the collaterals, from 10-200 μm in the absence of coronary artery disease to 100-800 μm in the presence of such underlying pathology. Under normal conditions, little flow passes through these vessels, due to their narrow diameter and correspondingly high resistance. However, in response to occlusion of one of the upstream supply arteries, collaterals can enlarge and accommodate a significant level of blood flow. This process is known as arteriogenesis (6), to distinguish it from the related process of capillary formation or angiogenesis.

Arteriogenesis is an adaptive physiological response to vascular obstruction which attempts to preserve blood flow to tissue at risk of ischemia by enlarging and remodeling preexisting collaterals to increase their flow capacity (Figure 1.1)(7;8). This survival mechanism aims to improve oxygen and nutrient supply to the affected tissue, thereby decreasing the chance of tissue damage due to vascular blockage. For instance, arteriogenesis helps to circumvent the blood flow limitation due to atherosclerosis in coronary arteries, which can otherwise lead to myocardial infarction. Thus, it is a natural bypass mechanism that can compensate for occlusion in a supply artery by enlarging preexisting collateral bridges originating from a non-occluded artery. In their unchallenged state, collaterals in the heart would be classified as elements of the microcirculation, ranging from 30 to 100 μm in diameter. This is comparable to 7.5% total cross sectional area of the normal coronary artery, with a flow capacity of 1.5% of normal arterial conduction in canine model (9). However,

during arteriogenesis an arteriole can remodel to become an artery of up to 12 times its original cross sectional area (10).Indeed, these vessels show a propensity to remodel into parts of the macrocirculation and can become arteries larger than 1000 μm in diameter when appropriately stimulated (11). Furthermore, this type of vessel structural remodeling is dependent on endothelial cell and smooth muscle proliferation, and is therefore distinct from vasodilation (6;8).

In addition to the arteriogenic response to pathologic vascular occlusions, increased metabolic demand of tissue due to physical activity can also induce arteriogenesis. For instance, exercise training increases collateral-dependent blood flow to tissues at risk of ischemia and enhances and demonstrate therapeutic benefits.

Key early findings on arteriogenesis

The presence of a collateral circulation in human tissues and its functional benefits were debated for decades. More than two centuries ago, William Heberden, a physician, described a patient who had been nearly cured of his angina pectoris by sawing wood daily which later described as first effort angina (12). This phenomenon highlighted the importance of coronary collaterals flow as an adaptive response to overcome myocardial ischemia (13). In 1911, Matas reported collateral-dependent limb blood flow in 145 human subjects by occluding major arteries with a tourniquet. The limb tissue below the arterial obstruction turned pale and colder; however, some patients were able to restore faint color and temperature even though the main artery remained occluded. The later observation was explained by the presence of efficient collateral circulation below the level of the occlusion (14). Later in 1946, Prinzmetal quantitatively demonstrated the existence of collateral vessels

in post-mortem hearts by perfusing them with radioactive erythrocytes and glass beads. This study reported the existence of collaterals with a diameter of 70-180 μm (15).

Functional benefits of collaterals have been demonstrated in many recent studies with acute myocardial infarction patients (Figure 1.2). The size of infarction following acute myocardial infarction was significantly affected by the presence or absence of well developed, angiographically documented collateral vessels in human patients. Beneficial effect of collateral flow was independent of site of coronary occlusion. Furthermore, cardiac function at hospital discharge, as measured by left ventricular ejection fraction, was significantly higher in patients with well-developed collateral vessels (16).

In a clinical study of 1164 cases of acute MI, Antoniucci et al. reported that the presence of well-developed collateral arteries increased the survival rate and decreased cardiac events after percutaneous coronary intervention, compared to patients with poorly developed collaterals (17). Significant majority of patients with well –developed collaterals were nondiabetic but demonstrated greater incidence of chronic angina, multi vessel disease and total chronic occlusion compared to that of patients without collateral arteries (17).

Another large scale study of 1697 patients with chronic total coronary artery occlusion (defined as >50% stenosis in ≥ 1 coronary artery) revealed the importance of the collateral circulation in preserving cardiac function despite the presence of coronary artery disease. Interestingly, previous myocardial infarction was not reported in 60% of this patient population, even though all patients had chronic total coronary artery occlusion. In addition, more than 50% of the patients had normal left ventricular function. Together, these observations indicate the existence of an efficient collateral circulation in many patients,

which serves as an alternative source of blood supply to the myocardium, preventing myocardial death (18).

In addition, survival of patients following percutaneous coronary intervention was significantly improved by the presence of coronary collaterals, indicating that coronary collaterals not only reduce myocardial death but also improve myocardial recovery following revascularization therapy. Wang et al reported survival rates in patients with acute myocardial infarction undergoing percutaneous coronary intervention within 12 hours of onset, grouped according to the presence of Angiographically evident collateral filling of the left anterior descending artery (LAD) (collateral group, n=78) and or absence of collateral filling of the LAD (no collateral group, n=111). During the one-year follow up study, the collateral group had significantly higher survival rates compared to no collateral group. However, there were no differences in the occurrence of reinfarction and stent thrombosis between the two groups (19).

Upstream occlusion is a key requirement for functional collateral development. In healthy human hearts, collaterals exist but these small, high-resistance vessels carry little flow compared to the more pronounced collateral arteries of patients with atherosclerosis (20;21). Severe stenosis has been recognized as a potent stimulator of collateral growth in humans (22). Moreover, the degree of occlusion proportionally influences the extent of collateral formation. As shown in dogs, only occlusions that develop a significant pressure gradient across the collateral pathway are capable of inducing outward remodeling. This knowledge has been applied to the development of experimental laboratory animal models to study externally induced arteriogenesis, including the arteriovenous fistula(23), surgical ligation (24-29), ameroid occlusion (29;30), and repetitive occlusion models (31;32).

Similar to the observations in the coronary collateral circulation, peripheral collaterals also remodel in response to arterial occlusion (33-35). Peripheral arterial stenosis animal models reported similar finding that a significant degree of stenosis is a prerequisite for collateral remodeling (36). However, some studies report functional collateral circulation in patients without coronary artery occlusion. Among 100 patients with angiographically normal coronary arteries (no stenosis), 25% had functional collateral flow to the level that there were no signs of MI during a brief experimental coronary occlusion period (37).

Diversity of arteriogenic responses among different animal species and strains

Understanding the diversity in collateral development in response to ischemia across species, as well as across strains within a particular species, has led to an appreciation of the role of genetic factors in arteriogenesis (38;39). Collateral dependent blood flow measurements in different species following acute coronary ligation revealed that considerable differences in the number of collateral conduits exist across species. For instance, the pig heart is sparse in collaterals to the extent that acute coronary ligation is generally lethal and results in widespread infarcted regions (40;41). In contrast, the dog heart has abundant epicardial collateral anastomoses, and acute coronary ligation results in a relatively smaller infarct size (42). Maxwell et al reported a quantitative comparison of coronary collateral circulation in acute myocardial ischemia across eight species as assessed by radioactive microspheres. Comparison of microsphere accumulation in ischemic tissue normalized to non-ischemic tissue showed that the size of the infarct region was directly proportional to the degree of collateral flow. Guinea pigs showed the highest collateral flow (which was as high as flow to non-ischemic tissue), followed by dogs and cats. The species with low collateral flow (in descending order) were rat, ferret, baboon, rabbit, and pig (43).

Other studies are consistent with the finding that subendocardial collateral flow is well-developed in dogs (44), whereas pigs have poor subepicardial and subendocardial collateral flow (45).

Interestingly, different mouse strains show diversity in collateral flow capacity and the extent of collateral remodeling (39;46;47). The collateral flow response to acute femoral artery ligation clearly differs (in order from greatest to least) between C57BL/6, 129S2/Sv, and BALB/c. The number of preexisting collaterals is also high in the C57BL/6 strain, which demonstrates complete flow recovery following femoral artery occlusion. Consequently, ischemic insult is minimal in C57BL/6(8). The opposite is seen in BALB/c, where the lowest number of collaterals, worst recovery, and highest ischemic damage is observed. Similar strain differences in the extent of collateral flow increase in response to femoral artery occlusion have been seen in rats (48). Although strain differences in arteriogenic capacity are not completely understood, they have been attributed to genetic differences in VEGF-A (38) and endothelial nitric oxide synthase (eNOS) expression (49) across strains.

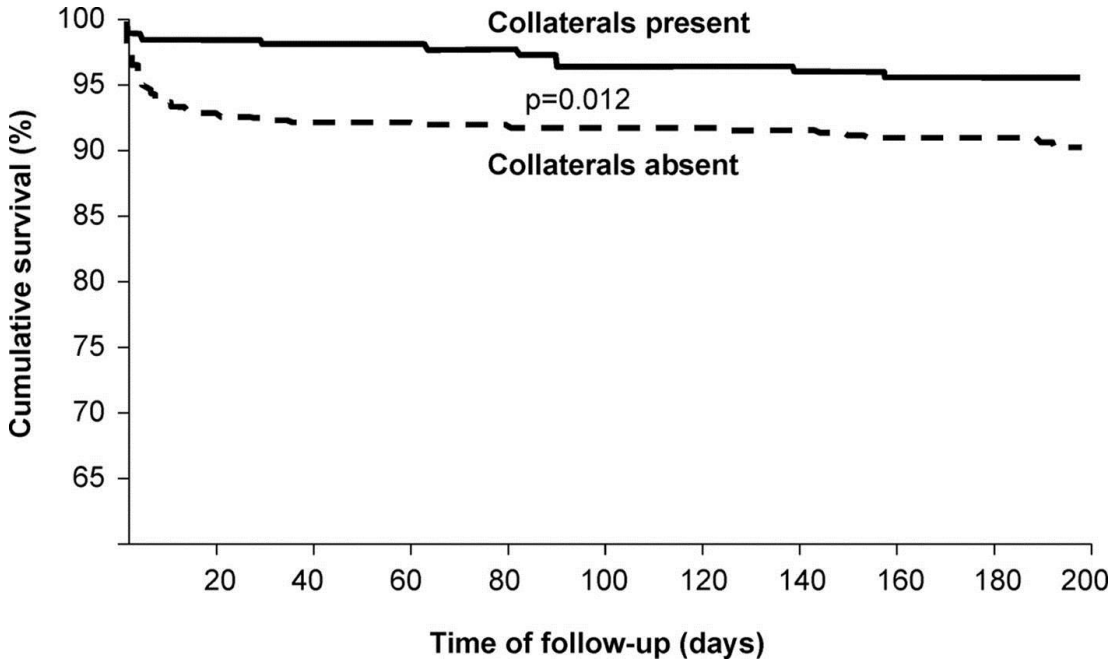

Figure 1.1: Cumulative survival of 264 patients following acute myocardial infarction, grouped according to the presence of coronary collaterals supplying the infarct area. Solid line represents patients with angiographically evident collaterals. Dashed line represents patients without collaterals (50).

Key changes at the cellular level during arteriogenesis

Structural reorganization of peripheral collaterals begins two to three days after an upstream occlusion (10). Several lines of evidence demonstrate that collateral enlargement is an active process involving cellular proliferation, rather than a passive vasodilation. Soon after upstream occlusion, endothelial cells (ECs) lining collateral vessels acquire an activated phenotype characterized by swollen and longitudinal bulges, as opposed to the flat inner surface of unchallenged collaterals (51;52).

Schaper *et al* observed DNA synthesis with thymidine labeling in coronary collateral arterioles of dogs subjected to progressive stenosis of the left circumflex coronary artery (53). The mitosis signal was more pronounced in narrow parts of the vessels and was seen in endothelial, medial and adventitial cells, with peak mitotic activity at 3 weeks post occlusion. Similar results were reported with BrdU/ TUNEL assay (53). Electron and confocal microscopy revealed both proliferating cells and apoptotic cells in narrow regions of the vessels, resulting in neointimal formation with luminal expansion. In rabbit hindlimb, a distinguishable neointimal layer was evident after 2 weeks, although the extracellular matrix was still actively rearranged. Smooth muscle cells of the secretory phenotype were observed in the neointima (54). Monocyte infiltration is another key early event, occurring during the first 1-2 weeks post-occlusion, and abnormalities in monocyte recruitment inhibit arteriogenesis (55). The role of monocytes in arteriogenesis will be discussed in detail later.

Vascular smooth muscle cells (SMC) undergo the most drastic changes during angiogenesis. In normal coronary arteries, SMC in the media are arranged in a circular and very regular structure. This is no longer the case in the growing artery, where the basement

membrane disappears. The number of SMC decreases in the media, mainly because of migration of SMC to the sub-endothelial layer. SMC with a contractile phenotype, characterized by abundant α-smooth muscle actin and scarcity of cellular organelles, switch to a secretory phenotype characterized by prominent rough endoplasmic reticulum, Golgi apparatus, and mitochondria, while contractile components are reduced (54). Furthermore, desmin, a principal intermediate filament protein, disappears in the secretory phenotype (56). SMC proliferation is mainly responsible for the outwardly remodeling pattern of collateral growth. The thickening of the vessel wall varies across species: approximately 3- fold in mice, 10-fold in rabbits, and 20-fold in dogs (57). As collateral growth reaches completion, SMC regain the contractile phenotype and are once again arranged in a typical pattern (58). Interestingly, SMC proliferation and wall thickening in pathological atherosclerosis results in inward remodeling that decreases the luminal diameter (59;60), in contrast to arteriogenesis, where outward remodeling of the vessel leads to elevated blood flow (61) to areas where the primary route has become obstructed. However, the underlying cause for this bidirectional response of blood vessel wall thickening is not clear. It is also important to note that arteriogenesis is a controlled process and self-limited in which collateral remodeling extends to partially restore blood flow sufficient to avoid tissue damage preserving vital tissues from ischemia (62;63). The development process cease and collaterals disappears following intervention of occlusion (64).

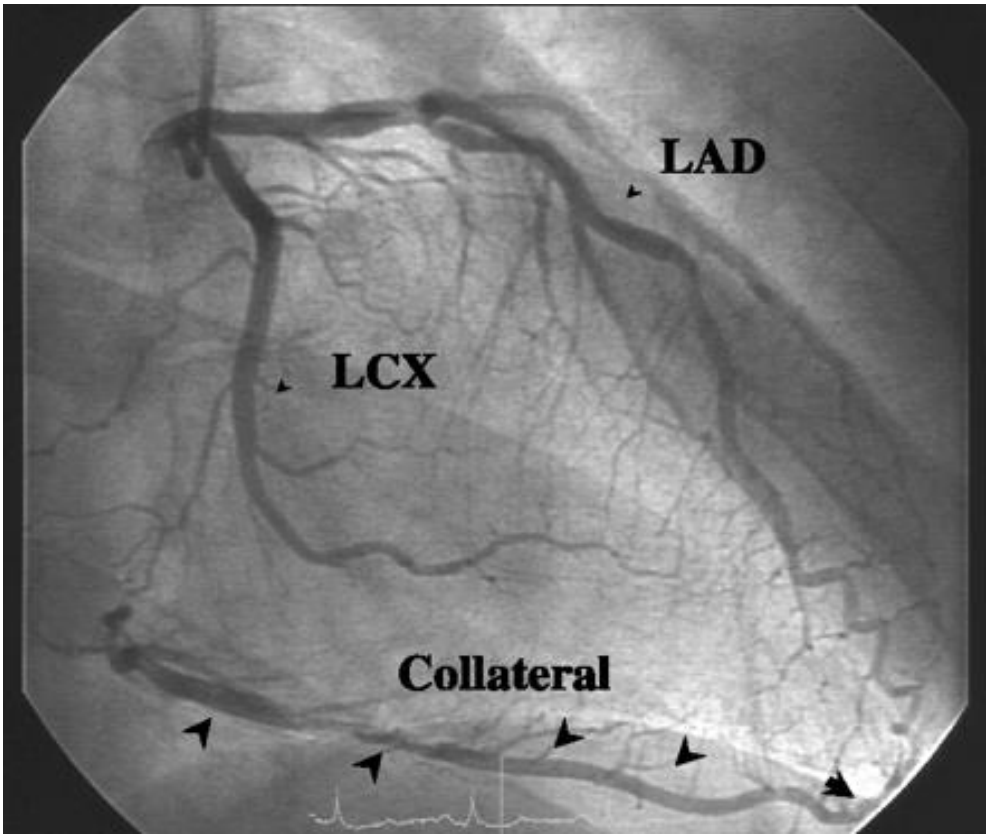

Figure 1.2: A coronary angiograph from a 42-year-old male with a completely occluded right coronary artery (65). As a physiological compensatory mechanism, Rentrop grade III collateral (large arrowheads) has developed from the non-occluded left anterior descending artery (LAD). Restoration of blood flow through the collateral pathway prevented the blocked right coronary from seriously compromising the functional capacity of the heart, as reflected by a left ventricular ejection fraction of 60%. LCX, left circumflex coronary artery.

Angiogenesis

Angiogenesis (proliferation of new blood vessels or capillary formation) is another important form of vascular growth by which both embryonic and adult vasculature acts to maintain oxygen homeostasis and tissue integrity. Sprouting angiogenesis is initiated by vascular leakage and a coordinated loosening of the extracellular matrix (ECM) by EC in response to a proangiogenic stimulus such vascular endothelial growth factor (VEGF-A), an endothelial cell mitogen. ECs proliferate and migrate toward gradients of VEGF-A, first forming immature vessels. These immature vessels eventually form lumens, mature, and adapt to the tissue-specific environment (66). As a physiological process, angiogenesis establishes a functional circulatory system during embryogenesis. Although it plays a vital role both in development and in maintenance of the vasculature, deregulation of angiogenesis is associated with many of the leading causes of mortality and morbidity in the modern world (67). Therefore, both angiogenic stimulation in ischemic cardiovascular disease and angiogenic inhibition in cancer are equally important areas for clinical research.

As previously mentioned, arteriogenesis is the outward growth of a mature collateral arteriole in response to stenosis or occlusion of a major conductance artery, and is driven by hemodynamic factors such as an increase in stretch and/or fluid shear stress on ECs. The process has the capacity to restore the blood supply to an extent that adverse ischemic damages are minimal. Angiogenesis is new capillary formation as oppose to remodeling of preexisting anastomoses and is triggered by different stimuli, with different functional consequences (68).

Angiogenesis is mainly triggered by hypoxia, which occurs in many pathological conditions such as cancer, stroke and ischemic vascular disease. Defective oxygenation of cells leads to activation and increased stability of the transcription factor hypoxia-inducible factor (HIF) one and two (69). HIF is a heterodimeric protein with a constitutively expressed and stable beta subunit and a hypoxia-induced alpha subunit (70). Even though cells constitutively synthesize the alpha subunit, it degrades rapidly by ubiquitination mediated protein degradation under normoxia. Lack of oxygen leads to block alpha subunit being ubiquitination there by increased stability of alpha subunit. This results HIF to form its transcriptionally active heterodimer to enhance the expression of hypoxia inducible genes associated with vascular growth (71). Therefore, HIF functions as a master regulator of oxygen homeostasis and its expression leads to an increase of the transcription of numerous "hypoxia-sensitive" genes including VEGF-A, VEGFR2, erythropoietin, and nitric oxide synthase (72;73).

A nitric oxide-mediated local increase in vascular diameter is one of the first phenomena of ischemia-stimulated angiogenesis which transiently facilitates increased blood supply to the tissue (66). Secondly, VEGF-A mediates hyperpermeability of the vessel wall. An increase in leakage of plasma proteins is observed, which helps forms a scaffold for EC migration after local degradation of the basement membrane of the mother vessel. Elevated permeability is achieved mainly via re-organization of adherens junctions proteins such as vascular endothelial (VE)-cadherin proteins of adjacent ECs, which form the principal permeability barrier throughout the majority of the vasculature. Src kinase signaling also appears to be important in VEGF-A dependent increased permeability, via inhibition of specific phosphatases (74). This is likely leads to increased tyrosine phosphorylation of VE-

cadherin, p120 and β-catenin. Phosphorylation-driven conformational changes alter protein-protein affinities, destabilizing the binding between members of the adherens junction and widening the gap between adjacent endothelial cells, promoting enhanced filtration of fluids and macromolecules (plasminogen, fibrinogen) from plasma to the interstitial space. It is hypothesized that these changes in permeability allow plasma components that promote the angiogenic cascade access to the interstitial matrix, where they stimulate the endothelial cell proliferative and migratory phenotype.

Under normal physiological conditions, capillary ECs are quiescent during their extended life span. However, angiogenic mitogens promote a switch to the EC proliferative phenotype by triggering the switch from the G0 to G1 stage of the cell cycle via signaling through the MAPK (ERK1/2, JNK1/2) axis (75). The initial decision of an EC to acquire a proliferative phenotype is associated with changes in the genetic and biochemical profile of EC which generally are considered to represent activated EC. EC activation refers to both functional and morphological alteration in ECs in response to inflammatory cytokines or hemodynamic forces.

Activated ECs enhance the expression and secretion of membrane type 1-matrix metalloproteinase (MMP), and plasminogen activators which facilitate the cleavage of adhesion proteins and matrix proteins, enabling EC to release contact with the BM (76).This change helps EC become exposed to interstitial collagen, which triggers signaling cascades in EC that are responsible for reorganization of the EC cytoskeleton to form filopodia and/or lamellipodia extensions to invade the interstitial matrix. A VEGF-A gradient drives the formation of a pioneering tip cell, which demonstrates directional chemotactic movement. The tip cell membrane is rich with VEGFR1, VEGFR2 (77) and delta-like ligand 4 (dll4)

(78). VEGF-A also induces mitosis of trailing stalk cells located proximally to the tip, forming a new vessel sprout. Excessive sprouting is limited by a mechanism by which dll4 on the tip membrane binds to its receptor, Notch, on adjacent stalk cells, promoting controlled development of new capillaries (79).

Recruitment of pericytes by EC plays a major role in regulating vascular maturation during angiogenesis, as pericytes act as a "turn off" switch for the proteolytic phenotype of the tip cell (80). Downregulation of tip cell MMP activity, termination of the EC proliferative phenotype, reestablishment of adherens junctions, recruitment of SMC, and generation of a BM mark the maturation process of the new vessels. Lumen formation occurs, mainly by fusion of intracellular vesicles with the plasma membrane. Vessels then stabilize to fulfill the tissue requirements by EC differentiation.

Arteriogenesis versus angiogenesis

In the postnatal organism, 2 types of vessel growth exist: angiogenesis, which involves the proliferation of capillaries mainly through effects on endothelial cells (EC), and arteriogenesis, defined as the development of collateral arteries from preexisting arteriolar connections, requiring the proliferation of both EC and smooth muscle cells (SMC). Angiogenesis and arteriogenesis are driven by two distinct initial triggers. During normal growth and development and in pathological tumor growth, capillary vascular network formation is initiated by hypoxia. It is a logical correlation, according to homeostasis principles, that O_2 stress in tissue should stimulate new capillary formation to restore normoxia. Cells respond to decreased O_2 tension by upregulating several hypoxia-inducible factors and other angiogenic molecules including the VEGF family. VEGF-A is a strong EC

mitogen which also increases EC permeability. Orchestration between pro-angiogenic factors (VEGF-A, FGF2, PDGF, TGFβ−1, ANGPT1) and inhibitory factors (ddl4, Notch, jagged1) that provide negative feedback to prevent pathological angiogenesis is vital for orderly vascular growth.

In contrast, arteriogenesis or remodeling of the collateral circulation can be induced independently from hypoxia (6;81). Ito et al. developed a rabbit hind limb femoral artery ligation model which allows simultaneous examination of both collateral vessel growth and capillary formation (6). In this model, the proximal region (thigh) in which the appearance of corkscrew collateral arteries occurs is normoxic, unlike the distal region (calf) where blood flow is reduced below the level of tissue demands resulting in hypoxia and capillary formation. These authors showed that collateral enlargement was associated with histochemical evidence for EC and SMC proliferation and resulted a 6-fold increase in collateral conductance, compared to non-ligated control. In contrast, a profound perfusion deficiency was observed in the calf region, with no signs of collateral vessel growth but extensive capillary sprouting. The reduced perfusion rates in the calf region clearly predict the hypoxia, while the several fold higher perfusion rates in thigh region are less likely to cause hypoxia. Therefore, this femoral artery occlusion model clearly demonstrated that arteriogenesis and angiogenesis are different in terms of their requirement to trigger by hypoxia.

Hindlimb ischemia models have been widely used in studying collateral development and have been validated extensively. In a separate study, time scale analysis of the mRNA expression profile of the rabbit femoral artery ligation induced arteriogenesis model clearly demonstrated that collateral artery growth is not associated with an increased expression of

VEGF-A (82). Direct measurements of mRNA and protein in growing collateral arteries and in skeletal muscle types which show consistent collateral supply failed to demonstrate an associated increase in VEGF-A, HIF and LDH-A, showing that molecular signature of collateral growth does not show characteristics of hypoxia in contrast to angiogenesis.

In another study, the femoral artery ligation model was used to assess the relative efficiency of collateral development across different mouse strains. Electron paramagnetic resonance oximetry, a technique which allows measuring regional pO_2, demonstrated in C57BL/6 mice that collateralization happened in well-oxygenated tissue. The authors concluded that local ischemia is not essential for collateral growth (47). The available evidence from femoral artery ligation models supports the idea that hypoxia alone is not likely to trigger arteriogenesis. However, in tissues where both types of vascular remodeling happen in an adjacent tissue environment, such as in the ischemic myocardium, where spatial separation of hypoxic territory and the area of collateral formation is limited, ischemia may have an influence on arteriogenesis. Thus, studies to assess the potential effect of hypoxia on collateral growth are best done using models other than hindlimb ischemia.

Physical forces such as altered shear stress through collateral vessels are considered to be the primary stimulus for arteriogenesis (6;10;83-85). When a supply artery is occluded, the blood flow is diverted through these preexisting collaterals, which have little or no flow under normal conditions. Occlusion in the major artery results in a large pressure difference along the collaterals, driving the flow through the collaterals, which originally possess narrow luminal diameters, about 30-100 μm. Since the blood flow resistance is inversely proportional to the fourth power of the vessel diameter, the elevated flow and low vessel diameter exerts high fluid shear stress on the collateral wall, activating the endothelium.

The altered physical shear forces on EC cause a marked increase in the expression of many genes, including monocyte chemoattractant protein-1 (MCP-1) (86) and endothelial surface receptors and adhesion molecules such as selectins, ICAM-1, and VCAM-1 (10) which are involved in monocyte tethering, rolling and migration to the adventitial space (53).

Monocyte adherence and subsequent transformation into macrophages is a pivotal event in the growth of collaterals, since monocytes produce various cytokine mediators, growth factors and proteases involved in arteriogenesis (10;24;53). Monocyte adherence to the growing collaterals was observed at day 3 post-occlusion using electron microscopy and immunohistochemistry in the rabbit hindlimb ischemia model and was associated with maximum cell proliferation in growing collaterals (24). The role of monocytes in the growth of collateral arteries after acute ligation of the femoral artery has been studied in both rabbits and mice. Heil and his group (87) reported that an increased circulating concentration of blood monocytes stimulates arteriogenesis, whereas collateral growth is diminished with reduced monocyte counts. The latter negative phenotype was reversed by injection of purified monocytes, demonstrating the concentration-dependent effect of monocytes on collateral growth.

Furthermore, monocytes accumulated in the area of collateral remodeling showed strong positive expression of bFGF and TNF alpha. The importance of TNF alpha in collateral formation was further supported by elevated collateral density and peripheral conductance in response to externally administered LPS, which is a well-known inducer of TNF alpha expression. Monocytes express chemokine receptor 2, which binds to MCP-1 on activated EC and initiates a cascade of events by producing a repertoire of cytokines including TNF alpha and b-FGF. TNF alpha further augments the monocyte response by

upregulating the expression of adhesion molecules on both ECs and monocytes and upregulation of granulocyte macrophage colony stimulating factor (GM-CSF). B-FGF is a mitogen for both ECs (88) and SMCs (89) and has been shown to potentiate the effect of VEGF (90).

In animal models of peripheral vascular disease, tissue ischemia does not appear to be an important controller of arteriogenesis, as the tissue in which the remodeling artery resides is not hypoxic. Furthermore, arteriogenesis is not associated with hypoxia-induced gene expression pattern or an ischemic metabolic profile. Rather, arteriogenesis is predominantly stimulated as a response to local hemodynamic changes. Thus, angiogenesis and arteriogenesis are governed by two different signaling pathways (91). In addition to their varying dependence on hypoxia, other key differences in signaling have been documented between these two processes. For instance, blockage of the nitric oxide synthase pathway has a profound inhibitory effect on exercise-induced collateral growth in femoral artery ligated rats, but does not prevent exercise-induced capillary proliferation (92).

Placenta growth factor

In 1991, Maglione *et al* isolated and named the placenta growth factor (PLGF) from a human placental cDNA library (93). Human PLGF was predicted as a 149 amino-acid-long protein displaying 53% sequence identity to the platelet-derived growth factor (PDGF)-like domain of VEGF-A. Subsequent gene mapping studies by the same author showed that it is located in human chromosome 14q24 (94). It is an N-glycosylated, secretory protein and a heterodimer in vivo.

PLGF is abundantly expressed in the placenta (95), with the primary site of synthesis in trophoblasts (96) which are the cells producing the outer layer of the blastocyst. Although it was first isolated from placenta, PLGF is expressed in other tissue types such as vascular EC and smooth muscle cells (97), heart, skeletal muscle (98), retina (99) and tumor cells (100).

Isoforms of PLGF

Pioneer studies revealed that, by alternative splicing of mRNA, the PLGF gene produces two different protein isoforms: $PLGF_{131}$ (PLGF-1) and $PLGF_{152}$ (PLGF-2). The latter carboxyl terminal has a 21 amino acid long insertion enriched with basic amino acids (101). The smaller isoform, PLGF-1, has a 20 amino acid signal sequence that is cleaved to yield mature $PLGF_{131}$. PLGF isoforms exist as homodimeric proteins showing different binding ability to heparin. PLGF-2 strongly binds to heparin, mainly due to its positively charged 21 amino acid insertion, whereas PLGF-1 lacks this insertion and does not bind to heparin (102). Mouse genome analysis revealed that there is a single PLGF gene in mice, which encodes for a 150 amino-acid protein which has 65% sequence similarity to human PLGF-2 (103).

Subsequent studies discovered two more isoforms of PLGF produced by alternative splicing of PLGF mRNA in humans, PLGF-3 (104) and PLGF-4 (105). PLGF-3 was also isolated from a human cDNA library prepared from term placenta tissue and consists of 221 amino acids. Similar to PLGF-1, PLGF-3 does not have 21-amino-acid insertion at the carboxyl terminal and therefore is unable to bind to heparin. Mammalian cells transiently expressing PLGF-3 secreted the polypeptide as both monomers and homodimers (104). The

fourth isomer, PLGF-4, was isolated from human trophoblasts and human umbilical cord vein EC, and the sequence was highly similar to PLGF-3 with the addition of the heparin binding domain (105).

Structure of PLGF

X–ray crystallography of PLGF demonstrated two α-helices and seven β-sheets with a cysteine knot (106) structural motif in which two disulfide bridges on β3 and β7 form a loop through which a third disulfide bond between two cystenes on β1 and β4 passes (107). Two additional disulfide bridges between Cys-59 and Cys-68 hold two PLGF monomers in an anti-parallel manner to form a PLGF homodimer (108).

The receptor binding specificity of PLGF for VEGF receptors has been tested for fms-like tyrosine kinase (Flt-1; VEGFR1) (109) and kinase domain region (KDR; VEGFR2) or the murine homologue of VEGFR2, fetal liver kinase-1 (Flk-1) (102;110;111). These tyrosine kinase receptors consist of a signal sequence, a transmembrane domain, an extracellular domain with seven immunoglobulin-like domains, and a consensus tyrosine kinase sequence. Competitive binding assays using chimeric receptor-IgGs and radiolabeled growth factors showed that ^{125}I-VEGF-A binding to Flt-1 was greatly reduced by PLGF, whereas no competition by PLGF was observed for KDR binding of ^{125}I-VEGF-A (102). Therefore, PLGF binds to the Flt-1 receptor, but not to the KDR receptor. In contrast, VEGF-A binds to both Flt and KDR (109;112). X-ray crystallography studies revealed that both PLGF and VEGF-A bind to the second of the seven immunoglobulin-like domains of Flt-1 (108). Flt-1 is essential for early embryonic vascular organization, with gene knockout causing death in utero at mid-somite stages (113). Abnormal vascular channel formation is

observed in the absence of Flt-1. Endothelial differentiation appeared to be normal, however. Flt-1 is expressed in many adult cells including EC (109), vascular SMC (114;115) and monocytes (116).

Site directed mutagenesis of PLGF revealed that mutations at Asp-72 and Glu-73, located in the β3-β4 loop, make PLGF unable to bind to the flt-1 receptor and activate downstream signaling. Thus, these residues are essential for Flt-1 binding (117). Flt-1 activation by PLGF homodimers requires receptor dimerization followed by trans-autophosphorylation of specific tyrosine residues on each Flt-1 cytoplasmic domain (118). As shown for other receptor tyrosine kinase receptors, the receptor transactivation is then able to transduce the signal to the cell interior by recruiting signaling molecules with phosphotyrosine binding domains (119).

PLGF functions

PLGF isoforms have little or no mitogenic activity on EC (100;102;111;120;121). Ligand binding to ECs demonstrated that PLGF binds to ECs through Flt-1 with high affinity and an uncharacterized low affinity binding was also detected (102). However, in this study PLGF binding to bovine adrenal cortex-derived capillary endothelial (ACCE) cells failed to induce tyrosine phosphorylation. PLGF failed to induce a mitogenic response in either cultured ACCE cells or human umbilical vein endothelial cells (HUVEC).

PLGF potentiates the effects of VEGF *in vitro* and *in vivo*. Interestingly, when ACCE or HUVE cells are co-treated with both VEGF (very low amounts) and PLGF, the mitogenic response to the combined growth factor treatment is about 50% higher than to VEGF-A treatment alone. Furthermore, this potentiation effect of PLGF is specific to VEGF, but not to

other growth factors such as bFGF (102;122). In agreement to the previous observation, the VEGF-dependent increase in vascular permeability was potentiated by PLGF in a dose-dependent manner *in vivo*. Altogether, these observations indicate that PLGF potentiates the biological activity of VEGF (102).

Naturally occurring PLGF/VEGF heterodimers have been reported in various human tumor cell lines (100;118;121). Heterodimers formed in vitro have increased mitogenic activity on EC, a higher degree of VEGFR2 binding and tyrosine phosphorylation, and greater promotion of EC chemotaxis, compared to PLGF homodimers. These observations support the idea that PLGF may modulate VEGF induced angiogenesis by forming PLGF/VEGF heterodimers in cells co-expressing both factors.

The femoral artery ligation method has been widely employed to study various aspects of arteriogenesis. PLGF treatment after femoral artery ligation in mice stimulates the growth and expansion of preexisting collateral arteries and also the precapillary arterioles, as a structural adaptation to reduce flow resistance (123). Furthermore, PLGF-boosted collateral expansion not only improves hind limb perfusion more than threefold, but also exercises tolerance as measured by a swim endurance exercise test. VEGF treatment under the same conditions fails to produce the same degree of beneficial structural adaptation and functional improvement in the ischemic hind limb (123).

PLGF knockout is not lethal in mice and PLGF knockout mice demonstrate normal embryonic vascular development (122). In contrast, VEGF is essential for embryonic development and loss of even a single allele of VEGF results embryonic lethality (124;125). However, PLGF knockout mice exhibit an impaired arteriogenic capacity compared to the

wild type in the presence of ischemia, wound healing and cancer, confirming the physiological role of PLGF as a mediator of adaptive collateral formation (122). Interestingly, this phenotype was reversed significantly by the transplantation of wild type bone marrow, demonstrating the importance of bone-marrow derived cells in PLGF-induced arteriogenesis (126).

Although the arteriogenic capacity of PLGF has been established *in vitro* and *in vivo*, little is known about the mechanisms by which the expression of PLGF is regulated. Since PLGF is arteriogenic, shear stress, a key initiator of arteriogenesis, would be a logical potential regulator of PLGF expression in vasculature. Indeed, *in vitro* co-culture studies with human coronary artery endothelial cells and human coronary artery vascular smooth muscle cells revealed that PLGF mRNA expression is increased in smooth muscle cells by high shear stress (physiologically relevant to 60% stenosis) and that PLGF protein levels are also increased (Rashdan and Lloyd, submitted) in agreement with this observation. PLGF has been shown to be upregulated by mechanical stimulation (127). When bronchial epithelial cells are exposed to cyclic stretch, increased levels of PLGF and NO are observed. The amount of PLGF produced is directly proportional to the magnitude of the cyclic stretch as well as the time length of exposure, (127) indicating that regulation of PLGF is sensitive to mechanical stress.

Furthermore, human patients with coronary artery disease and chronic total coronary occlusion showed higher levels of circulating PLGF in blood samples drawn from the distal coronary bed (which is fed by collaterals) (128). Importantly, the release of PLGF appeared to be related to increased shear stress, but not to the duration of the occlusion (with which the level of bFGF was shown to be correlated).

Studies focusing on hypoxia as an inducer of PLGF expression have produced conflicting results, in that some studies reported hypoxia did not stimulate PLGF expression (120) while others did find some evidence of hypoxia mediated upregulation of PLGF (99;129). Hypoxia in human choriocarcinoma cells did not alter PLGF mRNA levels, in contrast to VEGF, which showed a marked increase in both mRNA and protein (120). However, PLGF mRNA levels were about twice the background normoxic control, which may indicate a hypoxia induced PLGF mRNA stabilizing effect. However, when neonatal mice were exposed to hyperoxia for five days and then transferred to room air (which results in ischemic retina), PLGF mRNA was increased in retina. However, in this study VEGF and its isoforms were down regulated during hyperoxia (99). This study model represents retinal obliteration in immature cornea induced by means of hyperoxia, which may have a different pathological influence on PLGF expression as opposed to hypoxia generated as a consequence of vascular obstruction. In another report, cardiomyocyte PLGF expression was elevated by hypoxia in vitro and human allografts from myocardial ischemia patients. PLGF upregulated allografts were positively correlated with reduced ischemic damage suggesting a cardio protective effect of PLGF (130). Studies from our laboratory also found PLGF elevation by hypoxia in human coronary artery endothelial cells (131). Therefore, majority of evidence support hypoxia as a possible inducer for PLGF expression.

Unlike VEGF, which has been extensively studied, very little is known about signaling pathways by which PLGF exerts its biological function. Comparison of PLGF mediated signaling pathways in term trophoblasts and HUVEC showed differential downstream kinase activation (132) with common upstream mediators (133). In trophoblasts, Flt-1 activation of PLGF induced stress activated kinase, JNK and p38 with little induction of

ERK 1/2 signal transduction pathways. In contrast, in HUVEC PLGF induced strong ERK 1/2 signaling with little activation of JNK and p38 pathways. In addition, Gab2, a member of the IRS (insulin receptor substrate)/Gab family of adaptor proteins) and tyrosine phosphatase SHP-2 (Src homology 2 - domain containing tyrosine phosphatase) was uniquely upregulated in HUVEC. However, Nck and PLC γ activation was common in both types of cells. This suggested common signaling mediators in upstream signaling mechanism with divergent stimulation of various MAPK downstream, possibly influencing differential responses according to the cell type (133).

PLGF as a potential therapeutic agent

PLGF administration as a proangiogenic factor may provide a suitable strategy for treatment of ischemic injury secondary to vascular obstruction. PLGF gene transfer into mouse ear skin induced the formation of large, stable tortuous vessels via arteriogenesis (123). PLGF treatment also induces SMC proliferation as indicated by the increased thickness of the collateral branches (123). Furthermore, transgenic mice expressing mouse PLGF under a constitutively active keratin 14 promoter in skin have a hypervascularized phenotype with enlarged and tortuous vessels compared to background control mice (134). (134).

Despite the extensive knowledge of the physiological and pathological role of VEGF, attempts at therapeutic VEGF-A gene transfer in many occasions resulted in inconsistent and ineffective preclinical and clinical outcomes. Undesirable side effects such as an unorganized, leaky vascular phenotype and hemangioma formation have also been reported (123;135-137). For instance, administration of recombinant VEGF protein to induce

arteriogenesis in pig myocardium caused severe hypotension resulting in the death of 50% of the treated animals (138). In phase 2 human clinical trials employing a VEGF gene therapy approach for ischemic vascular disease have been unsuccessful (139;140). Percutaneous intramyocardial transfer of a plasmid encoding VEGF-A in patients with severe coronary artery disease was unable to produce a significant improvement in perfusion deficits (139). In a separate study, intramuscular delivery of an adenoviral vector encoding VEGF-A to the lower extremities of patients with peripheral artery disease failed to promote recovery from debilitating limb performance associated with disabling intermittent claudication (140). This inconsistent and unsatisfactory outcome of VEGF therapy for ischemic diseases suggests that further research is required on other potential growth factors to promote arteriogenesis to circumvent ischemia.

As previously mentioned, monocytes play a vital role in arteriogenesis and monocyte infiltration at sites of collateral expansion is a prelude to arteriogenesis. An important finding that suggested PLGF as a key mediator in arteriogenesis is its chemoattractant behavior towards monocytes (116;123;126;141;142). Interestingly, monocytes express only one type of VEGFR1, with which PLGF specifically interacts (116). This finding marks the first biological role reported for the VEGFR1 receptor in human monocyte recruitment and activation via PLGF. Moreover, PLGF promoted the production of the arteriogenic cytokines TNFα and monocyte chemoattractant protein (MCP-1) by recruiting monocytes/ macrophages (123). Inhibitors of MAPK/MEK and phosphoinositide-3 kinase, but not p38 kinase, abrogate the effects of PLGF on monocytes suggesting potential downstream signal pathways (143).

In vivo evidence also supports the importance of PLGF dependent monocyte attraction. The decreased arteriogenic response to femoral artery ligation in PLGF-/- mice was rescued by administration of human PLGF, and this recovery was shown to be associated with PLGF-induced elevated monocyte population at sites of collateral growth. Monocyte depletion by pharmacological methods prior to femoral artery ligation in rabbits completely abolished the PLGF dependent collateral expansion. Similarly, PLGF-/- mice with hampered arteriogenic ability recovered arteriogenesis upon infusion of exogenous PLGF, but exogenous PLGF failed to rescue arteriogenesis under conditions of depleted circulating monocyte levels (144). This was further supported by impaired arteriogenesis in response to femoral artery occlusion in monocyte/macrophage deficient mice (145). Consistent with the previous studies, these mice showed significant monocytophenia and demonstrated reduced blood flow perfusion seven days after femoral artery ligation, compared to control mice with normal circulating monocyte levels. Furthermore, monocyte deficiency related impairment of arteriogenesis was angiographically evident, with narrow collaterals and a diminished macrophage population as detected by immunohistology.

Effect of diabetes on arteriogenesis

Over 360 million adults are living with diabetes worldwide, and this number is projected to rise to over 550 million by 2030. Diabetes caused 4.6 million deaths in 2011, and cardiovascular complications are mainly responsible for the high morbidity and mortality of diabetes. In fact, more than 50% of diabetics die of cardiovascular complications of diabetes, and diabetes is an independent risk factor for cardiovascular disease. In addition, related disorders such as peripheral artery disease (PAD) and cerebrovascular disease are also complicated by diabetes (146;147).

Diabetes mellitus is one of the major risk factors for atherosclerosis mainly through promoting endothelial dysfunction (148) which in turn precedes the development of ischemic disease. For instance, diabetes patients without previous myocardial infarction (MI) are at 6 fold higher risk of MI than are non-diabetics without history of MI (149). Diabetes is also known to complicate the prognosis of coronary and peripheral artery disease, and the patients have a less favorable outcome after coronary stenting (150;151) or leg bypass surgery (152), and a higher incidence of restenosis (153).

Diabetes mellitus has been recognized as a negative predictor of collateral formation (154-158). Arteriogenesis, a natural adaptive response by outward remodeling of pre-existing collateral arteries when the primary pathway of blood supply is blocked, restores the blood flow to downstream tissue protecting it from ischemic damage. However, patients with coronary and peripheral artery disease demonstrate huge variability in the extent of collateral development. The observed variability in formation of functional coronary collateral vessels among patients with coronary artery disease reflects the fact that co-occurrence of additional disorders may have a role in suppressing the innate vascular response to arterial occlusion. A comparative angiographic assessment of the degree of coronary collateralization between diabetic patients with coronary artery disease and nondiabetic patients with comparable other baseline characteristics revealed a reduced extent of coronary collateral formation in the diabetic condition (154).

The limited ability to develop an effective coronary collateral network in response to occlusion in a primary supply artery may contribute to the observed high mortality of diabetes-associated coronary artery disease. A direct comparison of the relative effects of type 1 and type 2 diabetes on neovascularization following femoral artery ligation revealed

that type 2 diabetes more severely reduced collateral dependent blood flow, compared to type 1 diabetes (159). Therefore, impairments in arteriogenesis associated with type 1 diabetes appear to be less severe than those produced by type 2 diabetes.

Numerous studies have demonstrated a negative effect of diabetes on arteriogenesis. The mechanism by which diabetes inhibits arteriogenesis is largely unknown, however. As previously explained, monocyte migration is a key event in arteriogenesis, and several studies have attempted to evaluate the effect of diabetes on monocyte migration (160). When alloxan-induced diabetic New Zealand White rabbits were subjected to femoral artery ligation, they developed a reduced number of collateral vessels, with narrow luminal diameter. The vessels delivered less blood compared to collaterals of non-diabetic rabbits. Furthermore, when monocytes were collected from these two groups and tested for VEGF-A and MCP-1 mediated chemotaxis, the monocyte population from the diabetic rabbits showed significantly less migration compared to monocytes from control subjects. Reduced migratory response of monocytes from diabetic rabbits was also shown to be specific, as only VEGF-A and MCP-1 mediated migration was abrogated. When monocytes were stimulated with FormylMetLeuPhe (fMLP) peptide, a known chemoattractant for monocytes, no difference was observed between groups for migration.

Several other studies, including work on monocytes from diabetic human patients, demonstrated the same reduced chemotactic response of diabetic monocytes, indicating one negative aspect of diabetes that may explain, at least partly, the compromised arteriogenic phenotype in the diabetes disease environment (161). As current knowledge supports the notion that monocyte recruitment to growing collaterals is PLGF dependent, it is possible that diabetes negatively influences PLGF-dependent monocyte recruitment to sites of

arteriogenesis. Although the level of VEGF (162;163), and bFGF (164) has been shown to be suppressed and elevated respectively in diabetic cardiac tissue, no studies have evaluated the effect of diabetes on baseline expression of PLGF, which is a potent mediator of arteriogenesis. Therefore, more studies are needed to characterize the effect of diabetes on PLGF expression. This is one of the primary goals of this study.

Sudden elevation of blood flow through collateral vessels with narrow lumens causes a significant mechanical force on the endothelial lining of these vessels. As discussed above, shear stress sensed by the endothelium has been identified as a key initiating stimulus for arteriogenesis. Furthermore, endothelial dysfunction is related to the pathology of vascular diseases associated with diabetes (165;166). Therefore, impaired shear stress sensing by the endothelium may suggest a parallel mechanism of suppressed arteriogenesis in diabetes patients. In an experimental porcine model of left iliac artery ligation, when the right iliac artery was infused with glucose to mimic acute hyperglycemia, normal shear stress induced vessel dilation was inhibited while acetylcholine mediated vasodilation was preserved (165). In contrast, normoglycemic control vessels responded to both shear stress and acetylcholine dependent vasodilatation. The authors concluded that hyperglycemia impaired the endothelium dependent mechanotransduction of shear stress.

Endothelial dysfunction is a complex process, where loss of the optimal balance between the production and degradation of mediators of normal function is altered. One such pathological imbalance is oxidative stress, in which the normal balance of reactive oxygen species (ROS) generation and their neutralization is destroyed. Several studies have demonstrated a link between oxidative stress and impaired arteriogenesis (167-169). Indeed, these studies demonstrated the redox window concept, which states that optimal ROS

concentrations are critical for normal vascular function (169-171). For instance, repetitive ischemia (RI) in rat left anterior descending artery stimulated arteriogenesis as measured by collateral dependent blood flow (169). However, this phenotype was reversed by pharmacological inhibition of either NADPH oxidase, which activates the production of superoxide (O_2^{2-}) radical or specific inhibition of superoxide dismutase, which converts O_2^{2-} into H_2O_2. H_2O_2 is further metabolized into molecular O_2 and water by catalase (167). In vitro, VEGF driven human coronary artery EC and SMC proliferation (169) was highly sensitive to either increased or decreased ROS. This indicates that optimal ROS levels are critical for collateral growth, and that they have a physiological role in cell signaling (172).

RI induced arteriogenesis was shown to occur via a mechanism involving p38 kinase, as pharmacological inhibition of this signaling enzyme abrogated the arteriogenic response (167). In addition, p38 kinase was shown to be activated during normal EC proliferation, and this activation of p38 kinase is redox sensitive both *in vitro* and *in vivo* (167). Moreover, further studies added more candidates to this signaling mechanism, including Akt and Src, whose activity was sensitive to the cellular balance of ROS (169). Overall, both antioxidant treatment and pathological oxidative stress interfere with the physiological role of ROS in arteriogenesis, where the latter condition more resembles the diabetes/metabolic syndrome related suppression of collateral formation.

Research from our laboratory has demonstrated the signaling role of H_2O_2 as a mediator of intercellular communication between EC and SMC. Shear stress arising from increased blood flow through collaterals stimulates EC and triggers the production of various soluble mediators that act on underlying SMC. We hypothesize that SMC produce PLGF in response to mediators from shear-stimulated EC via H_2O_2 dependent cell signaling. When

EC-conditioned culture medium was added to SMC, there was a ~4.5 fold upregulation of PLGF gene expression compared to SMCs that were not exposed to EC-conditioned medium (173). This upregulation was determined to be due to increased H_2O_2 in the EC medium. Further studies in our lab demonstrated that externally added, physiologically relevant levels of H_2O_2 increase PLGF gene and protein expression as well as the mRNA half-life (174) of PLGF. Therefore EC produced H_2O_2 acts as a soluble mediator for arteriogenesis. This evidence suggests that proper regulation of ROS is vital to normal physiological arteriogenesis, and that disruption of the ROS balance can potentially cause impairments in arteriogenesis, as seen in diabetes.

The use of mouse models in diabetes research

Diabetes is characterized by absolute lack of insulin production (type 1) or insulin resistance and the inability of beta cells to compensate (type 2). Type 1 and type 2 diabetes represent diverse endocrine disorders that affect different bodily systems differently. In animal models of type 1 diabetes, lack of insulin production is achieved mainly by chemical disruption of the beta cells, leading to hyperglycemia. In contrast, type 2 diabetic animal models are mainly generated by naturally occurring genetic variations or genetic manipulation, leading to obesity associated insulin resistance. When selecting an animal model to study CVD complications associated with diabetes, it is important to consider the aspect of the disease being investigated. For instance, if the primary focus of the study to evaluate the effect of hyperglycemia on CVD, a type 1 diabetic model is suitable. If insulin resistance is the central aspect of the study, a type 2 diabetic model is appropriate (175).

Mouse models of type 1 diabetes

Streptozotocin model: Streptozotocin is administered i.p. or i.v. and accumulates in pancreatic beta cells through Glut-2 transporter uptake. Streptozotocin alkylates beta cell DNA, which in turn leads to NAD+ depletion (176;177). Subsequent reduction of beta cell ATP results in inhibition of insulin production. There are two major dose regimens: a single high dose (200 mg/kg) or multiple low doses (50 mg/kg daily for 5 days). One disadvantage of the single high dose regimen is that possible regeneration of beta cells can occur, which raises the need of appropriate controls for the experiment. Another unfavorable feature of this model is that non-targeted toxicity of streptozotocin to other organs can occur. This drawback can be partially overcome by using the multiple low-dose regimen (178). Depending on the duration and severity of diabetes, these mice are reported to develop systolic and diastolic dysfunction when associated with dyslipidemia (179). Increased fatty acid oxidation, reduced glucose oxidation and reduced pyruvate dehydrogenase activity has been observed in cardiac tissue of these mice (180).

Alloxan model: Alloxan is administered at a rate of 40-200 mg/kg, depending on the strain and route of administration. Alloxan is rapidly taken up by beta cells. Similar to streptozotocin, alloxan preferentially accumulates in pancreatic beta cells through GLUT 2 glucose transporter uptake. Alloxan is a free radical generator causing DNA fragmentation. Alloxan also oxidizes essential thiol groups in proteins, and disturbs intracellular calcium homeostasis. The reduced form of alloxan (dialuric acid) is then autoxidized to superoxide, hydrogen peroxide and hydroxyl radicals. These hydroxyl radicals induce beta cell death, since these cells have a low redox neutralization capacity (181). Selecting the proper alloxan dose is highly important, since it has a narrow diabetogenic dose range, and even slight overdosing can cause kidney toxicity.

Non-obese diabetic mice: The spontaneous development of autoimmune disease that disrupts pancreatic beta cell insulin production is the mechanism for development of type 1 diabetes in this rodent model (175). The non-obese diabetic (NOD) mouse is the most commonly used autoimmune model for type I diabetes. NOD mice develop insulitis at the age of one month, which causes partial beta cell disruption leading to pre-diabetes (182). The onset of overt diabetes occurs at around 3-4 months, when 90% of the pancreatic insulin production is affected. There is a high gender bias for development of diabetes in NOD mice, with diabetes more prevalent in females (60-90%) than males (10-30%). An important feature of the disease progression is that NOD mice tend to lose weight and require insulin treatment. Furthermore, MHC class 2 in NOD mice and human share high structural similarity. Therefore, NOD mice are useful in studying mechanisms of type 1 diabetes (183).

AKITA mice: AKITA mice were originally derived in Akita, Japan from a spontaneous mutation of the insulin 2 gene in the C57BL/6NSIc mouse (184). The mutation causes abnormal processing of pro-insulin, which causes accumulation of misfolded protein in the ER, leading to ER stress. The onset of insulin-dependent diabetes begins at one month of age and is associated with dyslipidemia (185). Homozygotes fail to survive more than three months if untreated. This model, combined with LDL receptor deficiency, has been used to study type 1 diabetes related macrovascular disease (186) and nephropathy (187).

Mouse models of type 2 diabetes

Lep$^{ob/ob}$ mice: The Lep$^{ob/ob}$ genetic model in the C57BL/6 background is characterized by a monogenic mutation that leads to leptin deficiency (188). Lack of leptin causes hyperphagia-related severe obesity. Significant weight gain is observed early as 2 weeks, and the mice

develop hyperglycemia by one month of age. Dyslipidemia, physical inactivity and infertility are characteristic features of this model (189). Insulin release is maintained without complete beta cell failure, and animals usually die at around 14 months of age. $Lep^{ob/ob}$ mice develop cardiac hypertrophy with subtle impairment of systolic function. It is accepted that $Lep^{ob/ob}$ mice recapitulate the metabolic phenotype and cardiac remodeling of obese humans with insulin resistance (190). However, the direct contribution of leptin deficiency in CVD has not been characterized yet.

$Lepr^{db/db}$ mice: The $Lepr^{db/db}$ genetic model in the C57BLKS/J background carries an autosomal recessive mutation in the leptin receptor (191). Similar to $Lep^{ob/ob}$ mice, excessive eating, obesity, hyperinsulinemia and hyperglycemia are observable in these mice starting at a very early age. $Lepr^{db/db}$ mice develop ketosis after a few months of age and have a relatively short life span. High levels of serum fatty acids and triglycerides are also observed (192). Cardiac hypotrophy and contractile dysfunction is more intense in $Lepr^{db/db}$ mice than in $Lep^{ob/ob}$ mice (193). Furthermore, increased fatty acid oxidation and lipid storage combined with reduced glucose uptake and oxidation is evident in these mice (194), similar to $Lep^{ob/ob}$ mice. Similar to $Lep^{ob/ob}$ mice, the effect of abnormal leptin receptor function on the CVD phenotype is incompletely understood. Interestingly, the gene transcription profile of $Lepr^{db/db}$ mice subjected to hindlimb ischemia revealed a different transcript signature, compared to the wild-type background strain (195).

Diet-induced altered glucose homeostasis: The first study reporting the use of high fat fed C57BL/6 mice as a model for diabetes was published in 1988 (196). This model allows researchers to circumvent the potential effects of altered leptin signaling. Characteristic features of this diet-induced model include obesity, hyperinsulinemia, dyslipidemia and

altered glucose homeostasis, in the absence of genetic manipulation. However, hyperglycemia and insulin resistance is not as severe in this model as has been observed in $Lep^{ob/ob}$ or $Lepr^{db/db}$ mice. Since the major inductive force is caloric excess rather than genetic manipulation, the diet-induced model is considered to mimic of the typical path of human diabetes progression. However, in order to observe irreversible metabolic dysfunction and impaired cardiac function, the high fat/high sucrose diet consumption needs to be maintained for over 20 weeks (197;198). Thus, studies of diet-induced diabetes are lengthy. Many different mouse strains have been used to study diet-induced alterations in glucose homeostasis. Care should be taken when selecting the mouse strain, since differential susceptibility to diet-induced disease across mouse strains has been reported (199;200). High fat and/or high sucrose feeding is often combined with genetic models to generate additive or gradient metabolic effects (201). Prolonged feeding of a high fat diet recapitulates the early phases of diabetic cardiomyopathy, in which reduction in contractility, cardiomyocyte hypertrophy, cardiac fibrosis, increased fatty acid consumption, reduced glucose oxidation and vasoconstriction all occur (202). In summary, high fat/ high sucrose feeding enables the researcher to model impaired glucose homeostasis and cardiovascular abnormalities in mice without the precipitative effects of genetic manipulation. Another major advantage of this model is that the availability of a wide range of customized diets allows for tailoring of the study to recapitulate the metabolic stage of interest.

Effect of diabetes on PLGF

Detailed characterization of the effect of diabetes on PLGF is yet to be described. However, limited reports in the published literature suggest that diabetes does have effects on PLGF. PLGF expression is upregulated during wound healing in epidermal cells,

keratinocytes and hyperplastic epidermis where new blood vessels grow (203), and the PLGF knockout mouse shows delayed wound healing (122). Similarly, streptozotocin induced diabetic mice demonstrate that the normal PLGF upregulation is abolished in diabetic wounds, contributing to delayed wound healing (204). Overexpression of PLGF by gene transfer rescued wound healing in diabetic animals. This therapeutic effect of PLGF gene transfer was associated with enhanced granulation tissue formation, vascularization and recruitment of monocytes/macrophages.

Acute hindlimb ischemia studies between healthy animals and experimentally induced diabetic animals revealed differences in PLGF expression. In healthy mice, temporal analysis of global gene expression profiles in acute hindlimb ischemia using the microarray technique revealed that PLGF mRNA increases ~3.5 fold at day 1 and ~1.75 fold at day three post ligation in the skeletal muscle surrounding growing collaterals (205). In a different study, vascular repair of acutely ligated hindlimb was impaired in the $Lepr^{db/db}$ type 2 diabetic mouse, and microarray analysis of gene expression revealed differences in vasculogenic molecules. Importantly, PLGF mRNA expression was down regulated at day 14 in these diabetic mice, compared to wild type control (206).

PLGF levels in proliferative diabetic retinopathy were reported to vary in the opposite direction of diabetic skeletal muscle and wound tissue. Vitreous fluid PLGF protein levels were higher in diabetic patients compared to non-diabetic individuals (207). Similarly, elevated PLGF was observed in akita diabetic mice and the PLGF knock out akita diabetic mouse did not show adverse signs of diabetic retinopathy such as diabetes induced retinal cell death, capillary degeneration. The mechanism of protective effect of PLGF -/- mice was associated with inhibition of HIF1 alpha – VEGF signaling (208).

In conclusion, it is well established that PLGF plays an important role in arteriogenesis and that arteriogenesis is inhibited in type II diabetes. Therefore, we hypothesized that regulation of PLGF expression is dysfunctional in type II diabetes and contributes to reduced arteriogenic capacity. To test this hypothesis, we assessed PLGF expression in cardiac, skeletal muscle, and vascular tissue of mice fed a Western-type diet to induce a metabolic phenotype similar to that of humans with type II diabetes determined whether PLGF was correlated with key metabolic variables. We further assessed the effect of a Western-type diet on the response of PLGF to hindlimb ischemia induced by gradual femoral artery occlusion. Lastly, we conducted in vitro studies to characterize cell-specific responses of PLGF expression to key metabolic variables.

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CHAPTER II

PLACENTA GROWTH FACTOR EXPRESSION IN MOUSE HEART AND PORCINE CORONARY ARTERIES CORRELATES WITH LONG TERM DIET INDUCED HYPERLIPIDEMIA AND OXIDATIVE STRESS

Abstract

Arteriogenesis (collateral artery remodeling) is a survival adaptation of the vasculature in response to occlusion, minimizing ischemia. Stimulation of arteriogenesis would be highly beneficial in diabetics, who are especially prone to develop ischemic cardiovascular disease. However, arteriogenesis is suppressed in diabetes by an undefined mechanism. Placenta growth factor (PLGF) is a key arteriogenic factor whose expression is elevated during myocardial ischemia. Furthermore, PLGF expression is sensitive to oxidative stress *in vitro*, and oxidative stress occurs in diabetes associated hyperglycemia and hyperlipidemia. Thus, we hypothesized that decreased PLGF levels might contribute to impaired arteriogenesis in diabetes. To test this hypothesis, we studied PLGF expression in cardiac tissue of C57BL/6J and ApoE-/- mice fed either a control diet or a Western-type diet high in fat, sucrose, and cholesterol for 6 mo (N=6-14/group). Male and female mice were studied separately to identify possible gender differences. Mouse phenotype was characterized by measuring plasma cholesterol, triglycerides, insulin, isoprostane (a biomarker of oxidative stress), and by performing intraperitoneal glucose tolerance testing. Cardiac PLGF protein expression was significantly decreased by ~ 40-50% in all Western diet fed groups, regardless of gender ($p< 0.005$). This decrease was specific to PLGF, as the related factor VEGF-A and VEGFR1 (the PLGF receptor) were not affected by diet. To study the mechanism for decreased PLGF expression, we performed regression analysis of PLGF protein levels vs metabolic parameters. Total cholesterol, triglycerides and isoprostane were well correlated ($r^2>0.75$, $p<0.001$) with reduced PLGF protein, while PLGF was poorly correlated with glucose, insulin, and the HOMA-IR index. These results provide strong support for our hypothesis that PLGF

expression is inhibited by hyperlipidemia and oxidative stress. In order to begin to characterize the cellular basis for the overall effects observed in cardiac tissue, we treated human coronary artery endothelial cells (HCAEC) with oxidized LDL (Ox-LDL, 50-200 μg/ml), advanced glycation end products (AGE, 300-1000 mg), and H_2O_2 (0.05-0.3 mM), since these factors increase during hyperlipidemia, hyperglycemia, or oxidative stress. Interestingly, hyperglycemia-related AGE, but not Ox-LDL or H_2O_2, significantly decreased PLGF protein levels by ~50% in HCAEC ($p<0.05$). Further experiments are underway to test the effect of these treatments on PLGF expression in cardiomyocytes.

Introduction

Atherosclerotic cardiovascular disease (CVD) is the leading cause of morbidity and mortality in patients with type II diabetes (1-5). Diabetes is an independent risk factor for coronary and peripheral artery disease and worsens patient prognosis (6;7). For instance, diabetics without previous myocardial infarction (MI) have a risk of infarction similar to that of nondiabetics with prior history of MI (8). Metabolic disorders such as hyperlipidemia, metabolic syndrome and obesity commonly co-occur with type II diabetes, and make the risk of CVD even higher (9). Current treatment strategies (e.g. coronary artery bypass grafting, stenting) for CVD are invasive. Diabetes also reduces the efficacy of coronary artery bypass grafting and percutaneous revascularization and increases the chance of restenosis (10-14). There is, therefore, a critical need for alternative treatment regimens for diabetics with ischemic CVD.

An important physiological mechanism that can protect tissue from ischemia due to upstream vascular stenosis is the enlargement of pre-existing collateral arteries, or

arteriogenesis (15-19). Arteriogenesis is an adaptive process of structural alteration that serves as a bypass mechanism to improve O_2 and nutrient supply to affected tissue, thereby decreasing the chance of damage due to ischemia. In their unchallenged state, collaterals in the heart (20) are elements of the microcirculation, ranging from 30-100 μm in diameter. Nevertheless, they can remodel into arteries larger than 1 mm in diameter when stimulated (21). When an upstream artery is occluded, a pressure gradient across the collaterals (linked to an adjacent supply artery) develops. Blood flow is then diverted through collaterals, generating high shear stress on the endothelium. This increase in shear stress is considered to be one key trigger for arteriogenesis, although other factors such as hypoxia may also play a role (22).

Arteriogenesis mainly depends on locally generated growth factors, vasoactive substances and hemodynamic stimuli (23). However, details on the key molecular players governing arteriogenesis and their regulation remain obscure, limiting therapeutic applications.

Placenta growth factor (PLGF), a vascular endothelial growth factor (VEGF)-related protein, is a key mediator of arteriogenesis (24-26). Although much work has been done on VEGF-A, little is known about mechanisms regulating PLGF expression in cardiac and vascular tissue. Although PLGF and VEGF-A share more than 50% sequence identity, they also show functional diversity. PLGF binds solely to VEGFR1, whereas VEGF-A binds to both VEGFR1 and VEGFR2 (27). The physiological effects of PLGF and VEGF-A are likewise distinct. PLGF is strongly arteriogenic and is thought to act primarily on monocytes (26;28), whereas VEGF-A is more angiogenic and acts mainly on endothelial cells (29). PLGF-/- mice exhibit impaired arteriogenic capacity compared

to wild type mice in ischemia, wound healing and cancer, confirming the physiological role of PLGF as a mediator of collateral formation (24). Furthermore, administration of PLGF following experimental vascular ligation significantly improves the arteriogenic response in mice and rabbits (25;26). Clinically, increased expression of PLGF following acute myocardial infarction predicts improved patient outcome, as assessed by increased left ventricular ejection fraction (30). Hence, pharmacological modulation of PLGF expression or signaling offers a potential therapeutic strategy to compensate for atherosclerotic occlusion by promoting the development of an efficient collateral circulation (31).

PLGF was first discovered in placenta, but is also expressed in skeletal muscle, heart, lung, thyroid, adipose tissue, tumors, and adult vasculature (24;32-34). In vessels, PLGF is expressed by both endothelial cells (EC) and smooth muscle cells (SMC). Previous studies from our lab showed that PLGF expression can be induced in SMC by H_2O_2, an important vascular signaling molecule produced by EC (25;35). PLGF expression is increased in collaterals following upstream ligation (36), suggesting that shear stress may be an important regulator of PLGF. Patients with ischemic CVD have significant variability in collateral formation, reflecting the fact that co-occurrence of additional disorders may suppress arteriogenesis. Importantly, diabetes and/or metabolic syndrome are negative predictors of collateral formation (37-43). Limited ability to develop an effective coronary collateral network in response to occlusion may contribute to the increased risk of MI in type 2 diabetics. The mechanism by which diabetes inhibits arteriogenesis is largely unknown. However, a direct comparison of the relative effects of type 1 and type 2 diabetes on peripheral collateralization in mice revealed that type 2

diabetes was more inhibitory than type 1 diabetes (44;45). Obesity and associated hyperlipidemia were also shown to be negatively associated with poor collateral growth (46). This finding suggests that factors associated with type II diabetes (hyperglycemia, hyperlipidemia, and hypercholesterolemia) may contribute to the suppression of arteriogenesis in type 2 diabetics. Indeed, mouse studies indicate that hyperlipidemia can significantly reduce collateral growth, and that the impairment is cholesterol dependent (44;47). During hyperlipidemia, low-density-lipoprotein (LDL) can be converted to oxidized LDL, which suppresses vascular remodeling by downregulating growth factor expression and increases the risk of atherogenesis by inducing inflammatory molecule production (48-50). Hyperglycemia can also negatively affect the collateral growth process, as shown by a reduction in arteriogenesis in an alloxan induced hyperglycemic diabetic rabbit (51). Persistent hyperglycemia leads to the generation of advanced glycation end products (AGE), which are formed by glycation of proteins in the presence of high glucose or aldehyde. Increased levels of AGE are considered to be a major contributor to impaired vascular growth in diabetic patients, as restoration of collateral growth was observed after inhibiting AGE formation (52).

Arteriogenesis is regulated by the interaction of growth factors generated locally in the ischemic myocardium in a temporal specific manner (53;54). Studies in chronic totally coronary occluded patients identified several growth factors that were elevated, including PLGF, basic fibroblast growth factor, macrophage chemoattractant protein-1, and transforming growth factor beta, and suggested that these factors could be induced by shear (55). Impaired growth factor signaling is considered to be one mechanism for poor collateral growth in diabetes and metabolic syndrome. However, the effect of these

conditions on PLGF regulation in the cardiovascular system has not been studied in detail. Therefore, the goal of this study was to identify metabolic parameters influencing PLGF expression in the cardiovascular system, and to identify the mechanism(s) by which type II diabetes-associated metabolic dysfunction modulates PLGF expression and signaling.

We have previously shown that reactive oxygen species (ROS) stimulate PLGF expression at low, physiological levels (35;56). However, a hallmark of the diabetic vasculature is oxidative stress, in which the normal balance of ROS generation and neutralization is lost. Several studies have demonstrated a link between oxidative stress and impaired arteriogenesis (57-59). Indeed, these studies led to the "redox window" concept, which states that optimal ROS concentrations are critical for arteriogenesis (58;60;61). We therefore hypothesize that PLGF is subject to the same redox window, with low ROS concentrations enhancing PLGF expression and arteriogenesis, whereas chronic oxidative stress inhibits PLGF expression and arteriogenesis.

Materials and Methods

Animals: C57BL/6J and ApoE$^{-/-}$ mice were either fed a Western-type diet or a control diet. The diet regimen was started when mice were 6-8 weeks of age and was continued for 24 weeks. Male and female mice were studied separately. Therefore, we studied 8 groups: C57BL/6J males fed the control diet (n=9), C57BL/6J females fed the control diet (n=8), C57BL/6J males fed the Western diet (n=14), C57BL/6J females fed the Western diet (n=8), ApoE$^{-/-}$ males fed the control diet (n=6), ApoE$^{-/-}$ females fed the control diet (n=6), ApoE$^{-/-}$ males fed the Western diet (n=6), and ApoE$^{-/-}$ females fed the

Western diet (n=6). All mice had access to food and water ad libitum and were housed in a temperature controlled facility with a 12 hour dark-light cycle. This study was approved by the Institutional Animal Care and Use Committee at Oklahoma State University.

Diets: The Western diet (TD 88137, Harlan) supplied 42% kcal from anhydrous milk fat and cholesterol, 42.7% kcal from carbohydrate, and 15.2% kcal from protein. The control diet (TD120336) supplied 13% kcal from fat, 67.9% from carbohydrate, and 19.2% from protein. The Western diet also has three times more sucrose by weight, compared to the control diet. The source of the relatively high carbohydrate content in the control diet is corn starch and maltodextrin, which are not readily available sources of sugar. Therefore, the Western diet used in this study is characterized by both high fat and high sucrose.

Metabolic phenotyping of the experimental groups: Body weight of each mouse was recorded weekly. Fasting (6 h) blood samples were obtained from all mice at the end of 24 week- diet regime for metabolic measurements. Glucose homeostasis was assessed by intraperitoneal glucose tolerance testing under fasting (6h) conditions in all mice just prior to sacrifice.

Cholesterol and triglycerides: Total plasma cholesterol was determined using the Amplex Red cholesterol assay (Molecular Probes). Total plasma triglycerides were measured using a triglyceride determination kit (Sigma).

Glucose and insulin: Intraperitoneal glucose tolerance testing (IPGTT) was performed on 6 h fasted, non-anesthetized mice by administering a 1g/kg ip D-glucose bolus (~30

mg/mouse in 300μL of water). Venous blood (0.05 mL/mouse/time point; total, 0.25 mL) was drawn from the facial vein for measurement of glucose and insulin at 0, 15, 30, 60, and 120 min. Plasma glucose levels were measured using a portable glucose meter (Life Scan, Inc.). Insulin was measured using a rat/mouse insulin ELISA kit (EMD Millipore, Germany).

Oxidative stress: Plasma 8-isoprostane was measured as a biomarker for oxidative stress using a competitive enzyme immunoassay (Cayman Chemical) according to the manufacturer's protocol.

Tissue isolation: After 24 weeks of the diet, mice were anesthetized with 5% isoflurane for ~5 min. After confirming a completely anesthetized state by applying mild pressure to the toes, the thoracic cavity was opened and the heart and aorta were excised. One third of the heart tissue (a longitudinal section, including both left atrium and the left ventricular region) was immediately immersed in liquid nitrogen and stored at -80°C. Heart tissues were homogenized in 1 mL of lysis buffer containing protease inhibitors (phenylmethylsulfonyl fluoride, sodium orthovanadate, leupeptin, benzamidine hydrochloride, aprotinin, and pepstatin A). Three one-minute homogenizations were done using a Bio-Gen Pro200 homogenizer (PRO Scientific). The homogenates were centrifuged at 700g for 5 minutes and the supernatants were transferred to clean microcentrifuge tubes and stored at -80°C.

Assessment of PLGF, VEGF, MCP-1 and VEGFR1 protein levels: PLGF, VEGF-A and VEGFR1 were quantified using ELISA (Mouse PLGF-2, VEGF-A, VEGFR-1 Quantikine ELISAs, R&D Systems). All samples were assayed in duplicate. Data were

normalized to total protein concentration, as determined by BCA assay (Pierce). All assay plates were read using a Biotek Synergy HT plate reader.

Real time PCR for gene expression analysis: Total RNA was isolated from the thoracic aorta and from cardiac tissue using RNeasy mini columns (Qiagen) following the manufacturer's directions. Following removal of genomic DNA from 1 μg of total RNA, reverse transcription was carried out using the QuantiTect reverse transcription kit (Qiagen). Real time PCR was performed on an ABI 7500 Fast instrument (Applied Biosystems) using PerfeCTa SYBR Green FastMix, Low ROX (Quanta Biosystems). The relative abundance of PLGF, VEGF, VEGFR1, NOX2, NOX4 and HO1 mRNA was determined using the comparative ΔΔCt method with target gene expression normalized to β-actin. Primer sequences are listed in table 1.

Large animal model: Archived porcine coronary artery samples from three separate studies (one in Yucatan miniature swine and two in Ossabaw swine) were used in this project (Lloyd PG and Sturek M, unpublished). The original animal studies were conducted at the University of Missouri (Columbia, MO) and at Indiana University School of Medicine (Indianapolis, IN). Detailed descriptions of the study protocols and characterization of the animals' metabolic phenotype has been previously reported (62;63). Pigs were divided into control (N=16 total) and high fat (N=20 total) groups. Pigs in the high fat groups were fed diets containing up to 78% kcal from fat for up to 55 wk. Control pigs received standard minipig diets. PLGF gene expression was assessed in archived frozen samples of right coronary artery and left anterior descending coronary artery. Similar PLGF gene expression results were found in each study. Thus, the results were combined for further analysis.

RNA isolation and RT-PCR: Porcine artery samples were powdered with a metal pestle in liquid nitrogen. The frozen powder was then transferred to tubes containing Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was then isolated according to manufacturer's protocol. RNA was treated with Turbo DNA Free (Ambion) to remove contaminating genomic DNA according to manufacturer's instructions and reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time quantitative PCR was performed on an ABI 7500 (Applied Biosystems, Foster City, CA) instrument. PCR was performed using TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA). PLGF mRNA was detected with TaqMan probe chemistry and was normalized to the signal of 18s rRNA. All primers were custom-synthesized by Invitrogen (Carlsbad, CA). PCR reactions were run in triplicate for 40 cycles.

Cell culture: Cell culture experiments were performed by Farzana Rouf. Human coronary artery endothelial cells (HCAEC) were purchased from Lonza (Walkersville, MD). Cells were grown to 85% confluence in 12 well plates at 37ºC in a humidified incubator containing 5% CO_2. HCAEC were maintained in endothelial cell basal medium (EBM) supplemented with EGM-2 MV SingleQuot growth factors (Lonza) and used at passage 6 for all experiments. Equal numbers of cells (~25000 cells/cm^2) were seeded on each well of the 12 well plates. The medium of the cells were refreshed the day after the cells were subcultured.

Advanced Glycation End Product (AGE) preparation: AGE was prepared through the glycation of bovine serum albumin (BSA) in the presence of D-(+) glucose or DL-glyceraldehyde. To make glucose derived AGE, 50 mg/mL BSA (Sigma-Aldrich, St.

Louis, MO) was incubated in the presence of 45 mg/mL D-(+) glucose (Sigma-Aldrich) in phosphate buffered saline (PBS, pH 7.4). The solution temperature was maintained at 37˚C for 8 weeks to fully glycate the albumin and to prepare irreversibly formed AGE. As a control, 50 mg/mL BSA was also incubated in PBS in the absence of D-(+) glucose for 8 weeks at 37˚C. Glyceraldehyde derived AGE was prepared by incubating 25 mg/mL BSA with 20 mM DL-glyceraldehyde (Sigma-Aldrich) in PBS at 37˚C for 1 week. After the incubation period, both the control and glucose- or glyceraldehyde-glycated samples were dialyzed in fresh PBS at 4˚C every 2 hours for a total of 4 changes. The glyceraldehyde derived AGE was centrifuged at 3,000 rpm for 30 min in Amicon concentrator tubes (Millipore, Billerica, MA) to concentrate the AGE solution. Total protein concentration in the control and AGE preparations was quantified using the BCA assay (Pierce, Rockford, IL). BSA and AGE samples were determined to be endotoxin free by testing with the LAL chromogenic Endotoxin Quantitation Kit (Life Technologies Corporation, Grand Island, NY).

Treatment of cells: HCAEC were serum starved in 2% serum media for 24 hours after reaching 85% confluence. Cells were then exposed to different treatment conditions for 24 hours. To study the effects of different metabolic parameters (hyperlipidemia, hyperglycemia, oxidative stress) associated with diabetes, cells were treated with different concentrations of oxidized LDL (Ox-LDL, 50, 100, and 150 µg/mL), AGE (glucose or glyceraldehyde derived, 300, 500, 750, and 1000 µg/mL), and hydrogen peroxide (H_2O_2, 0.05, 0.1, and 0.3 mM). Treatments were directly added to the cell medium. Cell medium was collected 4, 8, and 24 hours post-treatment in fresh tubes containing protease inhibitors for quantification of PLGF protein. Ox-LDL was

purchased from Alfa Aesar (Ward Hill, MA), and H_2O_2 was purchased from vWR International (Houston, TX).

ELISA: PLGF production by endothelial cells was quantified using a commercially available ELISA development kit (PLGF DuoSet, R&D Systems, McKinley Place, MN). Cell medium were added to microtiter plates coated with a PLGF capture antibody (prepared overnight) and PLGF present in the samples was quantified following manufacturer's instructions. In brief, the microtiter plates were first blocked with BSA (1%, RT, 1 h). Samples and PLGF protein standards were then added (RT, 2 h) followed by detection antibody (RT, 2 h), streptavidin-HRP (RT, 20 min, in the dark), substrate solution (RT, 20 min, in the dark). The reaction was then stopped using stop solution (provided in the ELISA kit) and absorbance was measured using a BioTek Synergy HT Multi-mode Plate Reader (BioTek Instruments, Winooski, VT) at 450 nm wavelength. Total protein in the samples was measured using the BCA assay at 562 nm wavelength.

Statistical analyses

Differences in mouse metabolic phenotype and mouse protein and mRNA expression were analyzed by 3-way ANOVA followed by post-hoc testing using SAS software. Values are mean ± SEM. * Diet effect by strain/gender; † strain effect by gender/diet; ‡ gender effect by strain/diet. The effects of different treatment conditions on endothelial cell PLGF and protein secretion were assessed using one-way ANOVA followed by Student-Newman-Keuls post hoc method for multiple comparisons. Primer of Biostatistics software (Version 4.02, McGraw Hill) was used for this purpose. In order to identify possible relationships between metabolic parameters and PLGF expression, linear regression and correlation analysis was performed. PLGF gene expression between

control and high fat fed pigs were analyzed by t-test using SigmaStat software. All differences were considered significant at $p<0.05$.

Table1.1 Primers used for gene expression analysis using real time PCR.

Primer	**Sequence 5' to 3'**
Pig PLGF forward	GGAGACGGTCAATGTCACCAT
Pig PLGF reverse	GAGAATGTCAGCTCCACGTAGGA
Probe for pig PLGF mRNA	CAGCTCCTGAAGATCCGCTCTGGG
Mouse PLGF forward	CTGCTGGGAACAACTCAAACAGA
Mouse PLGF reverse	GCGACCCCACACTTCGTT
VEGF forward	CCCTGGCTTTACTGCTGTACCT
VEGF reverse	CTTGATCACTTCATGGGACTTCTG
VEGFR1 forward	TGCGAAGCCACCGTCAA
VEGFR1 reverse	CTCGGCGGGCGTATTTG
HO-1 Forward	CAACATTGAGCTGTTTGAGGAGC
HO-1 reverse	ATCTTGCACCAGGCTAGCAG
NOX2 forward	CACACCGCCATCCACACAAT
NOX2 reverse	GTCACCGATGTCAGAGAGAGC
NOX4 Forward	TGCCCCAGTGTATCAGCATTAG
NOX4 reverse	CCGGAATCGTTCTGTCCAGTC
β-actin forward	AGTTCGCCATGGATGACGAT
β-actin reverse	TGCCGGAGCCGTTGTC

Results

In order to induce diverse metabolic characteristics associated with diabetes and to determine their effects on PLGF and related proteins, C57BL/6J and ApoE$^{-/-}$ mice were fed a Western diet for 6 months. C57BL/6J mice fed the Western diet showed profound hyperglycemia, hyperinsulinemia, a high HOMA-IR index, and obesity, consistent with published results (64-68), whereas the effects of the diet on these parameters were less pronounced in the ApoE$^{-/-}$ strain. We observed significantly elevated fasting blood glucose in the C57BL/6J Western diet groups (both male and female) from as early as week 3-4 onwards. In contrast, the ApoE$^{-/-}$ Western diet fed groups did not demonstrate any impairment in fasting blood glucose until week 13, after which it was inconsistently elevated (data not shown). Fasting glucose levels were elevated among all the Western diet fed groups ($p<0.05$) by week 23 (data not shown). Glucose clearance as measured by IPGTT (Figure 2.1) and presented as area under the curve (Figure 2.2) indicated that both male and female C57BL/6J mice fed the Western diet had impaired glucose tolerance (WD fed C57BL/6J males, 39959 ± 742 vs CD fed C57BL/6J males, 26852 ± 1126; WD fed C57BL/6J females, 44731 ± 2317 vs CD fed C57BL/6J females, 23003 ± 890, $p<0.01$). From all the Western diet fed groups C57BL/6J strain demonstrated highest glucose intolerance ($p< 0.0001$). In contrast, only male ApoE$^{-/-}$ mice fed the Western diet had slightly but significantly elevated glucose intolerance (WD fed ApoE$^{-/-}$ males, 29876 ± 1161 vs CD fed ApoE$^{-/-}$ males, 23524 ± 1344, $P<0.01$), whereas female WD fed ApoE$^{-/-}$ mice were not glucose intolerant. Therefore, IPGTT results confirmed that feeding of the Western diet disrupted glucose homeostasis. However, significant differences were noted for both sex (in ApoE$^{-/-}$) and

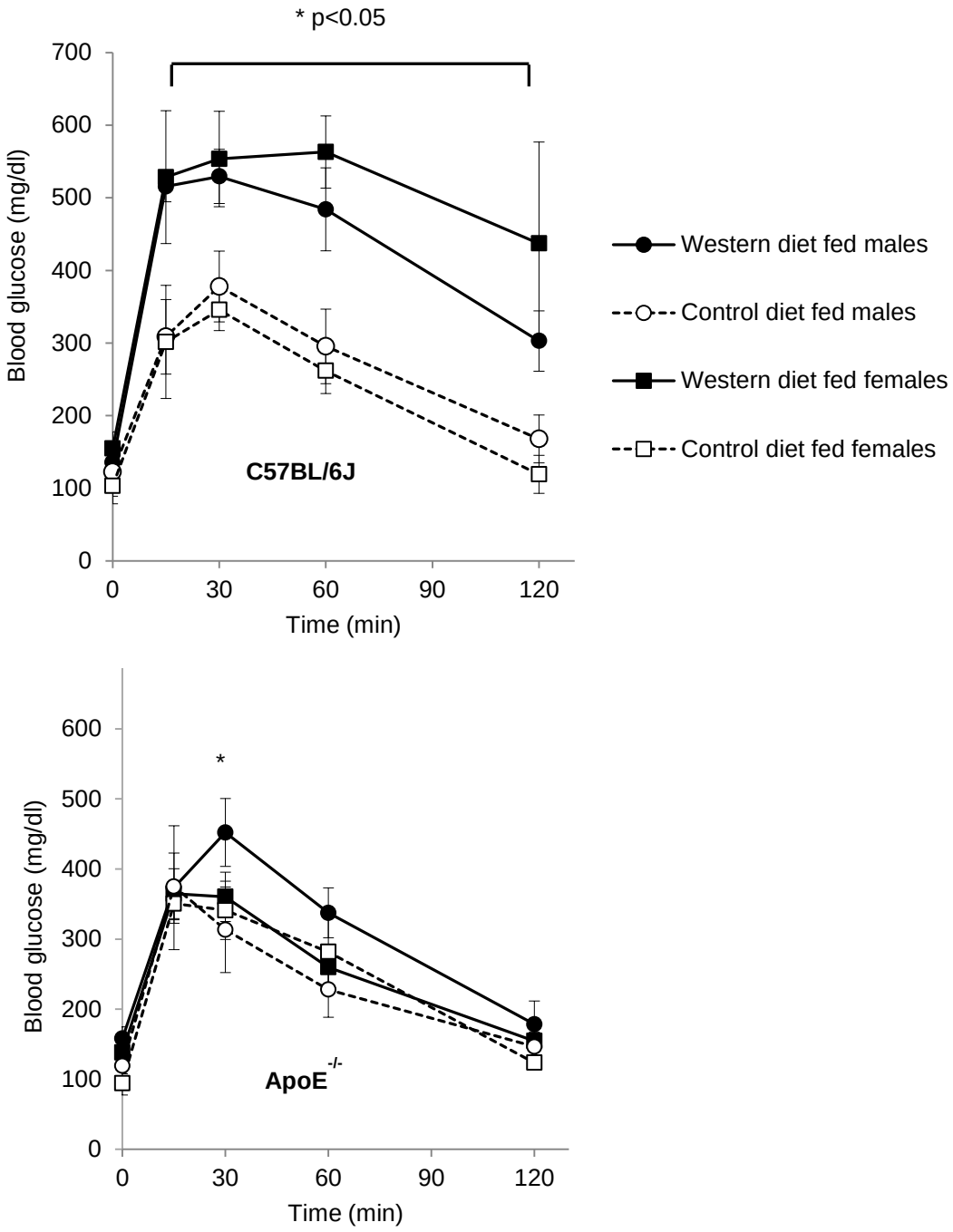

Figure 2.1: IPGTT in C57BL/6 mice (above) and ApoE-/- mice (below). *, p<0.05 for Western diet vs control diet group of same strain/gender at the same time point.

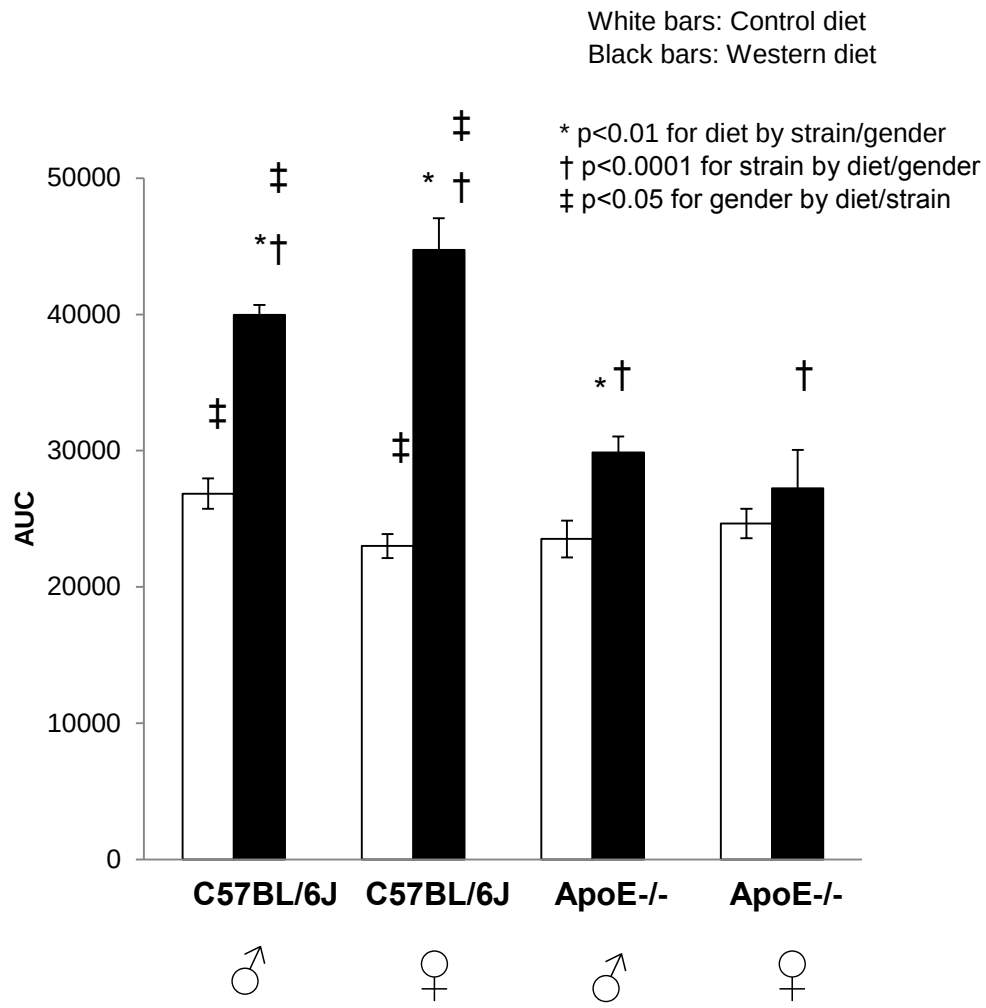

Figure 2.2: Area under the curve calculated from IPGTT in C57BL/6 mice and ApoE-/- mice fed with a control diet and a Western diet for six months.

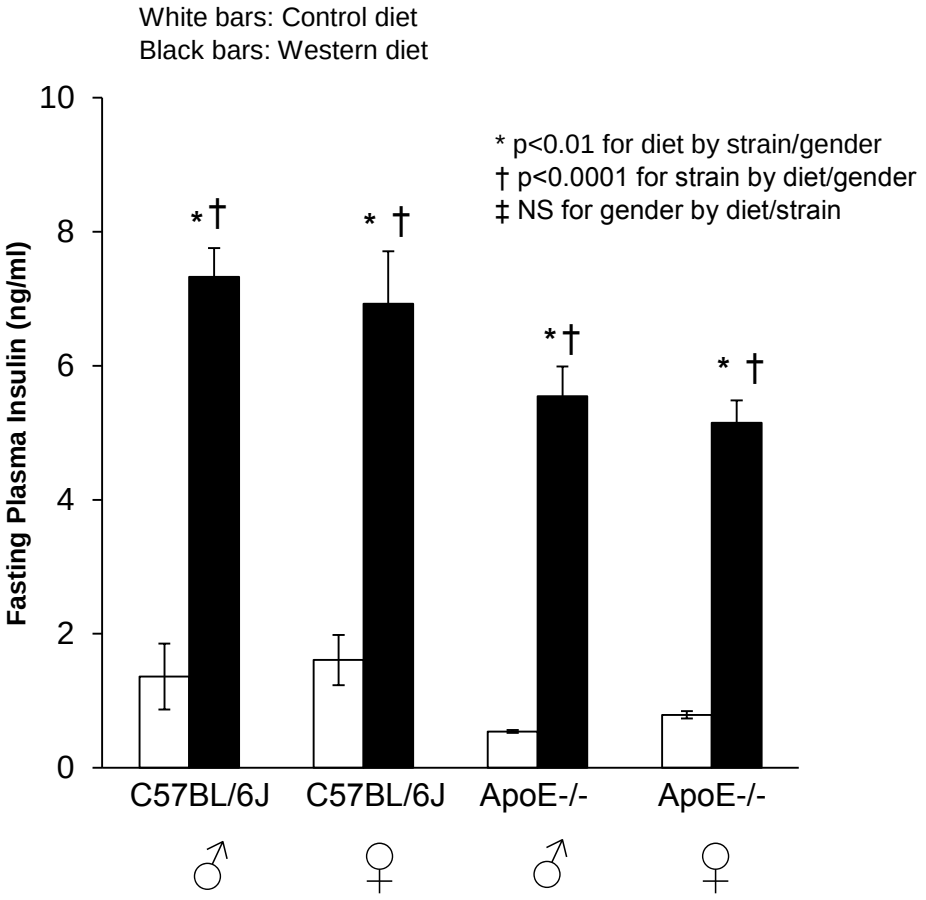

Figure 2.3: Feeding of a Western diet for 6 months increased plasma insulin in all groups (black bars) compared to the control diet (white bars).

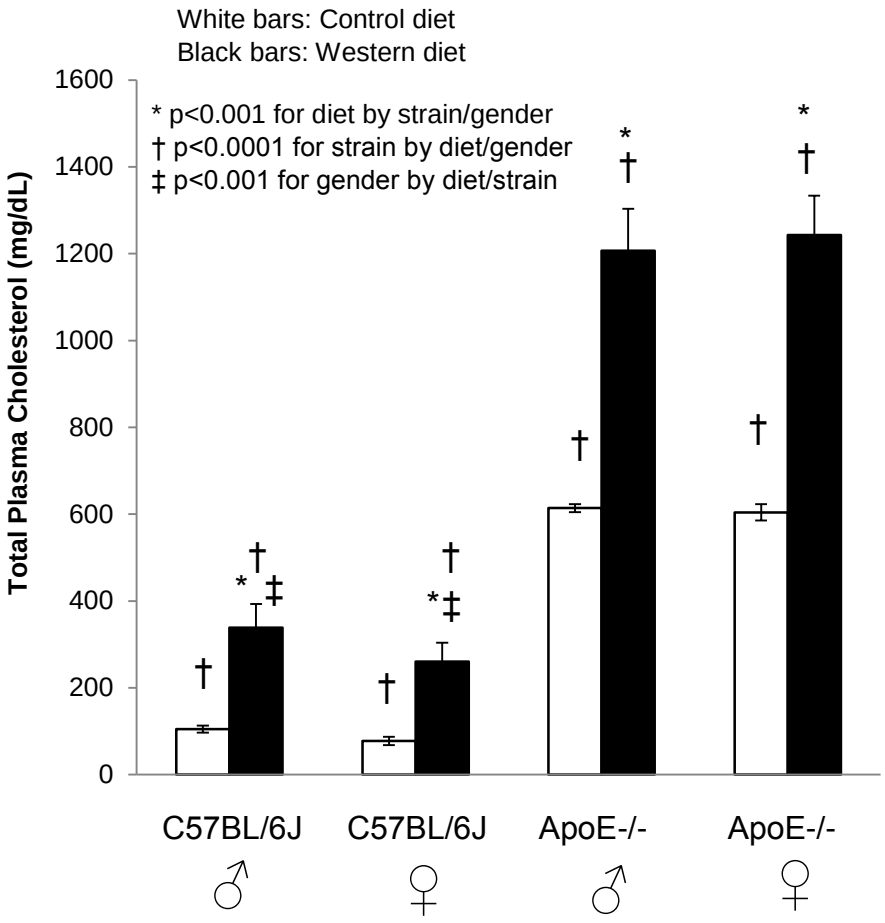

Figure 2.4: Feeding of a Western diet increased plasma cholesterol in all the treatment groups.

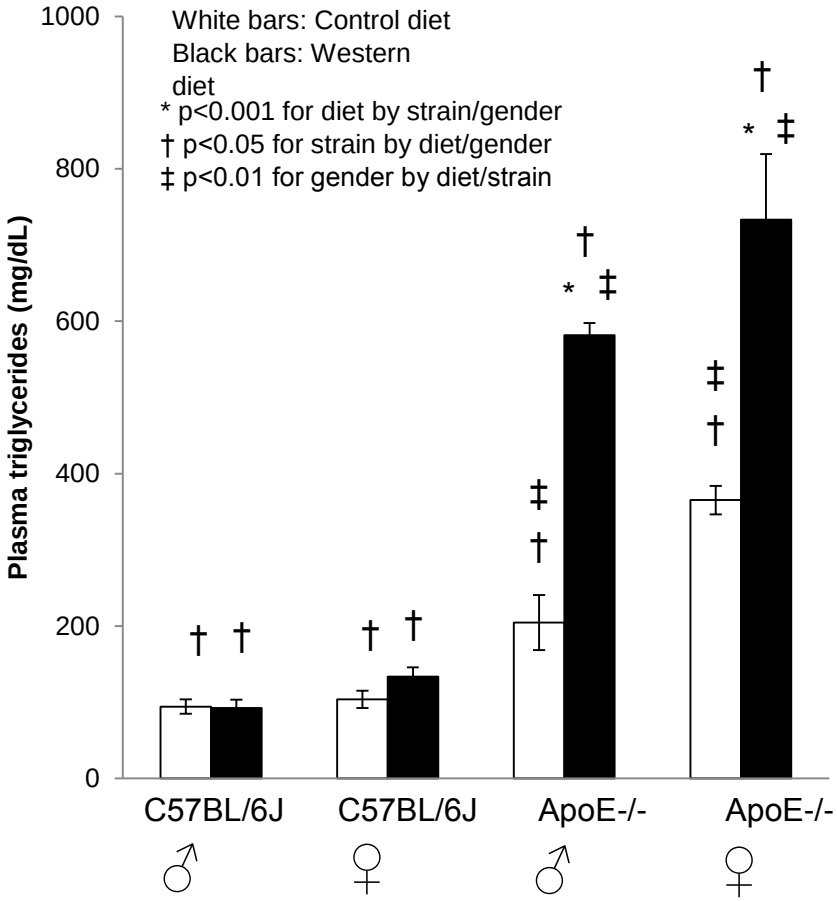

Figure 2.5: Western diet fed ApoE$^{-/-}$ mice had elevated plasma triglycerides compared to the low fat fed ApoE $^{-/-}$ mice, as well as all C57BL/6J groups

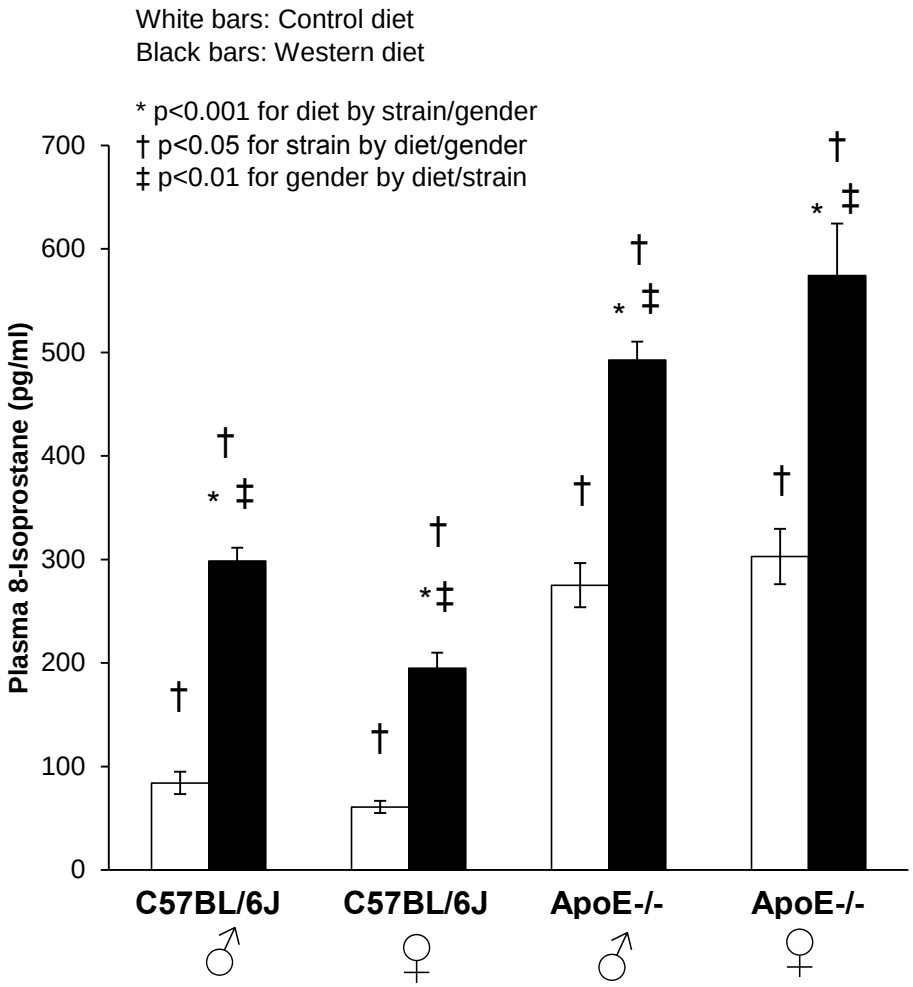

Figure 2.6: Plasma 8-isoprostane was measured as a biomarker for oxidative stress. Western diet fed mice showed increased oxidative stress, compared to control diet fed groups

strain. C57BL/6J males, regardless of diet, had elevated plasma glucose compared to ApoE$^{-/-}$ males on the same diet (data not shown, $p<0.05$).

All Western diet fed groups (both strains and sexes) were hyperinsulinemic (Figure 2.3, $p<0.0001$), in agreement with other published data (65;69). A significant ($p<0.0001$) strain difference existed, with Western diet fed ApoE$^{-/-}$ mice of both sexes showing less hyperinsulinemia than their C57BL/6J counterparts.

Among all the Western diet fed groups, ApoE$^{-/-}$ mice were significantly less obese than C57BL/6J mice, similar to other reports (65;70-72).

Feeding of a Western diet increased plasma cholesterol (Figure 2.4) in all groups relative to their matched strain/sex group on the control diet ($p<0.001$). However, significant effects of both sex ($p<0.001$) and strain ($p<0.0001$) were observed. The Western diet increased cholesterol in both male (338.30 ± 10.92 mg/dL) and female C57BL/6J mice (260.09 ± 19.26 mg/dL) relative to control diet fed male (104.94 ± 8.43 mg/dL) and female (77.61 ± 9.70 mg/dL) C57BL/6 mice, with a relatively greater effect in males. No sex differences were observed in the ApoE$^{-/-}$ strain. Cholesterol levels were sharply increased (by up to 4-5 fold) in all ApoE$^{-/-}$ groups regardless of diet and sex when compared to the corresponding C57BL/6J groups ($p<0.0001$; ApoE$^{-/-}$ male control, 604.13 ± 18.97 mg/dL; ApoE$^{-/-}$ female control, 614.20 ± 9.27 mg/dL; ApoE$^{-/-}$ male WD, 1207.00 ± 16.70 mg/dL; ApoE$^{-/-}$ female WD, 1242.73 ± 18.24 mg/dL). These findings are in agreement with those shown by others (65;72-74).

Feeding of a Western diet elevated triglyceride levels in both male and female ApoE$^{-/-}$ mice relative to control diet fed ApoE$^{-/-}$ mice ($p<0.001$), but had no effect in

C57BL/6J mice of either sex (Figure 2.5). Female ApoE$^{-/-}$ mice had higher triglyceride levels than male ApoE$^{-/-}$ mice on either diet ($p<0.01$). There was a clear strain difference, with all ApoE$^{-/-}$ groups showing significantly higher triglyceride levels than the corresponding C57BL/6J groups ($p<0.05$).

These metabolic data demonstrate that the Western diet induced different metabolic phenotypes, which mainly varied according to mouse strain. C57BL/6J mice fed a Western diet (C57-WD) were hyperglycemic, hyperinsulinemic, and hypercholesterolemic, while ApoE$^{-/-}$ mice fed the Western diet (ApoE-WD) were extremely hyperlipidemic and hyperinsulinemic. ApoE$^{-/-}$ mice receiving the control diet (ApoE-CD) were moderately hyperlipidemic, whereas C57BL/6J mice fed the control diet (C57-CD) were considered to be normoglycemic and normolipidemic and represent the controls for this study.

In order to characterize the oxidative stress levels associated with each of these metabolic phenotypes, we measured plasma 8-isoprostane, which widely used as an indicator of oxidative stress. The Western diet significantly increased 8-isoprostane levels across both strains and sexes (Figure 2.6, $p<0.001$) as previously reported (75-78). There was a significant strain effect, with all ApoE$^{-/-}$ mice demonstrating greater levels of oxidative stress than the corresponding C57BL/6J groups ($p<0.05$). A significant effect of sex on the response to the Western diet was seen in both strains. Interestingly, however, the effect varied in opposite directions by strain, with Western diet fed female C57BL/6J mice demonstrating lower 8-isoprostane levels than the corresponding male group, whereas Western diet fed female ApoE$^{-/-}$ mice had greater levels of oxidative stress than did the matched male group ($p<0.01$).

After characterizing the metabolic phenotypes of the mouse groups, we analyzed PLGF protein expression to determine whether baseline (unstimulated) PLGF levels are affected by metabolic phenotype. The Western diet significantly decreased PLGF protein in cardiac tissue by ~40-50% in both C57BL/6J and ApoE$^{-/-}$ mice ($p<0.001$), with no sex differences (Figure 2.7). There was a significant effect of strain to further decrease PLGF levels by ~40-50% in ApoE$^{-/-}$ mice in both the control and Western diet fed groups, relative to the same C57BL/6J groups. Since the most striking differences between the two strains were the higher cholesterol, triglyceride, and oxidative stress levels in ApoE$^{-/-}$ mice compared to C57BL/6J, our data suggest that these factors may act to inhibit PLGF expression.

In order to further investigate possible mechanisms behind the decreased PLGF levels in our mouse models of metabolic dysfunction, we performed Pearson correlation analysis of each individual animal's PLGF protein level with its plasma cholesterol, isoprostane, triglyceride, glucose, insulin and HOMA-IR values. This analysis revealed that cardiac PLGF levels were strongly and inversely correlated with plasma cholesterol, isoprostane, and triglyceride levels ($r^2 = 0.88$, 0.89, and 0.76, $p<0.0001$ respectively). However, plasma insulin, glucose and HOMA-IR were only weakly correlated with cardiac PLGF levels ($r^2 = 0.43$, 0.39, and 0.45, $p<0.001$) respectively).

In contrast to the marked effects of the Western diet on PLGF, expression of the related protein VEGF-A (Figure 2.8) and the PLGF receptor VEGFR1 (Figure 2.9) was not affected by diet. ApoE$^{-/-}$ males fed the control diet expressed slightly less VEGF-A than the corresponding C57BL/6J males ($p<0.05$), while all ApoE-/- groups expressed less VEGFR1 than did the matched C57BL/6J groups ($p<0.01$). This latter finding is

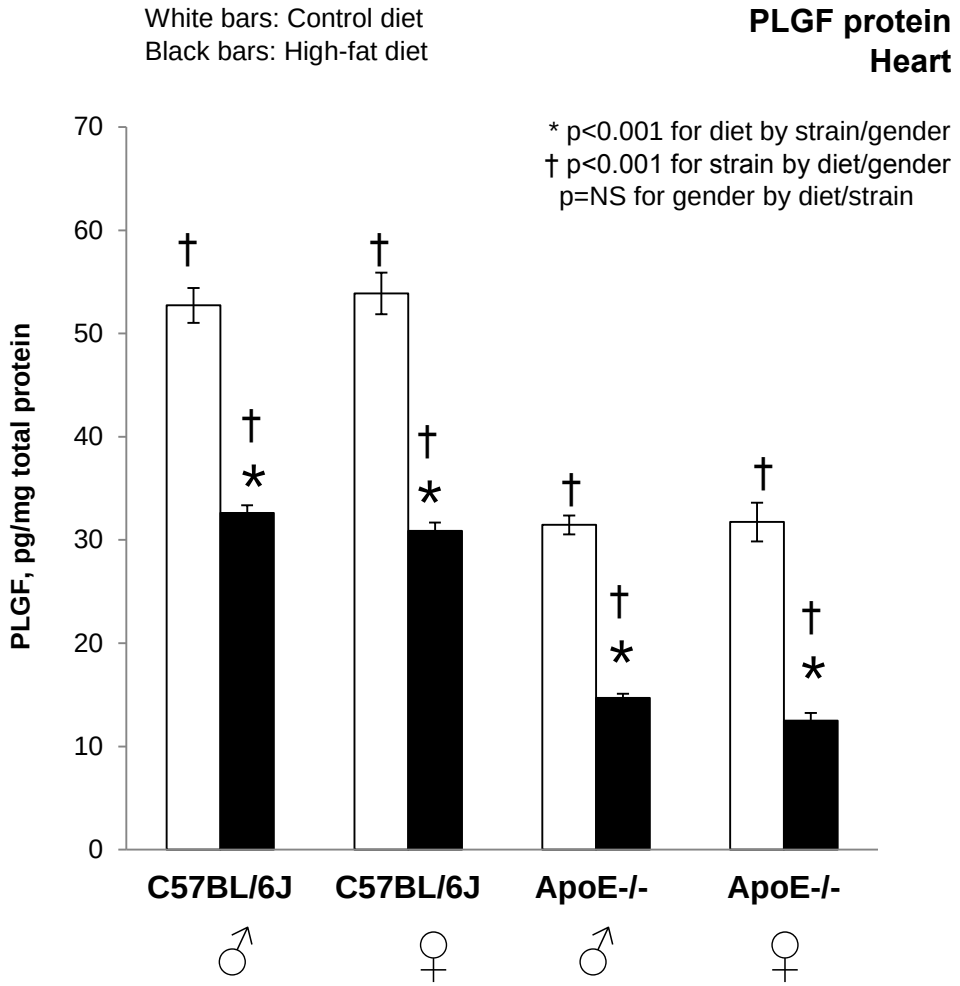

Figure 2.7: The HF diet significantly reduced PLGF protein expression in heart in all treatment groups.

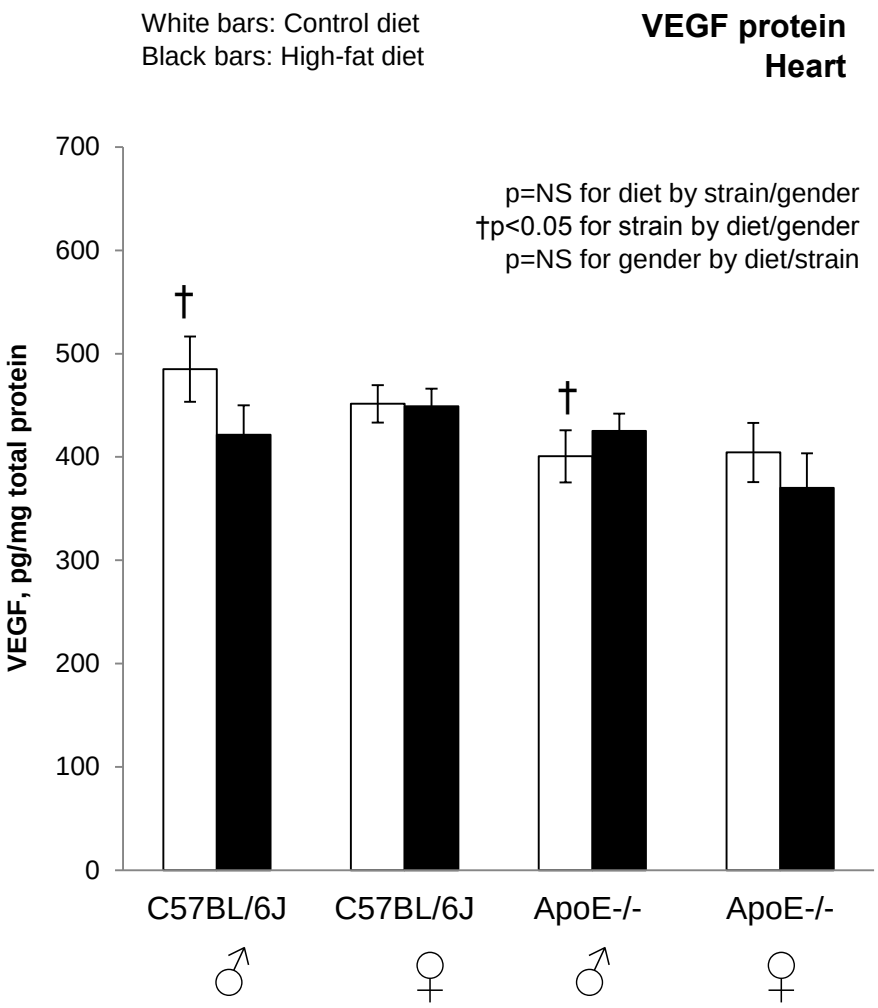

Figure 2.8: The Western diet did not affect VEGF-A protein levels in cardiac tissue.

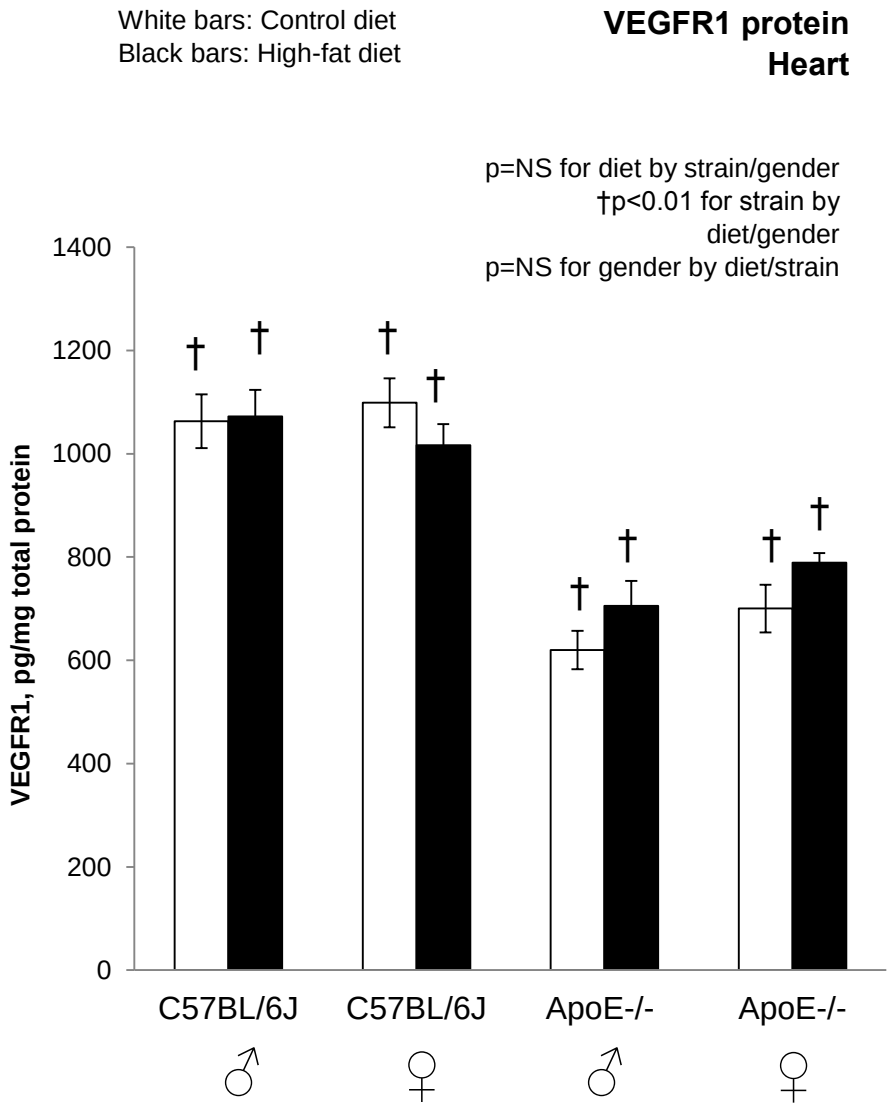

Figure 2.9: Western diet consumption did not affect VEGFR1 protein expression in cardiac tissue.

interesting, since it suggests that PLGF signaling in the ApoE$^{-/-}$ strain may be impaired at the level of the receptor as well as the ligand. The lack of effect of the Western diet on VEGF-A levels in cardiac tissue indicates that the effect of the diet on PLGF is specific.

We next assessed PLGF expression in the aorta to determine whether a similar pattern of response to a Western diet occurs in this tissue as it does in cardiac tissue. Interestingly, however, the aorta demonstrated the opposite diet effect, with increased PLGF mRNA levels in both C57BL/6J and ApoE$^{-/-}$ mice fed the Western diet (Figure 2.10, $p<0.05$). Similarly, an opposite strain effect was observed in aorta relative to cardiac tissue, with ApoE$^{-/-}$ mice having higher aortic PLGF levels than C57BL/6J mice ($p<0.05$). No effect of sex on aortic PLGF expression levels was observed. In addition to its beneficial role in arteriogenesis, PLGF is also known to play a pathological role in the development of atherosclerosis (79) (80) (81); thus, the increased expression of PLGF in aorta of mice fed a Western diet is a particularly interesting finding.

Both VEGF-A and VEGFR1 mRNA levels were increased in aorta by the Western diet, although only in the ApoE$^{-/-}$ strain (Figures 2.11 and 2.12; $p<0.05$ for both proteins). A strain difference also existed for VEGF-A expression in aorta, with ApoE-WD having higher expression than C57-WD ($p<0.05$). Strain differences in VEGFR1 levels were also seen for ApoE$^{-/-}$ males vs C57BL/6J males ($p<0.05$). No gender differences were detected.

Other studies in our lab have demonstrated that a signaling cascade involving Nox4 and heme oxygenase 1 contributes to regulation of PLGF (Rashdan and Lloyd, in preparation). In order to determine whether the effect of the Western diet on PLGF were

associated with alterations in the expression of these factors, we measured the relative abundance of Nox2, Nox4 and heme oxygenase 1 mRNA in cardiac tissue. No clear pattern of altered expression of these genes corresponding to our PLGF results could be identified (Figure 2.13).

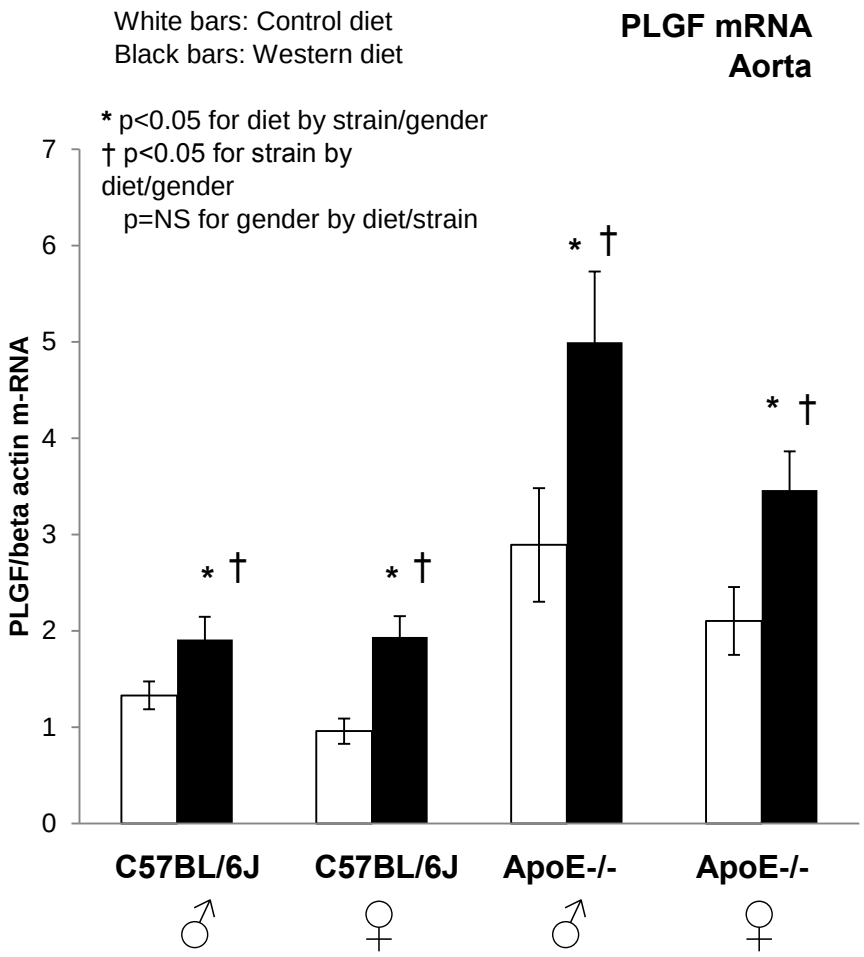

Figure 2.10: Western diet fed groups had elevated PLGF gene expression in aorta.

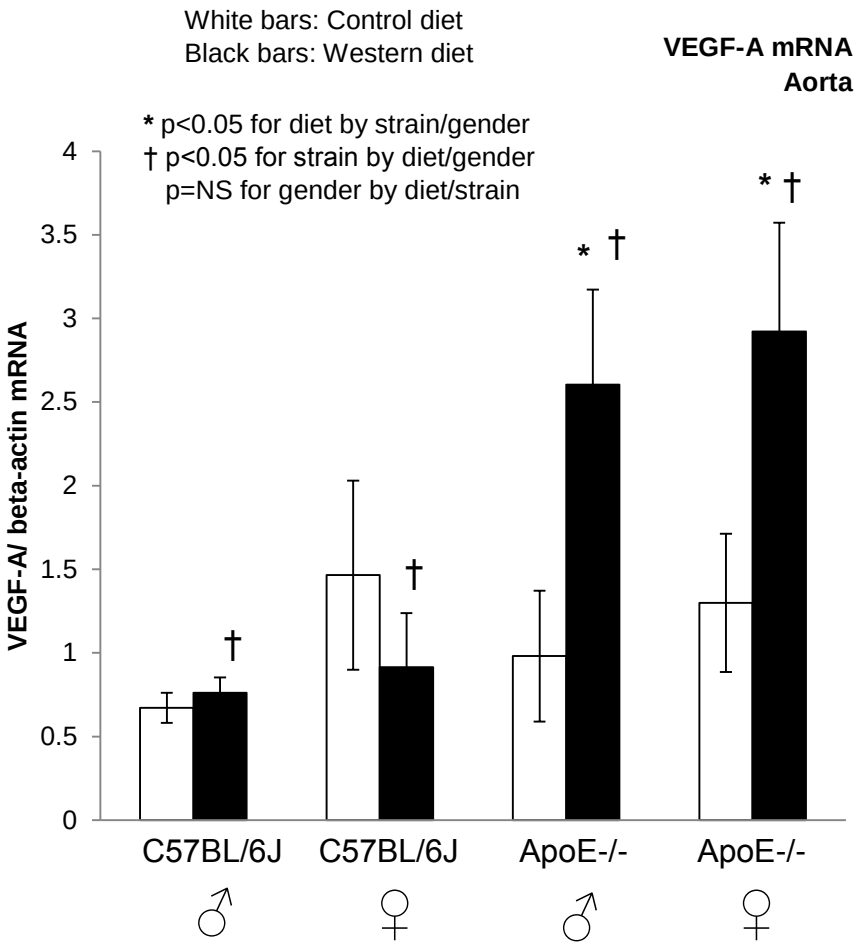

Figure 2.11: VEGF-A gene expression in aorta of ApoE$^{-/-}$ mice were elevated due to Western diet.

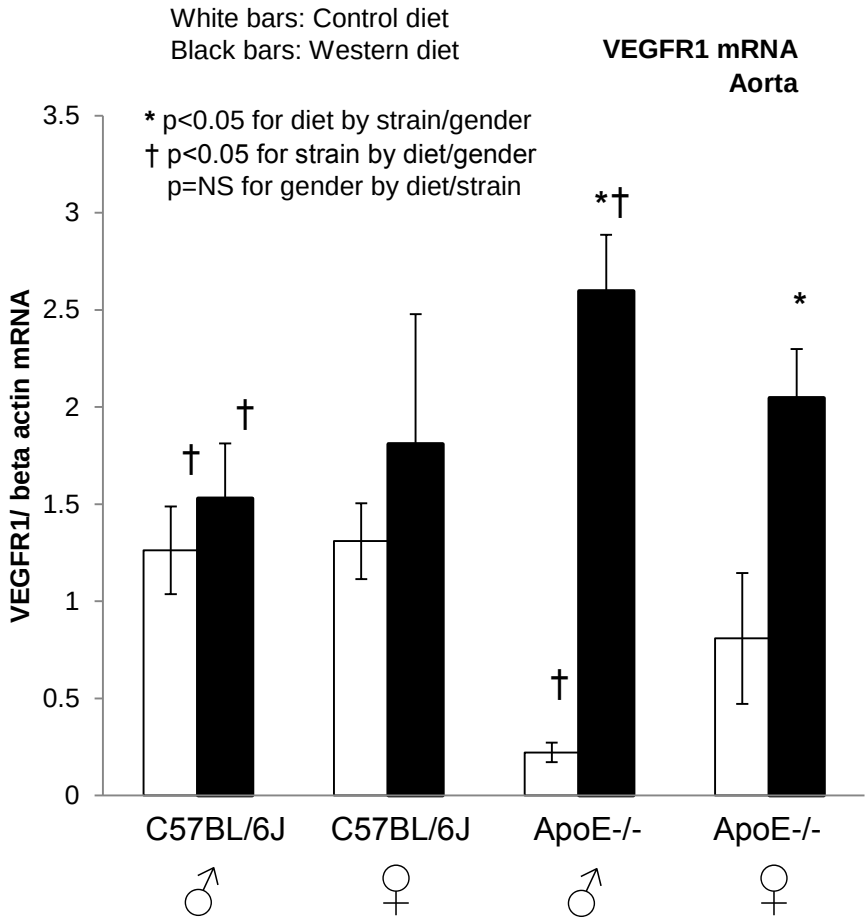

Figure2.12: Western diet consumption increased VEGFR1 gene expression in all ApoE$^{-/-}$ mice aorta.

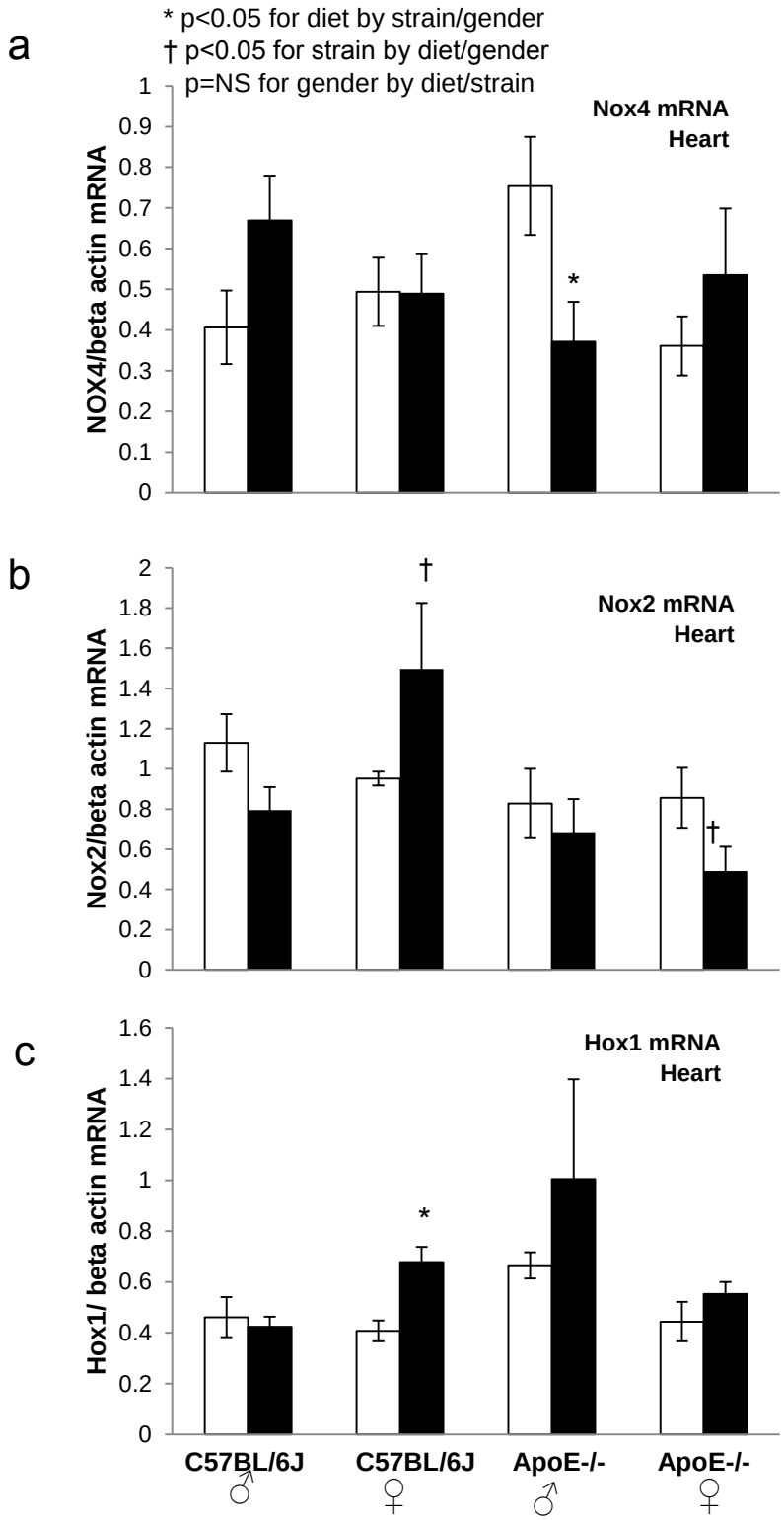

Figure 2.13: Redox sensitive gene expression in cardiac tissue. (a) Nox 4 mRNA levels were reduced in ApoE-/- males fed with Western diet. (b) Nox2 mRNA levels were not affected by diet. (c) Hox 1 mRNA levels were upregulated in male C57BL/6J fed with Western diet.

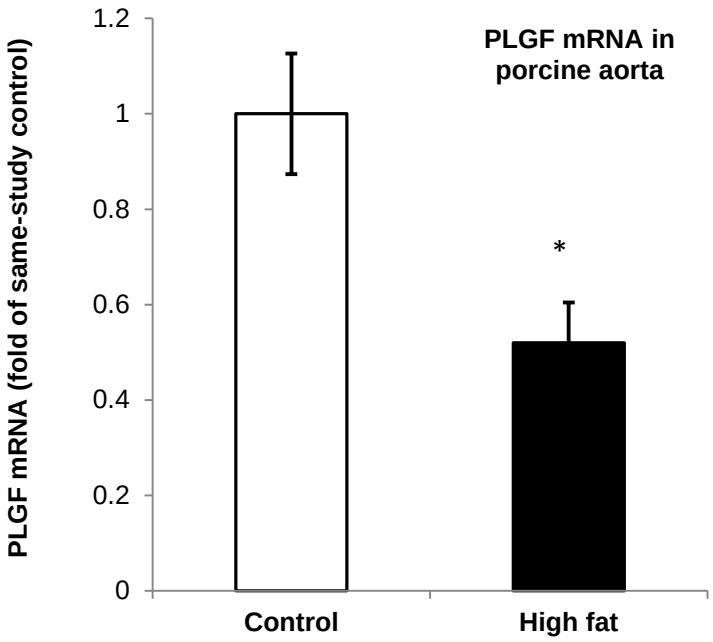

Figure 2.14: PLGF gene expression in porcine coronary artery is significantly decreased by feeding of a high fat diet (N=20), relative to expression in coronary arteries of control pigs (N=16). *, $p<0.01$. (Lloyd PG and Sturek M, unpublished).

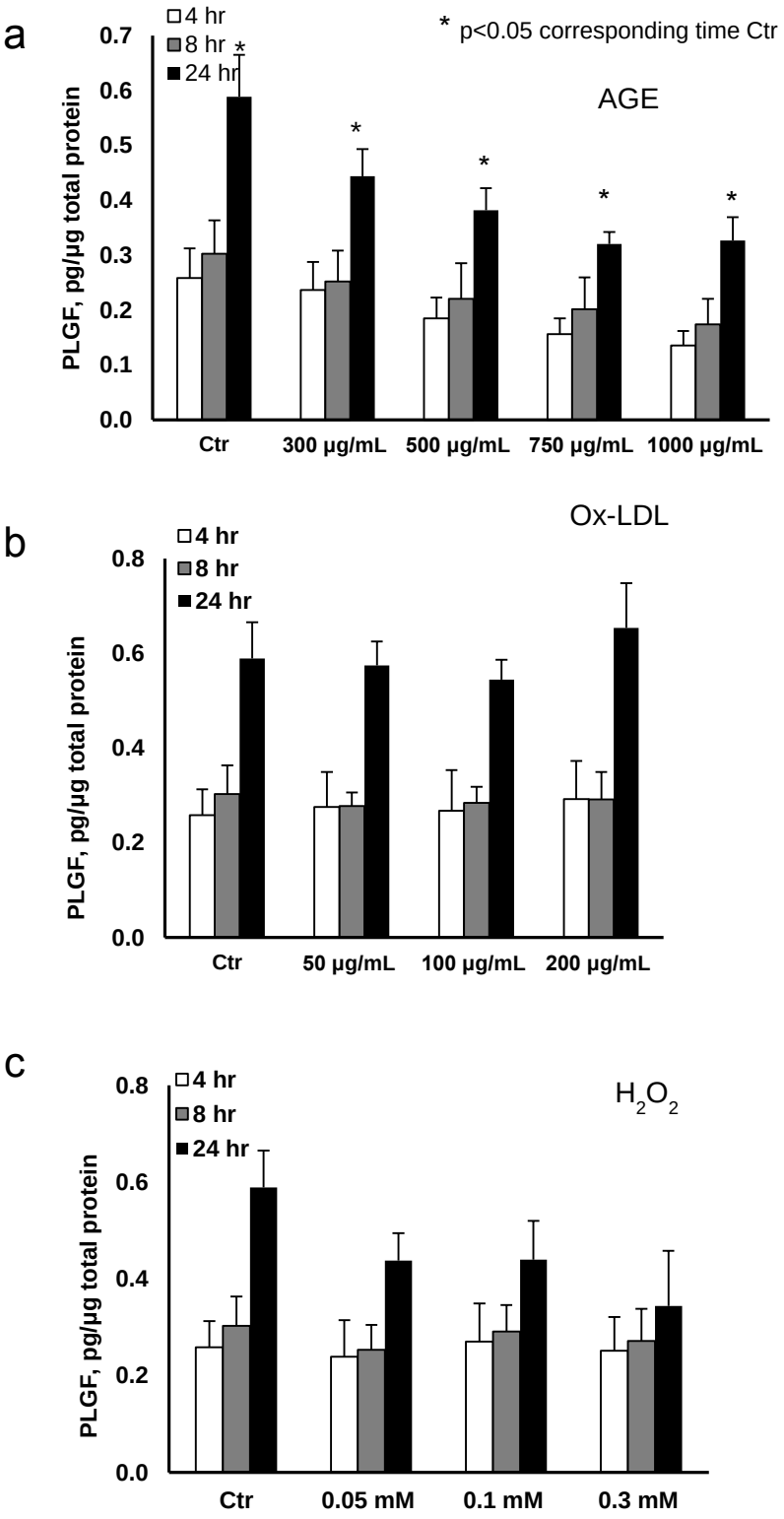

Figure 2.15: PLGF protein expression in HCAEC treated with different conc. of AGE, Ox-LDL and H_2O_2 for 4, 8 or 24 hours. (a) PLGF is reduced in AGE treated HCAEC for 24 hr at all doses tested. (b) PLGF expression in HCAEC was not affected by Ox-LDL or (c) H_2O_2

PLGF expression in porcine models of hyperlipidemia

Coronary artery PLGF gene expression was reduced to a similar extent in all three studies by a high fat diet (to 47 ± 17, 55 ± 13, and 67 ± 17% of the same-study control group). Thus, the data were combined for further analysis. Overall, PLGF gene expression in coronary arteries of high fat fed animals (N=20) was reduced to 52 ± 8% of the level in control animals (N=16) (Figure 2.14). Linear regression analysis was performed between PLGF mRNA levels and metabolic end points (plasma cholesterol, triglycerides, LDL and peak glucose value from glucose tolerance test). This analysis revealed that porcine coronary artery PLGF gene expression was strongly and inversely correlated to plasma cholesterol (r^2=0.475, $p<0.05$) than any other parameter in consistent with mouse cardiac PLGF protein expression.

The tissues examined in this study consist of multiple cell types (cardiac tissue: cardiac myocytes, endothelial cells, smooth muscle cells, and fibroblasts; aorta: endothelial cells and smooth muscle cells). It is unknown which of these cell types contributes to the alterations in PLGF expression which we observed in the mouse models of metabolic dysfunction. Our previous studies have shown that endothelial cells are a significant source of PLGF. Therefore, we next tested the effect of specific molecules associated with hyperglycemia, hypercholesterolemia, and oxidative stress (advanced glycation end products, AGE; oxidized LDL, Ox-LDL; and hydrogen peroxide, H_2O_2) on PLGF protein production by cultured HCAEC. Three different doses of each compound were tested across three exposure times (4, 8 and 24 h). Surprisingly, neither Ox-LDL nor H_2O_2 affected PLGF expression in HCAEC at the doses and times tested (Figure 14). However, AGE treatment produced a dose-dependent inhibition of

PLGF production in HCAEC at the 24 h time point ($p<0.05$). Further studies are underway to test the effects of these compounds on PLGF expression in other cell types.

Discussion

Diabetes is a negative predictor of arteriogenesis; however, the mechanism of inhibition is undefined. The central role of PLGF as a mediator of arteriogenesis led us to hypothesize that regulation of PLGF expression may be dysfunctional in type II diabetes. To test this hypothesis, we modeled type II diabetes-related metabolic abnormalities in two mouse strains fed either a control diet or a Western diet for six months. After characterizing the metabolic phenotypes, we assessed the level of PLGF in cardiac tissue. The related protein VEGF-A was also studied for comparison, along with VEGFR1. We found that a Western diet induces significant metabolic abnormalities and consistently decreases cardiac PLGF protein expression without affecting VEGF-A levels. Genetically-based metabolic dysfunction (in the $ApoE^{-/-}$ mice fed the control diet) also reduced PLGF protein levels and this affect was additive to the effect of the diet. Further analysis showed that cardiac PLGF protein levels were strongly and negatively correlated with plasma cholesterol and triglyceride levels, and with markers of oxidative stress. Interestingly, PLGF inhibition in a cholesterol dependent manner was conserved in porcine coronary arteries suggesting hyperlipidemia a common route of PLGF inhibition in cardiovascular system.

Diabetes associated oxidative stress is known to be the root cause for microvasculature and macrovascular defects of diabetes. Excess food intake along with sedentary life style results greater influx of glucose and fatty acids in to cellular catabolic

reactions. This leads to the generation of excess ROS, particularly through the mitochondrial electron-transport chain (82). ROS synthesis via AGE signaling also greatly contributes to excess oxidative stress in diabetes (83). In addition, hyperglycemia mediated elevation of circulatory inflammatory markers; TNF-α, IL-6(84), are known to initiate sequence of additional mechanisms for ROS generation by liver (85).

The redox window hypothesis of arteriogenesis states that an optimal physiological redox balance is critical for arteriogenesis, and that imbalance to either side inhibits arteriogenesis (86). Arteriogenesis involves redox sensitive signaling; Akt, Src in repetitive ischemia model and both overt oxidative stress and antioxidant treatments inhibit the signaling mediators resulting poor arteriogenesis (58). Diabetic hyperglycemia and consequential overt reactive oxidative species formation have been directly linked to growth factor abnormalities in microvascular complications including arteriogenesis. This is in line with our finding that inhibition of PLGF, a key arteriogenic factor is highly correlated with ROS.

Several studies have demonstrated that elevated cholesterol levels have an inhibitory effect on arteriogenesis (87;88). Hypercholesterolemic low-density lipoprotein receptor -/- and ApoB-48 -/- mice demonstrated down regulated FGF receptor 1, HIF1a, vascular cell adhesion molecule -1 along with reduced monocyte recruitment (89). Hyperlipidemia in high fat fed athymic rats also shown to reduce collateral remodeling via endothelial dysfunction and defective NO signaling (90) which is essential for successful arteriogenesis (91). Antioxidant treatment improved the reduced arteriogenesis in hypercholesterolemia.

Another line of evidence demonstrated hypercholesterolemia mediated sparse arteriogenic response in hypercholesterolemic rabbit and ApoE-/- mice (47). This is in accordance with our finding that highest inhibition of PLGF occurred in ApoE-WD group along with high negative correlation of PLGF and plasma total cholesterol in our study. Altogether, previous findings that support reduced arteriogenesis by impaired ROS generation and hypercholesterolemia represent the major link to PLGF inhibition in our study. Therefore, our results, PLGF inhibition by Western diet provide a mechanistic basis for reduced arteriogenesis found in previous studies.

Interestingly, the effect of the Western diet on PLGF expression in aorta trended in the opposite direction relative to cardiac tissue, with an increase in PLGF observed in aorta of Western diet fed mice. This may indicate tissue and/or cell-type specific responses to the diet. This finding is consistent with the previously reported pro-atherogenic role of PLGF. PLGF has been shown to be elevated in atherosclerotic lesions of hypercholesterolemic mice and rabbits (79) similarly to what we observed in our mice models. Indeed, $ApoE^{-/-}$ mice develop severe atherosclerosis in the thoracic aorta regardless of diet. Increased PLGF expression in the vascular wall may contribute to this effect. The mechanism for increased PLGF expression in aorta in response to a Western diet is an important area for future research, as is the basis for the apparently differing regulation of PLGF between cardiac and vascular tissue. It remains to be determined whether increased aortic PLGF reflects increased synthesis of PLGF by vascular cells, decreased PLGF degradation, or increased sequestration of PLGF in the vascular extracellular matrix.

Although our study is the first to examine the effect of Western-diet induced metabolic dysfunction on PLGF expression in cardiac tissue, other groups have reported decreased PLGF expression in other tissue types during diabetes. PLGF was shown to be decreased in wounds of diabetic mice relative to healthy mice, and this decrease was associated with impaired wound healing secondary to reduced neovascularization. When this PLGF deficit was overcome by PLGF adenoviral gene therapy, wound healing was improved (92). In another study, reduced collateralization in respond to hindlimb ischemia in diabetic mice was overcome by exogenous PLGF infusion (93). Diabetes also shown to cause reduced PLGF expression in rat placenta (94). All these studies were performed in streptozotocin induced diabetes in mice which represents type 1 diabetes.

Our finding that Western diet induced metabolic dysfunction reduces PLGF expression in cardiac tissue suggests that coronary collateral development would be diminished in these mice, compared to mice expressing normal levels of PLGF. This observation is consistent with the reduced arteriogenic capacity seen in human type II diabetes patients. We hypothesize that the inhibition of coronary arteriogenesis in type II diabetes may be due to reduced PLGF expression in diabetic cardiac tissue, leading to greater vulnerability of diabetic patients to life threatening MI. PLGF has been shown to be elevated during MI, possibly as a compensatory mechanism to acute occlusion, and PLGF treatment prior to experimental MI resulted larger vessel size and reduced ischemic damage in mice (95;96). However, these studies were performed in young healthy animals, which do not accurately represent the clinical population, where most MI patients are elderly and many have co-occurring diabetes or metabolic syndrome. Therefore, our study design and outcomes are highly relevant to the human clinical

situation and provide important considerations for therapeutic developments aimed at preventing MI by enhancing collateral artery growth.

Vascular related growth factor deficiency has been linked to reduced collateral growth and single growth factor supplementation has produced mixed results in clinical diabetes (Reviewed in (97)). Therefore, knowledge on the expression and the regulation of key arteriogenic growth factors in diabetic back ground has a high demand. To our knowledge, the effect of type II diabetes-associated metabolic abnormalities on cardiovascular PLGF levels has not previously been reported. Our findings from multiple animal species suggests that PLGF may be one of several key growth factors that are abnormally expressed in diabetes, making PLGF and/or its downstream signaling pathways potential therapeutic targets for enhancing collateralization in diabetic heart.

Our *in vitro* findings with HCAEC treated with AGE, Ox-LDL and H_2O_2 somewhat surprisingly showed that AGE, but not Ox-LDL or H_2O_2, dose-dependently decreased PLGF expression. This finding is in contrast to our results showing that cardiac PLGF is best correlated with plasma oxidative stress and cholesterol. There are several possible explanations for this disparity. First, there may be species differences between mouse and human underlying this effect. Second, it is possible that PLGF is regulated differently in the various cell types found in cardiac tissue. Third, it may be that endothelial cells are not the primary source of PLGF in cardiac tissue as a whole, and that another cell type is largely responsible for the effects we observed in cardiac tissue. Studies are ongoing in cardiac myocytes to determine what contribution this cell type makes to overall cardiac PLGF expression and whether PLGF regulation differs in this cell type as compared to EC.

In summary, we have provided the first demonstration that Western diet-induced metabolic abnormalities decrease PLGF mRNA and protein levels in mouse heart and porcine coronary arteries in a manner consistent with the type II diabetes associated impairment of arteriogenesis which has previously been reported. Hypercholesterolemia, hypertriglyceridemia, and oxidative stress appear to play a role in this inhibition, although the mechanism remains to be further defined. Interestingly, we found clear tissue-dependent differences in the effect of the Western diet on PLGF expression, with aortas of Western diet fed mice expressing higher levels of PLGF protein, consistent with the known pro-atherogenic effect of PLGF in the vascular wall. Studies to characterize cell-specific mechanisms controlling PLGF levels will provide a basis for development of therapeutic methods to normalize PLGF expression and enhance arteriogenesis in the diabetic heart and may also suggest ways in which PLGF expression could be inhibited in tissues in which it has a pathological role.

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CHAPTER III

PLACENTA GROWTH FACTOR EXPRESSION IN MOUSE SKELETAL MUSCLE CORRELATES WITH LONG TERM DIET INDUCED HYPERINSULINEMIA

Abstract

Arteriogenesis (collateral artery remodeling) is a vital adaptation of the vasculature in response to peripheral artery disease (PAD) which serves to improve O_2 and nutrient delivery to distal tissue, thereby decreasing risk of tissue damage due to ischemia. Stimulation of arteriogenesis would be highly beneficial in diabetics, who are especially prone to develop critical limb ischemia and limb loss. However, arteriogenesis is suppressed in diabetes by an undefined mechanism. Placenta growth factor (PLGF) is a key arteriogenic factor whose expression is sensitive to oxidative stress *in vitro*, and oxidative stress occurs in diabetes associated hyperglycemia and hyperlipidemia. Thus, we hypothesized that decreased PLGF levels might contribute to impaired arteriogenesis in diabetes. To test this hypothesis, we utilized three mouse models of long term (6 mo) diet-induced metabolic dysfunction: hyperglycemic + hyperlipidemic (Western diet fed C57BL/6J; n=21), hyperlipidemic (control diet fed ApoE$^{-/-}$, n=12) and extremely hyperlipidemic (Western diet fed ApoE$^{-/-}$, n=12). A normoglycemic + normolipidemic control group (control diet fed C57BL/6J, n=13) was also studied. Both males and females were studied to identify gender differences. Quadriceps femoris (thigh) and gastrocnemius/plantaris/soleus (GPS; calf) muscles were isolated and assayed separately for arteriogenic markers. During hindlimb ischemia, arteriogenesis occurs in the quadriceps, whereas vascular growth in the GPS is primarily angiogenesis. Mouse metabolic phenotype was confirmed by measuring plasma cholesterol, insulin, and isoprostane (biomarker for oxidative stress), performing intraperitoneal glucose tolerance testing, and tracking body weight. Quadriceps femoris muscle PLGF protein was significantly decreased in all Western diet fed groups, except C57BL/6J females ($p<$

0.01). VEGF-A protein levels were also affected by diet, but in a strain dependent manner. Interestingly, the Western diet increased PLGF in C57BL/6J ($p<0.0001$) but decreased it in ApoE$^{-/-}$ ($p<0.001$). A notable gender response was seen for VEGFR1, which was elevated only in females by the Western diet ($p<0.05$). To study the mechanism for decreased PLGF expression in the Western diet groups, we performed regression analysis of PLGF protein levels vs metabolic parameters. PLGF was best correlated with insulin ($r^2=0.66$, $P<0.0001$), in contrast to our results from cardiac tissue, in which PLGF was best correlated to total cholesterol and isoprostane. In the GPS muscle group, PLGF and VEGF-A expression was generally reduced by the Western diet ($p<0.05$). In contrast to our findings in quadriceps, VEGFR1 was decreased in ApoE$^{-/-}$ males by the Western diet ($p<0.05$). Regression analysis of PLGF protein vs metabolic variables in the GPS muscle group yielded similar results as were seen in quadriceps.

We conclude that PLGF protein is decreased in the mouse quadriceps and GPS muscles by a Western diet, and that this decrease is inversely correlated with insulin. Our previous results in cardiac tissue identified hyperlipidemia and oxidative stress as possible mediators of PLGF inhibition in diabetes. It is possible that plasma isoprostane does not accurately reflect the level of oxidative stress within skeletal muscle. Alternatively, regulation of PLGF may differ between cardiac and skeletal muscle.

Introduction

Over 21 million people are diabetic and approximately 40% of them have PAD in the United States (1;2). Atherosclerosis reduces the blood flow and perfusion pressure in diabetic PAD, leaving tissue at risk of ischemia. Adverse outcomes of PAD with underlying diabetes include critical limb ischemia, foot ulcers, and limb amputation.

Revascularization surgery is not an option for many patients, due to impaired wound healing ability (3).

The compensatory physiological response to gradual arterial occlusion is arteriogenesis, in which remodeling of preexisting collateral arteries results in a natural bypass of the occlusion, minimizing downstream ischemia (4;5). However, arteriogenesis is impaired in diabetes by a mechanism that is yet to be fully defined (6). Successful arteriogenesis requires a coordinated response involving sensing of signals of reduced blood flow, recruitment of circulating cells, and the expression of a variety of growth factors in a temporally specific manner (7;8). Growth factor abnormalities, altered monocyte function (9-11), and impaired shear stress signaling (12;13) have all been linked to poor arteriogenesis in diabetes (14). Levels of multiple growth factors have been reported to be altered in diabetes, including VEGF-A (10;15), bFGF-2(14;16), HGF (17), and GM-CSF (18). However, attempts to overcome reduced arteriogenesis by targeting single growth factors have had limited success (19). Development of efficient therapies to overcome the adverse effects of diabetes on arteriogenesis is hampered by the lack of knowledge regarding the mechanism(s) by which diabetes affects the behavior of key arteriogenic growth factors.

Placenta growth factor is a key arteriogenic growth factor which induces arteriogenesis primarily through a monocyte-mediated mechanism (20). PLGF overexpression induces formation of large stable vessels (21) whereas PLGF inhibition leads to poor collateral growth (22). PLGF infusion at the site of vessel occlusion has been shown to be more effective than VEGF-A at inducing arteriogenesis in animal models of PAD (20;23). Similarly, decreased PLGF expression is implicated in impaired

wound healing in diabetes, and administration of a PLGF adenoviral construct improves wound healing by increasing microvasculature in the wound (24). However, mechanisms regulating PLGF expression in skeletal muscle, where arteriogenesis is highly important, have not been characterized yet, nor has the effect of diabetes on skeletal muscle PLGF expression been examined to our knowledge. We hypothesized that PLGF levels would be decreased in skeletal muscle tissue under metabolic conditions associated with type II diabetes. To test this hypothesis, we examined the expression of PLGF, the related protein VEGF-A and the PLGF receptor VEGFR1 in skeletal muscle of mice exhibiting varying degrees of metabolic dysfunction produced by feeding of a Western diet. Similar to what others have reported for diabetic wounds, we found that PLGF protein levels are decreased in skeletal muscle by a Western diet. These results suggest type II diabetes associated metabolic dysfunction may result in PLGF deficiency in diabetic human patients, impairing their ability to undergo arteriogenesis. Exploration of the mechanistic basis of Western diet-induced PLGF inhibition may suggest novel therapeutic strategies to improve patient outcomes in diabetics with PAD.

Materials and Methods

Animals: C57BL/6J and ApoE$^{-/-}$ mice were either fed a Western-type diet or a control diet. The diet regimen was started when mice were 6-8 weeks of age and was continued for 24 weeks. Male and female mice were studied separately. Therefore, we studied 8 groups: C57BL/6J males fed the control diet (n=9), C57BL/6J females fed the control diet (n=8), C57BL/6J males fed the Western diet (n=14), C57BL/6J females fed the Western diet (n=8), ApoE$^{-/-}$ males fed the control diet (n=6), ApoE$^{-/-}$ females fed the control diet (n=6), ApoE$^{-/-}$ males fed the Western diet (n=6), and ApoE$^{-/-}$ females fed the

Western diet (n=6). All mice had access to food and water ad libitum and were housed in a temperature controlled facility with a 12 hour dark-light cycle. This study was approved by the Institutional Animal Care and Use Committee at Oklahoma State University.

Diets: The Western diet (TD 88137, Harlan) supplied 42% kcal from anhydrous milk fat and cholesterol, 42.7% kcal from carbohydrate, and 15.2% kcal from protein. The control diet (TD120336) supplied 13% kcal from fat, 67.9% from carbohydrate, and 19.2% from protein. The Western diet also has three times more sucrose by weight, compared to the control diet. The source of the relatively high carbohydrate content in the control diet is corn starch and maltodextrin, which are not readily available sources of sugar. Therefore, the Western diet used in this study is characterized by both high fat and high sucrose.

Metabolic phenotyping of the experimental groups: Body weight of each mouse was recorded weekly. Fasting (6 h) blood samples were obtained from all mice at the end of 24 week- diet regime for metabolic measurements. Glucose homeostasis was assessed by intraperitoneal glucose tolerance testing under fasting (6h) conditions in all mice just prior to sacrifice.

Cholesterol and triglycerides: Total plasma cholesterol was determined using the Amplex Red cholesterol assay (Molecular Probes). Total plasma triglycerides were measured using a triglyceride determination kit (Sigma).

Glucose and insulin: Intraperitoneal glucose tolerance testing (IPGTT) was performed on 6 h fasted, non-anesthetized mice by administering a 1g/kg ip D-glucose bolus (~30

mg/mouse in 300μL of water). Venous blood (0.05 mL/mouse/time point; total, 0.25 mL) was drawn from the facial vein for measurement of glucose and insulin at 0, 15, 30, 60, and 120 min. Plasma glucose levels were measured using a portable glucose meter (Life Scan, Inc.). Insulin was measured using a rat/mouse insulin ELISA kit (EMD Millipore, Germany).

Oxidative stress: Plasma 8-isoprostane was measured as a biomarker for oxidative stress using a competitive enzyme immunoassay (Cayman Chemical) according to the manufacturer's protocol.

Tissue isolation: After 24 weeks of the diet program, mice were euthanized by excision of the heart under deep isoflurane anesthesia. Two sets of skeletal muscles were isolated from the hindlimb: the quadriceps femoris muscle (from the thigh) and the gastrocnemius/plantaris/soleus muscle group (from the calf). Tissues were immediately immersed in liquid nitrogen and stored at -80°C until use. Muscle tissue was homogenized in 1 mL of lysis buffer containing protease inhibitors (PMSF, sodium orthovanadate, leupeptin, benzamidine hydrochloride, aprotinin, and pepstatin A). Three one minute homogenizations were done at speed 5 using a Bio-Gen Pro200 homogenizer (PRO Scientific).The homogenates were centrifuged at 700 x g for 5 minutes, and the supernatant was transferred to a clean microcentrifuge tube.

Assessment of PLGF, VEGF and VEGFR1 protein levels: PLGF, VEGF-A and VEGFR1 protein were quantified by ELISA (Mouse PLGF-2, VEGF-A, and VEGFR-1 Quantikine ELISAs, R&D Systems). All samples were assayed in duplicate. Data were normalized to total protein concentration, as determined by BCA assay (Pierce). All assay plates were read using a Biotek Synergy HT plate reader.

Results

In order to induce diverse metabolic characteristics associated with diabetes and to determine their effects on PLGF and related proteins, C57BL/6J and ApoE$^{-/-}$ mice were fed a Western diet for 6 months. C57BL/6J mice fed the Western diet showed profound hyperglycemia, hyperinsulinemia, a high HOMA-IR index, and obesity, consistent with published results (25-29), whereas the effects of the diet on these parameters were less pronounced in the ApoE$^{-/-}$ strain (See table 3.1 for metabolic data). We observed significantly elevated fasting blood glucose levels in the C57BL/6J Western diet groups (both male and female) from as early as week 3-4 onwards. In contrast, the ApoE$^{-/-}$ Western diet fed groups did not demonstrate any impairment in fasting blood glucose until week 13, after which it was inconsistently elevated (data not shown). Fasting glucose levels were elevated among all the Western diet fed groups ($p<0.05$) by week 23 (data not shown). Glucose clearance as measured by IPGTT and presented as area under the curve indicated that both male and female C57BL/6J mice fed the Western diet had impaired glucose tolerance (WD fed C57BL/6J males, 39959 ± 742 vs CD fed C57BL/6J males, 26852 ± 1126; WD fed C57BL/6J females, 44731 ± 2317 vs CD fed C57BL/6J females, 23003 ± 890, $p<0.01$). From all the Western diet fed groups C57BL/6J strain demonstrated highest glucose intolerance ($p< 0.0001$) as depicted by AUC of IPGTT. In contrast, only male ApoE$^{-/-}$ mice fed the Western diet had slightly but significantly elevated glucose intolerance (WD fed ApoE$^{-/-}$ males, 29876 ± 1161 vs CD fed ApoE$^{-/-}$ males, 23524 ± 1344, $P<0.01$), whereas female WD fed ApoE$^{-/-}$ mice were not glucose intolerant. Therefore, IPGTT results confirmed that feeding of the Western diet disrupted glucose homeostasis. However, significant differences were

noted for both sex (in ApoE$^{-/-}$) and strain. C57BL/6J males, regardless of diet, had elevated plasma glucose compared to ApoE$^{-/-}$ males on the same diet (data not shown, $p<0.05$).

All Western diet fed groups (both strains and sexes) were hyperinsulinemic ($p<0.0001$), in agreement with other published data (26;30). A significant ($p<0.0001$) strain difference existed, with Western diet fed ApoE$^{-/-}$ mice of both sexes showing less hyperinsulinemia than their C57BL/6J counterparts.

Among all the Western diet fed groups, ApoE$^{-/-}$ mice were significantly less obese than C57BL/6J mice, similar to other reports (26;31-33).

Feeding of a Western diet increased plasma cholesterol in all groups relative to their matched strain/sex group on the control diet ($p<0.001$). However, significant effects of both sex ($p<0.001$) and strain ($p<0.0001$) were observed. The Western diet increased cholesterol in both male (338.30 ± 10.92 mg/dL) and female C57BL/6J mice (260.09 ± 19.26 mg/dL) relative to control diet fed male (104.94 ± 8.43 mg/dL) and female (77.61 ± 9.70 mg/dL) C57BL/6 mice, with a relatively greater effect in males. No sex differences were observed in the ApoE$^{-/-}$ strain. Cholesterol levels were sharply increased (by up to 4-5 fold) in all ApoE$^{-/-}$ groups regardless of diet and sex when compared to the corresponding C57BL/6J groups ($p<0.0001$; ApoE$^{-/-}$ male control, 604.13 ± 18.97 mg/dL; ApoE$^{-/-}$ female control, 614.20 ± 9.27 mg/dL; ApoE$^{-/-}$ male WD, 1207.00 ± 16.70 mg/dL; ApoE$^{-/-}$ female WD, 1242.73 ± 18.24 mg/dL). These findings are in agreement with those shown by others (26;33-35).

Feeding of a Western diet elevated triglyceride levels in both male and female ApoE$^{-/-}$ mice relative to control diet fed ApoE$^{-/-}$ mice ($p<0.001$), but had no effect in C57BL/6J mice of either sex. Female ApoE$^{-/-}$ mice had higher triglyceride levels than male ApoE$^{-/-}$ mice on either diet ($p<0.01$). There was a clear strain difference, with all ApoE$^{-/-}$ groups showing significantly higher triglyceride levels than the corresponding C57BL/6J groups ($p<0.05$).

These metabolic data demonstrate that the Western diet induced different metabolic phenotypes, which mainly varied according to mouse strain. C57BL/6J mice fed a Western diet (C57-WD) were hyperglycemic, hyperinsulinemic, and hypercholesterolemic, while ApoE$^{-/-}$ mice fed the Western diet (ApoE-WD) were extremely hyperlipidemic and hyperinsulinemic. ApoE$^{-/-}$ mice receiving the control diet (ApoE-CD) were moderately hyperlipidemic, whereas C57BL/6J mice fed the control diet (C57-CD) were considered to be normoglycemic and normolipidemic and represent the controls for this study.

In order to characterize the oxidative stress levels associated with each of these metabolic phenotypes, we measured plasma 8-isoprostane, which widely used as an indicator of oxidative stress. The Western diet significantly increased 8-isoprostane levels across both strains and sexes ($p<0.001$) as previously reported (36-39). There was a significant strain effect, with all ApoE$^{-/-}$ mice demonstrating greater levels of oxidative stress than the corresponding C57BL/6J groups ($p<0.05$). A significant effect of sex on the response to the Western diet was seen in both strains. Interestingly, however, the effect varied in opposite directions by strain, with WD fed female C57BL/6J mice demonstrating lower 8-isoprostane levels than the corresponding male group, whereas

WD fed female ApoE$^{-/-}$ mice had greater levels of oxidative stress than did the matched male group ($p<0.01$).

Table 3.1: Summary of the key metabolic data

	C57BL6/J				ApoE-/-				
	Male		Female		Male		Female		P value
	Normal	High fat	Normal	High fat	Normal	High fat	Normal	High fat	
Total cholesterol (mg/dL)	104.94 ± 8.43†	338.30 ± 10.92*†‡	77.61 ± 9.70†	260.09 ± 19.26*†‡	614.20 ± 9.27†	1207.00 ± 16.70*†	604.13 ± 18.97†	1242.73 ± 18.24*†	*†‡<.0001
Plasma isoprostane (pg/mL)	84.17 ± 10.90†	298.40 ± 13.09*†‡	60.90 ± 5.85†	194.93 ± 15.20*†‡	275.13 ± 21.39†	492.70 ± 17.58*†‡	302.88 ± 26.73†	574.28 ± 50.10*†‡	*†<.0001 ‡<.05
Plasma triglycerides (mg/dL)	94.38 ± 9.40†	92.75 ± 10.74†	103.81 ± 11.23†	133.79 ± 12.27†	204.72 ± 35.96†‡	581.72 ± 15.94*†‡	365.31 ± 18.57†‡	733.31 ± 85.90*†‡	*<.0001 †<.05 ‡<.01
Fasting glucose wk0 (mg/dL)	137.33 ± 10.64	136.60 ± 6.72	117.50 ± 6.62*	121.43 ± 7.60	135.83 ± 5.06	136.67 ± 10.11	125.33 ± 4.14	128.67 ± 7.20	‡<.05 *†NS
Fasting glucose wk23 (mg/dL)	131.67 ± 3.00†	193.80 ± 3.63*†	121.00 ± 2.23†	194.71 ± 5.09*	155.17 ± 4.83†	175.33 ± 4.94*†	164.50 ± 4.09†	181.67 ± 6.24	*†<.05 ‡NS
Plasma insulin (ng/mL)	1.36 ± 0.49	7.33 ± 0.43*†	1.61 ± 0.38	6.93 ± 0.78*†	0.54 ± 0.02	5.54 ± 0.45*†	0.79 ± 0.06	5.15 ± 0.33*†	*<.0001 †<.001 ‡NS
Area under the curve	26852 ± 1126‡	39959 ± 742*†‡	23003 ± 890‡	44731 ± 2317*†‡	23524 ± 1344	29876 ± 1161*†	24654 ± 1078	27245 ± 2806†	*<.01 †<.0001 ‡<.05
HOMA-IR index	10.74 ± 1.01	60.31 ± 1.81*	10.29 ± 1.17	65.40 ± 7.94*†	4.51 ± 0.22	53.28 ± 4.34*	4.50 ± 0.30	42.66 ± 4.92*†	*†<.0001 ‡NS

Values are mean ± SEM. * Diet effect by strain/gender; † strain effect by gender/diet; ‡ gender effect by strain/diet (3-way ANOVA followed by post-hoc testing using SAS software. Analysis performed by Dr. Mark Payton, Professor & Head, Dept. of Statistics, Oklahoma State University).

Growth factor expression in quadriceps femoris muscle

We found that PLGF protein was significantly decreased by the Western diet in the quadriceps femoris muscle ($p<0.001$), except in C57-WD females (Figure 3.1). The magnitude of the reduction was similar to what we previously observed in cardiac muscle. Interestingly, the Western diet had a clear strain-dependent effect on VEGF-A protein levels in quadriceps femoris muscle (Figure 3.2). VEGF-A was increased by the Western diet in the C57BL/6J strain ($p<0.0001$), but decreased in the $ApoE^{-/-}$ strain ($p<0.0001$). No gender differences in the response of VEGF-A to the Western diet were detected. However, a clear gender-dependent difference in the response of VEGFR1 to the Western diet was observed in quadriceps muscle (Figure 3.3), with the females of both strains showing a diet-dependent increase in VEGFR1 protein ($p<0.05$). A strain-dependent difference in VEGFR1 expression in quadriceps was also noted, with overall higher expression levels in $ApoE^{-/-}$ females vs C57BL/6J females ($p<0.05$).

In order to understand the possible mechanism(s) behind the Western-diet induced reduction of PLGF in quadriceps femoris muscle, where arteriogenesis is possible due to the presence of pre-existing collaterals, we performed Pearson correlation analysis of each individual animal's PLGF protein level with its plasma cholesterol, isoprostane, triglyceride, glucose, insulin, HOMA-IR, and AUC values. The analysis revealed that quadriceps muscle PLGF levels were highly negatively correlated with plasma insulin and the HOMA-IR index ($r^2=0.66$ and 0.65, $p<0.0001$) respectively. Furthermore, there was a weaker but still significant negative correlation of PLGF with fasting glucose ($r^2=0.35$, $p<0.05$). However, plasma cholesterol, isoprostane, and triglycerides were not significantly correlated with quadriceps muscle PLGF levels. These findings are in

contrast to our results from cardiac tissue, in which PLGF was best correlated with plasma cholesterol and isoprostane. The basis for this tissue-specific difference remains to be determined.

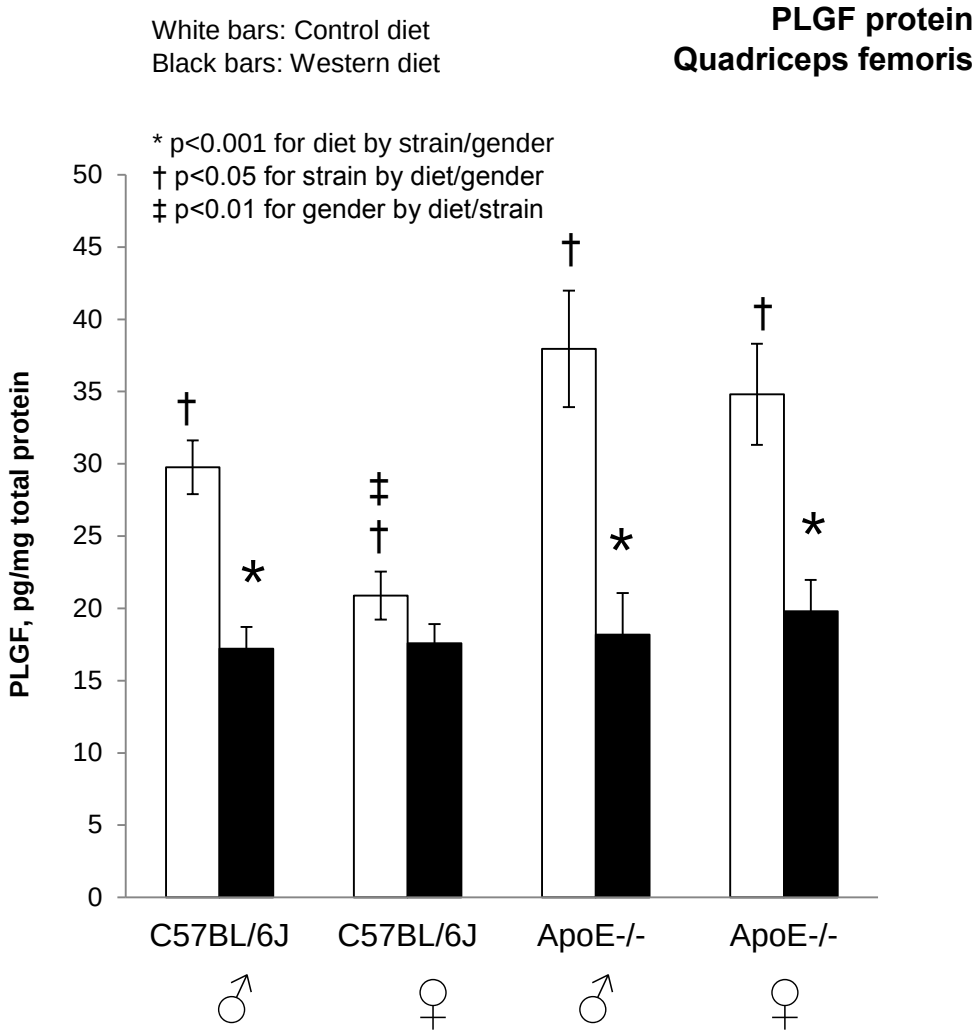

Figure 3.1: PLGF expression in quadriceps muscle was reduced by Western diet

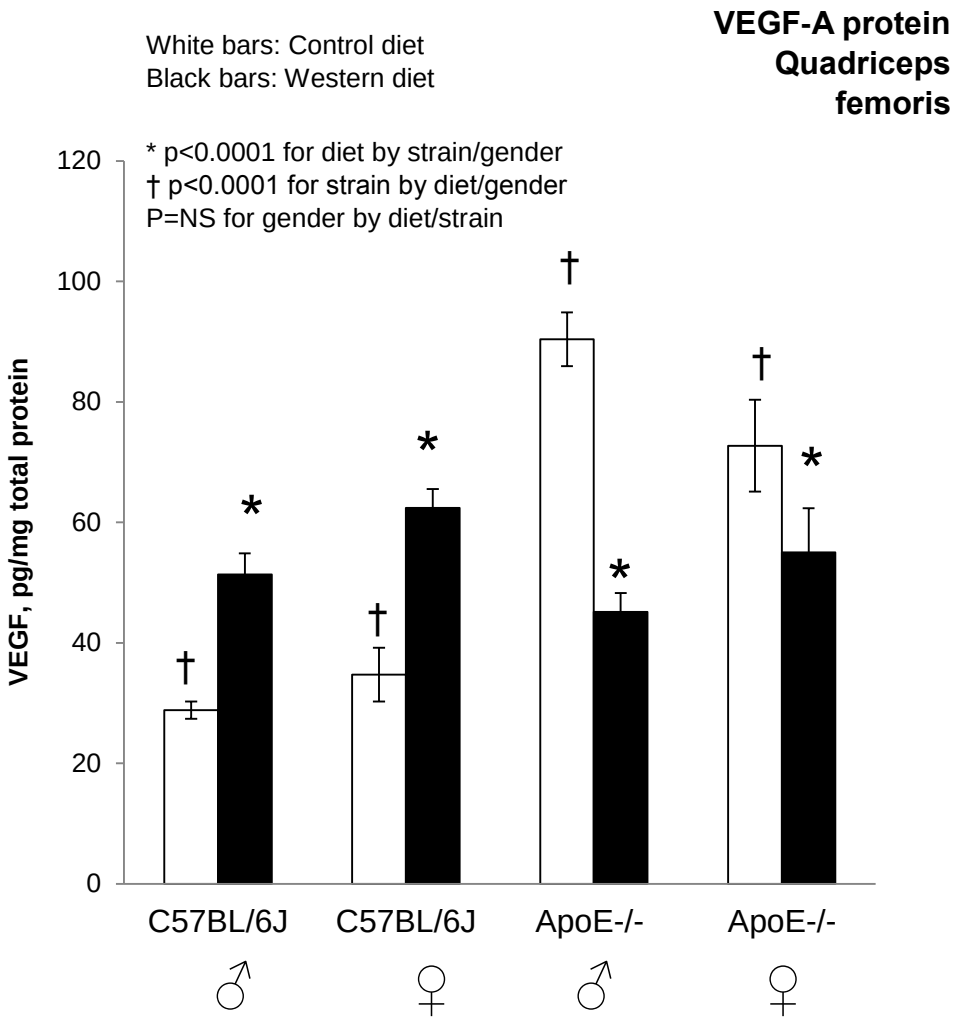

Figure 3.2: VEGF-A protein in quadriceps muscle was affected by Western diet in a mice strain dependent manner

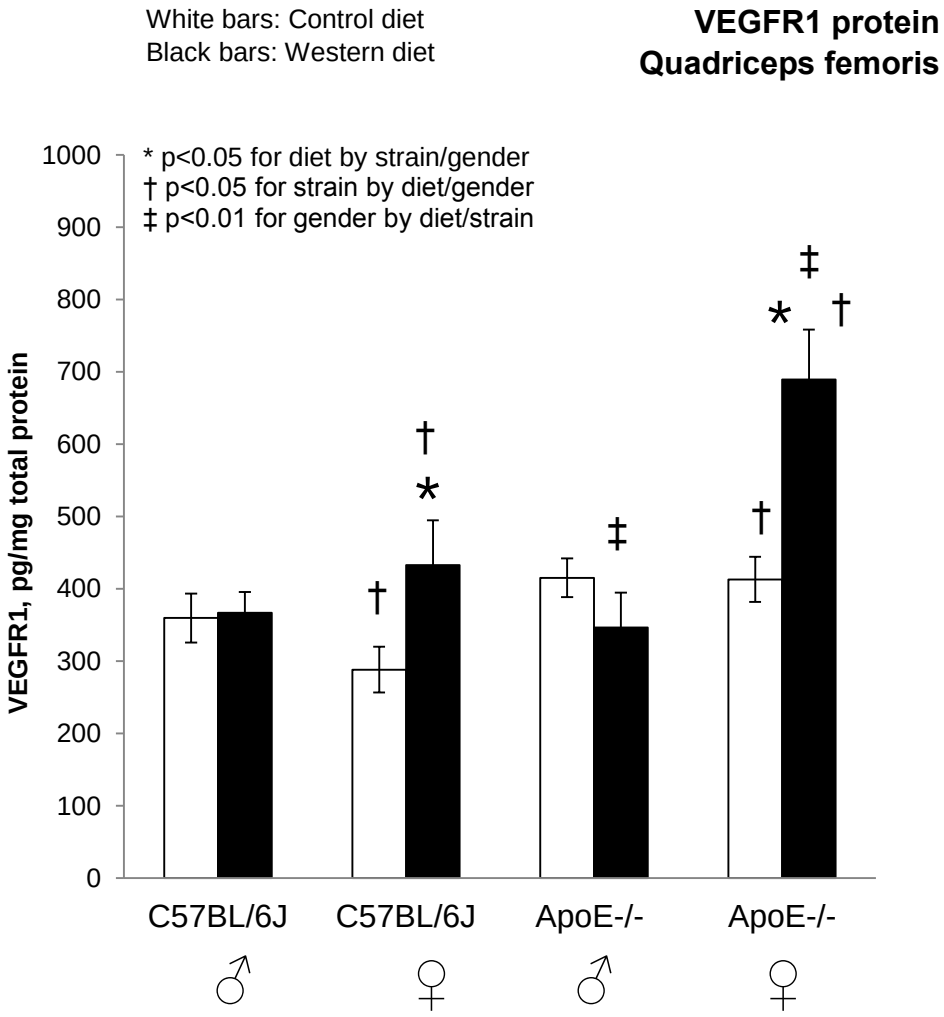

Figure 3.3: Western diet increased VEGFR1 protein in quadriceps of C57BL/6J and ApoE-/- males

Growth factor expression in the GPS muscle group

PLGF expression in the GPS muscle group was generally reduced by the Western diet, but the effect tended to be less than what we previously observed in cardiac and quadriceps femoris tissue, and only reached statistical significance in C57BL/6J males and ApoE$^{-/-}$ females ($p<0.05$) when compared to their respective control diet fed animals (Figure 3.4). A strain difference was observed in control diet fed females, with ApoE$^{-/-}$ females showing elevated PLGF protein levels compared to C57BL/6J females ($p<0.0001$). A gender difference was evident in the ApoE$^{-/-}$ strain, with females having elevated PLGF levels compared to males ($p<0.0001$).

VEGF-A protein expression in the GPS muscle group was decreased by the Western diet regardless of strain and gender ($p<0.05$). These results are in contrast to the opposing strain-dependent effects of the Western diet on VEGF-A expression in the quadriceps femoris muscle (Figure 3.5). Similar to what we observed for PLGF in the GPS group, a strain difference was observed for VEGF-A in control diet fed females, with ApoE$^{-/-}$ females showing elevated VEGF-A protein levels compared to C57BL/6J females ($p<0.05$). VEGF-A levels in the GPS also varied significantly with gender in the ApoE$^{-/-}$ strain, with ApoE$^{-/-}$ females expressing significantly higher VEGF-A levels compared to ApoE$^{-/-}$ males ($p<0.05$).

Western diet-dependent effects to decrease VEGFR1 expression in the GPS were only seen in ApoE$^{-/-}$ males (Figure 3.6). Strain-dependent differences in GPS VEGFR1 expression regardless of diet treatment were also observed, with ApoE$^{-/-}$ mice generally showing lower expression compared to C57BL/6J mice ($p<0.001$). Gender effects on

VEGFR1 expression were seen in the ApoE$^{-/-}$ strain, with lower VEGFR1 levels in females than in males on either diet ($p<0.05$).

Pearson correlation analysis was performed to compare each individual animal's PLGF protein level with its plasma cholesterol, isoprostane, triglyceride, glucose, insulin, HOMA-IR index, and AUC values. Interestingly, similar to our observations for PLGF in quadriceps, PLGF levels in the GPS were also best correlated with indices of hyperglycemia such as plasma insulin ($r^2=0.52$, $p<0.001$), HOMAIR ($r^2=0.51$, $p<0.001$), and fasting glucose ($r^2=0.32$, $p<0.05$). However, also similar to our findings in the quadriceps, but in contrast to results from cardiac tissue, PLGF was not significantly correlated with plasma cholesterol, isoprostane, or triglycerides in the GPS muscle group.

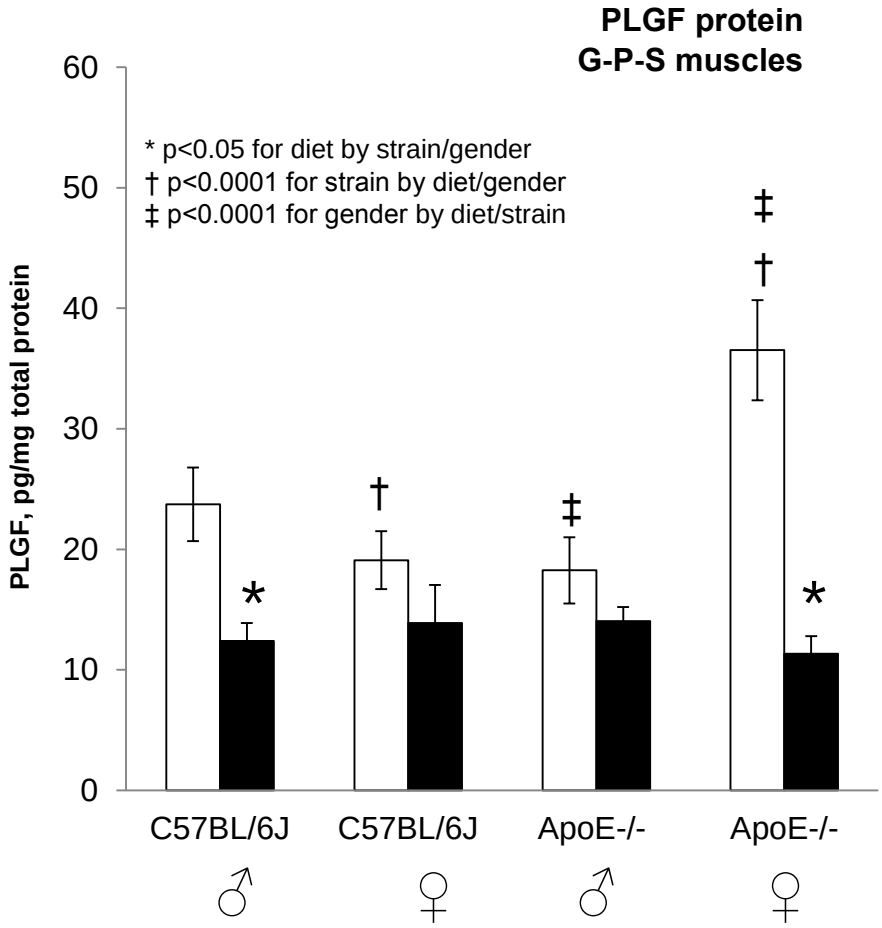

Figure 3.4: PLGF protein was reduced in G-P-S muscle of C57BL/6J males and ApoE$^{-/-}$ females fed Western diet

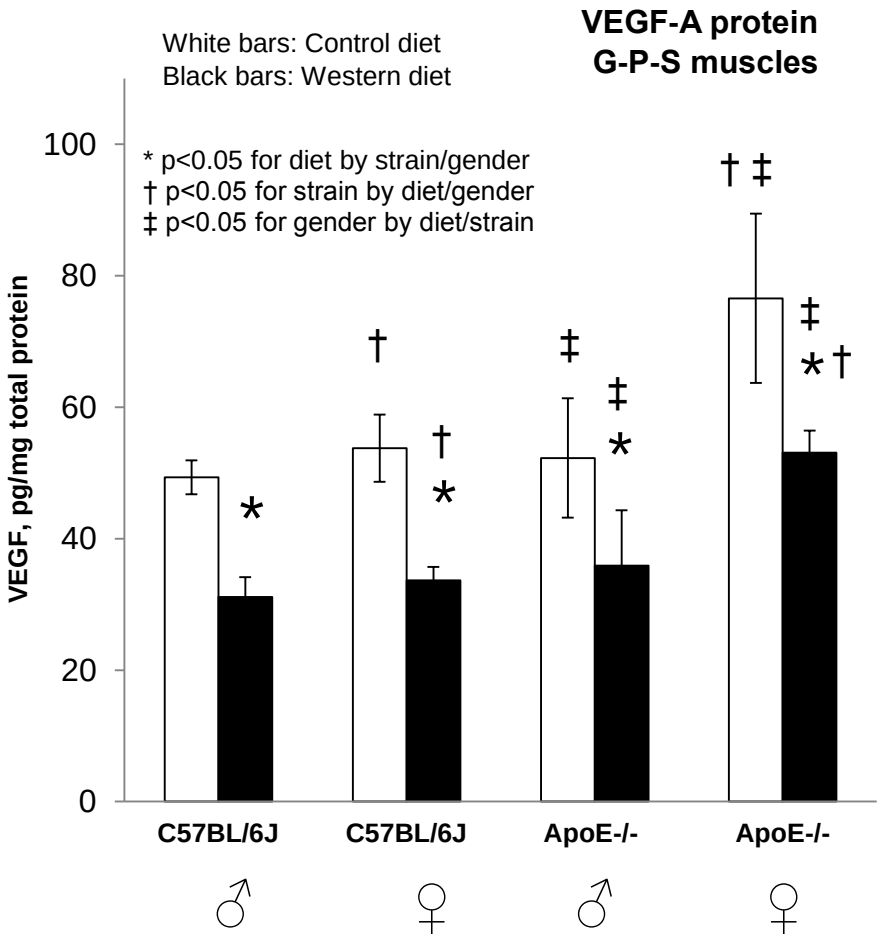

Figure 3.5: VEGF-A is reduced by Western diet in all groups

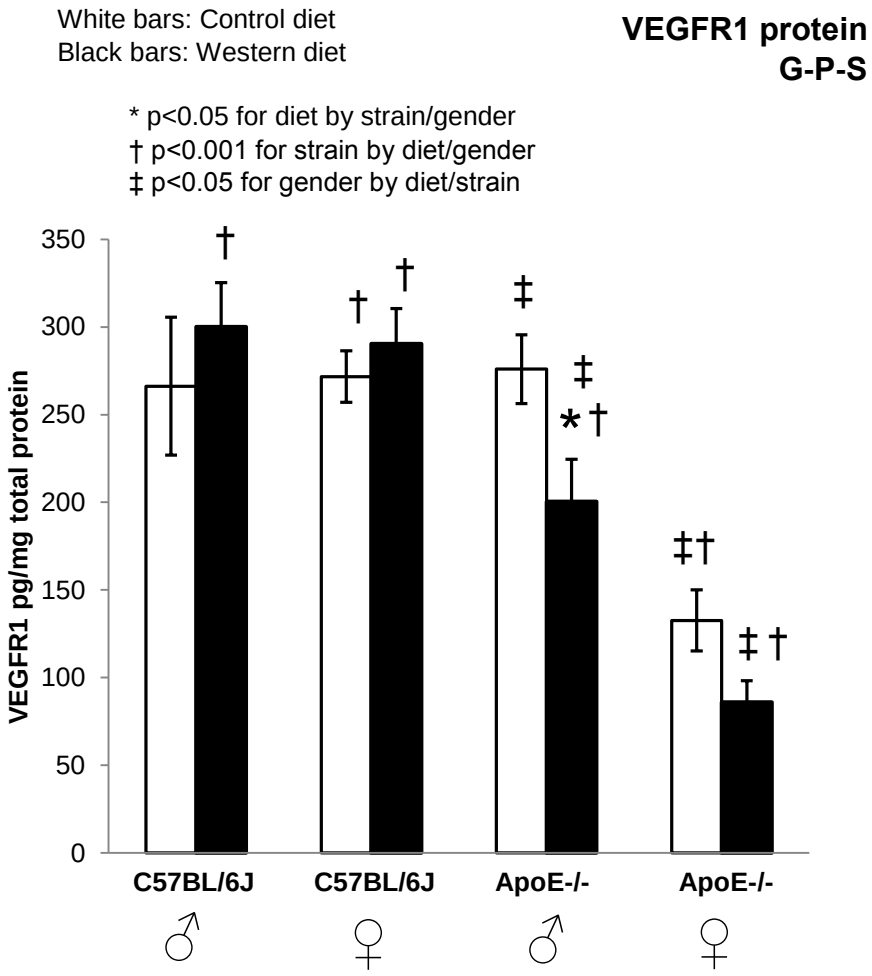

Figure 3.6: Western diet decreased VEGFR1 protein in quadriceps muscles of ApoE-/- males

Discussion

The primary goal of this study was to investigate the effect of diabetes related metabolic dysfunction on the expression and regulation of PLGF and related growth factors and receptors in skeletal muscle tissues, particularly those in which vascular remodeling occurs in peripheral artery disease. Long term feeding of a Western diet is a physiologically relevant model for human type II diabetes and studying its associated cardiovascular complications including PAD (25;40). Here we demonstrate that C57BL/6J mice develop significant hyperglycemia, hyperinsulinemia and hypercholesterolemia on a Western diet, whereas $ApoE^{-/-}$ mice develop extreme hyperlipidemia. Even when consuming a control diet, $ApoE^{-/-}$ mice developed significantly higher levels of hyperlipidemia than C57BL/6J mice. By studying two strains of mice with varying genetic susceptibility to diet-induced metabolic dysfunction, we were able to study PLGF expression under a wide range of metabolic phenotypes. In humans, PAD most commonly occurs in older patients with type II diabetes or other metabolic abnormalities; however, most animal studies aimed at characterizing signal pathways mediating collateral growth in the periphery have utilized young animals with a normal metabolic profile. Therefore, our results are more relevant to the typical clinical setting of human PAD.

We previously demonstrated that PLGF is reduced by a Western diet in cardiac tissue in a hyperlipidemia- and oxidative stress-associated manner, in agreement with the known inhibitory effect of type II diabetes on arteriogenesis. We therefore hypothesized that a similar inhibition may occur in skeletal muscle and contribute to reduced arteriogenesis in diabetic PAD.

PLGF was decreased in skeletal muscle from the quadriceps and GPS by a Western diet, similar to our findings in cardiac tissue. The Western diet had a greater inhibitory effect on PLGF expression in the quadriceps muscle than the GPS group, in agreement with the fact that arteriogenesis (which is regulated by PLGF) is the predominant type of vascular remodeling in the quadriceps in response to occlusion, whereas angiogenesis (which is primarily regulated by VEGF-A) is the main type of remodeling in skeletal muscle of the distal limb. Contrary to our results in cardiac tissue, the metabolic variable which showed the strongest statistical correlation to PLGF protein level in skeletal muscle was plasma insulin, as opposed to plasma cholesterol and oxidative stress for the heart. The basis for this observation remains to be determined. It is possible that changes in plasma oxidative markers do not accurately represent the muscle tissue redox balance as shown by others (41). Therefore, a stronger correlation of skeletal muscle PLGF levels might be obtained if a direct comparison to skeletal muscle oxidative stress was made. It is also possible that PLGF is differentially regulated in cardiac muscle vs skeletal muscle. Lastly, the PLGF expression which we observed in whole cardiac and muscle tissues may arise from different cell types. Further studies are needed to better characterize differences in PLGF expression and regulation between cardiac and skeletal muscle tissues.

Elevated levels of VEGF-A in skeletal muscle of high fat diet fed mice have been previously reported. The elevated VEGF-A observed was attributed to high fat diet induced increased fatty acid oxidation, resulting in high oxygen demand in skeletal muscles and capillaries resulting hypoxic environment (42). Our results were somewhat different. Although the Western diet did increase VEGF-A levels in the quadriceps

muscle of C57BL/6J mice, it decreased VEGF-A in the quadriceps muscle of ApoE-/- mice and in the GPS muscle group of both mouse strains. The strain dependent difference in the quadriceps muscle was quite striking. The mechanism behind this difference, as well as its functional significance to the collateral remodeling process, is an important area for future study.

The effect of a Western diet on VEGFR1 expression in skeletal muscle varied between quadriceps and the GPS group. The Western diet increased VEGFR1 in quadriceps of female mice of both strains, but reduced it in male and female $ApoE^{-/-}$ mice. VEGFR1 levels were unaffected by the diet in the remaining experimental groups. One previous report shows that reduced VEGFR1 in diabetic tibialis anterior muscle (43). It remains to be determined whether the differences we observed between the quadriceps muscle and the GPS group relate to muscle fiber type, the predominant form of vascular remodeling that occurs in each muscle (arteriogenesis in quadriceps vs angiogenesis in GPS), or other factors.

Although we observed a few gender-related differences, overall PLGF expression in skeletal muscle of female mice was similar to that in male mice. This finding is of interest due to the predominant role of PLGF in placental development and suggests that PLGF is equally important in mediating non-reproductive related vascular remodeling in both males and females.

In summary, this study focused on the effect of a Western diet on baseline (unstimulated) PLGF, VEGF-A, and VEGFR1 expression. We show that PLGF levels in skeletal muscle are generally reduced by a Western diet, suggesting that dysfunctional PLGF expression could contribute to impaired peripheral arteriogenesis in type II

diabetes. PLGF expression has been demonstrated to increase significantly above baseline in rat hindlimb following induction of experimental PAD (44). Therefore, the next question to be addressed is whether PLGF is normally upregulated in skeletal muscle of Western diet fed mice by the physiological stimulus of vascular occlusion, or whether the inhibitory effect of the Western diet on baseline PLGF expression also applies to upregulation of PLGF. Normalization of PLGF expression in skeletal muscle could potentially rescue impaired peripheral arteriogenesis in type II diabetes. Thus, our results have important clinical implications.

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CHAPTER IV

A WESTERN DIET DECREASES AND DELAYS PLACENTA GROWTH FACTOR UPREGULATION IN RESPONSE TO FEMORAL ARTERY LIGATION IN MICE

Abstract

Arteriogenesis (collateral remodeling) preserves blood flow distal to occlusions, ameliorating ischemic tissue damage in peripheral artery disease (PAD). Stimulation of arteriogenesis would be highly beneficial in diabetics, who are especially prone to develop PAD. However, arteriogenesis is reduced in diabetes by an undefined mechanism. Placenta growth factor (PLGF) is a key arteriogenic factor that induces the formation of large, stable vessels. PLGF is known to be upregulated following arterial occlusion, and PLGF therapy improves arteriogenesis. However, whether PLGF regulatory mechanisms function normally in diabetic peripheral tissues in response to occlusion is unknown. We previously showed that baseline PLGF protein levels are significantly decreased in mouse hindlimb skeletal muscle by a Western-type diet in a hyperinsulinemia-associated manner. Thus, we hypothesized that upregulation of PLGF in response to the physiological stimulus of upstream vascular occlusion may also be impaired by feeding of a Western diet. We hypothesized that both basal and stimulated PLGF expression might be abnormal by Western diet induced metabolic dysfunction, potentially contributing to impaired arteriogenesis in diabetic PAD. To test this hypothesis, we utilized three mouse models of long term (6 mo) diet-induced metabolic dysfunction: hyperlipidemic + hyperglycemic + hyperinsulinemic (Western diet fed C57BL/6J, C57-WD; n=58), very hyperlipidemic (control diet fed ApoE$^{-/-}$, ApoE-CD; n=42) and extremely hyperlipidemic + hyperinsulinemic (Western diet fed ApoE$^{-/-}$, ApoE-WD; n=45). A normolipidemic + normoglycemic + normoinsulinemic control group (control diet fed C57BL/6J, C57-CD; n=52) was also studied. After 24 weeks on the diet, animals were subjected to gradual femoral artery occlusion by placement of an

ameroid constrictor. Sham control animals (Total n= 20) were also studied. Hindlimb skeletal muscle was collected 3-28 days post-surgery (dps) for measurement of PLGF and related arteriogenic proteins. Flow recovery at 21dps was measured (n=4/group) by infusion of fluorescent microspheres. Mouse metabolic phenotype was confirmed by measuring relevant plasma parameters. PLGF was sharply elevated in skeletal muscle of CONT mice in response to femoral artery occlusion, peaking around 10 dps. PLGF upregulation by occlusion was both decreased and delayed in C57-WD group ($p<0.05$). Even more strikingly, the response to PLGF to occlusion was completely abolished in ApoE-CD and ApoE-WD groups. We conclude that metabolic dysfunction induced by a Western-type diet inhibits PLGF upregulation by the physiological stimulus of occlusion, in addition to reducing baseline PLGF levels as we previously described. Impaired PLGF regulation may therefore contribute to the poor arteriogenic response in diabetes.

Introduction

Peripheral artery disease is a major cause of morbidity and mortality in the US. Current treatment strategies include life style changes and pharmacological interventions aimed at controlling the metabolic conditions that promote atherosclerosis. However, these strategies are not optimally effective, and ~40% of patients with critical limb ischemia will eventually need limb amputations (approximately 150,000/year) (1).

Type II diabetes is a major predictor for development of PAD. Diabetic patients are five times more likely to have undergone limb amputation compared to nondiabetic patients (2). Furthermore, diabetic PAD patients have a poorer response to revascularization therapies (vascular reconstruction, stent placement or angioplasty), compared to non-diabetic PAD patients (3). Arteriogenesis is an innate mechanism by

which the vasculature can remodel to naturally bypass blood vessel occlusion. Since arteriogenesis results in an increase in blood flow capacity to the tissue, it is a more efficient mechanism of countering PAD-induced tissue ischemia than angiogenesis, which can decrease the diffusion distance for O_2 but does not alter overall blood flow to the tissue (4;5). However, aging and metabolic conditions that promote PAD also tend to inhibit arteriogenesis (6;7).

Type II diabetes and associated hyperlipidemia are the worst negative predictors of arteriogenesis (8-10). The mechanism of this inhibition is unclear, but oxidative stress, endothelial dysfunction, and abnormal growth factor signaling have all been implicated. Normalization of diabetes-associated abnormal arteriogenic growth factor signaling has long been regarded as a potential target for new PAD therapies (11). However, despite the promising arteriogenic and angiogenic effects of VEGF-A and FGF in preclinical studies, translation of these findings to clinical application has been disappointing (12-17). One explanation for the lack of success for such therapies is that multiple growth factors and cytokines are involved in orchestrating arteriogenesis, and supplying a single factor may not be sufficient, especially since multiple factors may be dysfunctional in diabetic PAD (18). Therefore, there is a critical need for knowledge of how additional growth factor and cytokine targets are altered in diabetes. Another major consideration is that the growth factors that have been tested for therapeutic benefit in PAD (e.g. VEGF-A) are either primarily angiogenic or have a mixed angiogenic/arteriogenic action, when the process that should be targeted to optimally improve tissue blood flow is arteriogenesis.

Placenta growth factor is a key arteriogenic growth factor. PLGF knockout mice show impaired arteriogenesis (19), while overexpression (20) or localized infusion of PLGF has positive arteriogenic effects through a monocyte-mediated mechanism. MCP-1, another important monocyte chemoattractant, is also arteriogenic in animal models of PAD; however, MCP-1 has a systemic atherogenic effect, limiting its potential therapeutic use (21).

PLGF has been recognized as a promising therapeutic cytokine target for compromised vascular remodeling in PAD (22). However, detailed characterization of PLGF expression and regulation in diabetic PAD has not yet been reported. PLGF treatment has shown consistent positive outcomes when administered to animals with experimental PAD. However, these studies have used healthy young animals in which acute hindlimb ischemia is induced; whereas clinical PAD patients are often elderly and suffer from underlying diabetes, and occlusion occurs gradually over a period of years. Therefore, one goal of this study was to study the regulation of PLGF in a more clinically relevant model system.

We characterized the temporal expression patterns of PLGF, VEGF-A, VEGFR1, and MCP-1 in mouse hindlimb skeletal muscle in response to gradual arterial occlusion. We sought to determine the detailed temporal expression profile of PLGF in response to gradual femoral artery occlusion, and to test whether that response is altered by a Western diet. The use of acute femoral artery ligation in healthy animals, as done in most studies of experimental PAD, does not accurately mimic the human clinical presentation of PAD. Therefore, we combined a Western diet-induced phenotype of Type II-diabetes associated metabolic dysfunction with gradual femoral artery occlusion, to more

accurately mimic the typical clinical scenario of PAD. The knowledge gained as a result of this study will reveal possible mechanisms contributing to the poor prognosis of PAD in diabetic patients and may provide potential therapeutic avenues.

Materials and Methods

Animals: C57BL/6J and ApoE$^{-/-}$ mice were either fed a Western-type diet or a control diet. The diet regimen was started when mice were 6-8 weeks of age and was continued for 24 weeks. Male and female mice were pooled together in each group. Therefore, we studied 4 groups: C57BL/6J fed control diet, n=52 (C57-CD), C57BL/6J fed Western diet, n=58 (C57-WD), ApoE-/- fed control diet, n=42 (ApoE-CD), ApoE-/- fed Western diet, n=45 (ApoE-WD). All mice had access to food and water ad libitum and were housed in a temperature controlled facility with a 12 hour dark-light cycle. This study was approved by the Institutional Animal Care and Use Committee at Oklahoma State University.

Diets: The Western diet (TD 88137, Harlan) supplied 42% kcal from anhydrous milk fat and cholesterol, 42.7% kcal from carbohydrate, and 15.2% kcal from protein. The control diet (TD120336) supplied 13% kcal from fat, 67.9% from carbohydrate, and 19.2% from protein. The Western diet also has three times more sucrose by weight, compared to the control diet. The source of the relatively high carbohydrate content in the control diet is corn starch and maltodextrin, which are not readily available sources of sugar. Therefore, the Western diet used in this study is characterized by both high fat and high sucrose.

Metabolic phenotyping of the experimental groups: Body weight of each mouse was recorded weekly. Fasting (6 h) blood samples were obtained from all mice at the end of

24 week- diet regime for metabolic measurements. Glucose homeostasis was assessed by intraperitoneal glucose tolerance testing under fasting (6h) conditions in all mice just prior to sacrifice.

Cholesterol and triglycerides: Total plasma cholesterol was determined using the Amplex Red cholesterol assay (Molecular Probes). Total plasma triglycerides were measured using a triglyceride determination kit (Sigma).

Glucose and insulin: Intraperitoneal glucose tolerance testing (IPGTT) was performed on 6 h fasted, non-anesthetized mice by administering a 1g/kg ip D-glucose bolus (~30 mg/mouse in 300□L of water). Venous blood (0.05 mL/mouse/time point; total, 0.25 mL) was drawn from the facial vein for measurement of glucose and insulin at 0, 15, 30, 60, and 120 min. Plasma glucose levels were measured using a portable glucose meter (Life Scan, Inc.). Insulin was measured using a rat/mouse insulin ELISA kit (EMD Millipore, Germany).

Oxidative stress: Plasma 8-isoprostane was measured as a biomarker for oxidative stress using a competitive enzyme immunoassay (Cayman Chemical) according to the manufacturer's protocol.

Femoral artery occlusion: After 24 weeks of the diet program, mice were subjected to gradual femoral artery occlusion by placement of an ameroid constrictor (0.25 mm inner/0.5 mm outer diameter, Research instruments SW, Figure 4.8a). Mice were anesthetized using continuous 2% isoflurane by inhalation. The hair over the thigh was shaved and the area was disinfected with Betadine. A one-to-two centimeter incision was made in the medial aspect of the thigh, and the femoral artery and vein were exposed.

The vein, artery and nerve were isolated from each other by blunt dissection under a surgical microscope (Olympus1X70 inverted microscope) (Figure 4.8b). An ameroid constrictor was placed around the femoral artery to produce gradual (~7-10 d) occlusion (Figure 4.8c). The skin was then closed with 3M VetBond (3M Animal Care Products) tissue adhesive and wound clips, and the incision site treated topically with 2% lidocaine ointment and bacitracin/polymyxin/neomycin antibiotic ointment. The right (unoperated) limb served as a paired control. Mice were returned to cages for recovery and closely observed for signs of discomfort or altered limb function. Wound clips were removed 7 d post-surgery if still present. Mice were returned to the same diet until the skeletal muscle tissue was harvested at 3, 5, 7, 10, 14, 21 or 28 days post ligation (dps; n=4-7/time point).

Sham operation: In a different set of mice, a sham surgery was performed (n=4/group/time point). The vessels were exposed and isolated as above, except for placing the ameroid constrictor on the femoral artery, and then the wound was closed as above. The right limb served as a paired control for these animals as well.

Tissue isolation: At the above mentioned time points, mice were euthanized by excision of the heart under deep isoflurane anesthesia, and the quadriceps femoris, biceps femoris and adductor muscles were isolated from the occluded and non-occluded leg separately. Tissues were homogenized in 1 mL of lysis buffer containing protease inhibitors (phenylmethylsulfonyl fluoride, sodium orthovanadate, leupeptin, benzamidine hydrochloride, aprotinin, and pepstatin A). Three one-minute homogenizations were done using a Bio-Gen Pro200 homogenizer (PRO Scientific). The homogenates were centrifuged at 700g for 5 minutes and the supernatants were transferred to clean microcentrifuge tubes and stored at -80°C.

Blood flow assessment following femoral artery occlusion: In a different set of mice, femoral artery occlusion was performed as described in the previous section. Hindlimb adductor muscle blood flow was assessed at 21dps (n=6/group) by infusion of fluorescent microspheres. Under isoflurane anesthesia, a PE-10 catheter was inserted into the right carotid artery proximal to the aortic bifurcation. Catheters were filled with saline containing 100 IU/mL heparin and 1g/L adenosine. Fluorescent microspheres (Red – orange, Excitation/Emission at 565/580 nm, 15 μm diameter, 5 x 10^5 microspheres, Molecular Probes) was infused through the carotid catheter in a rate of 100 μL/5 second. Three minutes after microsphere infusion, mice were euthanized under 5% isoflurane as above. Hindlimb thigh skeletal muscles (adductor, quadriceps and biceps femoris) were collected from each hindlimb separately for analysis of the accumulation of microspheres. Kidneys were also collected to verify even distribution of microspheres to both sides of the body.

Tissue samples were weighed, and then digested in 5 mL of 2.3M ethanolic KOH with 0.5% Tween 80 overnight in a shaker bath at 60°C. The samples were centrifuged at 2000g for 20 min, and the supernatant was removed. The tissue pellet was resuspended twice in distilled water with 0.5% Tween 80 and centrifuged, and the supernatant was discarded. The pellet was then resuspended in methanol, centrifuged, and decanted. The remaining methanol was removed by evaporative drying. The remaining microsphere residue was dissolved in 2 mL of 2-ethoxyethylacetate and fluorescence was measured by flow cytometry (Accuri C6; BD) using the recommended extinction and emission frequencies for microsphere fluorescence. Mean fluorescence counts from the occluded

and non-occluded leg were normalized to tissue weight and used to calculate the ratio of flow recovery due to arteriogenesis.

Assessment of PLGF, VEGF, MCP-1 and VEGFR1 protein levels: PLGF, VEGF-A, VEGFR1 and MCP-1 protein were quantified using ELISA (Quantikine, R&D Systems). All samples were assayed in duplicate. Protein levels in the occluded leg were normalized to the non-occluded leg in order to calculate a ratio for each factor. Data were also normalized to total protein concentration, as determined by BCA assay (Pierce). All assay plates were read using a Biotek Synergy HT plate reader.

Results

In order to induce diverse metabolic characteristics associated with diabetes and to determine their effects on PLGF and related proteins, C57BL/6J and ApoE$^{-/-}$ mice were fed a Western diet for 6 months. C57BL/6J mice fed the Western diet showed profound hyperglycemia, hyperinsulinemia, a high HOMA-IR index, and obesity, consistent with published results (23-27), whereas the effects of the diet on these parameters were less pronounced in the ApoE$^{-/-}$ strain. We previously observed significantly elevated fasting blood glucose levels in the C57BL/6J Western diet groups from as early as week 3-4 onwards. In contrast, the ApoE$^{-/-}$ Western diet fed groups did not demonstrate any impairment in fasting blood glucose until week 13, after which it was inconsistently elevated (data not shown). Fasting glucose levels were elevated among all the Western diet fed groups ($p<0.05$) by week 23 (data not shown). Glucose clearance as measured by IPGTT (Figure 4.1) and presented as area under the curve (Figure 4.2) indicated that C57BL/6J mice fed the Western diet had impaired glucose tolerance (WD fed C57BL/6J, 49950 ± 595 vs CD fed C57BL/6J, 31658 ± 754; WD,

p<0.01). From all the Western diet fed groups C57BL/6J strain demonstrated highest glucose intolerance (p< 0.001). In contrast, ApoE$^{-/-}$ mice fed the Western diet had slightly but significantly elevated glucose intolerance (WD fed ApoE$^{-/-}$, 33136 ± 626 vs CD fed ApoE$^{-/-}$, 29096 ± 591, P<0.01). Therefore, IPGTT results confirmed that feeding of the Western diet disrupted glucose homeostasis. However, significant differences were noted for strain.

All Western diet fed groups (both strains) were hyperinsulinemic (Figure 4.3, p<0.0001), in agreement with other published data (24;28). A significant (p<0.0001) strain difference existed, with Western diet fed ApoE$^{-/-}$ mice showing less hyperinsulinemia than their C57BL/6J counterparts.

Among all the Western diet fed groups, ApoE$^{-/-}$ mice were significantly less obese than C57BL/6J mice, similar to other reports (24;29-31).

Feeding of a Western diet increased plasma cholesterol (Figure 4.4) in all groups relative to their matched group on the control diet (p<0.001). However, significant effects of strain (p<0.0001) were observed. The Western diet increased cholesterol in C57BL/6J mice (282.32 ± 10.04 mg/dL) relative to control diet fed male (100.28 ± 4.44). Cholesterol levels were sharply increased (~4 fold) in all ApoE$^{-/-}$ groups regardless of diet when compared to the corresponding C57BL/6J groups (p<0.0001; ApoE$^{-/-}$ CD, 547.49 ± 23.76 mg/dL; ApoE$^{-/-}$ WD, 1104.58 ± 38.98 mg/dL). These findings are in agreement with those shown by others (24;31-33).

Feeding of a Western diet elevated triglyceride levels in both male and female ApoE$^{-/-}$ mice relative to control diet fed ApoE$^{-/-}$ mice (p<0.001), but had no effect in

C57BL/6J mice (Figure 4.5). There was a clear strain difference, with ApoE$^{-/-}$ showing significantly higher triglyceride levels than C57BL/6J groups ($p<0.05$).

These metabolic data demonstrate that the Western diet induced different metabolic phenotypes, which mainly varied according to mouse strain. C57BL/6J mice fed a Western diet (C57-WD) were hyperglycemic, hyperinsulinemic, and hypercholesterolemic, while ApoE$^{-/-}$ mice fed the Western diet (ApoE-WD) were extremely hyperlipidemic and hyperinsulinemic. ApoE$^{-/-}$ mice receiving the control diet (ApoE-CD) were moderately hyperlipidemic, whereas C57BL/6J mice fed the control diet (C57-CD) were considered to be normoglycemic and normolipidemic and represent the controls for this study.

In order to characterize the oxidative stress levels associated with each of these metabolic phenotypes, we measured plasma 8-isoprostane, which widely used as an indicator of oxidative stress. The Western diet significantly increased 8-isoprostane levels across both strains (Figure 4.6, $p<0.001$) as previously reported (34-37). There was a significant strain effect, with all ApoE$^{-/-}$ mice demonstrating greater levels of oxidative stress than C57BL/6J groups ($p<0.05$).

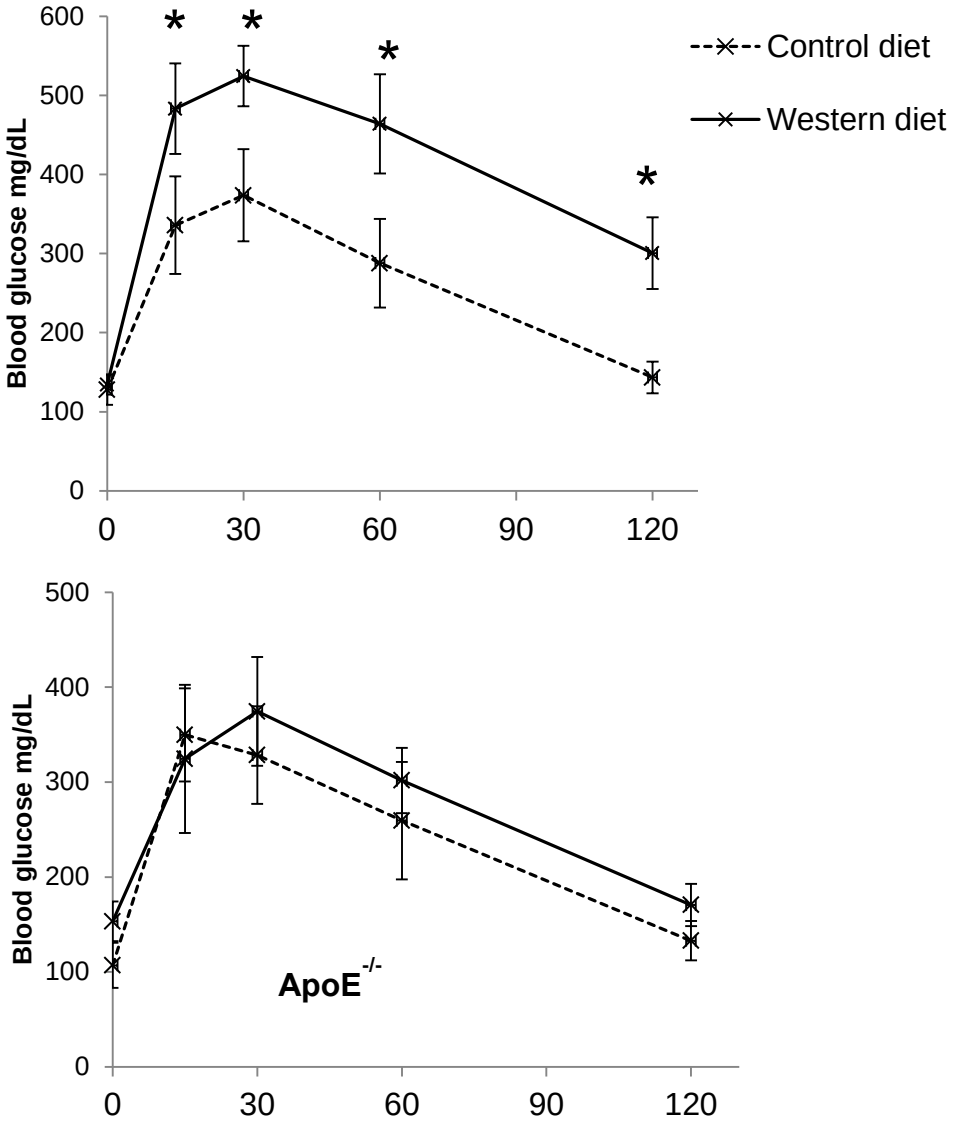

Figure 4.1: IPGTT in C57BL/6 mice (above) and ApoE-/- mice (below). *, $p<0.05$ for Western diet vs control diet group of same strain at the same time point.

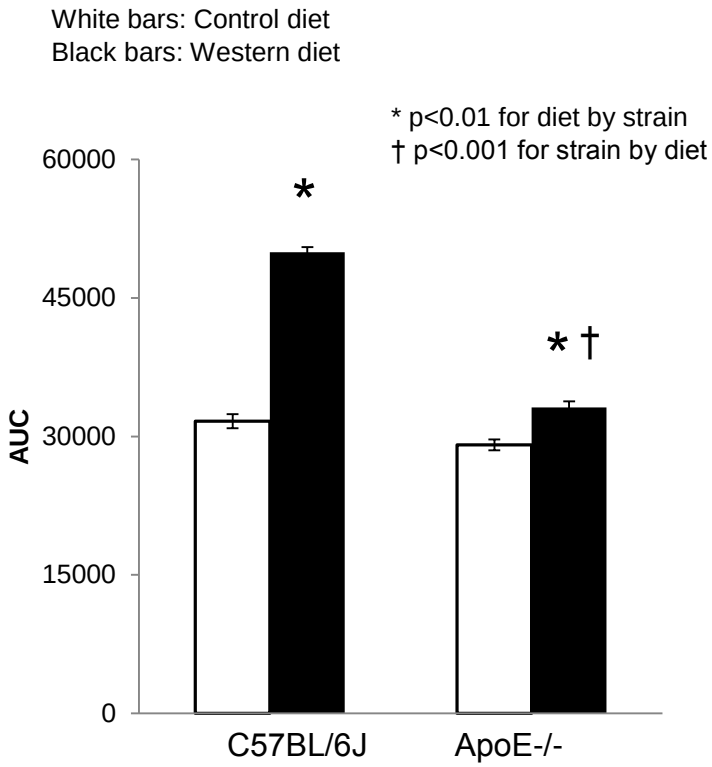

Figure 4.2: Area under the curve calculated from IPGTT in C57BL/6 mice and ApoE-/- mice fed with a control diet and a Western diet for six months.

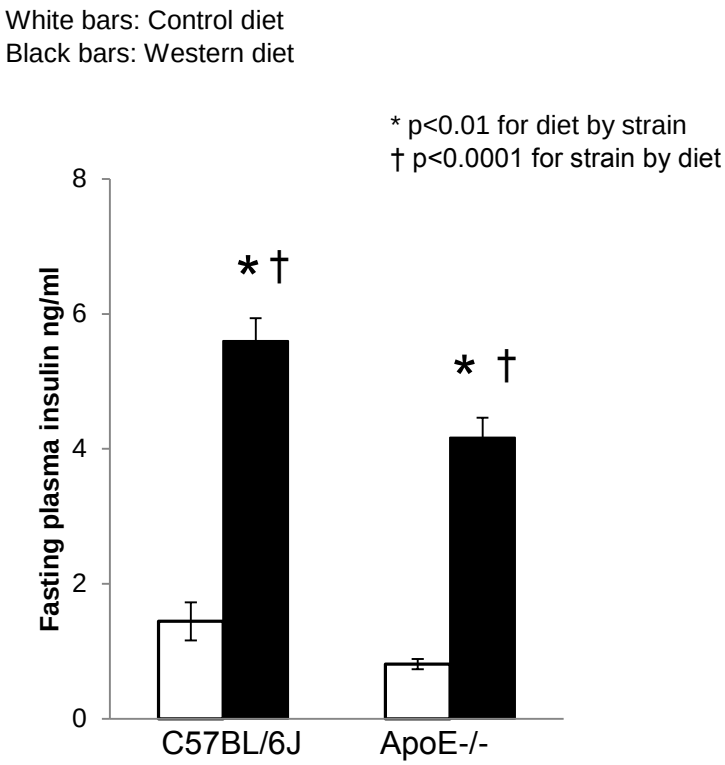

Figure 4.3: Feeding of a Western diet for 6 months increased plasma insulin in both strains.

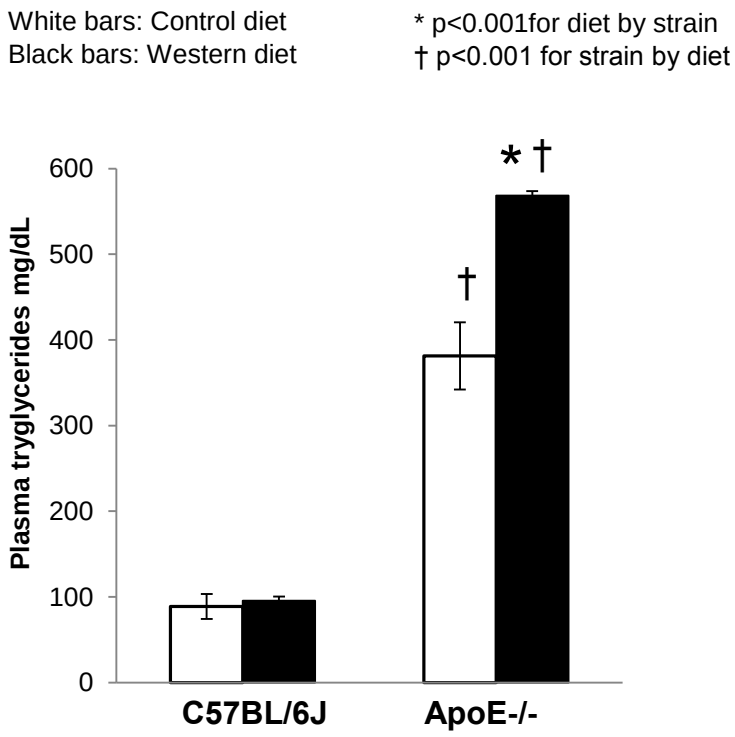

Figure 4.5: Western diet fed ApoE$^{-/-}$ mice had elevated plasma triglycerides compared to the low fat fed ApoE$^{-/-}$ mice, as well as all C57BL/6J groups

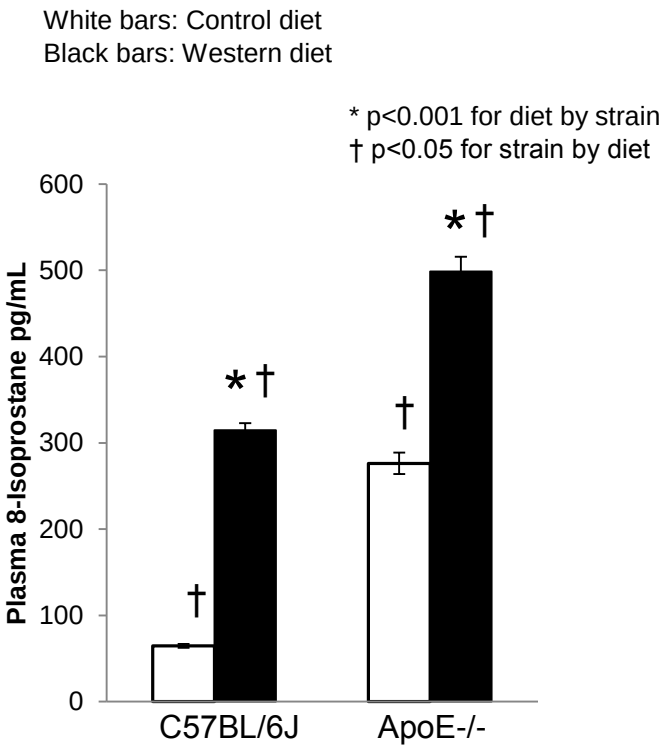

Figure 4.6: Plasma 8-isoprostane was measured as a biomarker for oxidative stress. Western diet fed mice showed increased oxidative stress, compared to control diet fed groups

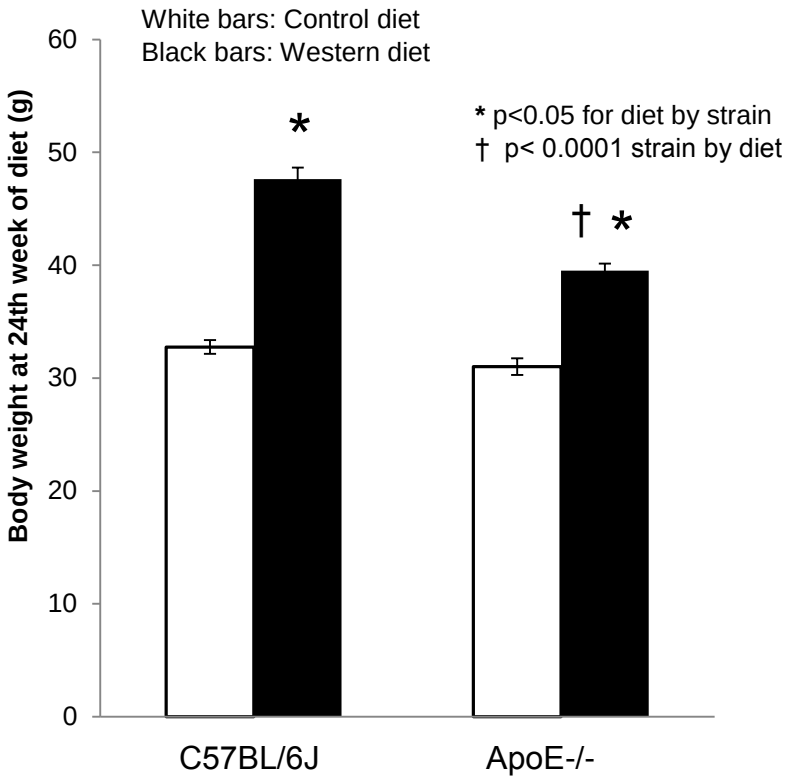

Figure 4.7: Body weights of Western diet fed mice were higher compared to control fed mice at 24th week. ApoE-/- Western diet fed animals were less obese compared to C57BL/6J fed with same diet.

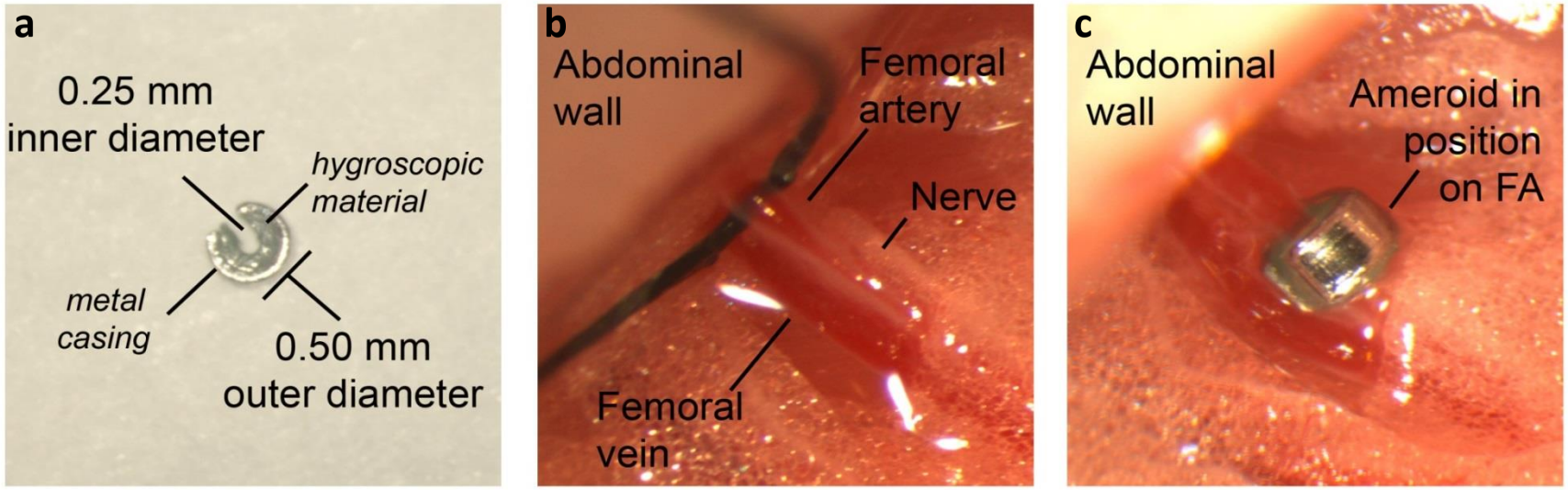

Figure 4.8: Ameroid constrictor **(a)**, isolation of femoral artery from femoral vein and nerve **(b)**, placement of ameroid constrictor on the femoral artery **(c)**.

Effect of a Western diet on PLGF, VEGF-A, VEGFR1 and MCP-1 levels following ameroid constrictor placement

After 6 months of the diet program, mice were subjected to gradual left femoral artery occlusion by placement of an ameroid constrictor, and the arteriogenic process was allowed to proceed for up to 28 dps. Analysis of blood perfusion measurements at 21-28 days following femoral artery occlusion reported almost complete flow recovery in mice hindlimb (38;39). In order to study the temporal expression pattern of the arteriogenic growth factor response in tissue in which arteriogenesis is occurring, we harvested thigh skeletal muscle from both limbs at 3, 5, 7, 10, 14, 21, or 28 days after ameroid placement and measured the protein levels. Data are presented as the ratio of each protein between the left (occluded) limb and the right (non-occluded) limb, which served as a paired control.

PLGF expression was significantly elevated beginning 3-10 dps in C57-CD and returned towards control levels thereafter (Figure 4.9). The peak PLGF expression was elevated ~2.5-fold in the occluded limb relative to the non-occluded limb at 10 dps. Sham operated animals failed to upregulate PLGF at 7 and 10 dps, confirming that the PLGF response is due to occlusion and not a nonspecific surgical effect (Figure 4.10). Critically, the upregulation of PLGF by occlusion was both delayed and reduced in magnitude in C57-WD, where peak expression was only ~1.5 fold of the level in the non-occluded limb and did not occur until up to 21 dps. Sham operated C57-WD animals also did not show any upregulation of PLGF indicating no effect due to surgery (Figure 4.11). Most interestingly, occlusion induced upregulation of PLGF was completely absent in the

ApoE$^{-/-}$ groups during all the time points tested, indicating that the greater the degree of metabolic dysfunction, the more PLGF is suppressed.

Since 7 and 10 dps were the time points at which PLGF expression was most sharply difference between the three metabolic dysfunction groups, compared to heathy control mice, we performed Pearson correlation analysis for PLGF protein levels at these two time points with the metabolic parameters of the same animal. The oxidative stress biomarker 8-isoprostane ($r=0.72$, $p<0.005$), along with insulin ($r=0.51$, $p<0.05$), showed the strongest inverse correlation with PLGF, whereas no significant correlations were found between PLGF and total cholesterol, triglycerides, or fasting glucose at 10 dps. Similar trend was observed at 7 dps (see Appendix for SAS output).

VEGF-A was also upregulated in C57-CD animals by femoral occlusion, reaching a peak expression of ~4-fold higher than the non-occluded limb at 14 dps. Similarly to what we observed for PLGF, this response was reduced or absent in the metabolic dysfunction groups (Figure 4.12). Sham operated animals did not show any response of VEGF-A to the procedure (Figure 4.13).

VEGFR1 expression in C57-CD animals began increasing 10 days after ameroid placement ($p<0.0001$) and continued to increase until the conclusion of the study at 21 d. However, this increase in VEGFR1 failed to occur in the C57-WD group or either of the ApoE$^{-/-}$ groups (Figure 14). From sham operated C57-CD animals at 7 and 10 dps only 10 dps was significantly different from the femoral artery occluded animals (Figure 4.15).

MCP-1 was elevated up to 30-fold as early as 3 dps in C57BL/6J mice (both CD and WD fed, $p<0.0001$), but not in either ApoE$^{-/-}$ group (Figure 4.16). MCP-1 levels in

sham operated animals failed to demonstrate significant elevation of MCP-1 levels (Figure 4.17 and 4.18).

When consider the PLGF level variation in operated leg and non-operated leg individually, non-operated leg tissue PLGF level stays unchanged from 3-28 dpl whereas operated leg PLGF level fluctuate with time. The same observation is true for VEGF-A, VEGFR1 and MCP-1 indicating that temporal regulation of these markers are solely due to the arterial occlusion (See appendix for the graphs that only includes protein levels from the operated leg. occlusion)

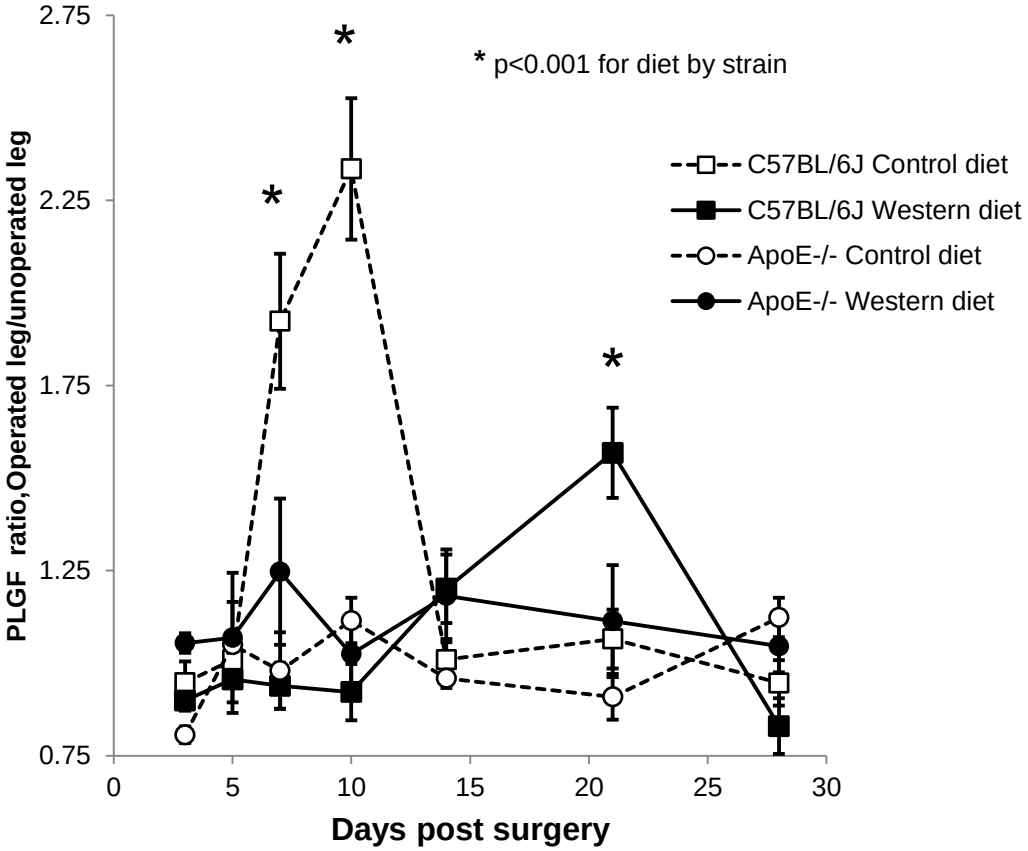

Figure 4.9: Effect of Western diet on the upregulation of PLGF in femoral artery occluded mice. Control diet fed C57BL/6J animals showed increased PLGF levels at 7 and 10 dps. *****, p<0.001 for Western diet vs control diet group of same strain at the same time point.

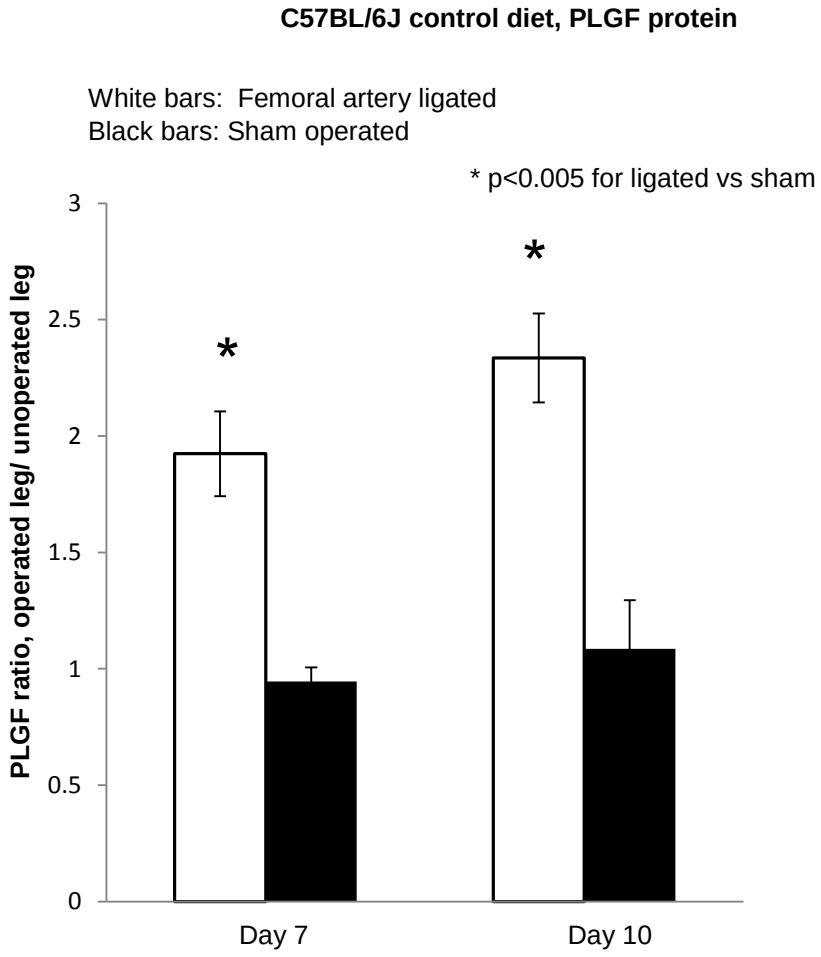

Figure 4.10: Control diet fed C57BL/6J sham operated animals failed to demonstrate occlusion associated PLGF induction at 7 and 10 dps compared to femoral artery occluded animals from same stain/diet/dps.

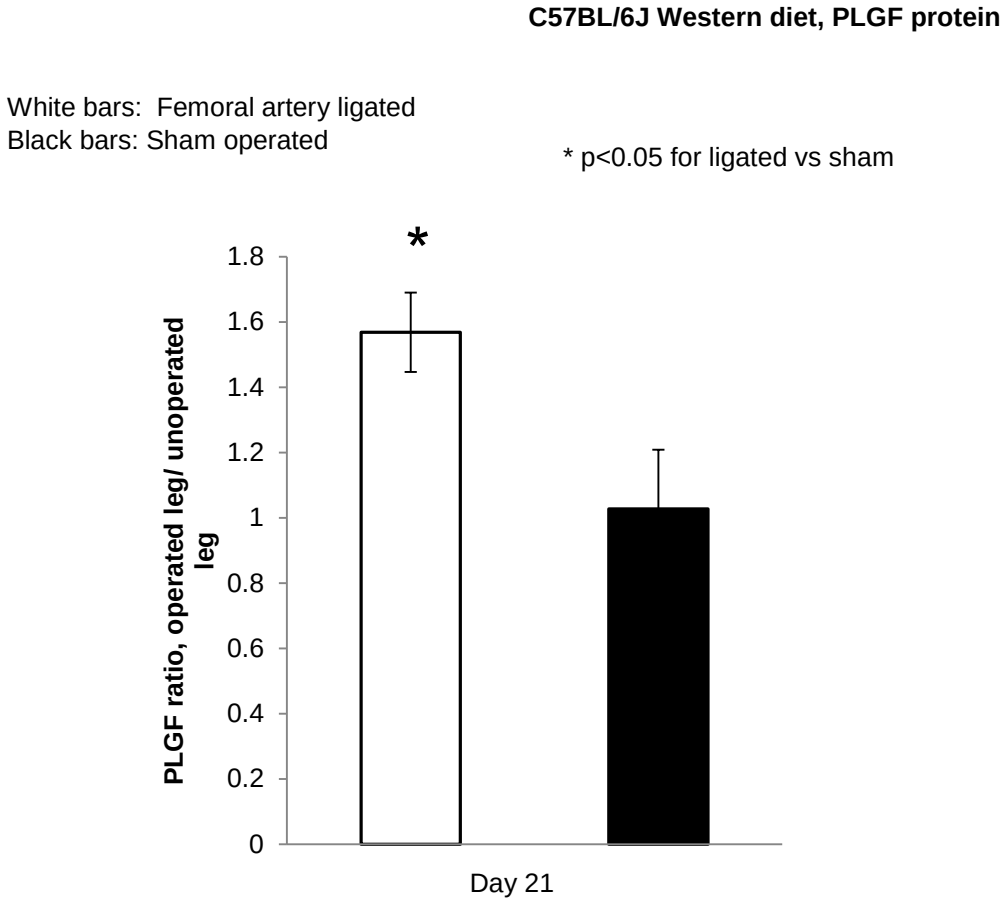

Figure 4.11: Western diet fed C57BL/6J sham operated animals failed to demonstrate occlusion associated PLGF induction at 21 dps compared to femoral artery occluded animals from same stain/diet/dps.

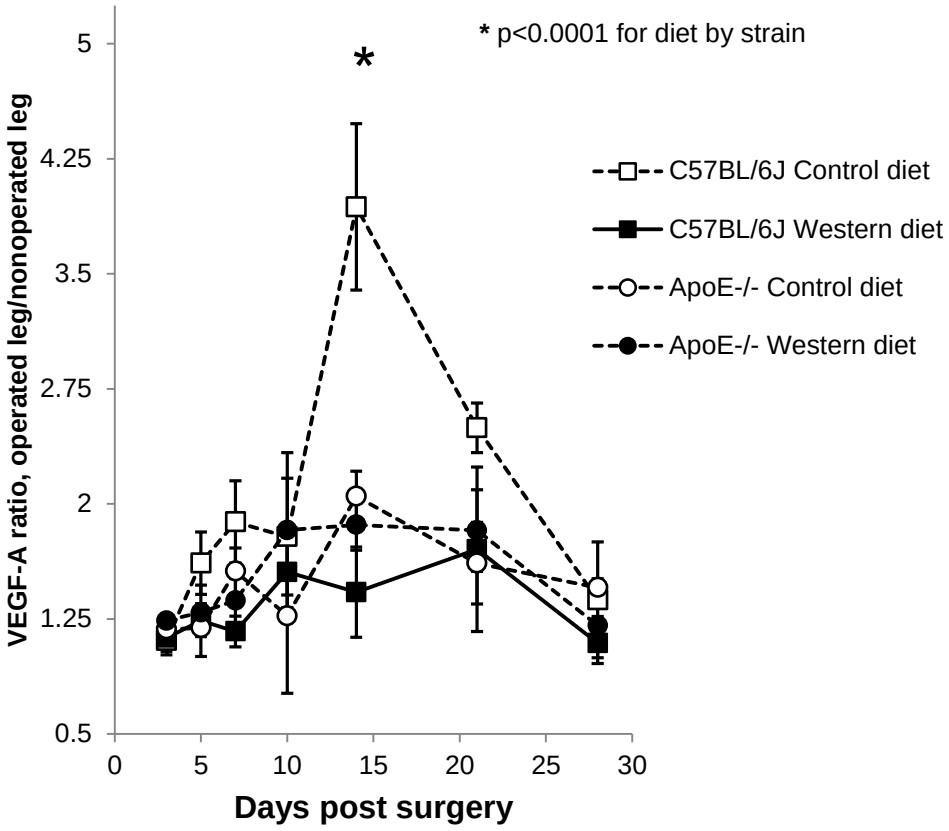

Figure 4.12: Effect of Western diet on the upregulation of VEGF in femoral artery occluded mice. Control diet fed C57BL/6J animals showed increased VEGF levels at 14 dps. *****, p<0.0001 for Western diet vs control diet group of same strain at the same time point.

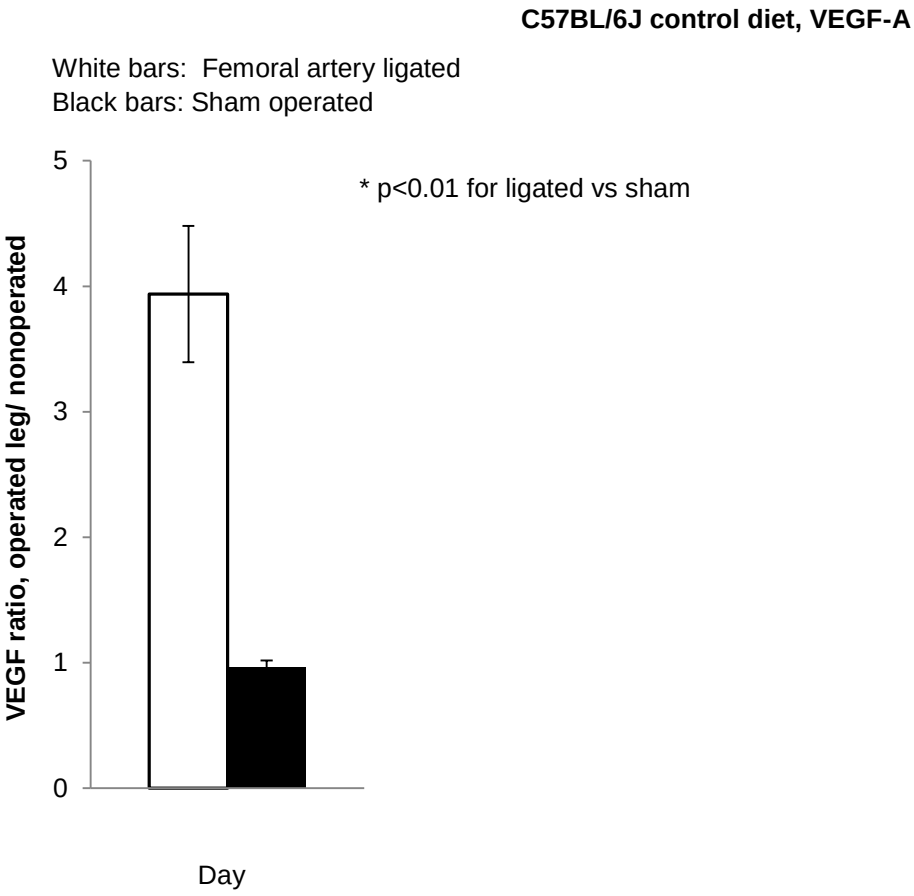

Figure 4.13: Control diet fed C57BL/6J sham operated animals failed to demonstrate occlusion associated VEGF-A induction at 14 dps compared to femoral artery occluded animals from same stain/diet/dps.

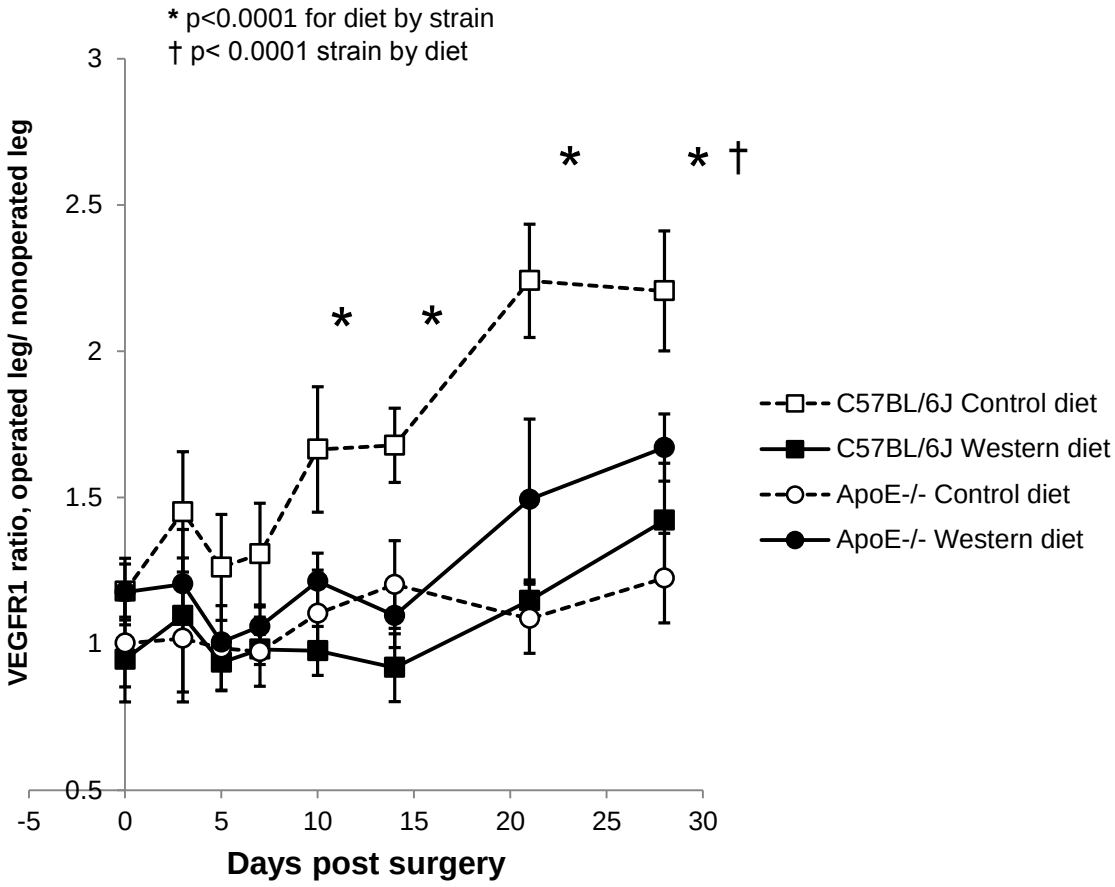

Figure 4.14: Effect of Western diet on the upregulation of VEGFR1 in femoral artery occluded mice. Control diet fed C57BL/6J animals showed increased VEGFR1 levels from 10 dps onwards. *****, p<0.001 for Western diet vs control diet group of

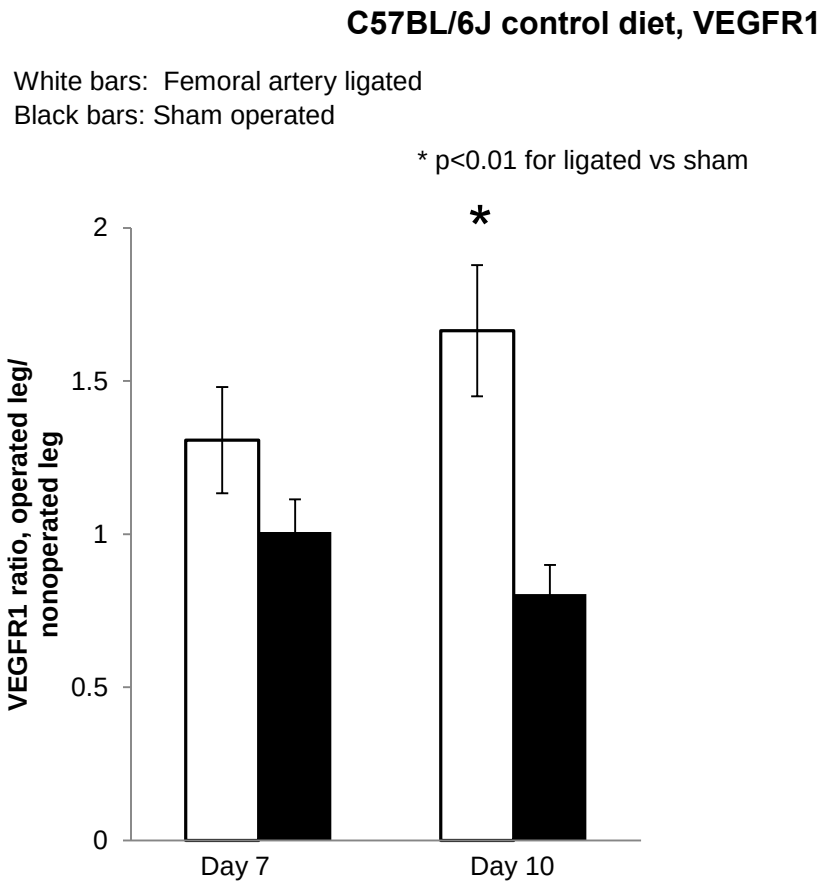

Figure 4.15: Control diet fed C57BL/6J sham operated animals failed to demonstrate occlusion associated VEGFR1 induction at 7 and 10 dps compared to femoral artery occluded animals from same stain/diet/dps.

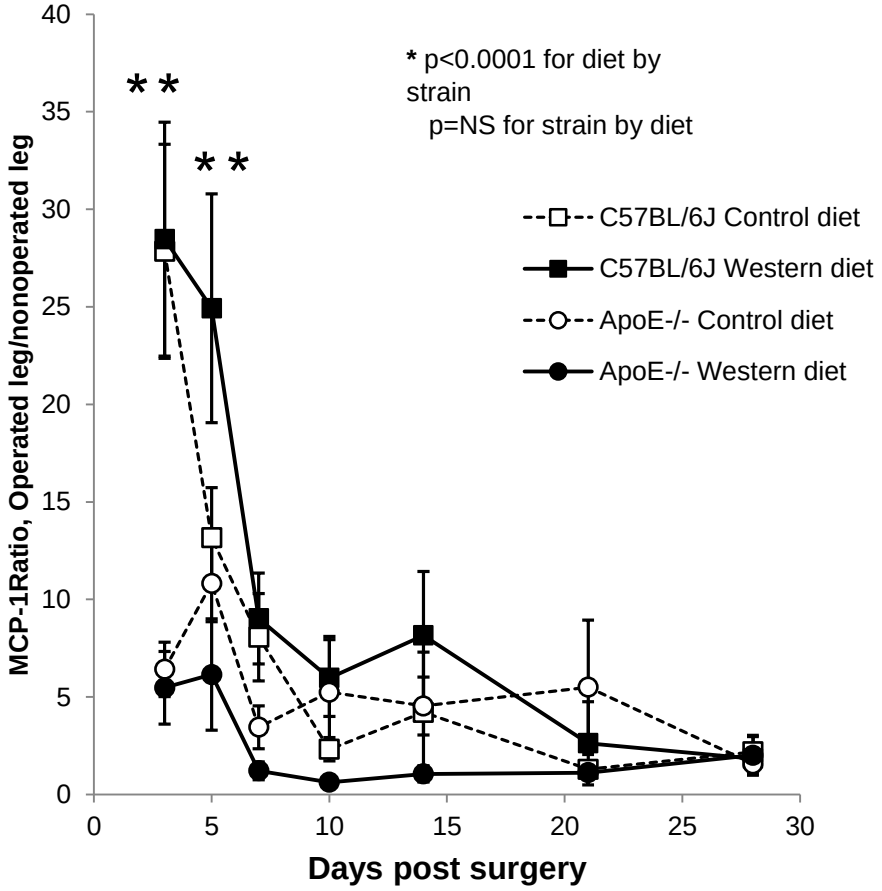

Figure 4.16: Effect of Western diet on the upregulation of MCP-1 in femoral artery occluded mice. Control diet fed C57BL/6J animals showed increased MCP-1 levels from 10 dps onwards. *****, p<0.0001 for Western diet vs control diet group of same strain at the same time point.

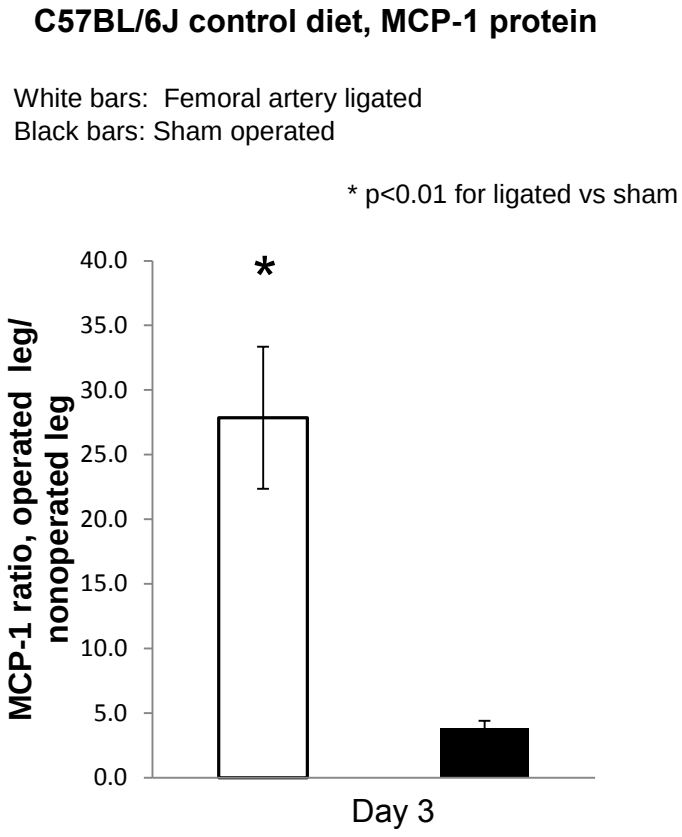

Figure 4.17: Control diet fed C57BL/6J sham operated animals failed to demonstrate occlusion associated MCP-1 induction at 3 dps compared to femoral artery occluded animals from same stain/diet/dps.

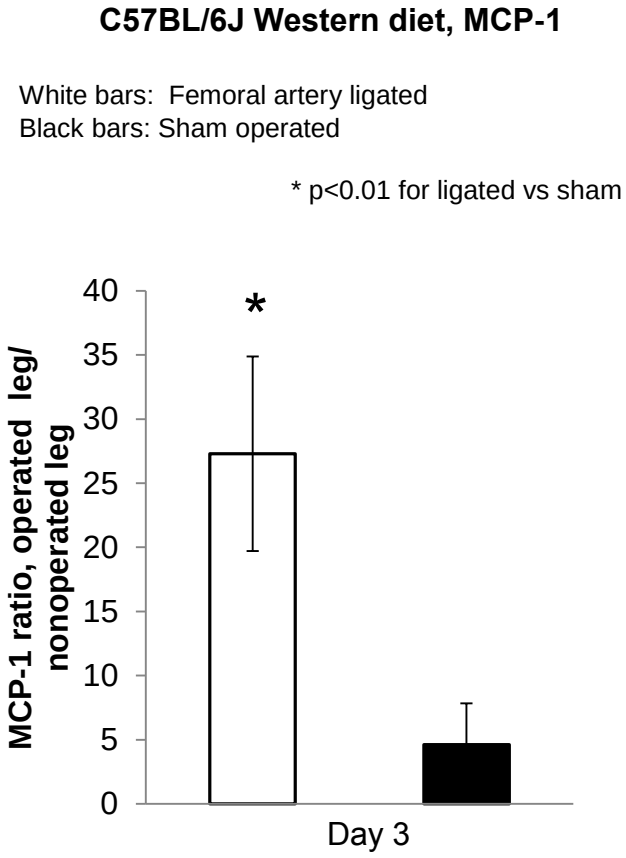

Figure 4.18: Western diet C57BL/6J sham operated animals failed to demonstrate occlusion associated MCP-1 induction at 3 dps compared to femoral artery occluded animals from same stain/diet/dps.

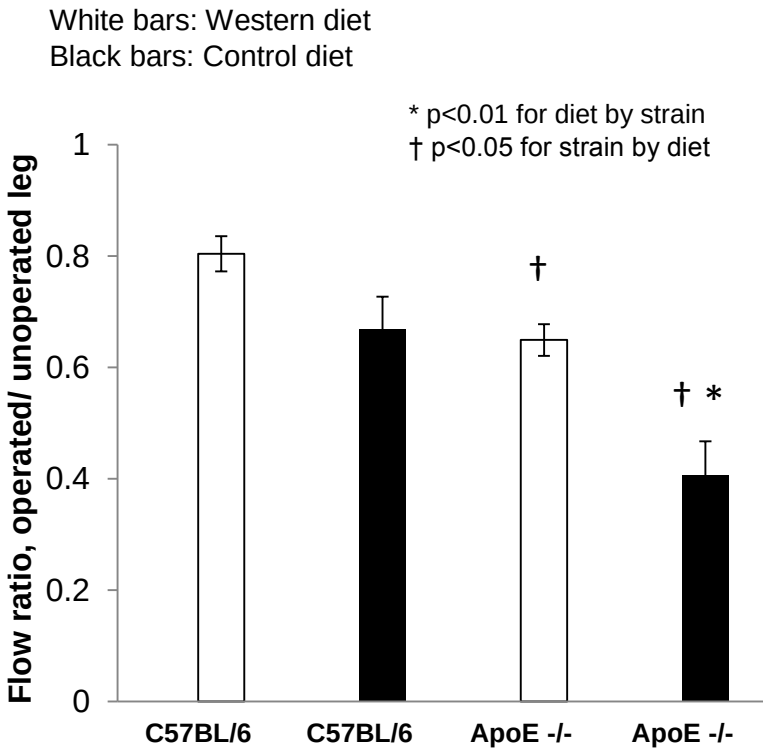

Figure 4.19: Impaired recovery of perfusion of G-P-S muscle at 21 dps in Western diet fed C57BL/6J and all ApoE-/- mice regardless of the diet

Recovery of perfusion following femoral artery occlusion

To study the effect of metabolic conditions on blood flow recovery in response to femoral artery occlusion, mice fed with respective diets for 24 weeks were subjected to unilateral femoral artery occlusion as described above. The degree of perfusion to G-P-S muscle was measured in both operated and unoperated limbs using fluorescence microsphere method and calculated the ratio. The ratio is higher and gets closer to 1 if the perfusion of the operated leg is well recovered due to strong collateral remodeling. Even distribution of microsphere in each side of the body was ensured by comparing the fluorescence accumulation among kidneys. Data were discarded if the kidney readings demonstrated < 10% variation coefficient. C57-WD, ApoE-CD and ApoE-WD showed reduced perfusion recovery compared to C57-CD (0.8 ± 0.03). However, statistically significant impaired perfusion was observed only in ApoE-/- mice regardless of the type of diet; ApoE-CD (0.65 ± 0.01, $p<0.01$) and ApoE-WD (0.41 ± 0.06, $p<0.001$). C57-WD demonstrated reduced perfusion recovery (0.66 ± 0.01, $p= 0.077$) which may reach statistical significance with increased number of samples.

Discussion

Type II diabetes promotes the pathogenesis of PAD. At the same time, however, the majority of diabetic PAD patients have limited ability to compensate for vascular occlusion via physiological survival mechanisms such as arteriogenesis. Therefore, these patients are at high risk for deleterious outcomes of PAD. Diabetes is a negative predictor of arteriogenesis with an undefined mechanism. In an attempt to gain insights into the mechanism by which diabetes inhibits arteriogenesis, we modeled Type II diabetes-

related metabolic abnormalities in two mouse strains fed either a control diet or a Western diet for six months. In order to better mimic clinical PAD, we performed gradual femoral artery occlusion in these animals. We then characterized the temporal expression pattern of arteriogenic growth factors in skeletal muscle in the thigh region, where arteriogenesis primarily occurs in this model. Coordinated temporal regulation of arteriogenic growth factors is essential in order for arteriogenesis to proceed. Here we present data showing that the normal response of PLGF, VEGF-A, VEGFR1, and MCP-1to vascular occlusion is hampered by Western diet-induced metabolic abnormalities in mice.

Gradual femoral artery occlusion using ameroid constrictors is novel in small animals and is a superior model system in which to study arteriogenesis compared to acute ligation, which often includes excision of the femoral artery and all of its branches, resulting in sudden severe ischemia (40). The method has been mainly limited to larger animal models due to technical difficulty in implementing it in small animals (41-43). We assessed the growth factor response in the thigh region because this is the primary site at which arteriogenesis occurs in hindlimb ischemia. The thigh region contains pre-existing collateral roots in the medial thigh, quadriceps femoris and biceps femoris muscles (44). Furthermore, we believe that the use of long term Western diet feeding to induce metabolic abnormalities is more physiologically relevant than use of pancreatic toxins (which mimic Type I diabetes) or genetic models of Type II diabetes, where diverse transcript signature profiles have been reported (45) according to the specific genetic background. Therefore, the outcomes of this study are highly relevant to the clinical pathology of PAD.

Chronic occlusion of the femoral artery alters the pressure gradient across preexisting collateral roots, leading to an increase in shear stress through these small vessels. Existing evidence suggests that increased shear stress is a key trigger for these small collaterals to undergo arteriogenesis to become functional arteries. The ameroid constrictors that we used to occlude the femoral artery in this study are known to close and thereby create maximum shear stress in collaterals by 7 dps. Other studies in our laboratory have revealed that PLGF is sensitive to shear stress, and increases with increasing shear stress *in vitro* (Rashdan and Lloyd, submitted). Therefore, the elevated PLGF expression we observed one week following ameroid placement in our model is consistent with other results from our laboratory.

Although a detailed investigation of the effect of experimental PAD on PLGF expression has not been conducted prior to this study to our knowledge, previous reports which studied genomic expression profiles using microarrays suggested that PLGF is an early responding growth factor in an acute femoral artery occlusion model, consistent with our findings (46;47).

Regional shear stress changes secondary to femoral artery occlusion have been studied using laser trans-illumination techniques (48). Shear stress dependent regulation of vascular growth factor expression has been studied for many factors including VEGF (49;50), PLGF (51), PDGF (52;53), and FGF (54). However, compelling evidence suggest that hypercholesterolemia alters shear stress sensing (55;56). Our finding that PLGF expression negatively correlates with hypercholesterolemia ($r = -0.45$, $p = 0.07$) is therefore consistent with potentially altered shear stress responses due to differential degrees of hypercholesterolemia in C57-WD, ApoE-CD and ApoE-WD.

VEGF-A expression is also elevated in C57-CD. However, it was delayed compared to PLGF. Previous studies which measured HIF-1 alpha, a hypoxia inducible transcription factor which is a key regulator of VEGF-A expression, showed that HIF-1 was not activated in adductor muscles at early time points after femoral artery ligation (46). It agreement with this result, is well accepted that the thigh region does not become hypoxic following femoral artery occlusion. This lack of hypoxia-inducible gene expression could partly explain the delayed response of VEGF-A compared to PLGF in our model. However, it contradicts other reported findings showing that VEGF-A is not induced in adductor muscles of a rabbit hindlimb ischemia model (57). As we reported in chapter 3, basal VEGF-A expression was increased in the C57-WD group, as previously reported by others (58). However, VEGF signaling was shown to be reduced as detected by reduced activation of key signaling intermediators in diabetes despite elevated protein levels (59).

Reduced PLGF expression in diabetes has previously been reported in diabetic wounds in mice and is associated with impaired wound healing due to reduced neovascularization, which can be reversed by PLGF supplementation (60). In another study, reduced collateralization in respond to hindlimb ischemia in diabetic mice was overcome by PLGF infusion (61). Both these studies were performed in streptozotocin treated mice, which represents type 1 diabetes. Growth factor deficiency has been linked to reduced collateral growth in and single growth factor supplementation has produced mixed results in clinical studies, reviewed in (62).

The role of MCP-1 in ischemia induced arteriogenesis has been widely studied and known to be associated with attraction and localization of monocytes to the sites of

arteriogenesis (17) (63). Skeletal muscles of insulin receptor deficient mice elevated MCP-1 gene expression along with other inflammatory markers (64). Humans with type 2 diabetes and high fat fed mice have elevated circulating MCP-1(65). Elevated MCP-1 levels in diabetes were attributed to increased baseline inflammatory state. This is accordance with C57-WD groups showing sharp upregulation of MCP-1 following ameroid placement similar to C57-CD. However, MCP-1 treatment increased collateral remodeling response which otherwise depressed in diabetic hindlimb ischemia models (17). Further, experiments are needed to determine the effect of increased MCP-1 on arteriogenesis of C57-WD.

Arteriogenesis is a vital source for perfusion recovery in response to femoral artery ligation and takes up to 2-4 weeks depending on the animal model and the type of surgical occlusion. Higher perfusion recovery in normal C57BL/6J mice have been previously reported while ApoE -/- demonstrated worst perfusion recovery attributed to hypercholesterolemia (66). In our study ApoE-CD and ApoE-WD demonstrated hypercholesterolemia associated impaired growth factor response of PLGF, VEGF, VEGFR1 and MCP-1 which further explains the mechanism of inhibited arteriogenesis and thereby reduced flow recovery.

In contrast to hypercholesterolemia, impaired glucose tolerance (as seen in C57-WD) considered least inhibitory for perfusion recovery following hindlimb ischemia (67). This is in accordance with our findings that C57-WD animals showing least affected perfusion recovery. The observation can be explained in part by our finding that C57-WD animals demonstrating partially reduced and delayed PLGF protein expression while intact MCP-1 response to femoral artery occlusion.

Our experiments shows that diet induced metabolic derangements negatively affect PLGF protein induction in the presence of gradual narrowing of femoral artery. Perfusion recovery was impaired in all the metabolic groups with abnormal PLGF response to femoral artery occlusion. To our knowledge, in depth assessment of the temporal regulation of PLGF levels in response to gradual femoral occlusion in diabetic conditions has not been reported previously. Our findings show that PLGF is abnormally regulated in the setting of Western diet induced metabolic dysfunction, suggesting that impaired PLGF production may contribute to the limited ability of diabetics with PAD to undergo arteriogenesis.

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APPENDICES

STATISTICAL ANALYSIS OUTPUTS FROM SAS PROGRAM PERFORMED BY DR. MARK PAYTON, DEPARTMENT OF STATISTICS, OKLAHOMA STATE UNIVERSITY

Chapter 2 and 3
Analyses of variance were conducted on the end point data. The program arranged these in groups by how the data were missing, so the order of the response variables is a bit different from what was on the spreadsheet. For each response variable, I've printed out the ANOVA table. Note that ordinarily we look at interactions to determine whether to analyze simple effects or main effects. More than half the time, it appears that simple effects are the proper analysis (enough interactions significant), therefore I analyzed simple effects for all response variables in an effort to remain consistent.

Class Level Information

Class	Levels	Values
Strain	2	ApoE-/- C57BL/6J
Gender	2	F M
Diet	2	HF LF

Dependent Variable: Chol

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	7493907.347	7493907.347	5713.32	<.0001
Gender	1	5736.524	5736.524	4.37	0.0411
Strain*Gender	1	15478.558	15478.558	11.80	0.0011
Diet	1	2439412.702	2439412.702	1859.80	<.0001
Strain*Diet	1	598000.074	598000.074	455.91	<.0001
Gender*Diet	1	23.222	23.222	0.02	0.8946
Strain*Gender*Diet	1	8403.724	8403.724	6.41	0.0143

Dependent Variable: Isopro8

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	910951.8122	910951.8122	287.09	<.0001
Gender	1	272.3414	272.3414	0.09	0.7706
Strain*Gender	1	50103.0656	50103.0656	15.79	0.0002
Diet	1	630181.3185	630181.3185	198.61	<.0001
Strain*Diet	1	17798.9767	17798.9767	5.61	0.0214
Gender*Diet	1	625.2371	625.2371	0.20	0.6589
Strain*Gender*Diet	1	16152.2839	16152.2839	5.09	0.0281

Dependent Variable: Glucose0

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	27.70842	27.70842	0.06	0.8151
Gender	1	1793.35951	1793.35951	3.57	0.0640
Strain*Gender	1	1740.84910	1740.84910	3.47	0.0679
Diet	1	19470.31277	19470.31277	38.78	<.0001
Strain*Diet	1	296.83674	296.83674	0.59	0.4452
Gender*Diet	1	1002.02404	1002.02404	2.00	0.1634
Strain*Gender*Diet	1	1744.61887	1744.61887	3.48	0.0676

Dependent Variable: Glucose15

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	26411.7433	26411.7433	5.58	0.0217
Gender	1	74.0925	74.0925	0.02	0.9009
Strain*Gender	1	676.0365	676.0365	0.14	0.7069
Diet	1	189854.6474	189854.6474	40.12	<.0001
Strain*Diet	1	147492.5343	147492.5343	31.17	<.0001
Gender*Diet	1	2473.2149	2473.2149	0.52	0.4728
Strain*Gender*Diet	1	79.1641	79.1641	0.02	0.8976

Dependent Variable: Glucose30

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	87749.2928	87749.2928	28.49	<.0001
Gender	1	1815.6722	1815.6722	0.59	0.4459
Strain*Gender	1	610.5719	610.5719	0.20	0.6579
Diet	1	268534.8540	268534.8540	87.20	<.0001
Strain*Diet	1	27279.8568	27279.8568	8.86	0.0043
Gender*Diet	1	1125.6268	1125.6268	0.37	0.5480
Strain*Gender*Diet	1	19413.8618	19413.8618	6.30	0.0150

Dependent Variable: Glucose60

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	205139.6845	205139.6845	70.35	<.0001
Gender	1	1550.1136	1550.1136	0.53	0.4690
Strain*Gender	1	856.0077	856.0077	0.29	0.5901
Diet	1	322438.9577	322438.9577	110.57	<.0001
Strain*Diet	1	120567.4633	120567.4633	41.34	<.0001

Gender*Diet	1	285.7787	285.7787	0.10
0.7554				
Strain*Gender*Diet	1	44773.3002	44773.3002	15.35
0.0002				

Dependent Variable: Glucose120

Source	DF	Type III SS	Mean Square	F Value
Pr > F				
Strain	1	164625.9383	164625.9383	53.50
<.0001				
Gender	1	1077.4021	1077.4021	0.35
0.5565				
Strain*Gender	1	16091.2283	16091.2283	5.23
0.0261				
Diet	1	237831.1830	237831.1830	77.28
<.0001				
Strain*Diet	1	139445.6629	139445.6629	45.31
<.0001				
Gender*Diet	1	28549.5091	28549.5091	9.28
0.0036				
Strain*Gender*Diet	1	31003.2212	31003.2212	10.07
0.0025				

Dependent Variable: AUC

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	768911981	768911981	50.21	<.0001
Gender	1	300063	300063	0.02	0.8892
Strain*Gender	1	5280489	5280489	0.34	0.5595
Diet	1	1723096927	1723096927	112.51	<.0001
Strain*Diet	1	602746639	602746639	39.36	<.0001
Gender*Diet	1	21229323	21229323	1.39	0.2441
Strain*Gender*Diet	1	137859634	137859634	9.00	0.0040

Dependent Variable: HOMAIR

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	1570.64506	1570.64506	18.06	<.0001
Gender	1	32.29587	32.29587	0.37	0.5448
Strain*Gender	1	209.61547	209.61547	2.41	0.1263
Diet	1	33005.61337	33005.61337	379.53	<.0001
Strain*Diet	1	283.19051	283.19051	3.26	0.0766
Gender*Diet	1	23.02020	23.02020	0.26	0.6090
Strain*Gender*Diet	1	234.43498	234.43498	2.70	0.1063

Dependent Variable: PLGFHeart

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	6071.644971	6071.644971	339.68	<.0001
Gender	1	0.000151	0.000151	0.00	0.9977
Strain*Gender	1	13.310404	13.310404	0.74	0.3919
Diet	1	5272.125335	5272.125335	294.95	<.0001
Strain*Diet	1	19.387357	19.387357	1.08	0.3022
Gender*Diet	1	7.356431	7.356431	0.41	0.5238

Strain*Gender*Diet	1	3.935696	3.935696	0.22
0.6408				

Dependent Variable: PLGFMT

Source	DF	Type III SS	Mean Square	F Value
Pr > F				
Strain	1	661.537373	661.537373	17.07
0.0001				
Gender	1	86.001192	86.001192	2.22
0.1420				
Strain*Gender	1	69.528523	69.528523	1.79
0.1860				
Diet	1	2279.109316	2279.109316	58.80
<.0001				
Strain*Diet	1	263.592029	263.592029	6.80
0.0117				
Gender*Diet	1	183.582693	183.582693	4.74
0.0338				
Strain*Gender*Diet	1	6.550808	6.550808	0.17
0.6826				

Dependent Variable: VEGFHeart

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	38519.87626	38519.87626	6.12	0.0165
Gender	1	2996.30731	2996.30731	0.48	0.4931
Strain*Gender	1	1829.04804	1829.04804	0.29	0.5920
Diet	1	5177.60821	5177.60821	0.82	0.3684
Strain*Diet	1	2833.59446	2833.59446	0.45	0.5051
Gender*Diet	1	4.35101	4.35101	0.00	0.9791
Strain*Gender*Diet	1	12960.62614	12960.62614	2.06	0.1570

Here are the least square means for each response variable for every combination of strain, gender and diet.

Strain	Gender	Diet	Chol LSMEAN	Isopro8 LSMEAN	Glucose0 LSMEAN	Glucose15 LSMEAN
ApoE-/-	F	HF	1242.73333	574.283333	158.000000	379.666667
ApoE-/-	F	LF	604.13333	302.883333	119.333333	350.583333
ApoE-/-	M	HF	1207.00000	492.700000	138.500000	373.333333
ApoE-/-	M	LF	614.20000	275.133333	94.500000	375.166667
C57BL/6J	F	HF	260.08571	194.928571	154.857143	528.285714
C57BL/6J	F	LF	77.61250	60.900000	103.250000	301.375000
C57BL/6J	M	HF	338.30000	298.400000	135.333333	512.933333
C57BL/6J	M	LF	104.94444	84.166667	122.444444	307.555556

Strain	Gender	Diet	Glucose30 LSMEAN	Glucose60 LSMEAN	Glucose120 LSMEAN	AUC LSMEAN
ApoE-/-	F	HF	388.666667	288.666667	151.833333	27245.0000
ApoE-/-	F	LF	341.166667	281.833333	123.583333	24654.3750
ApoE-/-	M	HF	452.000000	337.333333	178.500000	29876.2500
ApoE-/-	M	LF	313.333333	227.833333	146.500000	23523.7500
C57BL/6J	F	HF	553.571429	563.142857	437.142857	44731.0714

C57BL/6J	F	LF	345.500000	261.625000	119.125000	23002.5000
C57BL/6J	M	HF	530.400000	484.800000	304.066667	39959.0000
C57BL/6J	M	LF	378.111111	303.777778	168.000000	26851.6667

Strain	Gender	Diet	HOMAIR LSMEAN	PLGFHeart LSMEAN	PLGFMT LSMEAN	VEGFHeart LSMEAN
ApoE-/-	F	HF	42.6581733	12.5100353	20.8402415	369.950258
ApoE-/-	F	LF	4.4961977	31.7318010	34.8106831	404.353942
ApoE-/-	M	HF	53.2760010	14.7069360	18.1892269	425.125895
ApoE-/-	M	LF	4.5098664	31.4522967	37.9549114	400.595953
C57BL/6J	F	HF	65.3989540	33.3790243	16.8157298	448.956047
C57BL/6J	F	LF	10.2888841	53.8765289	20.8750461	451.396646
C57BL/6J	M	HF	60.3079949	32.6059043	17.2121057	421.545644
C57BL/6J	M	LF	10.7419053	52.7192948	29.7660119	485.119786

Here are the tests of simple effects. As an example, the p-value of 0.0931 refers to the comparison of cholesterol means of the two genders (M v. F) for strain = ApoE-/- and diet = HF. There are four comparisons of gender, one for each combination of strain and diet. Note that the only comparison that was significant was the C57BL/6J and the HF combination.

Strain*Gender*Diet Effect Sliced by Strain*Diet for Chol

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	3830.613333	3830.613333	2.92	0.0931
ApoE-/-	LF	1	304.013333	304.013333	0.23	0.6321
C57BL/6J	HF	1	29197	29197	22.26	<.0001
C57BL/6J	LF	1	3163.913734	3163.913734	2.41	0.1261

Here are the comparisons of diet (HF v. LF) for each combination of strain and gender:

Strain*Gender*Diet Effect Sliced by Strain*Gender for Chol

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	1223430	1223430	932.74	<.0001
ApoE-/-	M	1	1054236	1054236	803.74	<.0001
C57BL/6J	F	1	124307	124307	94.77	<.0001
C57BL/6J	M	1	306308	306308	233.53	<.0001

Here are the comparisons of strain for each combination of diet and gender:

Strain*Gender*Diet Effect Sliced by Gender*Diet for Chol

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	3119619	3119619	2378.38	<.0001
F	LF	1	950483	950483	724.64	<.0001
M	HF	1	3234170	3234170	2465.72	<.0001
M	LF	1	933628	933628	711.79	<.0001

Strain*Gender*Diet Effect Sliced by Strain*Diet for Isopro8

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	19968	19968	6.29	0.0151
ApoE-/-	LF	1	2310.187500	2310.187500	0.73	0.3972
C57BL/6J	HF	1	51098	51098	16.10	0.0002
C57BL/6J	LF	1	2292.724706	2292.724706	0.72	0.3990

Strain*Gender*Diet Effect Sliced by Strain*Gender for Isopro8

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	220974	220974	69.64	<.0001
ApoE-/-	M	1	142006	142006	44.75	<.0001
C57BL/6J	F	1	67064	67064	21.14	<.0001
C57BL/6J	M	1	258165	258165	81.36	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for Isopro8

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	464940	464940	146.53	<.0001
F	LF	1	200763	200763	63.27	<.0001
M	HF	1	161796	161796	50.99	<.0001
M	LF	1	131286	131286	41.38	<.0001

Strain*Gender*Diet Effect Sliced by Strain*Diet for Glucose0

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	1140.750000	1140.750000	2.27	0.1374
ApoE-/-	LF	1	1850.083333	1850.083333	3.69	0.0601
C57BL/6J	HF	1	1819.264069	1819.264069	3.62	0.0622
C57BL/6J	LF	1	1560.395425	1560.395425	3.11	0.0835

Strain*Gender*Diet Effect Sliced by Strain*Gender for Glucose0

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	4485.333333	4485.333333	8.93	0.0042
ApoE-/-	M	1	5808.000000	5808.000000	11.57	0.0013
C57BL/6J	F	1	9942.976190	9942.976190	19.81	<.0001
C57BL/6J	M	1	934.444444	934.444444	1.86	0.1780

Strain*Gender*Diet Effect Sliced by Gender*Diet for Glucose0

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	31.912088	31.912088	0.06	0.8019
F	LF	1	886.880952	886.880952	1.77	0.1893
M	HF	1	42.976190	42.976190	0.09	0.7709
M	LF	1	2811.211111	2811.211111	5.60	0.0215

Strain*Gender*Diet Effect Sliced by Strain*Diet for Glucose15

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	120.333333	120.333333	0.03	0.8739
ApoE-/-	LF	1	1813.020833	1813.020833	0.38	0.5385
C57BL/6J	HF	1	1124.910823	1124.910823	0.24	0.6278
C57BL/6J	LF	1	161.785131	161.785131	0.03	0.8540

Strain*Gender*Diet Effect Sliced by Strain*Gender for Glucose15

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	2537.520833	2537.520833	0.54	0.4671
ApoE-/-	M	1	10.083333	10.083333	0.00	0.9633
C57BL/6J	F	1	192224	192224	40.62	<.0001
C57BL/6J	M	1	237263	237263	50.14	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for Glucose15

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	71360	71360	15.08	0.0003
F	LF	1	8302.148810	8302.148810	1.75	0.1908
M	HF	1	83521	83521	17.65	<.0001
M	LF	1	16457	16457	3.48	0.0675

Strain*Gender*Diet Effect Sliced by Strain*Diet for Glucose30

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	12033	12033	3.91	0.0531
ApoE-/-	LF	1	2324.083333	2324.083333	0.75	0.3888
C57BL/6J	HF	1	2562.549351	2562.549351	0.83	0.3657
C57BL/6J	LF	1	4504.169935	4504.169935	1.46	0.2317

Strain*Gender*Diet Effect Sliced by Strain*Gender for Glucose30

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	6768.750000	6768.750000	2.20	0.1439
ApoE-/-	M	1	57685	57685	18.73	<.0001
C57BL/6J	F	1	161630	161630	52.48	<.0001
C57BL/6J	M	1	130454	130454	42.36	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for Glucose30

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	87856	87856	28.53	<.0001
F	LF	1	64.380952	64.380952	0.02	0.8856
M	HF	1	26342	26342	8.55	0.0050
M	LF	1	15106	15106	4.91	0.0309

Strain*Gender*Diet Effect Sliced by Strain*Diet for Glucose60

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	7105.333333	7105.333333	2.44	0.1243
ApoE-/-	LF	1	8748.000000	8748.000000	3.00	0.0889
C57BL/6J	HF	1	29293	29293	10.05	0.0025
C57BL/6J	LF	1	7525.510621	7525.510621	2.58	0.1139

Strain*Gender*Diet Effect Sliced by Strain*Gender for Glucose60

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	140.083333	140.083333	0.05	0.8273
ApoE-/-	M	1	35971	35971	12.33	0.0009
C57BL/6J	F	1	339409	339409	116.39	<.0001
C57BL/6J	M	1	184326	184326	63.21	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for Glucose60

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	243397	243397	83.46	<.0001
F	LF	1	1400.148810	1400.148810	0.48	0.4913
M	HF	1	93199	93199	31.96	<.0001
M	LF	1	20763	20763	7.12	0.0100

Strain*Gender*Diet Effect Sliced by Strain*Diet for Glucose120

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	2133.333333	2133.333333	0.69	0.4087
ApoE-/-	LF	1	1575.520833	1575.520833	0.51	0.4773
C57BL/6J	HF	1	84522	84522	27.47	<.0001
C57BL/6J	LF	1	10117	10117	3.29	0.0753

Strain*Gender*Diet Effect Sliced by Strain*Gender for Glucose120

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	2394.187500	2394.187500	0.78	0.3816
ApoE-/-	M	1	3072.000000	3072.000000	1.00	0.3221
C57BL/6J	F	1	377572	377572	122.69	<.0001
C57BL/6J	M	1	104142	104142	33.84	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for Glucose120

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	262990	262990	85.46	<.0001
F	LF	1	68.148810	68.148810	0.02	0.8822
M	HF	1	67573	67573	21.96	<.0001
M	LF	1	1664.100000	1664.100000	0.54	0.4652

Strain*Gender*Diet Effect Sliced by Strain*Diet for AUC

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	20770430	20770430	1.36	0.2492
ApoE-/-	LF	1	3834939	3834939	0.25	0.6188
C57BL/6J	HF	1	108687723	108687723	7.10	0.0101
C57BL/6J	LF	1	62750474	62750474	4.10	0.0478

Strain*Gender*Diet Effect Sliced by Strain*Gender for AUC

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	20134014	20134014	1.31	0.2565
ApoE-/-	M	1	121062769	121062769	7.91	0.0068
C57BL/6J	F	1	1762621714	1762621714	115.10	<.0001
C57BL/6J	M	1	966387303	966387303	63.10	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for AUC

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	987848704	987848704	64.50	<.0001
F	LF	1	9355512	9355512	0.61	0.4378
M	HF	1	435693632	435693632	28.45	<.0001
M	LF	1	39870106	39870106	2.60	0.1124

Strain*Gender*Diet Effect Sliced by Strain*Diet for HOMAIR

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	338.214793	338.214793	3.89	0.0536
ApoE-/-	LF	1	0.000560	0.000560	0.00	0.9980
C57BL/6J	HF	1	123.698900	123.698900	1.42	0.2381
C57BL/6J	LF	1	0.869202	0.869202	0.01	0.9207

Strain*Gender*Diet Effect Sliced by Strain*Gender for HOMAIR

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	4369.009150	4369.009150	50.24	<.0001
ApoE-/-	M	1	7134.407647	7134.407647	82.04	<.0001
C57BL/6J	F	1	11339	11339	130.38	<.0001
C57BL/6J	M	1	13819	13819	158.91	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for HOMAIR

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	1670.770029	1670.770029	19.21	<.0001
F	LF	1	115.046455	115.046455	1.32	0.2550
M	HF	1	211.924019	211.924019	2.44	0.1242
M	LF	1	139.817911	139.817911	1.61	0.2101

Strain*Gender*Diet Effect Sliced by Strain*Diet for PLGFHeart

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	14.479118	14.479118	0.81	0.3720
ApoE-/-	LF	1	0.234368	0.234368	0.01	0.9093
C57BL/6J	HF	1	2.852729	2.852729	0.16	0.6911
C57BL/6J	LF	1	5.671867	5.671867	0.32	0.5755

Strain*Gender*Diet Effect Sliced by Strain*Gender for PLGFHeart

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	1108.428828	1108.428828	62.01	<.0001
ApoE-/-	M	1	841.221315	841.221315	47.06	<.0001
C57BL/6J	F	1	1568.551395	1568.551395	87.75	<.0001
C57BL/6J	M	1	2275.585190	2275.585190	127.31	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for PLGFHeart

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	1407.047495	1407.047495	78.72	<.0001
F	LF	1	1681.333625	1681.333625	94.06	<.0001
M	HF	1	1373.027422	1373.027422	76.82	<.0001
M	LF	1	1628.226748	1628.226748	91.09	<.0001

Strain*Gender*Diet Effect Sliced by Strain*Diet for PLGFMT

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	21.083636	21.083636	0.54	0.4639
ApoE-/-	LF	1	29.658515	29.658515	0.77	0.3855
C57BL/6J	HF	1	0.749862	0.749862	0.02	0.8899
C57BL/6J	LF	1	334.796916	334.796916	8.64	0.0048

Strain*Gender*Diet Effect Sliced by Strain*Gender for PLGFMT

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	585.519718	585.519718	15.11	0.0003
ApoE-/-	M	1	1172.046859	1172.046859	30.24	<.0001
C57BL/6J	F	1	61.518049	61.518049	1.59	0.2130
C57BL/6J	M	1	886.503149	886.503149	22.87	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for PLGFMT

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	52.327782	52.327782	1.35	0.2503
F	LF	1	665.835357	665.835357	17.18	0.0001
M	HF	1	4.091853	4.091853	0.11	0.7465
M	LF	1	241.409075	241.409075	6.23	0.0156

Strain*Gender*Diet Effect Sliced by Strain*Diet for VEGFHeart

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	9133.052806	9133.052806	1.45	0.2335
ApoE-/-	LF	1	42.367440	42.367440	0.01	0.9349
C57BL/6J	HF	1	3585.894102	3585.894102	0.57	0.4536
C57BL/6J	LF	1	4816.588916	4816.588916	0.77	0.3855

Strain*Gender*Diet Effect Sliced by Strain*Gender for VEGFHeart

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	3550.840474	3550.840474	0.56	0.4558
ApoE-/-	M	1	1805.154119	1805.154119	0.29	0.5945
C57BL/6J	F	1	22.237682	22.237682	0.00	0.9528
C57BL/6J	M	1	22734	22734	3.61	0.0626

Strain*Gender*Diet Effect Sliced by Gender*Diet for VEGFHeart

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	20166	20166	3.20	0.0790
F	LF	1	7587.483453	7587.483453	1.21	0.2770
M	HF	1	54.935116	54.935116	0.01	0.9259
M	LF	1	25719	25719	4.09	0.0481

Here the analysis is performed for some of the response variable that had missing values and the program performed the analysis in an order determined by data structure.

Dependent Variable: Triglyc

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	1279517.781	1279517.781	325.81	<.0001
Gender	1	78903.211	78903.211	20.09	<.0001
Strain*Gender	1	41093.181	41093.181	10.46	0.0028
Diet	1	358849.712	358849.712	91.38	<.0001
Strain*Diet	1	308158.210	308158.210	78.47	<.0001
Gender*Diet	1	306.298	306.298	0.08	0.7818
Strain*Gender*Diet	1	989.463	989.463	0.25	0.6191

Strain	Gender	Diet	Triglyc LSMEAN
ApoE-/-	F	HF	733.305000
ApoE-/-	F	LF	365.305000
ApoE-/-	M	HF	581.723750
ApoE-/-	M	LF	204.716250
C57BL/6J	F	HF	133.787667
C57BL/6J	F	LF	103.811500
C57BL/6J	M	HF	92.753500
C57BL/6J	M	LF	94.379000

Strain*Gender*Diet Effect Sliced by Strain*Diet for Triglyc

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	45954	45954	11.70	0.0017
ApoE-/-	LF	1	51577	51577	13.13	0.0010
C57BL/6J	HF	1	5051.408502	5051.408502	1.29	0.2652
C57BL/6J	LF	1	266.916169	266.916169	0.07	0.7960

Strain*Gender*Diet Effect Sliced by Strain*Gender for Triglyc

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	270848	270848	68.97	<.0001
ApoE-/-	M	1	284269	284269	72.38	<.0001
C57BL/6J	F	1	2695.711704	2695.711704	0.69	0.4135
C57BL/6J	M	1	7.926751	7.926751	0.00	0.9644

Strain*Gender*Diet Effect Sliced by Gender*Diet for Triglyc

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	862610	862610	219.65	<.0001
F	LF	1	164109	164109	41.79	<.0001
M	HF	1	573821	573821	146.11	<.0001
M	LF	1	29218	29218	7.44	0.0103

Dependent Variable: Insulin

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	24.1665693	24.1665693	28.80	<.0001
Gender	1	0.0805987	0.0805987	0.10	0.7578
Strain*Gender	1	0.0000966	0.0000966	0.00	0.9915
Diet	1	381.5973755	381.5973755	454.71	<.0001
Strain*Diet	1	3.3044508	3.3044508	3.94	0.0523
Gender*Diet	1	1.4860639	1.4860639	1.77	0.1889
Strain*Gender*Diet	1	0.0000332	0.0000332	0.00	0.9950

Strain	Gender	Diet	Insulin LSMEAN
ApoE-/-	F	HF	5.15116667
ApoE-/-	F	LF	0.79050000
ApoE-/-	M	HF	5.54416667
ApoE-/-	M	LF	0.54233333
C57BL/6J	F	HF	6.92629714
C57BL/6J	F	LF	1.60803875
C57BL/6J	M	HF	7.32753571
C57BL/6J	M	LF	1.36202333

Strain*Gender*Diet Effect Sliced by Strain*Diet for Insulin

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	0.463347	0.463347	0.55	0.4607
ApoE-/-	LF	1	0.184760	0.184760	0.22	0.6408
C57BL/6J	HF	1	0.751298	0.751298	0.90	0.3483
C57BL/6J	LF	1	0.256335	0.256335	0.31	0.5828

Strain*Gender*Diet Effect Sliced by Strain*Gender for Insulin

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	57.046241	57.046241	67.98	<.0001
ApoE-/-	M	1	75.055010	75.055010	89.44	<.0001

C57BL/6J	F	1	105.593123	105.593123	125.82	<.0001
C57BL/6J	M	1	194.956721	194.956721	232.31	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for Insulin

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	10.180439	10.180439	12.13	0.0010
F	LF	1	2.291553	2.291553	2.73	0.1042
M	HF	1	13.357702	13.357702	15.92	0.0002
M	LF	1	2.418810	2.418810	2.88	0.0953

Dependent Variable: PLGFGPS

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	84.245441	84.245441	2.33	0.1353
Gender	1	105.860103	105.860103	2.93	0.0954
Strain*Gender	1	241.365591	241.365591	6.68	0.0138
Diet	1	1455.789675	1455.789675	40.28	<.0001
Strain*Diet	1	114.114857	114.114857	3.16	0.0838
Gender*Diet	1	153.239749	153.239749	4.24	0.0466
Strain*Gender*Diet	1	506.100276	506.100276	14.00	0.0006

Dependent Variable: VEGFGPS

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	1719.821577	1719.821577	10.26	0.0028
Gender	1	1614.628254	1614.628254	9.63	0.0037
Strain*Gender	1	822.862242	822.862242	4.91	0.0330
Diet	1	4217.998697	4217.998697	25.16	<.0001
Strain*Diet	1	1.634335	1.634335	0.01	0.9219

Gender*Diet	1	56.068177	56.068177	0.33	0.5666
Strain*Gender*Diet	1	18.736558	18.736558	0.11	0.7400

Strain	Gender	Diet	PLGFGPS LSMEAN	VEGFGPS LSMEAN
ApoE-/-	F	HF	11.3269904	53.0742181
ApoE-/-	F	LF	36.5279239	76.5676629
ApoE-/-	M	HF	14.0516715	35.8994214
ApoE-/-	M	LF	18.2546669	52.2784252
C57BL/6J	F	HF	13.8751880	33.6422992
C57BL/6J	F	LF	19.0996409	53.7598915
C57BL/6J	M	HF	12.4089562	31.1323589
C57BL/6J	M	LF	23.7250521	49.3478044

Strain*Gender*Diet Effect Sliced by Strain*Diet for PLGFGPS

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	20.246965	20.246965	0.56	0.4589
ApoE-/-	LF	1	801.388614	801.388614	22.17	<.0001
C57BL/6J	HF	1	6.449507	6.449507	0.18	0.6752
C57BL/6J	LF	1	64.183284	64.183284	1.78	0.1908

Strain*Gender*Diet Effect Sliced by Strain*Gender for PLGFGPS

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	1411.304549	1411.304549	39.04	<.0001
ApoE-/-	M	1	52.995509	52.995509	1.47	0.2336
C57BL/6J	F	1	81.884727	81.884727	2.27	0.1408
C57BL/6J	M	1	384.162080	384.162080	10.63	0.0024

Strain*Gender*Diet Effect Sliced by Gender*Diet for PLGFGPS

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	17.709029	17.709029	0.49	0.4883
F	LF	1	728.988111	728.988111	20.17	<.0001
M	HF	1	8.095541	8.095541	0.22	0.6388
M	LF	1	89.775343	89.775343	2.48	0.1235

Strain*Gender*Diet Effect Sliced by Strain*Diet for VEGFGPS

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	804.473569	804.473569	4.80	0.0349
ApoE-/-	LF	1	1415.920960	1415.920960	8.45	0.0061
C57BL/6J	HF	1	18.899400	18.899400	0.11	0.7389
C57BL/6J	LF	1	58.399539	58.399539	0.35	0.5586

Strain*Gender*Diet Effect Sliced by Strain*Gender for VEGFGPS

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	1226.537662	1226.537662	7.32	0.0103
ApoE-/-	M	1	804.815297	804.815297	4.80	0.0348
C57BL/6J	F	1	1214.152571	1214.152571	7.24	0.0106
C57BL/6J	M	1	995.407361	995.407361	5.94	0.0198

Strain*Gender*Diet Effect Sliced by Gender*Diet for VEGFGPS

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	1029.816750	1029.816750	6.14	0.0179
F	LF	1	1248.466644	1248.466644	7.45	0.0097
M	HF	1	68.174654	68.174654	0.41	0.5276
M	LF	1	25.765615	25.765615	0.15	0.6973

Dependent Variable: VEGFMT

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	10252.71548	10252.71548	74.95	<.0001
Gender	1	462.76395	462.76395	3.38	0.0714
Strain*Gender	1	8.27591	8.27591	0.06	0.8066
Diet	1	672.18880	672.18880	4.91	0.0309
Strain*Diet	1	15815.90631	15815.90631	115.63	<.0001
Gender*Diet	1	282.42701	282.42701	2.06	0.1565
Strain*Gender*Diet	1	2.91187	2.91187	0.02	0.8845

Strain	Gender	Diet	VEGFMT LSMEAN
ApoE-/-	F	HF	55.0435995
ApoE-/-	F	LF	90.4380726
ApoE-/-	M	HF	45.1709166
ApoE-/-	M	LF	90.4053364
C57BL/6J	F	HF	61.8675642
C57BL/6J	F	LF	31.3213305
C57BL/6J	M	HF	51.3727788
C57BL/6J	M	LF	28.8524134

Strain*Gender*Diet Effect Sliced by Strain*Diet for VEGFMT

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	292.409606	292.409606	2.14	0.1495
ApoE-/-	LF	1	0.003215	0.003215	0.00	0.9961
C57BL/6J	HF	1	525.670664	525.670664	3.84	0.0551
C57BL/6J	LF	1	24.001235	24.001235	0.18	0.6770

Strain*Gender*Diet Effect Sliced by Strain*Gender for VEGFMT

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	3758.306175	3758.306175	27.48	<.0001
ApoE-/-	M	1	6138.458220	6138.458220	44.88	<.0001

C57BL/6J	F	1	3265.753372	3265.753372	23.88	<.0001
C57BL/6J	M	1	2852.813580	2852.813580	20.86	<.0001

Strain*Gender*Diet Effect Sliced by Gender*Diet for VEGFMT

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	150.445596	150.445596	1.10	0.2990
F	LF	1	11291	11291	82.54	<.0001
M	HF	1	164.841837	164.841837	1.21	0.2772
M	LF	1	13640	13640	99.72	<.0001

Dependent Variable: VEGFR1Heart

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	1813495.372	1813495.372	91.28	<.0001
Gender	1	18274.471	18274.471	0.92	0.3418
Strain*Gender	1	29800.299	29800.299	1.50	0.2260
Diet	1	9118.308	9118.308	0.46	0.5010
Strain*Diet	1	54031.814	54031.814	2.72	0.1049
Gender*Diet	1	6878.961	6878.961	0.35	0.5587
Strain*Gender*Diet	1	7861.981	7861.981	0.40	0.5320

Dependent Variable: VEGFR1MT

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Strain	1	152724.9063	152724.9063	12.11	0.0010
Gender	1	98753.3635	98753.3635	7.83	0.0071
Strain*Gender	1	105285.4412	105285.4412	8.35	0.0055
Diet	1	113629.7210	113629.7210	9.01	0.0041
Strain*Diet	1	2730.2569	2730.2569	0.22	0.6436

Gender*Diet	1	204536.8782	204536.8782	16.22
0.0002				
Strain*Gender*Diet	1	37845.4243	37845.4243	3.00
0.0889				

Dependent Variable: VEGFR1GPS

Source	DF	Type III SS	Mean Square	F Value
Pr > F				
Strain	1	129688.7160	129688.7160	39.41
<.0001				
Gender	1	47302.0227	47302.0227	14.37
0.0005				
Strain*Gender	1	44289.0722	44289.0722	13.46
0.0008				
Diet	1	3319.8747	3319.8747	1.01
0.3217				
Strain*Diet	1	21077.6852	21077.6852	6.40
0.0158				
Gender*Diet	1	133.0642	133.0642	0.04
0.8417				
Strain*Gender*Diet	1	1345.3012	1345.3012	0.41
0.5265				

Least Squares Means

Strain	Gender	Diet	PLGFGPS LSMEAN	VEGFGPS LSMEAN	VEGFR1GPS LSMEAN
ApoE-/-	F	HF	11.3269904	53.0742181	86.054615
ApoE-/-	F	LF	36.5279239	76.5676629	132.591230
ApoE-/-	M	HF	14.0516715	35.8994214	200.367362
ApoE-/-	M	LF	18.2546669	52.2784252	275.932465
C57BL/6J	F	HF	13.8751880	33.6422992	290.483592
C57BL/6J	F	LF	19.0996409	53.7598915	271.692622
C57BL/6J	M	HF	12.4089562	31.1323589	300.172136
C57BL/6J	M	LF	23.7250521	49.3478044	266.243075

Strain*Gender*Diet Effect Sliced by Strain*Diet for VEGFR1Heart

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	20941	20941	1.05	0.3092
ApoE-/-	LF	1	19439	19439	0.98	0.3270
C57BL/6J	HF	1	13299	13299	0.67	0.4169
C57BL/6J	LF	1	5413.130859	5413.130859	0.27	0.6038

Strain*Gender*Diet Effect Sliced by Strain*Gender for VEGFR1Heart

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	23716	23716	1.19	0.2794
ApoE-/-	M	1	22116	22116	1.11	0.2961
C57BL/6J	F	1	23180	23180	1.17	0.2849
C57BL/6J	M	1	479.537285	479.537285	0.02	0.8771

Strain*Gender*Diet Effect Sliced by Gender*Diet for VEGFR1Heart

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	154966	154966	7.80	0.0072
F	LF	1	544230	544230	27.39	<.0001
M	HF	1	575772	575772	28.98	<.0001
M	LF	1	707006	707006	35.58	<.0001

Strain*Gender*Diet Effect Sliced by Strain*Diet for VEGFR1MT

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	352032	352032	27.91	<.0001
ApoE-/-	LF	1	13.869109	13.869109	0.00	0.9737
C57BL/6J	HF	1	18640	18640	1.48	0.2294
C57BL/6J	LF	1	21591	21591	1.71	0.1963

Strain*Gender*Diet Effect Sliced by Strain*Gender for VEGFR1MT

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	228696	228696	18.13	<.0001
ApoE-/-	M	1	14120	14120	1.12	0.2947
C57BL/6J	F	1	71665	71665	5.68	0.0207
C57BL/6J	M	1	293.760038	293.760038	0.02	0.8793

Strain*Gender*Diet Effect Sliced by Gender*Diet for VEGFR1MT

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	197167	197167	15.63	0.0002
F	LF	1	53433	53433	4.24	0.0444
M	HF	1	1756.060677	1756.060677	0.14	0.7105
M	LF	1	11124	11124	0.88	0.3518

Strain*Gender*Diet Effect Sliced by Strain*Diet for VEGFR1GPS

Strain	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	HF	1	35638	35638	10.83	0.0022
ApoE-/-	LF	1	49312	49312	14.98	0.0004
C57BL/6J	HF	1	281.603664	281.603664	0.09	0.7715
C57BL/6J	LF	1	89.092682	89.092682	0.03	0.8702

Strain*Gender*Diet Effect Sliced by Strain*Gender for VEGFR1GPS

Strain	Gender	DF	Sum of Squares	Mean Square	F Value	Pr > F
ApoE-/-	F	1	4812.569914	4812.569914	1.46	0.2342
ApoE-/-	M	1	17130	17130	5.21	0.0284
C57BL/6J	F	1	1059.301676	1059.301676	0.32	0.5739
C57BL/6J	M	1	3453.543564	3453.543564	1.05	0.3123

Strain*Gender*Diet Effect Sliced by Gender*Diet for VEGFR1GPS

Gender	Diet	DF	Sum of Squares	Mean Square	F Value	Pr > F
F	HF	1	113976	113976	34.63	<.0001
F	LF	1	46438	46438	14.11	0.0006
M	HF	1	29883	29883	9.08	0.0046
M	LF	1	281.652836	281.652836	0.09	0.7715

Obs	MNGlucose0	MNGlucose15	MNGlucose30	MNGlucose60	MNGlucose120	MNAUC
1	158.000	379.667	388.667	288.667	151.833	27245.00

```
 2    119.333      350.583       341.167       281.833        123.583
24654.38
 3    138.500      373.333       452.000       337.333        178.500
29876.25
 4     94.500      375.167       313.333       227.833        146.500
23523.75
 5    154.857      528.286       553.571       563.143        437.143
44731.07
 6    103.250      301.375       345.500       261.625        119.125
23002.50
 7    135.333      512.933       530.400       484.800        304.067
39959.00

Obs MNHOMAIR  MNPLGFHeart  MNPLGFMT  MNPLGFGPS  MNVEGFHeart  MNVEGFMT
MNVEGFGPS

 1   42.6582    12.5100     20.8402   11.3270     369.950     55.0436   53.0742
 2    4.4962    31.7318     34.8107   36.5279     404.354     90.4381   76.5677
 3   53.2760    14.7069     18.1892   14.0517     425.126     45.1709   35.8994
 4    4.5099    31.4523     37.9549   18.2547     400.596     90.4053   52.2784
 5   65.3990    33.3790     16.8157   13.8752     448.956     61.8676   33.6423
 6   10.2889    53.8765     20.8750   19.0996     451.397     31.3213   53.7599
 7   60.3080    32.6059     17.2121   12.4090     421.546     51.3728   31.1324

Obs MNVEGFR1Heart  MNVEGFR1MT  MNVEGFR1GPS   SEChol  SEIsopro8  SETriglyc
SEInsulin

 1      789.07       689.259      86.055    14.8956   50.0993    85.9041
0.33481
 2      700.16       413.158     132.591    15.4925   26.7278    18.5688
0.05668
 3      705.52       346.704     200.367    16.6961   17.5774    15.9382
0.44533
 4      619.67       415.308     275.932     9.2747   21.3873    35.9609
0.02338
 5     1016.35       432.896     290.484    19.2615   14.0688    12.2726
0.78329
 6     1098.58       288.320     271.693     5.3626    5.8541    11.2258
0.06057
 7     1072.06       366.946     300.172    10.9187   13.0943    10.7439
0.23215

Obs SEGlucose0   SEGlucose15   SEGlucose30   SEGlucose60   SEGlucose120
SEAUC

 1     6.9138      53.1304       44.8313       37.9857        15.3045
2806.28
 2     4.6380      11.4720       17.0087       21.5877         2.4847
1077.94
 3    11.1796      36.0506       19.7653       14.5434        13.5173
1161.49
 4     6.9988      19.4052       24.8860       16.1068         8.1271
1343.99
 5     8.5730      34.5768       24.8489       18.8129        52.8044
2316.71
 6     8.6968      27.5557       10.0410       10.9690         9.2359
889.86
 7     4.3256       6.0509        9.6203       14.7446        10.7062
741.57

Obs SEHOMAIR  SEPLGFHeart  SEPLGFMT  SEPLGFGPS  SEVEGFHeart  SEVEGFMT
SEVEGFGPS
```

1	4.91903	0.71864	2.04305	1.46519	33.5245	7.31494	3.3711
2	0.29696	1.88260	3.50230	4.15454	28.5883	7.60479	12.8640
3	4.34220	0.39783	2.86927	1.15330	16.8030	3.09466	3.9752
4	0.21986	0.91630	4.03060	2.74436	25.1693	4.46130	8.4258
5	7.94341	2.58252	1.35575	3.17576	17.1900	2.71272	2.0572
6	1.17259	2.00615	1.55331	2.40753	18.0059	2.73158	5.1175
7	1.80778	0.76270	1.45355	1.48013	27.4424	3.37245	3.0192

Obs	SEVEGFR1Heart	SEVEGFR1MT	SEVEGFR1GPS
1	18.4409	69.0787	13.2805
2	45.9867	31.1847	21.3363
3	48.1220	48.2502	24.1288
4	37.1239	26.7380	19.5606
5	40.8073	61.9346	19.9533
6	47.4857	31.6552	14.6888
7	49.6539	27.9292	25.1148

Obs	Strain	Gender	Diet	_TYPE_	_FREQ_	MNChol	MNIsopro8	MNTriglyc	MNInsulin
8	C57BL/6J	M	LF	0	9	104.94	84.167	94.379	1.36202

Obs	MNGlucose0	MNGlucose15	MNGlucose30	MNGlucose60	MNGlucose120	MNAUC
8	122.444	307.556	378.111	303.778	168.000	26851.67

Obs	MNHOMAIR	MNPLGFHeart	MNPLGFMT	MNPLGFGPS	MNVEGFHeart	MNVEGFMT	MNVEGFGPS
8	10.7419	52.7193	29.7660	23.7251	485.120	28.8524	49.3478

Obs	MNVEGFR1Heart	MNVEGFR1MT	MNVEGFR1GPS	SEChol	SEIsopro8	SETriglyc	SEInsulin
8	1062.83	359.720	266.243	8.4265	10.9007	9.3985	0.03895

Obs	SEGlucose0	SEGlucose15	SEGlucose30	SEGlucose60	SEGlucose120	SEAUC
8	11.2189	17.0507	16.2843	17.3748	10.9430	1125.92

Obs	SEHOMAIR	SEPLGFHeart	SEPLGFMT	SEPLGFGPS	SEVEGFHeart	SEVEGFMT	SEVEGFGPS
8	1.01347	1.68193	1.85635	3.05135	31.5769	1.44763	2.5796

Obs	SEVEGFR1Heart	SEVEGFR1MT	SEVEGFR1GPS
8	51.9654	33.8250	39.3643

Analysis of repeated measures data.

Dependent Variable Glucose

Class Level Information

Class	Levels	Values
Week	15	0 1 2 3 4 5 7 9 11 13 15 17 19 21 23
Strain	2	ApoE-/- C57BL/6J

Gender	2	F M
Diet	2	HF LF

The estimate of 0.4694 below is the correlation estimated in the autoregressive covariance matrix. This is a pretty strong correlation.

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
AR(1)	Mous*Stra*Gende*Diet	0.4694
Residual		289.70

This test below is testing whether the model is significant. A small p-value would indicate that the model is needed.

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	187.28	<.0001

For this analysis of variance, one could argue that the model could be simplified and main effects (at some level) be investigated. I've decided to keep the simple effect analysis, but we can do it differently. BW does need simple effects (coming later).

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Week	14	734	17.46	<.0001
Strain	1	143	32.95	<.0001
Week*Strain	14	734	0.83	0.6402
Gender	1	143	31.55	<.0001
Week*Gender	14	734	1.58	0.0800
Strain*Gender	1	143	15.25	0.0001
Week*Strain*Gender	14	734	0.51	0.9273
Diet	1	143	207.14	<.0001
Week*Diet	14	734	6.99	<.0001
Strain*Diet	1	143	75.42	<.0001
Week*Strain*Diet	14	734	1.82	0.0324
Gender*Diet	1	143	0.02	0.8913
Week*Gender*Diet	14	734	0.47	0.9511
Strain*Gender*Diet	1	143	0.26	0.6080
Week*Stra*Gende*Diet	14	734	0.48	0.9427

Tests of effect slices, where comparisons of one factor given fixed levels of all of the other factors are made. This is more complicated than the end point analyses since we are specifying week. If you concentrate on the diet effect, you see a really nice result. In the early weeks, the diets are not different. However, when you progress through the experiment, you see the diets start to have an effect, up until the point where the diets are highly significant at the end of the study.

Tests of Effect Slices

Effect	Strain	Gender	Diet	Week	Num DF	Den DF	F Value	Pr > F
Week*Stra*Gende*Diet	ApoE-/-		HF	0	1	542	0.66	0.4159
Week*Stra*Gende*Diet	ApoE-/-		LF	0	1	542	1.14	0.2858
Week*Stra*Gende*Diet	C57BL/6J		HF	0	1	542	3.79	0.0520
Week*Stra*Gende*Diet	C57BL/6J		LF	0	1	542	5.75	0.0168
Week*Stra*Gende*Diet	ApoE-/-		HF	1	1	542	0.21	0.6472
Week*Stra*Gende*Diet	ApoE-/-		LF	1	1	542	0.44	0.5086
Week*Stra*Gende*Diet	C57BL/6J		HF	1	1	542	5.78	0.0166
Week*Stra*Gende*Diet	C57BL/6J		LF	1	1	542	4.65	0.0315
Week*Stra*Gende*Diet	ApoE-/-		HF	2	1	542	0.35	0.5530
Week*Stra*Gende*Diet	ApoE-/-		LF	2	1	542	2.71	0.1005
Week*Stra*Gende*Diet	C57BL/6J		HF	2	1	542	6.84	0.0091
Week*Stra*Gende*Diet	C57BL/6J		LF	2	1	542	9.81	0.0018
Week*Stra*Gende*Diet	ApoE-/-		HF	3	1	542	0.01	0.9190
Week*Stra*Gende*Diet	ApoE-/-		LF	3	1	542	1.71	0.1921
Week*Stra*Gende*Diet	C57BL/6J		HF	3	1	542	7.88	0.0052
Week*Stra*Gende*Diet	C57BL/6J		LF	3	1	542	3.61	0.0578
Week*Stra*Gende*Diet	ApoE-/-		HF	4	1	542	0.00	0.9730
Week*Stra*Gende*Diet	ApoE-/-		LF	4	1	542	0.03	0.8654
Week*Stra*Gende*Diet	C57BL/6J		HF	4	1	542	19.56	<.0001
Week*Stra*Gende*Diet	C57BL/6J		LF	4	1	542	4.30	0.0386
Week*Stra*Gende*Diet	ApoE-/-		HF	5	1	542	1.75	0.1864
Week*Stra*Gende*Diet	ApoE-/-		LF	5	1	542	0.04	0.8388
Week*Stra*Gende*Diet	C57BL/6J		HF	5	1	542	7.28	0.0072

Week*Stra*Gende*Diet	C57BL/6J	LF	5	1	542	3.39 0.0662
Week*Stra*Gende*Diet	ApoE-/-	HF	7	1	542	0.61 0.4356
Week*Stra*Gende*Diet	ApoE-/-	LF	7	1	542	1.98 0.1598
Week*Stra*Gende*Diet	C57BL/6J	HF	7	1	542	23.43 <.0001
Week*Stra*Gende*Diet	C57BL/6J	LF	7	1	542	10.75 0.0011
Week*Stra*Gende*Diet	ApoE-/-	HF	9	1	542	0.12 0.7346
Week*Stra*Gende*Diet	ApoE-/-	LF	9	1	542	0.00 1.0000
Week*Stra*Gende*Diet	C57BL/6J	HF	9	1	542	12.90 0.0004
Week*Stra*Gende*Diet	C57BL/6J	LF	9	1	542	1.85 0.1743
Week*Stra*Gende*Diet	ApoE-/-	HF	11	1	542	0.03 0.8654
Week*Stra*Gende*Diet	ApoE-/-	LF	11	1	542	0.06 0.8124
Week*Stra*Gende*Diet	C57BL/6J	HF	11	1	542	13.10 0.0003
Week*Stra*Gende*Diet	C57BL/6J	LF	11	1	542	1.61 0.2054
Week*Stra*Gende*Diet	ApoE-/-	HF	13	1	542	0.15 0.6966
Week*Stra*Gende*Diet	ApoE-/-	LF	13	1	542	0.69 0.4063
Week*Stra*Gende*Diet	C57BL/6J	HF	13	1	542	6.15 0.0135
Week*Stra*Gende*Diet	C57BL/6J	LF	13	1	542	6.00 0.0146
Week*Stra*Gende*Diet	ApoE-/-	HF	15	1	542	0.00 0.9730
Week*Stra*Gende*Diet	ApoE-/-	LF	15	1	542	0.19 0.6594
Week*Stra*Gende*Diet	C57BL/6J	HF	15	1	542	5.50 0.0193
Week*Stra*Gende*Diet	C57BL/6J	LF	15	1	542	6.81 0.0093
Week*Stra*Gende*Diet	ApoE-/-	HF	17	1	542	0.12 0.7346
Week*Stra*Gende*Diet	ApoE-/-	LF	17	1	542	0.87 0.3513
Week*Stra*Gende*Diet	C57BL/6J	HF	17	1	542	0.90 0.3439
Week*Stra*Gende*Diet	C57BL/6J	LF	17	1	542	3.13 0.0773
Week*Stra*Gende*Diet	ApoE-/-	HF	19	1	542	0.09 0.7603
Week*Stra*Gende*Diet	ApoE-/-	LF	19	1	542	0.06 0.7993
Week*Stra*Gende*Diet	C57BL/6J	HF	19	1	542	0.81 0.3693
Week*Stra*Gende*Diet	C57BL/6J	LF	19	1	542	1.85 0.1743
Week*Stra*Gende*Diet	ApoE-/-	HF	21	1	542	0.13 0.7219
Week*Stra*Gende*Diet	ApoE-/-	LF	21	1	542	0.09 0.7603

Week*Stra*Gende*Diet	C57BL/6J		HF	21	1	542	0.13
0.7167							
Week*Stra*Gende*Diet	C57BL/6J		LF	21	1	542	2.85
0.0917							
Week*Stra*Gende*Diet	ApoE-/-		HF	23	1	542	0.42
0.5195							
Week*Stra*Gende*Diet	ApoE-/-		LF	23	1	542	0.90
0.3426							
Week*Stra*Gende*Diet	C57BL/6J		HF	23	1	542	0.01
0.9066							
Week*Stra*Gende*Diet	C57BL/6J		LF	23	1	542	1.66
0.1977							
Week*Stra*Gende*Diet	ApoE-/-	F		0	1	542	0.12
0.7346							
Week*Stra*Gende*Diet	ApoE-/-	M		0	1	542	0.01
0.9324							
Week*Stra*Gende*Diet	C57BL/6J	F		0	1	542	0.20
0.6558							
Week*Stra*Gende*Diet	C57BL/6J	M		0	1	542	0.01
0.9186							
Week*Stra*Gende*Diet	ApoE-/-	F		1	1	542	0.18
0.6717							
Week*Stra*Gende*Diet	ApoE-/-	M		1	1	542	0.39
0.5306							
Week*Stra*Gende*Diet	C57BL/6J	F		1	1	542	0.41
0.5221							
Week*Stra*Gende*Diet	C57BL/6J	M		1	1	542	0.83
0.3630							
Week*Stra*Gende*Diet	ApoE-/-	F		2	1	542	0.37
0.5417							
Week*Stra*Gende*Diet	ApoE-/-	M		2	1	542	2.76
0.0971							
Week*Stra*Gende*Diet	C57BL/6J	F		2	1	542	1.68
0.1957							
Week*Stra*Gende*Diet	C57BL/6J	M		2	1	542	0.67
0.4122							
Week*Stra*Gende*Diet	ApoE-/-	F		3	1	542	0.07
0.7862							
Week*Stra*Gende*Diet	ApoE-/-	M		3	1	542	0.87
0.3513							
Week*Stra*Gende*Diet	C57BL/6J	F		3	1	542	3.04
0.0818							
Week*Stra*Gende*Diet	C57BL/6J	M		3	1	542	8.98
0.0028							
Week*Stra*Gende*Diet	ApoE-/-	F		4	1	542	0.26
0.6111							
Week*Stra*Gende*Diet	ApoE-/-	M		4	1	542	0.09
0.7603							
Week*Stra*Gende*Diet	C57BL/6J	F		4	1	542	6.53
0.0109							
Week*Stra*Gende*Diet	C57BL/6J	M		4	1	542	30.79
<.0001							
Week*Stra*Gende*Diet	ApoE-/-	F		5	1	542	0.35
0.5530							
Week*Stra*Gende*Diet	ApoE-/-	M		5	1	542	2.93
0.0873							
Week*Stra*Gende*Diet	C57BL/6J	F		5	1	542	11.38
0.0008							
Week*Stra*Gende*Diet	C57BL/6J	M		5	1	542	24.49
<.0001							
Week*Stra*Gende*Diet	ApoE-/-	F		7	1	542	4.21
0.0406							

Week*Stra*Gende*Diet	ApoE-/-	M	7	1	542	2.03	0.1548
Week*Stra*Gende*Diet	C57BL/6J	F	7	1	542	25.27	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	7	1	542	58.50	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	9	1	542	0.75	0.3874
Week*Stra*Gende*Diet	ApoE-/-	M	9	1	542	1.45	0.2290
Week*Stra*Gende*Diet	C57BL/6J	F	9	1	542	30.36	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	9	1	542	82.71	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	11	1	542	3.54	0.0603
Week*Stra*Gende*Diet	ApoE-/-	M	11	1	542	2.18	0.1406
Week*Stra*Gende*Diet	C57BL/6J	F	11	1	542	32.70	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	11	1	542	90.02	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	13	1	542	6.22	0.0130
Week*Stra*Gende*Diet	ApoE-/-	M	13	1	542	4.21	0.0406
Week*Stra*Gende*Diet	C57BL/6J	F	13	1	542	45.87	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	13	1	542	66.93	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	15	1	542	7.18	0.0076
Week*Stra*Gende*Diet	ApoE-/-	M	15	1	542	5.17	0.0234
Week*Stra*Gende*Diet	C57BL/6J	F	15	1	542	52.96	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	15	1	542	71.78	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	17	1	542	3.42	0.0650
Week*Stra*Gende*Diet	ApoE-/-	M	17	1	542	5.96	0.0149
Week*Stra*Gende*Diet	C57BL/6J	F	17	1	542	49.59	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	17	1	542	58.26	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	19	1	542	3.61	0.0580
Week*Stra*Gende*Diet	ApoE-/-	M	19	1	542	1.80	0.1808
Week*Stra*Gende*Diet	C57BL/6J	F	19	1	542	48.35	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	19	1	542	63.09	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	21	1	542	3.05	0.0812
Week*Stra*Gende*Diet	ApoE-/-	M	21	1	542	1.18	0.2782
Week*Stra*Gende*Diet	C57BL/6J	F	21	1	542	72.84	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	21	1	542	66.17	<.0001

Week*Stra*Gende*Diet	ApoE-/-	F		23	1	542	3.05
0.0812							
Week*Stra*Gende*Diet	ApoE-/-	M		23	1	542	4.21
0.0406							
Week*Stra*Gende*Diet	C57BL/6J	F		23	1	542	70.03
<.0001							
Week*Stra*Gende*Diet	C57BL/6J	M		23	1	542	74.96
<.0001							
Week*Stra*Gende*Diet		F	HF	0	1	542	0.58
0.4450							
Week*Stra*Gende*Diet		F	LF	0	1	542	0.73
0.3945							
Week*Stra*Gende*Diet		M	HF	0	1	542	0.00
0.9935							
Week*Stra*Gende*Diet		M	LF	0	1	542	0.03
0.8673							
Week*Stra*Gende*Diet		F	HF	1	1	542	0.47
0.4912							
Week*Stra*Gende*Diet		F	LF	1	1	542	3.16
0.0761							
Week*Stra*Gende*Diet		M	HF	1	1	542	0.88
0.3494							
Week*Stra*Gende*Diet		M	LF	1	1	542	0.31
0.5775							
Week*Stra*Gende*Diet		F	HF	2	1	542	0.50
0.4786							
Week*Stra*Gende*Diet		F	LF	2	1	542	6.89
0.0089							
Week*Stra*Gende*Diet		M	HF	2	1	542	0.91
0.3411							
Week*Stra*Gende*Diet		M	LF	2	1	542	2.57
0.1093							
Week*Stra*Gende*Diet		F	HF	3	1	542	0.31
0.5752							
Week*Stra*Gende*Diet		F	LF	3	1	542	3.83
0.0507							
Week*Stra*Gende*Diet		M	HF	3	1	542	3.58
0.0588							
Week*Stra*Gende*Diet		M	LF	3	1	542	2.84
0.0927							
Week*Stra*Gende*Diet		F	HF	4	1	542	2.52
0.1132							
Week*Stra*Gende*Diet		F	LF	4	1	542	12.53
0.0004							
Week*Stra*Gende*Diet		M	HF	4	1	542	5.78
0.0165							
Week*Stra*Gende*Diet		M	LF	4	1	542	3.61
0.0578							
Week*Stra*Gende*Diet		F	HF	5	1	542	0.51
0.4739							
Week*Stra*Gende*Diet		F	LF	5	1	542	11.13
0.0009							
Week*Stra*Gende*Diet		M	HF	5	1	542	0.02
0.8808							
Week*Stra*Gende*Diet		M	LF	5	1	542	3.78
0.0523							
Week*Stra*Gende*Diet		F	HF	7	1	542	0.81
0.3671							
Week*Stra*Gende*Diet		F	LF	7	1	542	12.63
0.0004							
Week*Stra*Gende*Diet		M	HF	7	1	542	6.84
0.0092							

Week*Stra*Gende*Diet	M	LF	7	1	542	4.67
0.0311						
Week*Stra*Gende*Diet	F	HF	9	1	542	0.58
0.4465						
Week*Stra*Gende*Diet	F	LF	9	1	542	26.42
<.0001						
Week*Stra*Gende*Diet	M	HF	9	1	542	4.50
0.0344						
Week*Stra*Gende*Diet	M	LF	9	1	542	16.11
<.0001						
Week*Stra*Gende*Diet	F	HF	11	1	542	0.28
0.5977						
Week*Stra*Gende*Diet	F	LF	11	1	542	16.09
<.0001						
Week*Stra*Gende*Diet	M	HF	11	1	542	9.15
0.0026						
Week*Stra*Gende*Diet	M	LF	11	1	542	10.25
0.0014						
Week*Stra*Gende*Diet	F	HF	13	1	542	0.20
0.6565						
Week*Stra*Gende*Diet	F	LF	13	1	542	18.35
<.0001						
Week*Stra*Gende*Diet	M	HF	13	1	542	1.88
0.1711						
Week*Stra*Gende*Diet	M	LF	13	1	542	9.25
0.0025						
Week*Stra*Gende*Diet	F	HF	15	1	542	0.03
0.8623						
Week*Stra*Gende*Diet	F	LF	15	1	542	18.39
<.0001						
Week*Stra*Gende*Diet	M	HF	15	1	542	3.93
0.0479						
Week*Stra*Gende*Diet	M	LF	15	1	542	6.11
0.0138						
Week*Stra*Gende*Diet	F	HF	17	1	542	0.00
0.9619						
Week*Stra*Gende*Diet	F	LF	17	1	542	22.31
<.0001						
Week*Stra*Gende*Diet	M	HF	17	1	542	1.84
0.1750						
Week*Stra*Gende*Diet	M	LF	17	1	542	4.78
0.0292						
Week*Stra*Gende*Diet	F	HF	19	1	542	0.36
0.5498						
Week*Stra*Gende*Diet	F	LF	19	1	542	16.13
<.0001						
Week*Stra*Gende*Diet	M	HF	19	1	542	3.63
0.0572						
Week*Stra*Gende*Diet	M	LF	19	1	542	9.86
0.0018						
Week*Stra*Gende*Diet	F	HF	21	1	542	2.27
0.1326						
Week*Stra*Gende*Diet	F	LF	21	1	542	22.65
<.0001						
Week*Stra*Gende*Diet	M	HF	21	1	542	3.30
0.0699						
Week*Stra*Gende*Diet	M	LF	21	1	542	13.35
0.0003						
Week*Stra*Gende*Diet	F	HF	23	1	542	1.90
0.1688						
Week*Stra*Gende*Diet	F	LF	23	1	542	22.39
<.0001						

Week*Stra*Gende*Diet	M	HF	23	1	542	5.04 0.0251
Week*Stra*Gende*Diet	M	LF	23	1	542	6.86 0.0090

Analysis of body weight response:

Dependent Variable BW

Wow, the 0.83 correlation is HUGE!

Covariance Parameter Estimates

Cov Parm	Subject	Estimate
AR(1)	Mous*Stra*Gende*Diet	0.8390
Residual		3.7940

Null Model Likelihood Ratio Test

DF	Chi-Square	Pr > ChiSq
1	947.19	<.0001

Here the four factor interaction is significant, so we must analyze simple effects.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Week	14	755	164.88	<.0001
Strain	1	76.5	27.12	<.0001
Week*Strain	14	755	10.30	<.0001
Gender	1	76.5	254.48	<.0001
Week*Gender	14	755	7.87	<.0001
Strain*Gender	1	76.5	2.67	0.1062
Week*Strain*Gender	14	755	3.21	<.0001
Diet	1	76.5	268.41	<.0001
Week*Diet	14	755	19.61	<.0001
Strain*Diet	1	76.5	1.90	0.1725
Week*Strain*Diet	14	755	6.04	<.0001
Gender*Diet	1	76.5	2.20	0.1419
Week*Gender*Diet	14	755	2.36	0.0033
Strain*Gender*Diet	1	76.5	1.09	0.2994
Week*Stra*Gende*Diet	14	755	1.74	0.0434

Tests of Effect Slices

Effect	Strain	Gender	Diet	Week	Num DF	Den DF	F Value	Pr > F
Week*Stra*Gende*Diet	ApoE-/-		HF	0	1	191	17.22	<.0001
Week*Stra*Gende*Diet	ApoE-/-		LF	0	1	191	22.35	<.0001
Week*Stra*Gende*Diet	C57BL/6J		HF	0	1	191	5.50	0.0200
Week*Stra*Gende*Diet	C57BL/6J		LF	0	1	191	6.80	0.0098
Week*Stra*Gende*Diet	ApoE-/-		HF	1	1	191	3.01	0.0845
Week*Stra*Gende*Diet	ApoE-/-		LF	1	1	191	21.11	<.0001
Week*Stra*Gende*Diet	C57BL/6J		HF	1	1	191	6.39	0.0123
Week*Stra*Gende*Diet	C57BL/6J		LF	1	1	191	14.60	0.0002
Week*Stra*Gende*Diet	ApoE-/-		HF	2	1	191	1.41	0.2372
Week*Stra*Gende*Diet	ApoE-/-		LF	2	1	191	25.54	<.0001
Week*Stra*Gende*Diet	C57BL/6J		HF	2	1	191	9.90	0.0019
Week*Stra*Gende*Diet	C57BL/6J		LF	2	1	191	18.36	<.0001
Week*Stra*Gende*Diet	ApoE-/-		HF	3	1	191	0.04	0.8359
Week*Stra*Gende*Diet	ApoE-/-		LF	3	1	191	22.35	<.0001
Week*Stra*Gende*Diet	C57BL/6J		HF	3	1	191	6.91	0.0093
Week*Stra*Gende*Diet	C57BL/6J		LF	3	1	191	21.11	<.0001
Week*Stra*Gende*Diet	ApoE-/-		HF	4	1	191	0.27	0.6046
Week*Stra*Gende*Diet	ApoE-/-		LF	4	1	191	19.11	<.0001
Week*Stra*Gende*Diet	C57BL/6J		HF	4	1	191	10.15	0.0017
Week*Stra*Gende*Diet	C57BL/6J		LF	4	1	191	26.61	<.0001
Week*Stra*Gende*Diet	ApoE-/-		HF	5	1	191	0.49	0.4869
Week*Stra*Gende*Diet	ApoE-/-		LF	5	1	191	23.77	<.0001
Week*Stra*Gende*Diet	C57BL/6J		HF	5	1	191	12.67	0.0005
Week*Stra*Gende*Diet	C57BL/6J		LF	5	1	191	32.08	<.0001
Week*Stra*Gende*Diet	ApoE-/-		HF	7	1	191	10.15	0.0017
Week*Stra*Gende*Diet	ApoE-/-		LF	7	1	191	23.20	<.0001
Week*Stra*Gende*Diet	C57BL/6J		HF	7	1	191	15.63	0.0001

Week*Stra*Gende*Diet	C57BL/6J	LF	7	1	191	29.37
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	HF	9	1	191	44.28
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	LF	9	1	191	33.75
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	HF	9	1	191	20.25
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	LF	9	1	191	34.96
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	HF	11	1	191	69.62
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	LF	11	1	191	37.28
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	HF	11	1	191	16.12
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	LF	11	1	191	45.98
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	HF	13	1	191	75.43
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	LF	13	1	191	40.42
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	HF	13	1	191	18.08
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	LF	13	1	191	47.02
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	HF	15	1	191	89.13
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	LF	15	1	191	40.05
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	HF	15	1	191	31.69
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	LF	15	1	191	64.88
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	HF	17	1	191	97.13
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	LF	17	1	191	51.45
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	HF	17	1	191	43.71
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	LF	17	1	191	78.82
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	HF	19	1	191	92.52
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	LF	19	1	191	52.09
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	HF	19	1	191	45.09
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	LF	19	1	191	83.79
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	HF	21	1	191	88.01
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	LF	21	1	191	52.31
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	HF	21	1	191	70.51
<.0001						
Week*Stra*Gende*Diet	C57BL/6J	LF	21	1	191	89.14
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	HF	23	1	191	92.52
<.0001						
Week*Stra*Gende*Diet	ApoE-/-	LF	23	1	200	48.36
<.0001						

Week*Stra*Gende*Diet	C57BL/6J		HF	23	1	191	96.91
<.0001							
Week*Stra*Gende*Diet	C57BL/6J		LF	23	1	198	85.81
<.0001							
Week*Stra*Gende*Diet	ApoE-/-	F		0	1	191	0.39
0.5344							
Week*Stra*Gende*Diet	ApoE-/-	M		0	1	191	0.00
0.9646							
Week*Stra*Gende*Diet	C57BL/6J	F		0	1	191	0.87
0.3517							
Week*Stra*Gende*Diet	C57BL/6J	M		0	1	191	0.47
0.4927							
Week*Stra*Gende*Diet	ApoE-/-	F		1	1	191	10.92
0.0011							
Week*Stra*Gende*Diet	ApoE-/-	M		1	1	191	0.20
0.6571							
Week*Stra*Gende*Diet	C57BL/6J	F		1	1	191	2.36
0.1258							
Week*Stra*Gende*Diet	C57BL/6J	M		1	1	191	0.05
0.8204							
Week*Stra*Gende*Diet	ApoE-/-	F		2	1	191	17.84
<.0001							
Week*Stra*Gende*Diet	ApoE-/-	M		2	1	191	0.13
0.7225							
Week*Stra*Gende*Diet	C57BL/6J	F		2	1	191	2.89
0.0907							
Week*Stra*Gende*Diet	C57BL/6J	M		2	1	191	0.32
0.5724							
Week*Stra*Gende*Diet	ApoE-/-	F		3	1	191	36.56
<.0001							
Week*Stra*Gende*Diet	ApoE-/-	M		3	1	191	2.33
0.1285							
Week*Stra*Gende*Diet	C57BL/6J	F		3	1	191	6.12
0.0142							
Week*Stra*Gende*Diet	C57BL/6J	M		3	1	191	0.35
0.5524							
Week*Stra*Gende*Diet	ApoE-/-	F		4	1	191	49.98
<.0001							
Week*Stra*Gende*Diet	ApoE-/-	M		4	1	191	4.75
0.0306							
Week*Stra*Gende*Diet	C57BL/6J	F		4	1	191	7.84
0.0056							
Week*Stra*Gende*Diet	C57BL/6J	M		4	1	191	0.91
0.3421							
Week*Stra*Gende*Diet	ApoE-/-	F		5	1	191	47.70
<.0001							
Week*Stra*Gende*Diet	ApoE-/-	M		5	1	191	7.44
0.0070							
Week*Stra*Gende*Diet	C57BL/6J	F		5	1	191	11.04
0.0011							
Week*Stra*Gende*Diet	C57BL/6J	M		5	1	191	2.00
0.1587							
Week*Stra*Gende*Diet	ApoE-/-	F		7	1	191	42.33
<.0001							
Week*Stra*Gende*Diet	ApoE-/-	M		7	1	191	23.77
<.0001							
Week*Stra*Gende*Diet	C57BL/6J	F		7	1	191	15.67
0.0001							
Week*Stra*Gende*Diet	C57BL/6J	M		7	1	191	8.45
0.0041							
Week*Stra*Gende*Diet	ApoE-/-	F		9	1	191	20.84
<.0001							

Week*Stra*Gende*Diet	ApoE-/-	M	9	1	191	29.26	<.0001
Week*Stra*Gende*Diet	C57BL/6J	F	9	1	191	26.37	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	9	1	191	19.14	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	11	1	191	20.84	<.0001
Week*Stra*Gende*Diet	ApoE-/-	M	11	1	191	46.28	<.0001
Week*Stra*Gende*Diet	C57BL/6J	F	11	1	191	60.83	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	11	1	191	37.43	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	13	1	191	22.07	<.0001
Week*Stra*Gende*Diet	ApoE-/-	M	13	1	191	49.35	<.0001
Week*Stra*Gende*Diet	C57BL/6J	F	13	1	191	99.21	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	13	1	191	79.93	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	15	1	191	18.73	<.0001
Week*Stra*Gende*Diet	ApoE-/-	M	15	1	191	55.35	<.0001
Week*Stra*Gende*Diet	C57BL/6J	F	15	1	191	135.32	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	15	1	191	123.38	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	17	1	191	20.70	<.0001
Week*Stra*Gende*Diet	ApoE-/-	M	17	1	191	52.31	<.0001
Week*Stra*Gende*Diet	C57BL/6J	F	17	1	191	169.14	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	17	1	191	166.66	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	19	1	191	23.92	<.0001
Week*Stra*Gende*Diet	ApoE-/-	M	19	1	191	53.17	<.0001
Week*Stra*Gende*Diet	C57BL/6J	F	19	1	191	211.91	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	19	1	191	213.42	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	21	1	191	31.22	<.0001
Week*Stra*Gende*Diet	ApoE-/-	M	21	1	191	59.85	<.0001
Week*Stra*Gende*Diet	C57BL/6J	F	21	1	191	222.14	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	21	1	191	273.24	<.0001
Week*Stra*Gende*Diet	ApoE-/-	F	23	1	191	38.56	<.0001
Week*Stra*Gende*Diet	ApoE-/-	M	23	1	200	74.69	<.0001
Week*Stra*Gende*Diet	C57BL/6J	F	23	1	197	215.40	<.0001
Week*Stra*Gende*Diet	C57BL/6J	M	23	1	191	327.10	<.0001

Week*Stra*Gende*Diet	F	HF	0	1	191	0.43
0.5120						
Week*Stra*Gende*Diet	F	LF	0	1	191	0.20
0.6550						
Week*Stra*Gende*Diet	M	HF	0	1	191	3.92
0.0491						
Week*Stra*Gende*Diet	M	LF	0	1	191	5.36
0.0216						
Week*Stra*Gende*Diet	F	HF	1	1	191	2.41
0.1220						
Week*Stra*Gende*Diet	F	LF	1	1	191	0.21
0.6464						
Week*Stra*Gende*Diet	M	HF	1	1	191	2.15
0.1441						
Week*Stra*Gende*Diet	M	LF	1	1	191	1.08
0.3001						
Week*Stra*Gende*Diet	F	HF	2	1	191	4.51
0.0349						
Week*Stra*Gende*Diet	F	LF	2	1	191	0.49
0.4866						
Week*Stra*Gende*Diet	M	HF	2	1	191	0.78
0.3788						
Week*Stra*Gende*Diet	M	LF	2	1	191	0.76
0.3847						
Week*Stra*Gende*Diet	F	HF	3	1	191	9.52
0.0023						
Week*Stra*Gende*Diet	F	LF	3	1	191	0.84
0.3614						
Week*Stra*Gende*Diet	M	HF	3	1	191	1.72
0.1915						
Week*Stra*Gende*Diet	M	LF	3	1	191	0.00
0.9957						
Week*Stra*Gende*Diet	F	HF	4	1	191	13.50
0.0003						
Week*Stra*Gende*Diet	F	LF	4	1	191	1.19
0.2774						
Week*Stra*Gende*Diet	M	HF	4	1	191	0.35
0.5548						
Week*Stra*Gende*Diet	M	LF	4	1	191	1.17
0.2805						
Week*Stra*Gende*Diet	F	HF	5	1	191	9.65
0.0022						
Week*Stra*Gende*Diet	F	LF	5	1	191	1.00
0.3195						
Week*Stra*Gende*Diet	M	HF	5	1	191	1.08
0.3006						
Week*Stra*Gende*Diet	M	LF	5	1	191	0.82
0.3673						
Week*Stra*Gende*Diet	F	HF	7	1	191	2.33
0.1284						
Week*Stra*Gende*Diet	F	LF	7	1	191	2.52
0.1139						
Week*Stra*Gende*Diet	M	HF	7	1	191	3.32
0.0702						
Week*Stra*Gende*Diet	M	LF	7	1	191	1.82
0.1794						
Week*Stra*Gende*Diet	F	HF	9	1	191	3.12
0.0789						
Week*Stra*Gende*Diet	F	LF	9	1	191	3.16
0.0769						
Week*Stra*Gende*Diet	M	HF	9	1	191	2.74
0.0997						

Week*Stra*Gende*Diet	M	LF	9	1	191	0.83
0.3644						
Week*Stra*Gende*Diet	F	HF	11	1	191	21.29
<.0001						
Week*Stra*Gende*Diet	F	LF	11	1	191	4.66
0.0321						
Week*Stra*Gende*Diet	M	HF	11	1	191	0.73
0.3943						
Week*Stra*Gende*Diet	M	LF	11	1	191	3.15
0.0775						
Week*Stra*Gende*Diet	F	HF	13	1	191	44.91
<.0001						
Week*Stra*Gende*Diet	F	LF	13	1	191	5.67
0.0183						
Week*Stra*Gende*Diet	M	HF	13	1	191	1.87
0.1731						
Week*Stra*Gende*Diet	M	LF	13	1	191	3.23
0.0740						
Week*Stra*Gende*Diet	F	HF	15	1	191	68.68
<.0001						
Week*Stra*Gende*Diet	F	LF	15	1	191	4.06
0.0452						
Week*Stra*Gende*Diet	M	HF	15	1	191	12.93
0.0004						
Week*Stra*Gende*Diet	M	LF	15	1	191	6.55
0.0112						
Week*Stra*Gende*Diet	F	HF	17	1	191	93.03
<.0001						
Week*Stra*Gende*Diet	F	LF	17	1	191	5.46
0.0205						
Week*Stra*Gende*Diet	M	HF	17	1	191	31.29
<.0001						
Week*Stra*Gende*Diet	M	LF	17	1	191	7.41
0.0071						
Week*Stra*Gende*Diet	F	HF	19	1	191	109.38
<.0001						
Week*Stra*Gende*Diet	F	LF	19	1	191	4.21
0.0416						
Week*Stra*Gende*Diet	M	HF	19	1	191	47.78
<.0001						
Week*Stra*Gende*Diet	M	LF	19	1	191	6.95
0.0091						
Week*Stra*Gende*Diet	F	HF	21	1	191	93.59
<.0001						
Week*Stra*Gende*Diet	F	LF	21	1	191	2.74
0.0994						
Week*Stra*Gende*Diet	M	HF	21	1	191	62.19
<.0001						
Week*Stra*Gende*Diet	M	LF	21	1	191	6.14
0.0141						
Week*Stra*Gende*Diet	F	HF	23	1	191	87.89
<.0001						
Week*Stra*Gende*Diet	F	LF	23	1	197	4.30
0.0395						
Week*Stra*Gende*Diet	M	HF	23	1	191	74.48
<.0001						
Week*Stra*Gende*Diet	M	LF	23	1	202	8.97
0.0031						

Here are the means and standard errors:

Obs	Week	Strain	Gender	Diet	_TYPE_	_FREQ_	MNGlucose	MNBW	SEGlucose	SEBW
1	0	ApoE-/-	F	HF	0	6	128.667	16.7167	7.2003	0.51343
2	0	ApoE-/-	F	LF	0	6	125.333	16.0167	4.1446	0.62579
3	0	ApoE-/-	M	HF	0	6	136.667	21.3833	10.1116	0.75251
4	0	ApoE-/-	M	LF	0	6	135.833	21.3333	5.0558	0.40716
5	0	C57BL/6J	F	HF	0	7	121.429	17.4286	7.5997	0.63983
6	0	C57BL/6J	F	LF	0	8	117.500	16.4875	6.6171	0.49224
7	0	C57BL/6J	M	HF	0	15	136.600	19.5200	6.7222	0.45010
8	0	C57BL/6J	M	LF	0	9	137.333	18.9556	10.6406	0.43593
9	1	ApoE-/-	F	HF	0	6	129.667	19.9833	5.0509	0.25484
10	1	ApoE-/-	F	LF	0	6	133.833	16.2667	13.1591	0.54508
11	1	ApoE-/-	M	HF	0	6	134.167	21.9333	10.2710	0.87965
12	1	ApoE-/-	M	LF	0	6	140.333	21.4333	9.5347	0.38093
13	1	C57BL/6J	F	HF	0	7	123.143	18.3000	6.8571	0.90816
14	1	C57BL/6J	F	LF	0	8	117.500	16.7500	5.7508	0.38219
15	1	C57BL/6J	M	HF	0	15	141.867	20.5533	4.9539	0.41552
16	1	C57BL/6J	M	LF	0	9	135.333	20.3667	10.3789	0.43397
17	2	ApoE-/-	F	HF	0	6	136.000	21.6167	6.0992	0.43083
18	2	ApoE-/-	F	LF	0	6	142.000	16.8667	9.5044	0.53208
19	2	ApoE-/-	M	HF	0	6	141.833	22.9500	9.1921	1.01251
20	2	ApoE-/-	M	LF	0	6	158.167	22.5500	4.8745	0.38449
21	2	C57BL/6J	F	HF	0	7	129.286	19.3143	4.3409	0.52027
22	2	C57BL/6J	F	LF	0	8	117.875	17.6000	3.2151	0.47056
23	2	C57BL/6J	M	HF	0	15	149.667	22.1200	5.6220	0.34808
24	2	C57BL/6J	M	LF	0	9	143.778	21.6556	6.6558	0.52125
25	3	ApoE-/-	F	HF	0	6	145.167	24.5000	5.0294	0.61101
26	3	ApoE-/-	F	LF	0	6	142.500	17.7000	6.1577	0.56095
27	3	ApoE-/-	M	HF	0	6	146.167	24.7333	4.0201	0.85894
28	3	ApoE-/-	M	LF	0	6	155.333	23.0167	6.0424	0.34873
29	3	C57BL/6J	F	HF	0	7	139.857	21.1571	5.6334	0.65205
30	3	C57BL/6J	F	LF	0	8	124.500	18.6625	4.0576	0.49531

31	3	C57BL/6J	M	HF	0	15	161.733	23.5000	5.0948
0.25448									
32	3	C57BL/6J	M	LF	0	9	140.222	23.0111	5.5297
0.48317									
33	4	ApoE-/-	F	HF	0	6	161.167	26.1667	3.3408
0.75351									
34	4	ApoE-/-	F	LF	0	6	156.167	18.2167	1.8514
0.49694									
35	4	ApoE-/-	M	HF	0	6	160.833	25.5833	5.3380
0.80433									
36	4	ApoE-/-	M	LF	0	6	157.833	23.1333	2.3298
0.31376									
37	4	C57BL/6J	F	HF	0	7	146.143	22.1857	5.6671
1.08922									
38	4	C57BL/6J	F	LF	0	8	123.625	19.3625	3.2179
0.41702									
39	4	C57BL/6J	M	HF	0	15	180.600	25.0267	6.0481
0.23993									
40	4	C57BL/6J	M	LF	0	9	140.778	24.2444	5.6070
0.49836									
41	5	ApoE-/-	F	HF	0	6	163.500	26.6667	5.4206
0.81432									
42	5	ApoE-/-	F	LF	0	6	157.667	18.9000	2.9851
0.52978									
43	5	ApoE-/-	M	HF	0	6	176.500	27.4500	7.2146
0.41773									
44	5	ApoE-/-	M	LF	0	6	159.667	24.3833	6.0369
0.55403									
45	5	C57BL/6J	F	HF	0	7	156.714	23.3000	3.7526
0.95319									
46	5	C57BL/6J	F	LF	0	8	127.000	19.9500	2.7646
0.55485									
47	5	C57BL/6J	M	HF	0	15	177.733	26.4733	7.6479
0.35637									
48	5	C57BL/6J	M	LF	0	9	142.222	25.3111	3.2905
0.52213									
49	7	ApoE-/-	F	HF	0	6	171.833	26.6833	3.9951
0.47848									
50	7	ApoE-/-	F	LF	0	6	151.667	19.3667	4.8212
0.59759									
51	7	ApoE-/-	M	HF	0	6	179.500	30.2667	4.6673
0.56667									
52	7	ApoE-/-	M	LF	0	6	165.500	24.7833	5.0448
0.41906									
53	7	C57BL/6J	F	HF	0	7	163.286	25.0286	2.2542
1.14844									
54	7	C57BL/6J	F	LF	0	8	119.000	21.0375	2.8661
0.38957									
55	7	C57BL/6J	M	HF	0	15	201.000	28.5533	6.0063
0.59240									
56	7	C57BL/6J	M	LF	0	9	146.111	26.1667	3.6150
0.59114									
57	9	ApoE-/-	F	HF	0	6	177.500	25.1000	2.5917
0.66282									
58	9	ApoE-/-	F	LF	0	6	169.000	19.9667	3.6968
0.61950									
59	9	ApoE-/-	M	HF	0	6	180.833	32.5833	3.4585
0.90753									
60	9	ApoE-/-	M	LF	0	6	169.000	26.5000	3.6968
0.47258									
61	9	C57BL/6J	F	HF	0	7	170.286	27.0143	3.5571
1.21976									

62	9	C57BL/6J	F	LF	0	8	121.750	21.8375	4.0916
0.39683									
63	9	C57BL/6J	M	HF	0	15	198.267	31.0267	4.4126
0.58305									
64	9	C57BL/6J	M	LF	0	9	133.000	27.4333	4.5826
0.63944									
65	11	ApoE-/-	F	HF	0	6	182.000	25.3000	4.7889
0.68944									
66	11	ApoE-/-	F	LF	0	6	163.500	20.1667	6.2650
0.60974									
67	11	ApoE-/-	M	HF	0	6	180.333	34.6833	4.4920
1.33402									
68	11	ApoE-/-	M	LF	0	6	165.833	27.0333	6.7795
0.37918									
69	11	C57BL/6J	F	HF	0	7	177.000	30.3000	5.6904
0.67893									
70	11	C57BL/6J	F	LF	0	8	126.625	22.4375	5.2574
0.34689									
71	11	C57BL/6J	M	HF	0	15	205.200	33.8800	6.7154
0.41129									
72	11	C57BL/6J	M	LF	0	9	137.111	28.8556	5.06196
0.78388									
73	13	ApoE-/-	F	HF	0	6	182.500	26.1667	9.42249
0.59423									
74	13	ApoE-/-	F	LF	0	6	158.000	20.8833	4.67618
0.67103									
75	13	ApoE-/-	M	HF	0	6	186.333	35.9333	7.00793
1.25875									
76	13	ApoE-/-	M	LF	0	6	166.167	28.0333	5.51009
0.41846									
77	13	C57BL/6J	F	HF	0	7	178.286	33.4286	3.59705
1.71904									
78	13	C57BL/6J	F	LF	0	8	118.625	23.3875	4.89146
0.32974									
79	13	C57BL/6J	M	HF	0	15	197.600	37.2200	7.77713
0.50824									
80	13	C57BL/6J	M	LF	0	9	138.889	29.8778	6.67176
0.85955									
81	15	ApoE-/-	F	HF	0	6	183.500	26.5333	2.45967
0.63805									
82	15	ApoE-/-	F	LF	0	6	157.167	21.6667	6.10146
0.56135									
83	15	ApoE-/-	M	HF	0	6	183.833	37.1500	7.78638
1.26905									
84	15	ApoE-/-	M	LF	0	6	161.500	28.7833	8.65929
0.43160									
85	15	C57BL/6J	F	HF	0	7	181.857	35.5143	4.92046
1.53164									
86	15	C57BL/6J	F	LF	0	8	117.750	23.7875	4.95606
0.28312									
87	15	C57BL/6J	M	HF	0	15	200.133	40.5333	4.69535
0.64429									
88	15	C57BL/6J	M	LF	0	9	139.333	31.4111	3.08221
0.94007									
89	17	ApoE-/-	F	HF	0	6	183.833	26.9333	6.52900
0.66767									
90	17	ApoE-/-	F	LF	0	6	165.667	21.8167	5.54176
0.62898									
91	17	ApoE-/-	M	HF	0	6	180.500	38.0167	3.96443
1.08272									
92	17	ApoE-/-	M	LF	0	6	156.500	29.8833	4.89047
0.45049									

93	17	C57BL/6J	F	HF	0	7	184.286	37.3857	2.23302
1.84796									
94	17	C57BL/6J	F	LF	0	8	122.250	24.2750	3.25549
0.33793									
95	17	C57BL/6J	M	HF	0	15	191.667	43.2800	3.96853
0.66578									
96	17	C57BL/6J	M	LF	0	9	136.889	32.6778	3.94914
0.87967									
97	19	ApoE-/-	F	HF	0	6	179.333	28.1667	4.47710
0.45436									
98	19	ApoE-/-	F	LF	0	6	160.667	22.6667	3.50872
0.53831									
99	19	ApoE-/-	M	HF	0	6	176.333	38.9833	2.13957
1.20234									
100	19	ApoE-/-	M	LF	0	6	163.167	30.7833	5.72955
0.43005									
101	19	C57BL/6J	F	HF	0	7	185.000	39.5000	3.35942
1.19960									
102	19	C57BL/6J	F	LF	0	8	123.750	24.8250	3.47311
0.37261									
103	19	C57BL/6J	M	HF	0	15	192.000	45.4867	7.31990
0.63710									
104	19	C57BL/6J	M	LF	0	9	135.000	33.4889	3.98957
0.74320									
105	21	ApoE-/-	F	HF	0	6	177.167	29.6167	4.16667
0.65849									
106	21	ApoE-/-	F	LF	0	6	160.000	23.3333	2.14476
0.53333									
107	21	ApoE-/-	M	HF	0	6	173.667	40.1667	5.43855
1.05977									
108	21	ApoE-/-	M	LF	0	6	163.000	31.4667	1.84391
0.44397									
109	21	C57BL/6J	F	HF	0	7	191.429	40.1000	5.63094
1.19363									
110	21	C57BL/6J	F	LF	0	8	116.250	25.0750	3.46281
0.32500									
111	21	C57BL/6J	M	HF	0	15	188.600	47.5867	5.62207
0.65530									
112	21	C57BL/6J	M	LF	0	9	130.222	34.0111	2.73241
0.74451									
113	23	ApoE-/-	F	HF	0	6	181.667	30.7833	6.24322
0.59856									
114	23	ApoE-/-	F	LF	0	6	164.500	23.8000	4.08860
0.62397									
115	23	ApoE-/-	M	HF	0	6	175.333	41.6000	4.93739
1.21874									
116	23	ApoE-/-	M	LF	0	6	155.167	31.5800	4.82643
0.59783									
117	23	C57BL/6J	F	HF	0	7	194.714	40.9429	5.09301
1.23940									
118	23	C57BL/6J	F	LF	0	8	121.000	25.9857	2.22807
0.46824									
119	23	C57BL/6J	M	HF	0	15	193.800	49.7200	3.63344
0.67020									
120	23	C57BL/6J	M	LF	0	9	131.667	34.8667	3.00463
0.59442									

Chapter 4

Strain	Diet	MNCholesterol	SECholesterol	PVALUE
ApoE -/-	HF	1104.58 a	38.9848	<.0001
ApoE -/-	LF	547.49 b	23.4126	
C57BL6/J	HF	282.32 c	10.0409	
C57BL6/J	LF	80.36 d	4.4490	

Strain	Diet	MNIsoprostane	SEIsoprostane	PVALUE
ApoE -/-	HF	498.707 a	16.8268	<.0001
ApoE -/-	LF	314.718 b	12.4189	
C57BL6/J	HF	276.226 c	8.1880	
C57BL6/J	LF	64.717 d	2.1724	

Strain	Diet	MNTriglyceride	SETriglyceride	PVALUE
ApoE -/-	HF	568.480 a	39.1558	<.0001
ApoE -/-	LF	226.850 b	14.5049	
C57BL6/J	HF	95.479 c	5.2049	
C57BL6/J	LF	88.779 c	4.6307	

Strain	Diet	MNInsulin	SEInsulin	PVALUE
ApoE -/-	HF	4.16909 b	0.28544	<.0001
ApoE -/-	LF	0.81188 c	0.07702	
C57BL6/J	HF	5.59904 a	0.33356	
C57BL6/J	LF	1.44417 c	0.05238	

Strain	Diet	MNBW24th Week	SEBW24th Week	PVALUE
ApoE -/-	HF	41.7559 b	0.60774	<.0001
ApoE -/-	LF	31.0162 d	0.42266	
C57BL6/J	HF	47.6417 a	0.73334	
C57BL6/J	LF	33.3029 c	0.47260	

Strain	Diet	MNAUC	SEAUC	PVALUE
ApoE -/-	HF	33136.29 b	658.084	<.0001
ApoE -/-	LF	29096.46 c	556.949	
C57BL6/J	HF	49950.66 a	577.844	
C57BL6/J	LF	31657.74 b	766.646	

Day	Strain	Diet	MNPLGFlig	SEPLGFlig	PVALUE
3	ApoE -/-	HF	21.3593 ab	3.03914	0.0119
3	ApoE -/-	LF	27.0317 a	3.10836	
3	C57BL6/J	HF	16.2020 b	1.86411	
3	C57BL6/J	LF	28.5958 a	3.63877	
5	ApoE -/-	HF	18.6036 b	3.07157	<.0001
5	ApoE -/-	LF	31.4602 a	3.15487	
5	C57BL6/J	HF	15.7600 b	2.53840	
5	C57BL6/J	LF	29.1990 a	3.41256	
7	ApoE -/-	HF	19.9932 b	1.38722	<.0001
7	ApoE -/-	LF	31.9086 a	2.54776	
7	C57BL6/J	HF	13.3684 b	1.95309	
7	C57BL6/J	LF	50.0469 a	2.83439	
10	ApoE -/-	HF	18.1821 c	1.20157	<.0001
10	ApoE -/-	LF	26.8077 b	1.25451	
10	C57BL6/J	HF	9.2315 d	1.20539	
10	C57BL6/J	LF	49.2405 a	3.80469	
14	ApoE -/-	HF	18.3432 b	1.87402	0.0374
14	ApoE -/-	LF	28.8627 a	2.95398	
14	C57BL6/J	HF	24.2022 ab	3.03842	
14	C57BL6/J	LF	27.7423 a	2.42431	
21	ApoE -/-	HF	17.3372 c	1.58567	<.0001
21	ApoE -/-	LF	31.4154 b	1.61761	
21	C57BL6/J	HF	48.1325 a	4.46425	
21	C57BL6/J	LF	24.5627 bc	3.07373	
28	ApoE -/-	HF	20.6537 a	2.11636	<.0001
28	ApoE -/-	LF	26.5103 a	0.92185	
28	C57BL6/J	HF	8.0697 b	1.43146	
28	C57BL6/J	LF	20.2665 a	2.93964	

Day	Strain	Diet	MNPLGFratio	SEPLGFratio	PVALUE
3	ApoE -/-	HF	0.85479 a	0.05046	0.7557
3	ApoE -/-	LF	1.05441 a	0.02668	
3	C57BL6/J	HF	0.93872 a	0.04398	
3	C57BL6/J	LF	0.94751 a	0.05713	
5	ApoE -/-	HF	1.05403 a	0.08174	0.9065
5	ApoE -/-	LF	1.06889 a	0.15646	
5	C57BL6/J	HF	0.95613 a	0.09038	
5	C57BL6/J	LF	1.00893 a	0.04073	
7	ApoE -/-	HF	0.98010 b	0.10360	<.0001
7	ApoE -/-	LF	1.24717 b	0.17690	
7	C57BL6/J	HF	0.93891 b	0.06204	
7	C57BL6/J	LF	1.92363 a	0.18251	
10	ApoE -/-	HF	1.11521 b	0.06155	<.0001
10	ApoE -/-	LF	1.02503 b	0.08356	
10	C57BL6/J	HF	0.92203 b	0.07595	
10	C57BL6/J	LF	2.33510 a	0.30947	
14	ApoE -/-	HF	0.95900 a	0.02572	0.3904
14	ApoE -/-	LF	1.18202 a	0.11194	
14	C57BL6/J	HF	1.20103 a	0.09239	
14	C57BL6/J	LF	1.02134 a	0.04462	
21	ApoE -/-	HF	0.90920 b	0.06148	0.0015
21	ApoE -/-	LF	1.11396 b	0.13528	
21	C57BL6/J	HF	1.56815 a	0.12176	
21	C57BL6/J	LF	1.07958 b	0.08076	
28	ApoE -/-	HF	1.06799 a	0.06699	0.4416
28	ApoE -/-	LF	1.04616 a	0.06331	
28	C57BL6/J	HF	0.83000 a	0.07500	
28	C57BL6/J	LF	0.89852 a	0.06207	

Day	Strain	Diet	MNVEGFlig	SEVEGFlig	PVALUE
3	ApoE -/-	HF	37.660 a	5.3056	0.1968
3	ApoE -/-	LF	50.480 a	8.3818	
3	C57BL6/J	HF	37.105 a	1.3777	
3	C57BL6/J	LF	24.847 a	1.9228	
5	ApoE -/-	HF	38.419 b	2.8210	0.0023
5	ApoE -/-	LF	74.454 a	11.0378	
5	C57BL6/J	HF	33.157 b	3.2893	
5	C57BL6/J	LF	34.338 b	0.7038	
7	ApoE -/-	HF	46.597 b	12.7684	<.0001
7	ApoE -/-	LF	100.347 a	16.4300	
7	C57BL6/J	HF	32.576 b	4.7982	
7	C57BL6/J	LF	42.844 b	5.9600	
10	ApoE -/-	HF	58.198 b	13.7757	<.0001
10	ApoE -/-	LF	89.899 a	11.4078	
10	C57BL6/J	HF	27.700 c	3.9486	
10	C57BL6/J	LF	26.968 c	4.6076	
14	ApoE -/-	HF	65.241 b	8.4806	<.0001
14	ApoE -/-	LF	114.928 a	14.5129	
14	C57BL6/J	HF	39.926 c	8.1809	
14	C57BL6/J	LF	68.564 b	7.3678	
21	ApoE -/-	HF	56.839 bc	13.5634	<.0001
21	ApoE -/-	LF	94.144 a	6.7208	
21	C57BL6/J	HF	33.218 c	8.8264	
21	C57BL6/J	LF	59.850 b	9.4865	
28	ApoE -/-	HF	50.938 b	7.3574	<.0001
28	ApoE -/-	LF	107.712 a	7.9333	
28	C57BL6/J	HF	32.517 b	3.3465	
28	C57BL6/J	LF	36.488 b	9.2520	

Day	Strain	Diet	MNVEGFratio	SEVEGFratio	PVALUE
3	ApoE -/-	HF	1.23929 a	0.03854	0.9851
3	ApoE -/-	LF	1.18323 a	0.06518	
3	C57BL6/J	HF	1.09739 a	0.06906	
3	C57BL6/J	LF	1.13100 a	0.09181	
5	ApoE -/-	HF	1.19895 a	0.10259	0.3675
5	ApoE -/-	LF	1.08740 a	0.16441	
5	C57BL6/J	HF	1.23888 a	0.08464	
5	C57BL6/J	LF	1.75546 a	0.20428	
7	ApoE -/-	HF	1.37039 a	0.15202	0.1759
7	ApoE -/-	LF	1.56165 a	0.25579	
7	C57BL6/J	HF	1.16866 a	0.10803	
7	C57BL6/J	LF	1.88258 a	0.26770	
10	ApoE -/-	HF	1.82969 a	0.50455	0.4451
10	ApoE -/-	LF	1.26964 a	0.12698	
10	C57BL6/J	HF	1.55569 a	0.24150	
10	C57BL6/J	LF	1.78642 a	0.38083	
14	ApoE -/-	HF	1.86372 b	0.16437	<.0001
14	ApoE -/-	LF	2.04895 b	0.48101	
14	C57BL6/J	HF	1.42494 b	0.31844	
14	C57BL6/J	LF	3.93659 a	0.54305	
21	ApoE -/-	HF	1.82735 a	0.26636	0.0823
21	ApoE -/-	LF	1.61343 a	0.25921	
21	C57BL6/J	HF	1.70393 a	0.53604	
21	C57BL6/J	LF	2.49681 a	0.16116	
28	ApoE -/-	HF	1.20807 a	0.05941	0.7715
28	ApoE -/-	LF	1.45430 a	0.11522	
28	C57BL6/J	HF	1.09254 a	0.15778	
28	C57BL6/J	LF	1.37487 a	0.37781	

Day	Strain	Diet	MNVEGFR1lig	SEVEGFR1lig	PVALUE
3	ApoE -/-	HF	327.247 a	12.8602	0.6013
3	ApoE -/-	LF	422.040 a	59.1821	
3	C57BL6/J	HF	391.805 a	56.8594	
3	C57BL6/J	LF	402.401 a	33.3849	
5	ApoE -/-	HF	320.159 a	26.2540	0.8334
5	ApoE -/-	LF	348.158 a	16.3432	
5	C57BL6/J	HF	329.581 a	55.9341	
5	C57BL6/J	LF	383.415 a	27.4992	
7	ApoE -/-	HF	339.245 a	15.8918	0.3200
7	ApoE -/-	LF	469.531 a	49.2973	
7	C57BL6/J	HF	391.232 a	36.8743	
7	C57BL6/J	LF	408.544 a	36.0741	
10	ApoE -/-	HF	362.187 b	48.3677	0.0214
10	ApoE -/-	LF	517.196 a	67.0749	
10	C57BL6/J	HF	380.819 b	48.5201	
10	C57BL6/J	LF	520.354 a	28.8945	
14	ApoE -/-	HF	390.082 bc	53.3636	<.0001
14	ApoE -/-	LF	465.545 b	49.3842	
14	C57BL6/J	HF	309.332 c	31.1990	
14	C57BL6/J	LF	617.460 a	43.1947	
21	ApoE -/-	HF	398.451 b	13.9570	<.0001
21	ApoE -/-	LF	434.563 b	36.1775	
21	C57BL6/J	HF	370.455 b	19.5865	
21	C57BL6/J	LF	704.986 a	64.9496	
28	ApoE -/-	HF	488.526 b	23.7721	0.0011
28	ApoE -/-	LF	530.441 b	45.7456	
28	C57BL6/J	HF	673.007 a	84.0295	
28	C57BL6/J	LF	733.440 a	96.1104	

Day	Strain	Diet	MNVEGFR1ratio	SEVEGFR1ratio	PVALUE
3	ApoE -/-	HF	1.20441 a	0.08899	0.2655
3	ApoE -/-	LF	1.01824 a	0.18283	
3	C57BL6/J	HF	1.09634 a	0.29500	
3	C57BL6/J	LF	1.45082 a	0.20546	
5	ApoE -/-	HF	1.00600 a	0.01890	0.4953
5	ApoE -/-	LF	0.98527 a	0.14584	
5	C57BL6/J	HF	0.93658 a	0.09438	
5	C57BL6/J	LF	1.26160 a	0.18065	
7	ApoE -/-	HF	1.05954 a	0.05964	0.4330
7	ApoE -/-	LF	0.97313 a	0.11836	
7	C57BL6/J	HF	0.98117 a	0.05146	
7	C57BL6/J	LF	1.30703 a	0.17392	
10	ApoE -/-	HF	1.21362 b	0.08647	0.0073
10	ApoE -/-	LF	1.10361 b	0.11554	
10	C57BL6/J	HF	0.97622 b	0.08406	
10	C57BL6/J	LF	1.66448 a	0.21396	
14	ApoE -/-	HF	1.09679 b	0.09762	0.0029
14	ApoE -/-	LF	1.20292 b	0.17656	
14	C57BL6/J	HF	0.91889 b	0.11566	
14	C57BL6/J	LF	1.67837 a	0.12681	
21	ApoE -/-	HF	1.49350 b	0.24603	<.0001
21	ApoE -/-	LF	1.08618 b	0.09813	
21	C57BL6/J	HF	1.14786 b	0.06235	
21	C57BL6/J	LF	2.24112 a	0.19360	
28	ApoE -/-	HF	1.67076 b	0.11469	<.0001
28	ApoE -/-	LF	1.22471 c	0.11853	
28	C57BL6/J	HF	1.42181 bc	0.19552	
28	C57BL6/J	LF	2.20620 a	0.20487	

Day	Strain	Diet	MNMCP1lig		SEMCP1lig	PVALUE
3	ApoE -/-	HF	132.178	b	45.2449	0.0052
3	ApoE -/-	LF	95.692	b	20.1969	
3	C57BL6/J	HF	155.040	b	33.1480	
3	C57BL6/J	LF	229.604	a	34.9374	
5	ApoE -/-	HF	113.266	c	42.2936	0.0007
5	ApoE -/-	LF	253.370	a	47.8718	
5	C57BL6/J	HF	189.125	ab	46.8542	
5	C57BL6/J	LF	134.718	bc	22.0139	
7	ApoE -/-	HF	37.864	a	16.4324	0.2029
7	ApoE -/-	LF	80.215	a	26.0223	
7	C57BL6/J	HF	96.472	a	30.7498	
7	C57BL6/J	LF	37.964	a	14.5126	
10	ApoE -/-	HF	16.978	a	8.0202	0.1587
10	ApoE -/-	LF	81.839	a	35.6486	
10	C57BL6/J	HF	63.724	a	20.7988	
10	C57BL6/J	LF	16.956	a	4.4160	
14	ApoE -/-	HF	19.721	a	11.3389	0.2885
14	ApoE -/-	LF	68.223	a	19.1366	
14	C57BL6/J	HF	69.115	a	16.6501	
14	C57BL6/J	LF	17.550	a	4.8655	
21	ApoE -/-	HF	24.608	a	12.4486	0.8331
21	ApoE -/-	LF	47.695	a	20.4045	
21	C57BL6/J	HF	35.942	a	14.9533	
21	C57BL6/J	LF	17.044	a	3.8523	
28	ApoE -/-	HF	33.587	a	14.5222	0.8033
28	ApoE -/-	LF	19.369	a	8.8730	
28	C57BL6/J	HF	45.609	a	16.6643	
28	C57BL6/J	LF	13.658	a	2.5836	

Day	Strain	Diet	MNMCP1ratio	SEMCP1ratio	PVALUE
3	ApoE -/-	HF	5.4704 b	1.85573	<.0001
3	ApoE -/-	LF	6.4195 b	1.39473	
3	C57BL6/J	HF	28.4734 a	5.99182	
3	C57BL6/J	LF	27.8457 a	5.49411	
5	ApoE -/-	HF	6.1443 b	2.85516	<.0001
5	ApoE -/-	LF	10.8204 b	1.95703	
5	C57BL6/J	HF	24.9312 a	5.86281	
5	C57BL6/J	LF	13.1568 b	2.57029	
7	ApoE -/-	HF	1.2136 a	0.46419	0.0829
7	ApoE -/-	LF	3.4466 a	1.09619	
7	C57BL6/J	HF	9.0269 a	2.32790	
7	C57BL6/J	LF	8.0670 a	2.24110	
10	ApoE -/-	HF	0.6241 a	0.20494	0.4077
10	ApoE -/-	LF	5.2428 a	2.86344	
10	C57BL6/J	HF	5.9708 a	1.96509	
10	C57BL6/J	LF	2.3151 a	0.59751	
14	ApoE -/-	HF	1.0566 a	0.41186	0.2752
14	ApoE -/-	LF	4.5376 a	1.47988	
14	C57BL6/J	HF	8.1537 a	3.27730	
14	C57BL6/J	LF	4.2094 a	3.08941	
21	ApoE -/-	HF	1.1208 a	0.24256	0.5921
21	ApoE -/-	LF	5.5007 a	3.44555	
21	C57BL6/J	HF	2.6289 a	2.13553	
21	C57BL6/J	LF	1.2871 a	0.15056	
28	ApoE -/-	HF	2.0149 a	1.02341	0.9985
28	ApoE -/-	LF	1.5679 a	0.57467	
28	C57BL6/J	HF	1.8440 a	0.73839	
28	C57BL6/J	LF	2.1831 a	0.77706	

Day	TRT	MNPLGFRATIO	SEPLGFRATIO	PVALUE
7	SHAM	0.94470	0.06042	0.0004
7	TRT	2.16628	0.22291	
10	SHAM	1.08488	0.20970	0.0014
10	TRT	2.13462	0.30436	
21	SHAM	1.02716	0.18112	0.8944
21	TRT	1.06468	0.10247	

TRT	MNVEGFRATIO	SEVEGFRATIO	PVALUE
SHAM	0.96243	0.05453	0.0016
TRT	3.93659	0.54305	

Day	TRT	MNVEGFR1RATIO	SEVEGFR1RATIO	PVALUE
7	SHAM	1.00748	0.10623	0.2394
7	TRT	1.35688	0.23564	
10	SHAM	0.80425	0.09564	0.0078
10	TRT	1.64761	0.27536	

Diet	TRT	MNMCP1RATIO	SEMCP1RATIO	PVALUE
HF	SHAM	4.6145	3.22657	0.0072
HF	TRT	27.2898	7.58299	
LF	SHAM	3.8147	0.57906	0.0050
LF	TRT	27.8457	5.49411	

---------- Day=3 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
PLGFratio	1.00000	-0.13526	0.13140	0.21887	-0.19882
		0.6175	0.6407	0.4154	0.5149
	16	16	15	16	13
VEGFratio	-0.13526	1.00000	0.05448	-0.33924	0.36926
	0.6175		0.8412	0.1684	0.1447
	16	20	16	18	17
VEGFR1ratio	0.13140	0.05448	1.00000	0.23717	-0.26004
	0.6407	0.8412		0.3765	0.3909
	15	16	16	16	13
MCP1ratio	0.21887	-0.33924	0.23717	1.00000	-0.64862
	0.4154	0.1684	0.3765		0.0066
	16	18	16	19	16

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
PLGFratio	-0.19489	-0.26189	-0.44042	-0.11524	-0.32447
	0.5234	0.3657	0.1519	0.6709	0.2380
	13	14	12	16	15
VEGFratio	0.27784	0.30492	-0.07618	-0.23150	-0.01558
	0.2802	0.2186	0.7792	0.3261	0.9495
	17	18	16	20	19

VEGFR1ratio	-0.33294	-0.02504	-0.22128	-0.00137	-0.09740
	0.2663	0.9323	0.4895	0.9960	0.7298
	13	14	12	16	15
MCP1ratio	-0.63099	-0.53579	0.30327	0.48124	0.55321
	0.0088	0.0266	0.2718	0.0370	0.0172
	16	17	15	19	18

---------- Day=3 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
Cholesterol	-0.19882	0.36926	-0.26004	-0.64862	1.00000
	0.5149	0.1447	0.3909	0.0066	
	13	17	13	16	18
Isoprostane	-0.19489	0.27784	-0.33294	-0.63099	0.95390
	0.5234	0.2802	0.2663	0.0088	<.0001
	13	17	13	16	18
Triglyceride	-0.26189	0.30492	-0.02504	-0.53579	0.89651
	0.3657	0.2186	0.9323	0.0266	<.0001
	14	18	14	17	18
Insulin	-0.44042	-0.07618	-0.22128	0.30327	0.08073
	0.1519	0.7792	0.4895	0.2718	0.7581
	12	16	12	15	17
BW24thWeek	-0.11524	-0.23150	-0.00137	0.48124	-0.13723
	0.6709	0.3261	0.9960	0.0370	0.5871
	16	20	16	19	18
AUC	-0.32447	-0.01558	-0.09740	0.55321	-0.13955
	0.2380	0.9495	0.7298	0.0172	0.5808
	15	19	15	18	18

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
Cholesterol	0.95390	0.89651	0.08073	-0.13723	-0.13955

	<.0001	<.0001	0.7581	0.5871	0.5808
	18	18	17	18	18
Isoprostane	1.00000	0.87527	0.27039	0.04497	0.01783
		<.0001	0.2939	0.8594	0.9440
	18	18	17	18	18
Triglyceride	0.87527	1.00000	0.12417	0.01269	-0.05227
	<.0001		0.6349	0.9589	0.8368
	18	19	17	19	18
Insulin	0.27039	0.12417	1.00000	0.70192	0.85748
	0.2939	0.6349		0.0017	<.0001
	17	17	17	17	17
BW24thWeek	0.04497	0.01269	0.70192	1.00000	0.82388
	0.8594	0.9589	0.0017		<.0001
	18	19	17	21	20
AUC	0.01783	-0.05227	0.85748	0.82388	1.00000
	0.9440	0.8368	<.0001	<.0001	
	18	18	17	20	20

---------- Day=5 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
PLGFratio	1.00000	-0.12539	-0.13817	0.09471	0.05651
		0.6201	0.6098	0.7085	0.8294
	18	18	16	18	17
VEGFratio	-0.12539	1.00000	0.03330	0.06909	-0.36050
	0.6201		0.9026	0.7853	0.1552
	18	18	16	18	17

VEGFR1ratio	-0.13817	0.03330	1.00000	-0.28668	-0.21115
	0.6098	0.9026		0.2817	0.4500
	16	16	16	16	15
MCP1ratio	0.09471	0.06909	-0.28668	1.00000	-0.37616
	0.7085	0.7853	0.2817		0.1367
	18	18	16	19	17

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
PLGFratio	0.09151	0.33758	-0.36886	-0.23964	-0.12030
	0.7269	0.2185	0.1761	0.3382	0.6344
	17	15	15	18	18
VEGFratio	-0.46574	-0.25020	-0.13720	-0.13718	-0.09227
	0.0595	0.3684	0.6258	0.5873	0.7158
	17	15	15	18	18
VEGFR1ratio	-0.44266	-0.21348	-0.32038	-0.08888	-0.18499
	0.0985	0.4637	0.2641	0.7434	0.4928
	15	14	14	16	16
MCP1ratio	-0.08681	-0.32207	0.44906	0.28610	0.72582
	0.7404	0.2417	0.0931	0.2351	0.0006
	17	15	15	19	18

---------- Day=5 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
Cholesterol	0.05651	-0.36050	-0.21115	-0.37616	1.00000
	0.8294	0.1552	0.4500	0.1367	
	17	17	15	17	17
Isoprostane	0.09151	-0.46574	-0.44266	-0.08681	0.85774
	0.7269	0.0595	0.0985	0.7404	<.0001
	17	17	15	17	17
Triglyceride	0.33758	-0.25020	-0.21348	-0.32207	0.78460
	0.2185	0.3684	0.4637	0.2417	0.0005
	15	15	14	15	15
Insulin	-0.36886	-0.13720	-0.32038	0.44906	0.18505
	0.1761	0.6258	0.2641	0.0931	0.5091
	15	15	14	15	15
BW24thWeek	-0.23964	-0.13718	-0.08888	0.28610	0.14995
	0.3382	0.5873	0.7434	0.2351	0.5657
	18	18	16	19	17
AUC	-0.12030	-0.09227	-0.18499	0.72582	-0.36015
	0.6344	0.7158	0.4928	0.0006	0.1556
	18	18	16	18	17

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
Cholesterol	0.85774	0.78460	0.18505	0.14995	-0.36015

	<.0001	0.0005	0.5091	0.5657	0.1556
	17	15	15	17	17
Isoprostane	1.00000	0.79084	0.46137	0.32171	-0.06897
		0.0004	0.0834	0.2080	0.7925
	17	15	15	17	17
Triglyceride	0.79084	1.00000	0.08352	-0.01278	-0.47131
	0.0004		0.7673	0.9639	0.0762
	15	15	15	15	15
Insulin	0.46137	0.08352	1.00000	0.73087	0.69626
	0.0834	0.7673		0.0020	0.0039
	15	15	15	15	15
BW24thWeek	0.32171	-0.01278	0.73087	1.00000	0.72377
	0.2080	0.9639	0.0020		0.0007
	17	15	15	19	18
AUC	-0.06897	-0.47131	0.69626	0.72377	1.00000
	0.7925	0.0762	0.0039	0.0007	
	17	15	15	18	18

---------- Day=7 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
PLGFratio	1.00000	0.46993	0.67798	0.19865	-0.37737
		0.0316	0.0039	0.3880	0.0917
	22	21	16	21	21
VEGFratio	0.46993	1.00000	0.69405	0.01521	-0.33545
	0.0316		0.0029	0.9478	0.1371
	21	22	16	21	21

VEGFR1ratio	0.67798	0.69405	1.00000	0.15793	-0.15242
	0.0039	0.0029		0.5449	0.5731
	16	16	17	17	16
MCP1ratio	0.19865	0.01521	0.15793	1.00000	-0.54183
	0.3880	0.9478	0.5449		0.0112
	21	21	17	22	21

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
PLGFratio	-0.57133	-0.39376	-0.64021	-0.43639	-0.40204
	0.0085	0.1059	0.0042	0.0423	0.0981
	20	18	18	22	18
VEGFratio	-0.54345	-0.25974	-0.58009	-0.50125	-0.23197
	0.0133	0.2979	0.0116	0.0175	0.3393
	20	18	18	22	19
VEGFR1ratio	-0.20187	-0.20471	-0.25302	-0.25159	-0.04365
	0.4706	0.4827	0.3828	0.3300	0.8822
	15	14	14	17	14
MCP1ratio	-0.49326	-0.55432	-0.15144	0.16315	0.36777
	0.0271	0.0170	0.5486	0.4682	0.1213
	20	18	18	22	19

---------- Day=7 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
Cholesterol	-0.37737	-0.33545	-0.15242	-0.54183	1.00000
	0.0917	0.1371	0.5731	0.0112	
	21	21	16	21	22
Isoprostane	-0.57133	-0.54345	-0.20187	-0.49326	0.90066
	0.0085	0.0133	0.4706	0.0271	<.0001
	20	20	15	20	21
Triglyceride	-0.39376	-0.25974	-0.20471	-0.55432	0.86698
	0.1059	0.2979	0.4827	0.0170	<.0001
	18	18	14	18	19
Insulin	-0.64021	-0.58009	-0.25302	-0.15144	0.22087
	0.0042	0.0116	0.3828	0.5486	0.3635
	18	18	14	18	19
BW24thWeek	-0.43639	-0.50125	-0.25159	0.16315	0.15436
	0.0423	0.0175	0.3300	0.4682	0.4928
	22	22	17	22	22
AUC	-0.40204	-0.23197	-0.04365	0.36777	-0.17021
	0.0981	0.3393	0.8822	0.1213	0.4995
	18	19	14	19	18

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
Cholesterol	0.90066	0.86698	0.22087	0.15436	-0.17021

	<.0001	<.0001	0.3635	0.4928	0.4995
	21	19	19	22	18
Isoprostane	1.00000	0.69912	0.55506	0.41592	0.16603
		0.0009	0.0136	0.0608	0.5242
	21	19	19	21	17
Triglyceride	0.69912	1.00000	0.23830	0.12833	-0.23638
	0.0009		0.3259	0.6006	0.3963
	19	19	19	19	15
Insulin	0.55506	0.23830	1.00000	0.60729	0.59187
	0.0136	0.3259		0.0058	0.0201
	19	19	19	19	15
BW24thWeek	0.41592	0.12833	0.60729	1.00000	0.77954
	0.0608	0.6006	0.0058		<.0001
	21	19	19	23	19
AUC	0.16603	-0.23638	0.59187	0.77954	1.00000
	0.5242	0.3963	0.0201	<.0001	
	17	15	15	19	19

---------- Day=10 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
PLGFratio	1.00000	0.20235	0.67320	-0.23917	-0.44730
		0.4061	0.0016	0.3241	0.0718
	19	19	19	19	17
VEGFratio	0.20235	1.00000	0.26722	-0.17814	0.00922
	0.4061		0.2687	0.4656	0.9720

	19	19	19	19	17
VEGFR1ratio	0.67320	0.26722	1.00000	-0.11239	-0.17127
	0.0016	0.2687		0.6371	0.4968
	19	19	20	20	18
MCP1ratio	-0.23917	-0.17814	-0.11239	1.00000	-0.21293
	0.3241	0.4656	0.6371		0.3963
	19	19	20	20	18

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
PLGFratio	-0.71689	-0.31500	-0.51496	-0.39563	-0.20342
	0.0018	0.2528	0.0495	0.0936	0.4036
	16	15	15	19	19
VEGFratio	-0.03473	0.20694	-0.08728	0.24648	0.02853
	0.8984	0.4593	0.7571	0.3090	0.9077
	16	15	15	19	19
VEGFR1ratio	-0.48071	-0.08307	-0.29019	-0.29287	-0.13654
	0.0508	0.7685	0.2941	0.2102	0.5659
	17	15	15	20	20
MCP1ratio	-0.03498	-0.37691	0.05226	-0.03416	0.21576
	0.8940	0.1661	0.8532	0.8863	0.3609
	17	15	15	20	20

---------- Day=10 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
Cholesterol	-0.44730	0.00922	-0.17127	-0.21293	1.00000
	0.0718	0.9720	0.4968	0.3963	
	17	17	18	18	18
Isoprostane	-0.71689	-0.03473	-0.48071	-0.03498	0.89848
	0.0018	0.8984	0.0508	0.8940	<.0001
	16	16	17	17	17
Triglyceride	-0.31500	0.20694	-0.08307	-0.37691	0.94594
	0.2528	0.4593	0.7685	0.1661	<.0001
	15	15	15	15	15
Insulin	-0.51496	-0.08728	-0.29019	0.05226	0.33736
	0.0495	0.7571	0.2941	0.8532	0.2188
	15	15	15	15	15
BW24thWeek	-0.39563	0.24648	-0.29287	-0.03416	0.29484
	0.0936	0.3090	0.2102	0.8863	0.2349
	19	19	20	20	18
AUC	-0.20342	0.02853	-0.13654	0.21576	-0.26192
	0.4036	0.9077	0.5659	0.3609	0.2937
	19	19	20	20	18

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
Cholesterol	0.89848	0.94594	0.33736	0.29484	-0.26192
	<.0001	<.0001	0.2188	0.2349	0.2937

	17	15	15	18	18
Isoprostane	1.00000	0.77716	0.40891	0.46920	-0.09740
		0.0007	0.1302	0.0574	0.7100
	17	15	15	17	17
Triglyceride	0.77716	1.00000	0.24252	0.23826	-0.34307
	0.0007		0.3838	0.3925	0.2106
	15	15	15	15	15
Insulin	0.40891	0.24252	1.00000	0.86772	0.73809
	0.1302	0.3838		<.0001	0.0017
	15	15	15	15	15
BW24thWeek	0.46920	0.23826	0.86772	1.00000	0.68776
	0.0574	0.3925	<.0001		0.0008
	17	15	15	20	20
AUC	-0.09740	-0.34307	0.73809	0.68776	1.00000
	0.7100	0.2106	0.0017	0.0008	
	17	15	15	20	20

---------- Day=14 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
PLGFratio	1.00000	-0.59905	0.06237	0.11557	-0.06773
		0.0067	0.7998	0.6479	0.7829
	20	19	19	18	19
VEGFratio	-0.59905	1.00000	0.50852	-0.26109	-0.34018
	0.0067		0.0312	0.2953	0.1672
	19	19	18	18	18

VEGFR1ratio	0.06237	0.50852	1.00000	-0.20044	-0.34759
	0.7998	0.0312		0.4252	0.1448
	19	18	20	18	19
MCP1ratio	0.11557	-0.26109	-0.20044	1.00000	-0.37900
	0.6479	0.2953	0.4252		0.1209
	18	18	18	19	18

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
PLGFratio	-0.01811	-0.08992	0.06918	0.16729	0.27392
	0.9413	0.7143	0.7919	0.4808	0.2565
	19	19	17	20	19
VEGFratio	-0.55018	-0.21188	-0.42487	-0.52577	-0.49249
	0.0180	0.3986	0.1009	0.0208	0.0379
	18	18	16	19	18
VEGFR1ratio	-0.60443	-0.11031	-0.38898	-0.43634	-0.48877
	0.0061	0.6530	0.1228	0.0544	0.0337
	19	19	17	20	19
MCP1ratio	-0.18261	-0.27419	0.34428	0.11133	0.38353
	0.4683	0.2709	0.1760	0.6500	0.1161
	18	18	17	19	18

---------- Day=14 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
Cholesterol	-0.06773	-0.34018	-0.34759	-0.37900	1.00000
	0.7829	0.1672	0.1448	0.1209	
	19	18	19	18	20
Isoprostane	-0.01811	-0.55018	-0.60443	-0.18261	0.88696
	0.9413	0.0180	0.0061	0.4683	<.0001
	19	18	19	18	20
Triglyceride	-0.08992	-0.21188	-0.11031	-0.27419	0.75494
	0.7143	0.3986	0.6530	0.2709	0.0001
	19	18	19	18	20
Insulin	0.06918	-0.42487	-0.38898	0.34428	0.07177
	0.7919	0.1009	0.1228	0.1760	0.7772
	17	16	17	17	18
BW24thWeek	0.16729	-0.52577	-0.43634	0.11133	0.13167
	0.4808	0.0208	0.0544	0.6500	0.5800
	20	19	20	19	20
AUC	0.27392	-0.49249	-0.48877	0.38353	-0.19038
	0.2565	0.0379	0.0337	0.1161	0.4214
	19	18	19	18	20

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
Cholesterol	0.88696	0.75494	0.07177	0.13167	-0.19038
	<.0001	0.0001	0.7772	0.5800	0.4214

	20	20	18	20	20
Isoprostane	1.00000	0.63794	0.31199	0.36096	0.11743
		0.0025	0.2075	0.1179	0.6220
	20	20	18	20	20
Triglyceride	0.63794	1.00000	-0.13014	-0.04585	-0.39216
	0.0025		0.6068	0.8478	0.0872
	20	20	18	20	20
Insulin	0.31199	-0.13014	1.00000	0.90263	0.82130
	0.2075	0.6068		<.0001	<.0001
	18	18	18	18	18
BW24thWeek	0.36096	-0.04585	0.90263	1.00000	0.70336
	0.1179	0.8478	<.0001		0.0005
	20	20	18	21	20
AUC	0.11743	-0.39216	0.82130	0.70336	1.00000
	0.6220	0.0872	<.0001	0.0005	
	20	20	18	20	20

---------- Day=21 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
PLGFratio	1.00000	-0.12461	-0.16453	0.22495	-0.28830
		0.6113	0.5009	0.3545	0.2618
	19	19	19	19	17
VEGFratio	-0.12461	1.00000	0.49873	0.04567	-0.16614
	0.6113		0.0297	0.8527	0.5239
	19	19	19	19	17

VEGFR1ratio	-0.16453	0.49873	1.00000	-0.17633	-0.18558
	0.5009	0.0297		0.4571	0.4610
	19	19	20	20	18
MCP1ratio	0.22495	0.04567	-0.17633	1.00000	0.00091
	0.3545	0.8527	0.4571		0.9971
	19	19	20	20	18

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
PLGFratio	-0.15365	-0.25226	0.10055	0.28745	0.74137
	0.5560	0.4289	0.7558	0.2328	0.0004
	17	12	12	19	18
VEGFratio	-0.25218	-0.06627	0.04020	-0.07388	-0.14609
	0.3288	0.8379	0.9013	0.7637	0.5630
	17	12	12	19	18
VEGFR1ratio	-0.45579	0.25324	-0.33728	-0.14365	-0.28289
	0.0573	0.4271	0.2837	0.5457	0.2406
	18	12	12	20	19
MCP1ratio	-0.10583	0.00836	-0.03067	-0.20819	-0.00351
	0.6760	0.9794	0.9246	0.3784	0.9886
	18	12	12	20	19

---------- Day=21 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
Cholesterol	-0.28830	-0.16614	-0.18558	0.00091	1.00000
	0.2618	0.5239	0.4610	0.9971	
	17	17	18	18	18
Isoprostane	-0.15365	-0.25218	-0.45579	-0.10583	0.86011
	0.5560	0.3288	0.0573	0.6760	<.0001
	17	17	18	18	18
Triglyceride	-0.25226	-0.06627	0.25324	0.00836	0.93133
	0.4289	0.8379	0.4271	0.9794	<.0001
	12	12	12	12	12
Insulin	0.10055	0.04020	-0.33728	-0.03067	0.32355
	0.7558	0.9013	0.2837	0.9246	0.3049
	12	12	12	12	12
BW24thWeek	0.28745	-0.07388	-0.14365	-0.20819	0.34875
	0.2328	0.7637	0.5457	0.3784	0.1561
	19	19	20	20	18
AUC	0.74137	-0.14609	-0.28289	-0.00351	-0.19765
	0.0004	0.5630	0.2406	0.9886	0.4470
	18	18	19	19	17

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
Cholesterol	0.86011	0.93133	0.32355	0.34875	-0.19765

	<.0001	<.0001	0.3049	0.1561	0.4470
	18	12	12	18	17
Isoprostane	1.00000	0.75614	0.56986	0.39200	0.07427
		0.0044	0.0531	0.1076	0.7770
	18	12	12	18	17
Triglyceride	0.75614	1.00000	0.27316	0.31347	-0.07620
	0.0044		0.3903	0.3211	0.8238
	12	12	12	12	11
Insulin	0.56986	0.27316	1.00000	0.73645	0.64015
	0.0531	0.3903		0.0063	0.0339
	12	12	12	12	11
BW24thWeek	0.39200	0.31347	0.73645	1.00000	0.72094
	0.1076	0.3211	0.0063		0.0005
	18	12	12	20	19
AUC	0.07427	-0.07620	0.64015	0.72094	1.00000
	0.7770	0.8238	0.0339	0.0005	
	17	11	11	19	19

---------- Day=28 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
PLGFratio	1.00000	0.00975	-0.24920	-0.01288	0.43575
		0.9694	0.3187	0.9596	0.1568
	18	18	18	18	12
VEGFratio	0.00975	1.00000	0.30965	-0.05520	0.00934
	0.9694		0.1970	0.8224	0.9758

	18	19	19	19	13
VEGFR1ratio	-0.24920	0.30965	1.00000	-0.05178	-0.54159
	0.3187	0.1970		0.8332	0.0559
	18	19	19	19	13
MCP1ratio	-0.01288	-0.05520	-0.05178	1.00000	-0.16812
	0.9596	0.8224	0.8332		0.5830
	18	19	19	19	13

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
PLGFratio	0.21042	0.65431	-0.45570	-0.22071	-0.44335
	0.4703	0.0401	0.2177	0.3946	0.1123
	14	10	9	17	14
VEGFratio	0.13187	0.45675	-0.46773	-0.25290	-0.48965
	0.6395	0.1579	0.1728	0.3113	0.0755
	15	11	10	18	14
VEGFR1ratio	-0.41186	-0.69068	0.13052	-0.08784	0.05587
	0.1272	0.0186	0.7193	0.7289	0.8495
	15	11	10	18	14
MCP1ratio	0.07799	0.06690	-0.01044	0.03662	0.04957
	0.7823	0.8450	0.9772	0.8853	0.8664
	15	11	10	18	14

---------- Day=28 ----------

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	PLGFratio	VEGFratio	VEGFR1ratio	MCP1ratio	Cholesterol
Cholesterol	0.43575	0.00934	-0.54159	-0.16812	1.00000
	0.1568	0.9758	0.0559	0.5830	
	12	13	13	13	13
Isoprostane	0.21042	0.13187	-0.41186	0.07799	0.78087
	0.4703	0.6395	0.1272	0.7823	0.0027
	14	15	15	15	12
Triglyceride	0.65431	0.45675	-0.69068	0.06690	0.94585
	0.0401	0.1579	0.0186	0.8450	<.0001
	10	11	11	11	10
Insulin	-0.45570	-0.46773	0.13052	-0.01044	-0.45399
	0.2177	0.1728	0.7193	0.9772	0.2196
	9	10	10	10	9
BW24thWeek	-0.22071	-0.25290	-0.08784	0.03662	0.01433
	0.3946	0.3113	0.7289	0.8853	0.9629
	17	18	18	18	13
AUC	-0.44335	-0.48965	0.05587	0.04957	-0.17093
	0.1123	0.0755	0.8495	0.8664	0.6153
	14	14	14	14	11

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	Isoprostane	Triglyceride	Insulin	BW24th Week	AUC
Cholesterol	0.78087	0.94585	-0.45399	0.01433	-0.17093

	0.0027	<.0001	0.2196	0.9629	0.6153
	12	10	9	13	11
Isoprostane	1.00000	0.42567	0.05558	0.25354	0.00762
		0.1918	0.8788	0.3619	0.9803
	15	11	10	15	13
Triglyceride	0.42567	1.00000	-0.71739	-0.53358	-0.63909
	0.1918		0.0195	0.0909	0.0639
	11	11	10	11	9
Insulin	0.05558	-0.71739	1.00000	0.94199	0.88781
	0.8788	0.0195		<.0001	0.0032
	10	10	10	10	8
BW24thWeek	0.25354	-0.53358	0.94199	1.00000	0.86763
	0.3619	0.0909	<.0001		<.0001
	15	11	10	18	14
AUC	0.00762	-0.63909	0.88781	0.86763	1.00000
	0.9803	0.0639	0.0032	<.0001	
	13	9	8	14	14

VITA

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