8-2010

Regional Differences in Adipose Tissue of the Sinclair Minipig

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To the Graduate Council:

I am submitting herewith a thesis written by Suzanne Lauren Booker entitled "Regional Differences in Adipose Tissue of the Sinclair Minipig." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Naima Moustaid-Moussa, Major Professor

We have read this thesis and recommend its acceptance:

Cheryl J. Kojima, Kelly R. Robbins

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Regional Differences in Adipose Tissue of the Sinclair Minipig

A Thesis Presented for the Master of Science Degree
at the University of Tennessee

Suzanne Lauren Booker
August 2010

Dedication

This thesis is dedicated to my mother whose endearing strength and courage inspires me daily, and to my brother and sister who make life worth living.

Acknowledgements

I would first like to thank all of the generations of Native Americans, women rights advocates, anti-slavery and civil rights warriors who sacrificed everything including their lives to give me a better life and the freedom to receive a quality education. I would like to thank all the women in my family, my mom Karen, my grandmothers Geraldine and Marsha, and my great-grandmother Flora who were the best examples of strength, courage, and femininity a young girl to have. I would like to thank my grandfather George, my brother Nathan, and my sister Diane for their unconditional love. I would also like to thank Mike for his support and intellectual advice.

I would like to thank my advisors Dr. Naima Moustaid-Moussa and Cheryl Kojima whose constant support academically and personally made this study possible. I would also like to thank Kelly Robbins whose instruction went beyond the classroom. I would like to thank Nishan Kalupahana, Nalin Siriwardhana, and Sarah Melton who taught me so much in the lab and otherwise. I would like to extend appreciation to Wenting Xin, Cristina Campistol, Karianne Chung, Tabitha Sawyer-Cooper, Ken, Roger, and Tammy who were always there to lend a hand. Thanks to the minipigs who made the ultimate sacrifice for science. Finally, I would like to thank the animal science department for providing me the opportunity to study.

Abstract

Adipose tissue is an endocrine organ, and its homeostatic mechanisms in normal weight, overweight and obese subjects must be elucidated. We sought to determine the basal adipose tissue biology of visceral (VIF) and subcutaneous (SQF) fat depots in 8 month old Sinclair minipigs, an animal that has been shown to be physiologically similar to humans.

Metabolic analysis showed a decrease in LDL, white blood cells (WBC), and lymphocyte percentages as the minipigs aged from 6 to 8 months ($p < 0.0001$ and $= 0.0046$ and 0.0165 respectively). There were no significant changes in triglycerides, HDL, VLDL, and neutrophil percentages. There was a trend in insulin increase ($P=0.0722$).

Microarray analysis was performed to determine transcriptome differences between VIF and SQF. When VIF was compared to SQF, expression of a total of 788 transcript ID's differed: were 240 up-regulated and 548 down-regulated. Examples included hydroxysteroid 11-beta dehydrogenase 2, fatty acid synthase, IL-18, and platelet factor 4 which were all up-regulated in VIF vs. SQF. The down-regulated transcripts included estrogen receptor 1, insulin-like growth factor binding protein 5, and platelet derived growth factor D.

When SQF was compared to VIF, a total of 598 transcript IDs were up or down-regulated by more than a 2 fold difference ($P<0.05$). From this subset of the transcriptome, we found 471 IDs were up-regulated in SQ fat, and 127 were down- regulated. Interestingly, the up-regulated genes included prostaglandin F2 receptor negative regulator, estrogen receptor 1, thrombospondin 1, lipoprotein related receptor protein 2, and platelet derived growth factor D. Down-regulated genes in SQF compared to VIF included IL-18, platelet factor 4, cyclooxygenase, and fatty acid synthase. We found no significant difference in gene expression between SQF and VIF TNF alpha, TLR 4, and adiponectin in our study.

Immunofluorensce (IF) assay revealed that SQF expressed more CD 163 positive (alternatively activated) macrophages than VIF, and little to no CD 68 (classically activated) positive macrophages. Additionally, VIF expressed more CD 68 positive macrophages compared to SQF.

The data from this study is consistent with the human and rodent literature which states that VIF is more metabolically active and pro-inflammatory compared to SQF.

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Abbreviations

Apo E - Apolipoprotein E
ATM - Adipose Tissue Macrophages
BAT- Brown Adipose Tissue
BMI - Body Mass Index
CCD - Cooled Charge- Coupled Device camera
CD 68 - Cluster of Differentiation 68
CD 163- Cluster of Differentiation 163
CTP - Cytidine Triphosphate
DAVID - Database for Annotation, Visualization and Integrated Discovery
DP - Domestic Pig
DXA - Dual Energy X-Ray Absorptiometry
EASE - Expression Analysis Systematic Explorer
ELISA - Enzyme-Linked-Immunosorbent Serologic Assay
ERα -Estrogen Receptor alpha
ERβ -Estrogen Receptor beta
E2 - Estradiol
FAS - Fatty Acid Synthase
FDR – False Discovery Rate
FTO - Fat Mass and Obesity associated genes
GCRMA - Guanine Cytosine Robust Multi-Array Analysis
HDL- High Density Lipoptrotein
IF - Immunofluorescence
IκB- Inhibitor Complex kappa B Inhibitor Complex
Ikk - IkB kinase complex
Il- 1 - Interleukin 1
Il- 1α - Interleukin 1α
Il- 1β - Interleukin 1β
Il- 6 - Interleukin 6
Il- 8 - Interleukin 8
Il- 10 - Interleukin 10
Il- 18 - Interleukin 18
Kg - Kilogram
LDL - Low Density Lipoprotein
LPS – Lipopolysaccharide
M1- Classically Activated Macrophages
M2- Alternatively Activated Macrophages
mL- Mililiter
MP – Minipig
$NADH_2$- Nicotinamide Adenine Dinucleotide
$FADH_2$- Flavin Adenine Dinucleotide
MCP-1- Monocyte Chemotactic Protein 1

NO- Nitric Oxide
NEFA- Non-Esterfied Fatty Acid
NFκB- Nuclear Factor kappa B
NPY - Neuropeptide Y
PAI-1 - Plasminogen Activator Inhibitor 1
PPAR - Peroxisome Proliferation Agonist Receptor
PPARγ - Peroxisome Proliferation Agonist Receptor gamma
PUFA - Polyunsaturated Fatty Acid
qRT-PCR - Quantitative Real-Time Polymerase Chain Reaction
ROS - Radical Oxygen Species
RPM - Rotations Per Minute
SFA- Saturated Fatty Acid
SQF - Subcutaneous Fat
T2D - Type 2 Diabetes
TBS- Tris Buffered Saline
TBST- Tris Buffered Saline with 0.1% Tween
T-reg - Regulatory T- cells; Suppressor T - cells
Th1 - T-Helper Cell
Th2 - T-Helper Cell
TLR 2 - Toll-Like Receptor 2
TLR 4 - Toll-Like Receptor 4
TNFα - Tumor Necrosis Factor alpha
TNFR1 - Tumor Necrosis Factor Receptor 1
TNFR2 - Tumor Necrosis Factor Receptor 2
μL - Microliter
UCP-1 - Uncoupling Protein 1
UTP- Uridine-5'-triphosphate
VLDL - Very Low Density Lipoprotein
VIF - Visceral Fat
WAT- White Adipose Tissue

Introduction

Adipose tissue is now widely accepted to be an endocrine organ, secreting many different cytokines, adipokines, proteins, and other circulating factors important in homeostasis regulation. Adipose tissue is also a heterogeneous tissue composed of adipocytes (fat cells), pre-adipocytes (mesechymal stem cells), endothelial cells, and immune cells (macrophages and neutrophils). The heterogeneous nature of adipose tissue is due in part to its role as a major regulator of whole body energy homeostasis.

The excessive adiposity in obesity leads to dysregulation of the adipose tissue biology and a disruption of whole body energy homeostasis. Obesity has been well characterized as a state of chronic low grade inflammation.

The body is not designed to have a prolonged exposure to immune cells, cytokines, hormones, and peptides associated with inflammation. Although the levels of these cells and factors never reach those seen during an acute phase response, chronic low levels of inflammation caused by obesity can lead to disease if the body is exposed to them over a long period of time.

In order conduct clinically relevant studies in adipose tissue biology and the metabolic consequences of obesity, a physiologically relevant model needs to be employed. The traditional model of choice has been that of the rodent model due to their prolific nature, small size, well supported analytical tools, and the ability to develop transgenic and knockout models. A major disadvantage of using rodent models is some of their metabolic and physiologic characteristics do not always allow for a direct correlation with human metabolism and physiology. It is therefore necessary to use an intermediate model to bridge the gap between rodent data and human clinical. We contend that the the Sinclair minipig is ideally suited to be this model.

The pig is an excellent model for human studies due to its gastrointestinal and cardiovascular similarities as well as a similar lipid profile and metabolism. Like any animal model, there are advantages and disadvantages to using a pig including a variety sub-species,

domestic and mini, which have different suitability in human studies. One disadvantage in using the domestic pig is the differential deposition of fat. With a change in consumer preferences, the domestic pig has been genetically selected to deposit lean mass preferentially therefore creating an obese domestic pig would require a great deal of feed and housing space. An alternative would be the miniature "mini" pigs. Minipigs have retained the feral phenotype and preferentially deposit fat in positive energy balance. Additionally, minipigs are smaller and therefore require less housing and feed. The Sinclair minipig, on breed of minipig, was developed in the 1940's and has been used a human model for studies including osteoporosis, toxicology, cardiovascular disease, and dermatology.

Based on its previously demonstrated clinical relevance to humans, we propose that the Sinclair minipig would be a good model to conduct adipose tissue and obesity studies. The Sinclair minipig adipose tissue has been relatively under characterized. In this study we hope to characterize the basic biology of subcutaneous (SQF) and visceral (VIF) of the Sinclair minipig through transcriptome analysis, measures of macrophage infiltration and phenotype, adipocyte and macrophage number, and analysis of key proteins relevant to adipocyte biology, inflammation, and metabolic disorders.

Chapter I – Literature Review

Section 1: An Overview of Obesity

1.1 What is Obesity?

Obesity is defined as an excess of body fat or adiposity. It is commonly assessed using the Body Mass Index (BMI), a measure of adult adiposity that is calculated by dividing a person's weight (in kg) by their height (in m^2)[1]. An individual is classified as normal weight with a BMI between 18.5 to 24, overweight with a BMI from 25 to 30, and obese with a BMI of 30 or greater. A third and fourth category of BMI have been added, morbidly obese and super obese, to classify a person with a BMI over 40 and 50 respectively [2]. Alternately, overweight and obesity can be classified by grade: 1 (BMI of 25-30), grade 2 (BMI 30-35), and grade 3 (BMI 5-40) obesity. The BMI was agreed upon as a common method of weight categorization because it was an evidence-based, practical, less invasive and cost effective method that could be used clinically by primary care physicians. There are however, other more accurate ways to asses obesity by directly measuring the amount and distribution of adipose tissue such as waist circumference, waist to hip ratio, and dual energy X-ray absorptiometry (DXA) scanning[3] [4].

Classifying people as normal weight or obese relates a person's risk for developing a number of co-morbidities associated with a higher weight status. Individuals with a weight status above 30 are at higher risk for a number of diseases including type 2 diabetes, cardiovascular disease, osteoarthritis, and certain types of cancer[5].

1.2 Obesity – Not Just for the Wealthy Anymore

Historically, obesity was an affliction of the rich, and therefore was an external signal of social status to strive toward. Until the last century, food supply was often inconsistent to the majority of the population so the ability to overeat enough to gain weight was an indication of excess and true social superiority. However, obesity has become more prevalent in those with a lower socioeconomic status in westernized societies in the 20th and 21st centuries [6]. The

incidence of obesity has greatly increased in the last 20 years (Figure 1.1); however, the trend of an increase in sedentary lifestyle, one postulated cause of the rise in obesity, has been noted since the 1950's [7]. In the United States, 67% of the adult population is overweight or obese and 33% of all adult Americans are obese[8].

This trend began primarily in westernized societies, but has spread to become a problem globally. Currently, the most rapid increases in obesity are occurring in Mexico, China, and Thailand[6]. Taking into account the rise in the rate of obesity, the projected life expectancy is expected to plateau or decline despite advances in medical technology that would otherwise confer a longer life expectancy[9].

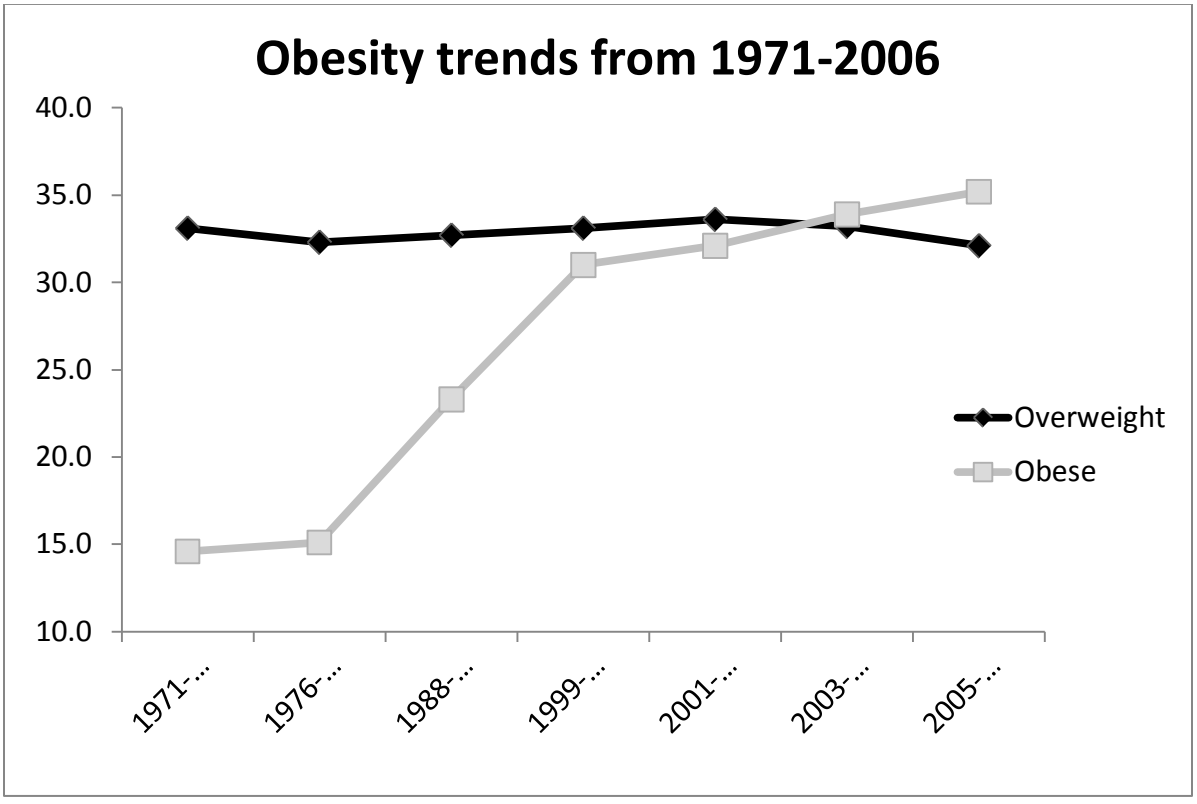


Figure 1.1: The increasing rate of obesity in the United States from 1971-2006 based on NHANES survey data

*Adapted from the CDC "Health, United States 2009" report

1.3 Early Obesity

Rates of childhood and adolescent obesity have increased coincidentally with adult obesity. Approximately 30% of the children in the United States are overweight, and as many as 39% are overweight in western Europe [5]. Having a higher than normal BMI, especially for those under the age of 30, has been shown to reduce life expectancy by as much as 20 years [10]. Additionally, overweight and obese children have lower plasma adiponectin concentrations and higher circulating concentrations of pro-inflammatory markers such as IL-6, and C-reactive protein, indicating a metabolically unfavorable profile [11]. Moreover, overweight and obese children are more likely to be overweight and obese in adulthood, and consequently are at an increased risk for obesity related diseases [5, 11].

The maternal environment is now recognized to be a causative factor in childhood, adolescent, and adult obesity. A child born to an overweight or obese mother has increased risk childhood obesity, a higher relative fat mass at birth, and a higher birth weight; all of which place the child at risk for obesity, the metabolic syndrome, and diabetes in adulthood [12]. Given this information, it is now imperative to begin lifestyle interventions in women of childbearing age, as one approach to combat the rise in obesity.

1.4 Nature vs. Nurture – Why is Obesity on the Rise?

A significant body of literature suggests that the increase in the availability of energy dense food along with the increase in a sedentary lifestyle is responsible for the increase in overweight and obesity; the other suggests that genetic and/or epigenetic changes have lead to the increase in obesity [13, 14].

Epigenetic changes and genetic polymorphisms have been demonstrated to alter genetic susceptibility to obesity. It has been postulated that 40-60% of obesity-related phenotypes may be heritable [15]. The Pima Indians of southwestern United States and the Sierra Madre mountains in Mexico are a well documented example of a genetic predisposition towards obesity and its co-morbidities.

The population had become well adapted to living in an arid, desert environment with the majority of their food being gathered from the landscape vegetation. It is postulated that the Pima Indians as well as other indigenous peoples have become so adept at extracting and storing nutrients from the food they eat that upon consumption of the energy dense food that is available today, they are at a greater risk for obesity and type 2 diabetes [16]. The Pima Indians that live in the United States are 3-10 times more likely to be obese for males and females respectively, and 5.5 times more likely to have diabetes compared to the Pima Indian population that still lives in Mexico [16].

One specific genetic influence leading to the rise in obesity rates are polymorphisms in the fat mass and obesity associated (FTO) genes [17]. The FTO gene is hypothesized to be involved in appetite control in relation to whole body energy homeostasis [18]. Individual genetic differences confer differing susceptibility to obesity its co-morbidities in conjunction with a sedentary lifestyle [19].

An entirely genetic explanation for the rise in obesity is an insufficient explanation of the current obesity rates because allelic frequencies within a population do not change as quickly as the rate of obesity has increased. The alleles making one susceptible to obesity have been present for quite some time, so there must be alternative reasons for the rise in obesity rates. Indeed, the literature suggests that the rise in obesity rates is due to an increasingly sedentary lifestyle coupled with an increase in the availability and consumption of energy dense food [16, 20]. In contrast to current nutritional trends, hypocaloric diets have been shown to increase both life expectancy and quality of life (i.e. decrease in degeneration of tissues and disease processes) in c.elegans, mice, rats, and primates [9, 21, 22].

Given the association between lifestyle, genetics, and obesity, scientists have begun to explore a third mechanism driving the increased rates of obesity, epigenetics. Epigenetics, as defined by Jaenich et al, refers to heritable differences in gene expression that are not caused by changes in the DNA sequence; namely DNA methylation of CpG, histone modifications, chromatin modifications, and autoregulatory DNA binding proteins expression [23, 24]. Epigenetic changes are normal, and can be advantageous in terms of natural selection. However, in instances such as obesity, the environmental factors contributing to obesity are thought to

change the epigenetic profile predisposing the offspring of obese individuals to obesity [24]. Given all of this information, the causality of obesity is truly an interaction of genes and the environment necessitating a multidimensional solution.

1.5 Obesity – Is it all a Mental Battle?

The regulation of appetite is essential for the survival of every organism. It is critical for a organism to properly regulate when it feeds, and what it feeds upon. In mammals and other animals, appetite and satiety are thought to be controlled by a combination of cues from the gastrointestinal system, adipose tissue, and the hypothalamus [25]. In humans and many other animals including human, appetite regulation is based upon satiety cues, time of day, and the time of year [26, 27].

Neuropeptide Y (NPY) is a neuropeptide secreted from the hypothalamus in response to body cues indicating low nutritional status, and it is essential in appetite regulation [28]. Neuropeptide Y stimulates an increase in appetite, especially in carbohydrate rich foods [29]. Interestingly, NPY secretion is inhibited by leptin, an adipokine whose levels are positively correlated with adiposity. Paradoxically, overweight and obese patients have been found to have increased levels of leptin and NPY, but this may be due to a phenomenon called leptin resistance which will be discussed later in this thesis [30]. Additionally, it is theorized that the obese person has an altered physiological set point, a determinant of the point in which the body detects critical food intake deficit [31]. Therefore during the course of weight loss, the body preferentially stores nutrients as fat in anticipation of a prolonged decrease in nutrient availability;, in other words, the body senses starvation rather than healthy weight loss and releases NPY [32].

1.6 Obesity as a Risk Factor for Disease

As is often the case, a financial incentive was the first motivation to examine the health risks of obesity. As early as the 1950's the life insurance industry recognized obesity as a risk factor for death and charged higher premiums accordingly [7]. Whatever causes the onset of obesity in an individual (Figure 2.1), the resultant diseases costly both physiologically and financially. The metabolic syndrome is a group of metabolic and physical abnormalities including central obesity, elevated triglycerides, reduced HDL cholesterol, elevated VLDL cholesterol elevated blood pressure, and elevated fasting glucose, which predispose and individual to the development cardiovascular disease and/or type 2 diabetes (T2D) [33]. The prevalence of the metabolic syndrome in the United States is 24% in some European countries it is as high as 30.9% [33]. In addition to the increased risk for T2D, cardiovascular disease, and the metabolic syndrome, obesity increases an individual's risk for gallbladder disease, arthritis, gout, various gastrointestinal disorders, renal disease, acute pancreatitis, stroke, and even death [7, 34, 35]. Additionally, obesity is a standalone risk factor for colorectal, gallbladder, prostate, uterine, endometrial, cervical, breast, ovarian, pancreatic, esophageal, and renal cancers as well as non- Hodgkins lymphoma [33-35]. Alarmingly, the highest obesity- related mortality rates are in those individuals younger than 30 [36].

Special attention must be given to obese women of reproductive age, for obesity makes pregnancy a more precarious endeavor. Maternal obesity is associated with an increased risk for preterm delivery, preeclampsia, and gestational diabetes, postpartum hemorrhage, lactation difficulties, cesarean section and delivery complications [12].

1.7 The Financial Burden of Obesity

The financial burden of obesity, its co-morbidities and related disorders is shared by employers, the government, and the individual. An overweight or obese person will face real discrimination from potential employers. Studies have shown an employer is less likely to hire an overweight or obese person with similar qualifications to a normal weight individual [37]. Furthermore, an individual with a higher weight status is statistically more likely to file a worker's compensation claim, be absent more often, and have lower work productivity due to an obesity related disease [38, 39]. Finally, an overweight or obese individual, on average, earns 1-6% less than a comparable normal employee [40]. An equally important financial ramification is the increase in healthcare costs associated with obesity and related co-morbid conditions. If the rate of obesity continues to increase as it is projected, the healthcare costs for obesity and obesity related diseases will to double ever 10 years [41]. Overall, the cost of healthcare for obesity related diseases is lower than initial projections due to the increased mortality of these individuals; however, their average annual cost is estimated at $140 billion a year [42]. A simultaneously alarming and potentially large contributing factor to the cost of obesity is the rise in childhood obesity; a trend which has resulted in a tripling of pediatric healthcare costs of obesity since 1979 [43].

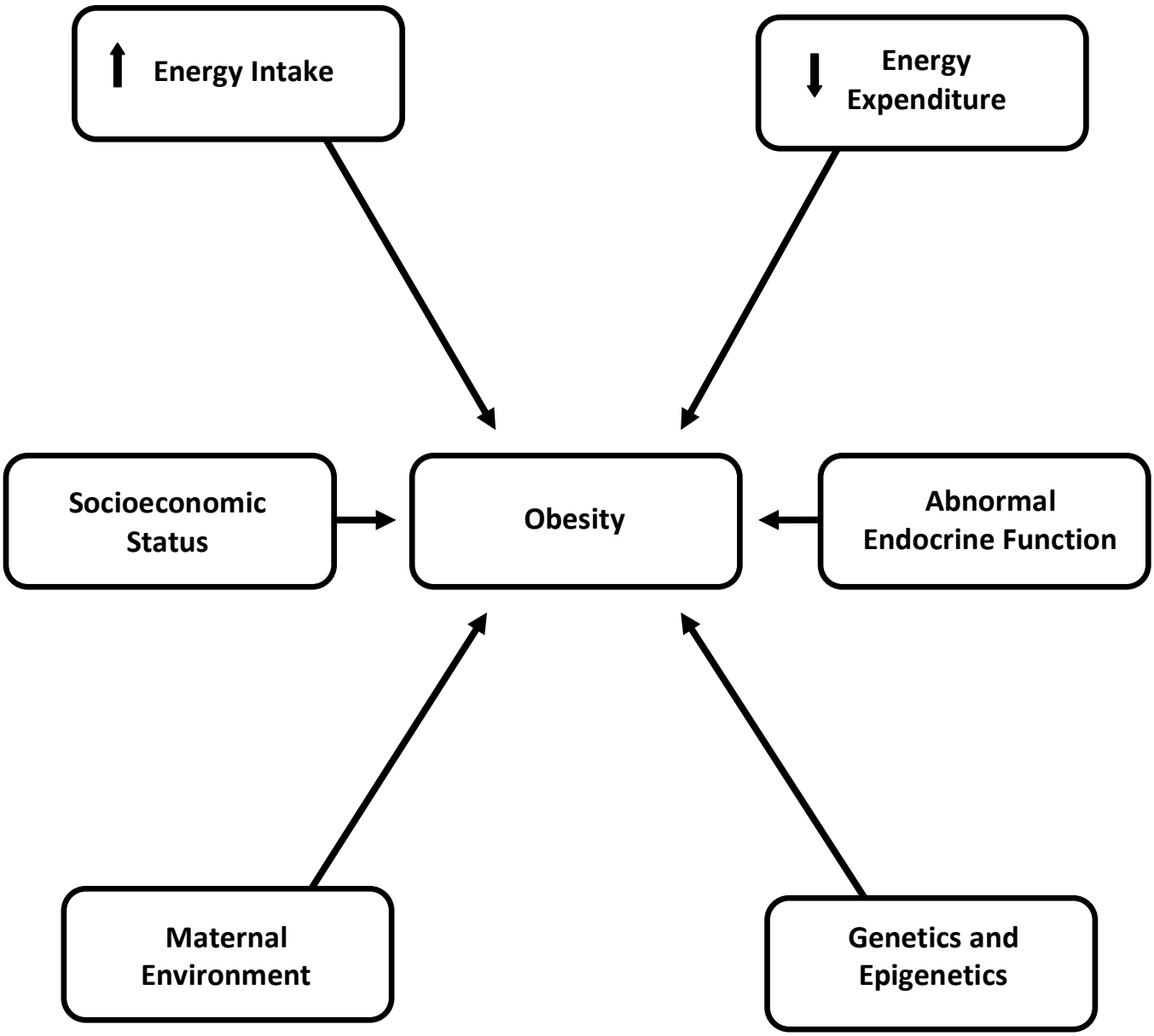


Figure 1.2: The predominant causes of obesity

*Modified from obesityinyouth.org

1.8 From Problem to Solutions – Potential Obesity Treatments

Now that obesity has been established as a serious problem of the 21st century, solutions and intervention targets must be identified. There are multiple life stages where programs could intervene to prevent or treat obesity.

First, diet and behavior modification needs to be a cornerstone in an intervention. Behavior interventions aimed at reducing caloric intake, increasing physical activity, and increasing the consumption of nutritious foods (and possibly supplements such as botanical extracts for fish oil), are critical and fundamental to reducing or reversing the obesity trend [44-46].

Second, health education to women of reproductive age about the importance of the fetal environment is imperative; educating potential mothers about the benefits of weight loss to both themselves and their unborn child is critical [47, 48].

Finally, governmental policy needs to be changed to promote positive health behavior. The relative low cost and high availability of energy dense, processed, nutrient poor food products contribute to the problem. Potential solutions include taxes on sugary foods and sweetened beverages, subsidies for healthier foods, incentives for quality supermarkets in low income areas, and mandates for healthier food choices in schools [14].

There are many potential interventions for obesity ranging from governmental policy to personal education that can be used to facilitate a healthier, leaner population.

Section 2: Adipose Tissue – An Endocrine Organ

2.1 Adipose Tissue – a Composition

2.1.1 Adipocytes

Adipocytes are the major cellular component (60%) of adipose tissue (Figure 1.3), and the major mechanism of the balance between lipid storage and release to maintain nutritional homeostasis [49, 50]. Moreover, adipocyte dysfunction is a major mechanism leading to the onset of the metabolic syndrome.

The adipocyte is responsible for the secretion of pro-inflammatory and anti-inflammatory cytokines and adipokines including leptin, TNFα, IL-6 and adiponectin. In obesity, the adipocyte becomes a pro-inflammatory cell; a net result of many physiological processes [51]. As the adipocyte expands due to over nutrition (hypertrophy), there is an increase in lipolysis, pro-inflammatory cytokine secretion (IL-6 and TNFα), immune cell infiltration, and a decrease in the secretion of anti-inflammatory adipokines and cytokines (adiponectin and IL-10) [52, 53]. When the hypertrophic adipocyte becomes overly lipid laden, the extracellular matrix that surrounds it is unable to compensate by expansion leading to a hypoxic state, an increase in pro-inflammatory cytokine release, and eventually cell death. This leads to macrophages and other immune cells of into adipose tissue, and a further increase in pro-inflammatory cytokine secretion [52, 54]. Therefore, the adipocyte (as well as the tissue that surrounds it) contributes significantly to disease pathogenesis which leading to the metabolic dysfunction associated with obesity [53].

2.1.2 Pre-Adipocytes

Pre-adipocytes are pluripotent mesenchymal stem cells that have the ability to differentiate into bone, cartilage, and adipocytes depending on the factors present [55, 56]. Pre-adipocyte proliferation or hyperplasia is a mechanism by which the adipose tissue allows for plasticity within itself to compensate for an increase in lipid accumulation. The ability to compensate for excessive lipid accumulation confers protection to the individual from developing the metabolic

diseases associated with adipocyte hypertrophy in obesity [57, 58]. The major feature of these metabolically normal obese subjects is the ability to compensate for the increased fat mass in by increasing the number of pre-adipocytes rather than enlarging the mature adipocytes [52]. The ability of the pre-adipocytes to differentiate is a major potentiator of metabolically favorable obesity. Ominously, pre-adipocytes have been shown to change their phenotype to one that resembles a macrophage in response to an increase in inflammation in the adipose tissue [58]. Therefore, pre-adipoctye biology plays an important role in the development of obesity as well as obesity associated immune dysfunction.

2.1.3 Macrophages

The third major cell population in adipose tissue is resident macrophages, a common feature in most other tissues in the body. The number of adipose tissue macrophages (ATM) is directly correlated with the amount of adipose tissue present in an individual. There are two major macrophage phenotypes expressed in adipose tissue; classically activated macrophages (M1) and alternatively activated macrophages (M2); the predominant phenotype present depends on the depot as well as the metabolic status of the individual [58, 59].

The pro-inflammatory M1 macrophages are stimulated by bacteria lipopolysaccharide (LPS) as well as saturated fatty acids [60]. The pro-inflammatory nature of M1 macrophages is considered to be a major contributor to the pathogenesis of the metabolic syndrome due to the release of pro-inflammatory cytokines such as TNF alpha, IL-6, nitric oxide, and reactive oxygen species[61 60, 62]. These macrophages are found in greater number both in obese subjects and in VIF relative to SQF [63]. In addition, the M1 macrophages have been shown to surround dead and dying adipocytes forming “crown like structures” [64-66].

The M2 macrophages are considered to be anti-inflammatory and are induced by monounsaturated fatty acids, peroxisome proliferator activated receptor gamma and delta, IL-4, IL-13, and release the anti-inflammatory cytokine IL-10 [67, 68]. These macrophages are the predominant phenotype in lean individuals, and have been directly correlated with increased sensitivity to insulin, oxidative phosphorylation, and fatty acid beta oxidation [60].

2.1.4 Other Cell Types

Total white blood cells count is positively correlated with BMI [69]. Further, endothelial cells and T-lymphocytes are also present in adipose tissue[69, 70] . Additionally, neutrophils are components of the innate immune system, often present in the initial stages of an inflammatory response, and are considered part of an acute phase response [71] . Interestingly, neutrophils have been found to infiltrate VIF as early as one day after high fat feeding in rodents, providing additional evidence that saturated fatty acids are inflammatory mediators [72]. Adipose tissue secretes IL-8, a chemokine that is necessary and sufficient to recruit and maintain neutrophil infiltration [70, 72].

Endothelial cells become important in neutrophil recruitment because secretion of IL-8 from the endothelial cells allows for adherence and migration of the neutrophils into host tissues.

T-lymphocyte phenotype is influenced by the macrophage population and cytokine profile in the adipose tissue. In obesity there is a change in lymphocyte phenotypes from T-regulatory (T-reg) and Th2 (induced by M2 macrophages) to Th1 (induced by M1 macrophages), or from an anti to pro inflammatory state [59]. In general, the obese state causes a change in the physiology of the adipose tissue as well as the whole body due in part to the change in the cellular composition of the adipose tissue, its remodeling, and the corresponding change in secreted factors.

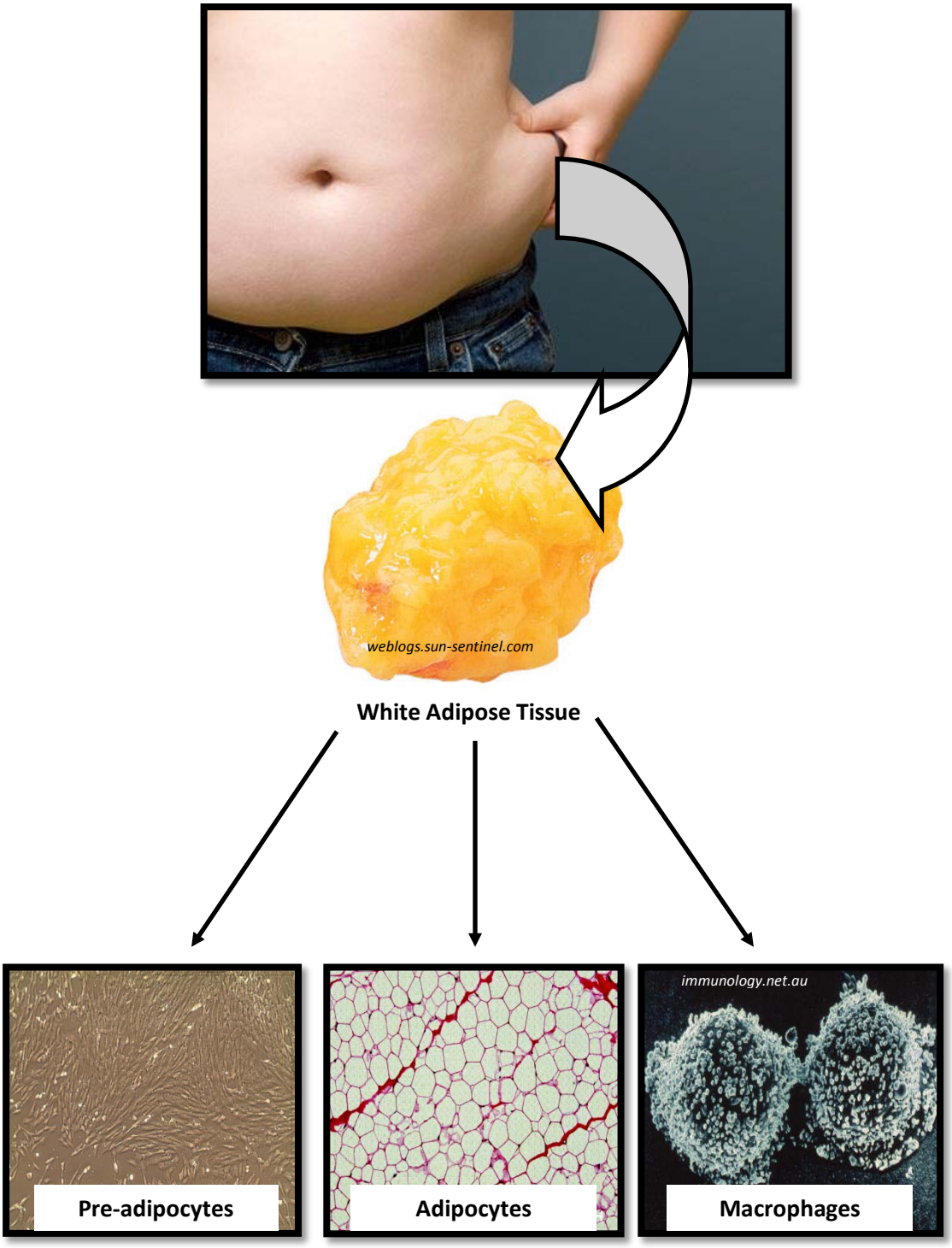


Figure 1.3: The predominant cell types of adipose tissue: adipocytes, pre-adipocytes, and macrophages

2.2 Why Do We Need Fat? – The Role of Adipose Tissue

With the dramatic rise in obesity rates over the last 30 years, it would be prudent to ask why we need adipose tissue. Additionally, if an excess of adipose tissue seems to be the problem, a logical solution would be to remove it entirely, and thus, remove the problem. Superficially, this sounds like a logical solution; however, adipose tissue is more than a storage mechanism in our body. Adipose tissue is, in fact, considered an endocrine organ and a major homeostatic mechanism in animals [73]. In fact, removal of the adipose tissue by liposuction results in a an increase in adipose tissue mass, especially visceral, long term[74]. Both excess adiposity (obesity) as well as lipodystrophy lead to metabolic disorders [75, 76]

As an endocrine organ, adipose tissue secretes cytokines, adipokines, and other peptides with autocrine, paracrine, and endocrine function. Some of these cytokines and peptides are found in other tissues (TNFα, IL-6,angiotensinogen. etc), others are adipose tissue specific (adiponectin)[77].

Adipose tissue is used as an internal barometer of sorts for other physiologic and metabolic processes; a role that is intuitive when one considers that the location and consumption of food is a primary focus of life. Thus, many physiologic processes are directly and indirectly influenced by nutrition status as indicated by the afore mentioned adipose tissue secretions [78]. Numerous studies have shown that nutrition status as indicated by adipose tissue influences processes as diverse as reproduction, satiety, immune function, and insulin sensitivity [77-79]. Once the almost ubiquitous nature of adipose secretions is considered, the ramifications of its dysregulation (obesity or lipodystrophy) are understandably far reaching.

In addition to its endocrine and metabolic roles, adipose tissue is a storage depot as well as a means for thermoregulation. To complicate matters further, there are two types of adipose tissue, brown and white, with different physiologic properties and metabolic profiles [78]. Adding a final layer of complexity, white adipose tissue has been characterized as having different metabolic and endocrine functions depending if the depot is subcutaneous or visceral [80]. In exploring causative elements of obesity, it is critical to explore the crux of the problem, the adipose tissue

2.3 If it's Brown, Lay it Down – The Brown Fat Paradox

The classification of obesity indicates the degree of adiposity, and more specifically a qualitative measurement of the amount of white adipose tissue (WAT) present in the body. However, there is another type of adipose tissue, brown adipose tissue (BAT), present at varying quantities and stages of life in many mammalian species [81].

Brown adipose tissue is biochemically and morphologically different than WAT, releasing energy in the form of heat rather than just storing it in the form of triglycerides. Additionally, it is more closely related to skeletal muscle than the other type of adipose tissue WAT [82] . Brown adipose tissue is affectionately named for its darker appearance due to the large number of mitochondria present; it functions primarily to generate heat via non-shivering thermogenesis [83, 84]. Heat generation is accomplished by what is termed "proton leakage" or "proton conductance" (Figure 1.4) [84], a process that is specific to BAT [81]. A mitochondrial specific protein, uncoupling protein 1 (UCP-1) facilitates proton movement back into the inner mitochondrial membrane; however, instead of generating the normal ATP, energy is released as heat [84-86]. It is noteworthy to mention that pigs do not have brown fat. It is estimated that UCP-1 expression was lost approximately 20 million years ago[87]. Moreover, there is limited evidence for the presence of brown fat in adult human beings.

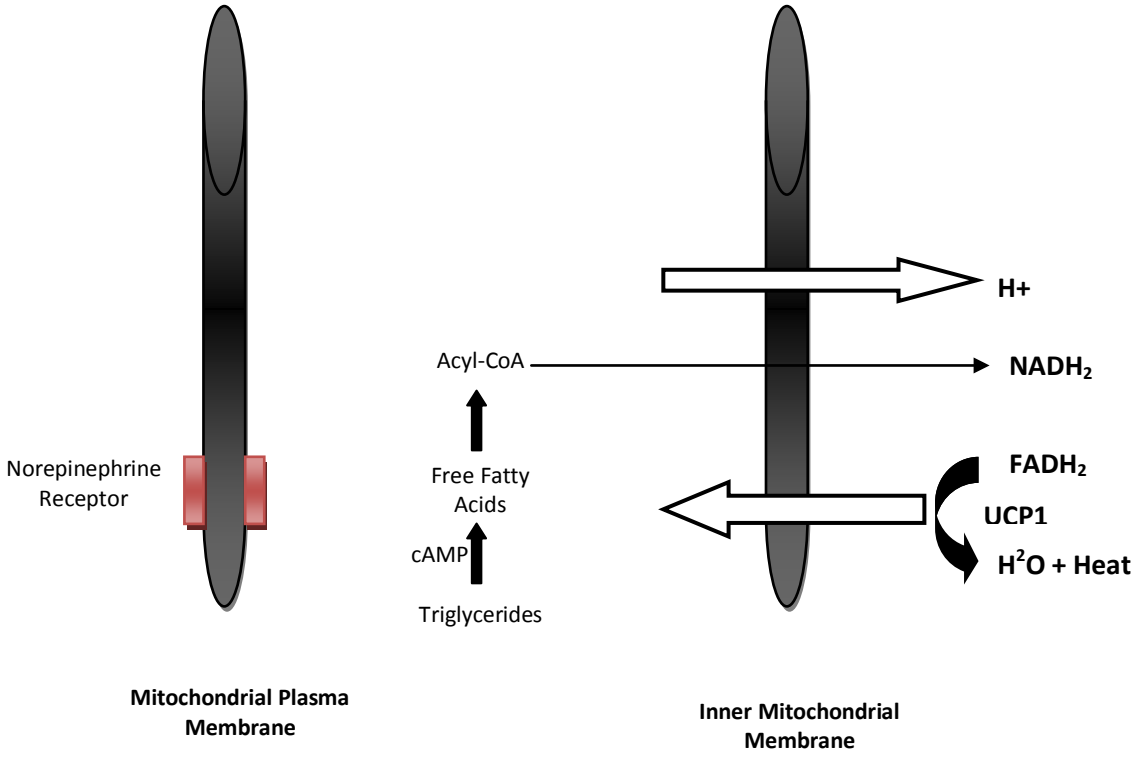


Figure 1.4: Thermogenesis via proton uncoupling in brown adipose tissue (adapted from [84])

2.4 A Tale of Two Depots – Subcutaneous Fat

Until recently, white adipose tissue was thought to be an inert storage depot, a physiological savings account to keep us nourished in times of famine and save energy in times of excess. Adipose tissue has in fact been discovered to be quite metabolically active, though specific effects on the body are determined by depot location, proximity to organs and vasculature, the amount of adiposity in the body, type of food consumed, and immune cell population [88]. In addition to the above conditions of function, there are metabolic and physiologic differences based on the location of the fat depots. Subcutaneous fat generally has favorable effects on obesity related complications by secreting anti-inflammatory adipokines such as adiponectin, but excess subcutaneous fat has also been linked with up-regulations of pro-inflammatory markers such as NFκB [89]. Further, studies have demonstrated that there are gender and age differences in relation to the deposition of VIF and SQF [90, 91].

In general, women preferentially store fat subcutaneously rather than viscerally until menopause, when storage tendencies switch to visceral [92]. Interestingly, weight loss in SQF is harder to achieve due to its decreased sensitivity to lipolysis [93].

Previously, it was thought that the adipose tissue closest to the vital organs, VIF, was the most metabolically active, and hence responsible for many, if not most, of the negative health risks associated with obesity [94]. Evidence now suggests errors in another paradigm related to obesity and white adipose tissue; SQF may play a more active role in obesity complications than previously believed. Studies have demonstrated metabolic and histological differences between deep and superficial SQF [95]. The metabolic and histological differences associated with the different sub layers of subcutaneous fat may account for the reported discrepancies in various studies involving SQF. Deep SQF arises from the mesenchyme and accumulates faster than superficial SQF [96].

It has been thought that the regional differences in SQF arise from potential differences in function. The superficial layer is hypothesized to be deposited for thermoregulation, whereas the deep layer is hypothesized may function in a more metabolic capacity that is comparable to visceral fat [96]. To further complicate matters, the different subcutaneous sub layers have differing correlations to insulin resistance, cardiovascular disease, and therapeutic treatments. The debate continues as to whether, is visceral or abdominal adiposity is more closely associated with the metabolic consequences of obesity [95, 97].

2.5 A Tale of Two Depots – Visceral Fat

Visceral fat has often been characterized as the more metabolically active WAT depot to its high synthesis and secretion of pro-inflammatory cytokines (TNFα, IL-6, PAI-1, MCP-1), low levels of anti-inflammatory cytokines and adipokines (IL-10 and adiponectin), and high correlation with the incidence of T2D, insulin resistance, hypertriglyceridemia and cardiovascular disease [94, 98]. However, there is some debate as to whether abdominal or visceral adiposity specifically is responsible for the negative metabolic effects associated with overweight and obesity. Abdominal obesity includes distinct regions of deep subcutaneous adipose tissue as well as visceral [76]. Moreover, waist to hip ratio as well has waist circumference has been shown to correlate more accurately with the metabolic consequences of overweight and obesity than has BMI [99, 100]. For simplicity, the remainder of this section will focus on visceral adiposity and its metabolic consequences.

One major contributor to obesity related complications is the release of non-esterfied fatty acids (NEFA) [101]. Visceral fat has been shown to release more NEFA, a relationship that is directly correlated with weight status. Paradoxically, there is an increase in VIF lipolysis, with a concomitant increase in lipogenic activity that further serves to increase the level of NEFA in the body. The NEFA's released from VIF are directly released into hepatic portal circulation leading to an increase of NEFA's in general circulation [89]. Increases in circulating NEFA's may lead to increased hepatic and skeletal muscle steatosis, potential contributors to insulin resistance, and increasing the risk for T2D [102].

Both SQF and VIF can be infiltrated by immune cells in overweight and obese states, however, the quantity and phenotype of immune cell infiltration is generally pro-inflammatory [69, 103]. Additionally, some environmental and metabolic factors lead to an increase in the expansion of VIF; these include age, low levels of estrogen, lack of physical activity, increases in circulating NPY, and a high fat and/or high sugar diet [29].

Section 3: Adipose Tissue Secretions – Adipokines

3.1 What are Adipokines?

By definition, adipokines are cytokines secreted specifically from adipose tissue. Although a number have been identified in recent years (resistin, visfatin, adipsin, adiponectin, and leptin) [104-106], we will focus here on leptin and adiponectin the most well characterized, well known, and metabolically relevant adipokines.

3.2 Adiponectin

Adiponectin is the most abundantly expressed adipokine in white adipose tissue, concentrations of adiponectin will decrease as adiposity (as measured by waist – to-hip ratio) increase [106]. Adiponectin exerts a receptor-mediated insulin sensitizing effect on the body. More specifically, it decreases glucose production in the liver while simultaneously increasing adipose and muscle glucose uptake and as well as increasing muscle fatty acid oxidation, via an AMPK-mediated mechanism, resulting in up-regulation of the genes coding for acyl Co A oxidase and uncoupling protein 2 [105, 106]. Another benefit of adiponectin is its ability to attenuate the activity of NFκB (discussed in detail later) via degradation of IκBα and inhibition of TNFα, the NFκB inhibitor [107]. Finally, adiponectin has been shown to be anti-atherogenic by being positively correlated with HDL cholesterol, inhibiting the expression vascular adhesion molecules, suppressing the conversion of macrophages to foam cells, and generally modulating the endothelial inflammatory response [106-108].

3.2 Leptin

Leptin, an adipokine released exclusively from white adipose tissue, is responsible for satiety in mammals ranging from humans to cats [104]. The notion of an adipose tissue specific factor that regulated satiety was first postulated by Kennedy in 1953 when he noted that "hyperphagic" mice ate large amounts of food, and preferentially chose to consume high fat oil over their chow when compared to lean mice [109]. However, it was not until the early to mid 1990's that the discovery of the ob gene in adipose tissue, and its secreted product leptin, were discovered to be one of the major satiety mechanisms of the body [110, 111].

The mice that Kennedy was studying, eventually named ob/ob mice for their genotype at the leptin locus, were severely obese, extremely hyperphagic, and had underdeveloped reproductive tracts, rendering them infertile. This set of phenotypes and related ob gene mutations have been observed in humans as well [112]. After administration of leptin, the abnormal phenotype of affected mice and humans was completely reversed [113]. Leptin supplementation was briefly thought to be the panacea cure for obesity, however, it was soon discovered that the majority of obese subjects had higher rather than lower levels of circulating leptin compared to their lean counterparts. In fact, levels of circulating leptin are positively correlated with BMI, leading to the concept of leptin resistance [114]. Further work has elucidated that leptin is demonstrated a highly metabolically active protein, having a pivotal role in reproduction, hematopoiesis, lipid metabolism, insulin sensitivity, inflammation, and energy balance [114-116].

Section 4: Adipose Tissue Secretions – Cytokines

4.1 Introduction to Obesity Related Cytokines

Circulating concentrations of adipokines and other cytokines are modulated by adipose tissue obesity. Many of the cytokines altered in the obese state are pro-inflammatory in nature, and are more often associated with protecting the body against illness or injury[11]. Conventionally, there is negative feedback mechanisms activated when the immune system is

engaged in repairing injuries and combat pathogens in order to prevent harm befalling the body. In obesity, these negative feedback mechanisms are not activated leading to overall chronic inflammation. This is because the levels of pro-inflammatory cytokines are substantially lower than those in an acute immune response, but higher than normal values. There is also a decrease in anti-inflammatory cytokines with a net result of an environment ripe for the development of diseases such as cardiovascular disease and T2D [117, 118]

4.2 Tumor Necrosis Factor alpha

Tumor necrosis factor alpha (TNFα) was first discovered in cancer patients in the late 1800's physician William Coley. He noted that some cancer patients underwent spontaneous tumor regression during an infective illness [119] He subsequently attempted to use killed bacteria cultures (known as Coley's toxins) as a treatment for cancer [120]. Following these trials it was discovered that is was not the bacteria, but rather a factor in blood stimulated the presence of bacteria, TNFα, that produced the anti-cancer, pro-inflammatory effect [120]. As an activator of NFκB (a central transcription factor of pro-inflammatory cytokines) TNFα has been implicated as a major contributor in the inflammatory pathway [121]. Tumor necrosis factor alpha has been demonstrated to be a major mediator of insulin resistance in obesity due to its ability to increase the amount of circulating pro-inflammatory cytokines, decrease adiponectin trigger enhanced lipolysis, inhibit lipoprotein lipase, and its ability to impair insulin receptor function and insulin signaling[122, 123]. Adipocyte derived TNFα release in proportional the amount of abdominal adipose tissue. There are two known receptors for TNFα (TNFR1 and TNFR2) [122, 124]. Once TNFα is bound, these receptors have the ability to solubilize, the soluble form of TNFR2 has been found to be a good measure of insulin resistance and obesity [124].

4.3 Interleukins 1, 6, and 10

Interleukin 1, 6, and 10 are primarily secretory components of the innate immune system. IL-1 and IL-6 are secreted from macrophages and are an important part of the pro-inflammatory response of the body. Their secretion by adipose tissue in obesity is due in large part to the increased infiltration of macrophages [117].

Along with TNFα IL- 6 is a major mediator of the acute phase pro-inflammatory response of the body, and is one of the cytokines produced by NFκB activation [118, 125]. Levels of IL-6 have been correlated with insulin resistance, and plasma ceramide levels [126].

There are two isoforms of IL- 1 (as mentioned above is pro-inflammatory), IL-1α and IL-1β. Interleukin1β is associated with obesity related macrophage infiltration and its ability to induce an inflammatory response in adipocytes [127, 128].

Interleukin-10 differs from IL-1β and IL-6 in that it is generally considered to be anti-inflammatory [125]. Adiponectin has been shown to increase synthesis in IL-10. In obesity, circulating levels of IL-10 are decreased due in part to low levels of adiponectin [129]. Indeed, weight loss increases levels of both adiponectin and IL-10 [117].

4.4 Nuclear Factor kappa B

Nuclear Factor kappa B (NFκB) is a nuclear transcription factor, induced by receptors such as toll-like receptor (TLR) 4, which is responsible for the modulation of a number of pro-inflammatory cytokines such as TNFα and IL-6 (Figure 1.5) [130]. Its role is pivotal in the innate immune system because it is considered to be one of the first mechanisms in the immune response [131].

Nuclear Factor kappa B is activated by the removal kappa B inhibitor complex, IκB, which is dissociated via phosphorylation by IkB kinase complex (Ikk) [132, 133]. Activated IKK is triggered by initial immune system activity such as TLR binding and high levels of circulating pro-inflammatory cytokines, leading to the recruitment of immune cells that will combat an immune challenge.

In obesity, the up-regulation of NFκB is a major mechanism by which adipose tissue increases the amount of pro-inflammatory cytokines in the body [121, 134]. The up-regulation of NFκB is due in part to low levels of adiponectin, a known TNFα and NFκB suppressor, as well as an increase in M1 macrophages that secrete pro-inflammatory cytokines [135, 136]. It is therefore not surprising that dysregulation of NFκB is a major mediator of several inflammatory disease states, including obesity, asthma, some cancers, and inflammatory bowel disease [137, 138].

Section 5: Adipose Tissue Hormone Receptors and Cell Signaling

5.1 Introduction

As mentioned above, adipose tissue is a very metabolically active organ, but its secretions are not its only mechanism of endocrine function. Adipose tissue also expresses a number of receptors that have local as well as global implications in whole body physiology and homeostasis. The major receptors in adipose tissue are estrogen receptors alpha and beta, toll-like receptors 2 and 4, and peroxisome proliferation agonist receptors.

5.2 Estrogen Receptors alpha and beta

Steroid hormones, predominantly estrogen but also testosterone and potentially progesterone, have been implicated as potential mediators of the sexual dimorphic properties of adipose tissue [139]. Estrogen and its receptors has been subject of a great amount of research given its importance in reproduction in humans and animals. Moreover, an observation that many post-menopausal women assume a more masculine pattern abdominal weight gain has generated a large amount of interest in the estrogen-obesity relationship [140].

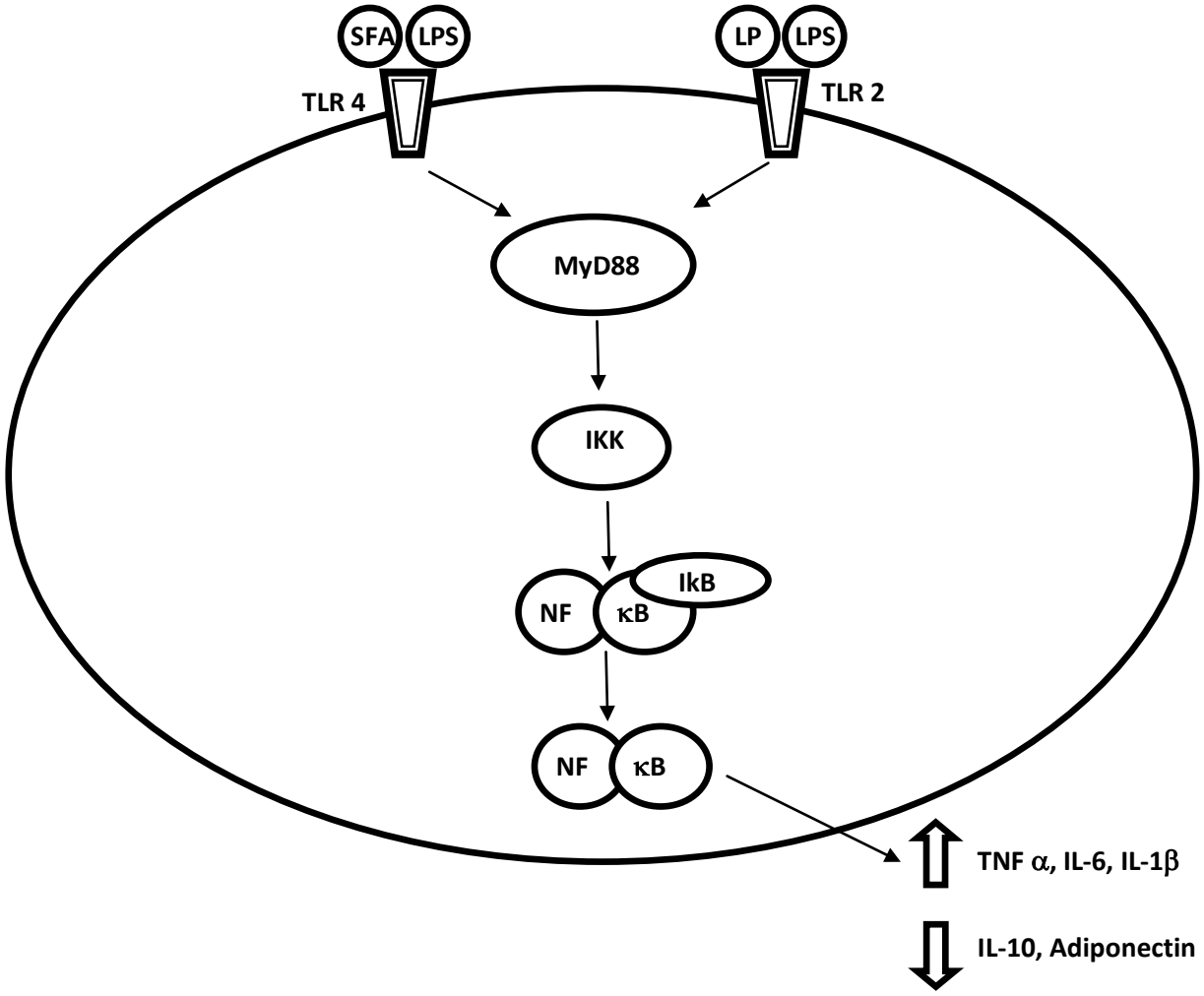


Figure 1.5: The relationship between TLR's, NFκB, and adipokine secretion patterns in obesity. Adipocyte TLR 2 and 4 binding increases NFκB activity which leading to an increase in pro-inflammatory (TNFα, IL-6, andIL-1b) and decrease in anti-inflammatory (IL-10 and adiponectin) cytokine and adipokine signaling upon the binding of LPS, saturated fatty acids (SFA), and lipoproteins (LP)

Estrogen has been shown to be a modulator of the immune system [141]. The concentration of circulating estrogen (specifically estradiol or E2) is the major physiologic mediator in immune function and adipose tissue physiology [141]. Intriguingly, both immune cells (macrophages, neutrophils, T-cells, and B-cells) as well as adipocytes express estrogen receptors [141, 142]. To further complicate matters, two known estrogen receptors, ER α and ER β have have different metabolic effects in adipose tissue [141, 143]. Evidence suggests that the ratio of ERα to ERβ estrogen receptors may play a significant role in both the development of obesity as well as some of the metabolic consequences associated with it [144]. Estrogen Receptor α has been shown to confer insulin sensitivity in the face of high fat feeding in rodents [145].

5.3 PPAR gamma

The peroxisome is an organelle responsible for the degradation of fatty acids via beta oxidation [146]. Peroxisome Proliferation Agonist Receptors (PPAR's), so named for their discovery via the use of peroxisome proliferator pharmaceutics, are important in cell proliferation, metabolism, and nutrient sensing [146, 147]. These receptors, specifically PPARγ, have a major role in adipocyte biology [148].

Peroxisome proliferation agonist receptors γ is a major transcription factor in the terminal differentiation of adipocytes through mechanisms not completely known, improves insulin sensitively while simultaneously increasing adipose mass, especially subcutaneously [148] [147]. This latter function may be due in part to the known adipogenic and lipogenic effects of PPARγ.

One hypothesis of PPARγ's insulin sensitizing action is down-regulation of pro-inflammatory cytokines such as TNFα while simultaneously increasing levels of anti-inflammatory and insulin sensitizing adiponectin [149, 150]. Another potential benefit of PPARγ is its ability to facilitate the remodeling of the adipose tissue extra cellular matrix thus allowing for the accommodation of the excess lipid without causing adipocyte hypertrophy [151]. Increasing adipose tissue mass by increasing the number rather than the size may confer resistance to the onset of the metabolic syndrome (as seen in the 20% of the obese population with a normal metabolic profile).

Polymorphisms in the PPARγ gene have been discovered which to confer resistance to T2D [152]. Allowing this expansion of adipose tissue via the increased differentiation of adipocytes into pre-adipocytes is a compensatory mechanism for an increased lipid influx [153, 154]. The class of anti-diabetes drug, thiazolidinediones improves insulin sensitivity and glucose homeostasis by acting as PPAR agonists [155].

5.4 Toll Like Receptors 2 and 4

Toll-like receptors (TLR) are a major mechanism of pathogen recognition for the innate immune system [156]. It is therefore not surprising that the adipocytes in adipose tissue, an immunologically active endocrine organ, have been found to express toll-like receptors 1-9, two of these TLR 2 and 4 appear to be most predominant in adipocytes[157]. The ultimate downstream effect of these TLRs is to increase the production of pro-inflammatory cytokines via activation of NFkB upon stimulation of adipocytes with LPS or saturated fatty acids (SFA) {Akira, 2000 #602;Tsukumo, 2007 #758}.

One may raise the question of the relevance of LPS stimulation to obesity, but studies have shown that endogenous LPS levels are higher in obese individuals. This may be due in part to the increase in synthesis of LPS by commensal bacteria as well as a decreased uptake of LPS in the liver (due to ectopic lipid deposition) [158]. Therefore, TLR 2 and 4 are also potential mediators of the low grade inflammation in obesity.

Section 6 – Adipose Tissue and the Immune System

6.1 Introduction to the Immune System

The immune system is responsible for the protection of the body from infection and injury that could lead to the disruption of normal body function. Inflammation, as defined by Baumann and Gauldie , is a series of reactions to prevent tissue damage, rid the body of pathogens, and activate repair mechanisms in the body [159]. Inflammation is characterized by

the influx of immune cells as well as an increase in the circulation of pro-inflammatory cytokines (which serve as chemotactic cues), and the generation of antioxidative molecules (superoxides, radical oxygen species, nitric oxide) which are designed to kill pathogens and repair wounds [160].

Inflammation itself is a disruption of homeostasis and itself can lead to disease, sepsis and death if unregulated [161]. The original nature of inflammation was to be acute and ephemeral, however, in some disease states such as asthma, inflammatory bowel disease, and obesity, the inflammation is chronic, but low grade [137, 138].

6.2 Acute vs. Chronic Inflammation

A distinction must be made between acute and chronic inflammation. Acute inflammation is characterized by a drastic increase in pro-inflammatory molecules such as TNFα and IL-6 as well as an infiltration of phagocytic and immune cells types [162]. The simultaneous increase in immune cells as well as pro-inflammatory cytokines leads to the 5 classic symptoms of inflammation -heat, redness, swelling, pain, and finally loss of function [163, 164]. Due to the potential for systemic damage, the inflammatory process is tightly regulated. The high levels of pro-inflammatory cytokines trigger a glucocorticoid (cortisol) mediated down-regulation of these cytokines [164]. Without cortisol, the pro-inflammatory cytokines would continue to circulate eventually leading to septic shock [161].

Chronic inflammation is usually considered to be low grade, meaning the levels of pro-inflammatory cytokines do not achieve the high levels in present in acute inflammation [165]. In chronic inflammation glucocortiocids do not effectively suppress the cytokine levels (lower than that of acute inflammation) and can result in disease over time [166, 167]. Obesity is considered a chronic low grade inflammatory state.

6.3 Atherosclerosis

Atherosclerosis is an insidious inflammatory disease resulting in the buildup of plaques derived from the combination of oxidized low density lipoprotein (LDL) and macrophages that adhere to walls of arteries [168, 169]. The development of atheromatous plaques place the individual at risk for heart attack and stroke because these plaques can cause either expansion or narrowing of the arteries, and vessel damage leading to clot formation [170]. The formation of clots is a major risk factor and cause of heart attack and stroke.

The development of atherosclerosis begins as early as adolescence, and can remain problematic throughout the life of the individual despite changes in diet and activity level. [171]. However, lifestyle changes greatly reduce the risk of vascular disease [172].

Obese individuals are found to have high levels of LDL and low levels of high density lipoprotein (HDL) which scavenges the LDL sequestering it to the liver where it is broken down and excreted [173]. Obesity has been shown to be a major risk factor for the development of atherosclerosis in children, adolescents, and adults [174, 175]. Insulin resistance, hyperinsulinemia, hypercholesterolemia, an increase in adipose derived pro- thrombotic factors, and abdominal adiposity all place a person at risk for developing atherosclerosis [169, 175].

6.4 Insulin Resistance

Insulin is a hormone secreted from the pancreatic β cells that is responsible for glucose uptake in peripheral tissues including adipose tissue, skeletal muscle, and the liver [102, 176]. Insulin is also responsible for decreasing lipolysis and hence the circulating levels of non-esterfied fatty acids (NEFA) [177]. Insulin resistance, as defined by Schenk et al, is an inadequate response by insulin target tissues to the physiologic effects of circulating insulin [102]. Insulin resistance is a major metabolic outcome of dysregulation of adipose tissue (both obesity and lipodystrophy) [178]. Additionally, insulin resistance is shown to be in part due to a simultaneous increased secretion of pro-inflammatory cytokines, such as TNFα, and decreased secretion of insulin sensitizing and anti-inflammatory adipokines such as adiponectin [178, 179]A causal link between VIF and insulin resistance has been observed [180]. The correlation between VIF and

insulin resistance is due in part to a higher rate of secretion and release of NEFA and pro-inflammatory cytokine release by VIF [181]. Moreover, insulin resistance has been causally linked to hypertension and T2D [182]

Section 7: Models of Human Obesity Studies

7.1 Introduction

Ideally, one must study the organism in question in order to best ascertain the mechanistic and physiologic properties of a disease, illness, or injury. However, using the organism of interest in some studies may be impractical for a number of reasons including ethical issues , limited number of subjects, safety and mortality concerns [183]. Therefore it is necessary to use a model exhibiting similarities to the subject/organism, disease, or other physiologic process of interest.

7.2 Rodent Models

Rodent models have been the model of choice for human studies due to their small size, prolific nature, relative ease of handling, and the ability to create transgenic and knockout species [184, 185]. The scope of this review will discuss the five most commonly used rodent models for human obesity.

7.2.1 ob/ob Mouse

Cloning of the ob gene and subsequent studies of the ob/ob mouse genotype discovery initiated an intensive effort to elucidate the effects of what seemed to be the panacea cure for obesity, leptin [186-188]. The ob gene mutation in the ob/ob mouse (also reported in humans) resulted in leptin deficiency in this model. Obesity, reproductive, and other metabolic dysfunctions in this model were completely reversed by central or peripheral injections of leptin [189]. Subsequent studies by Farooqi et al have demonstrated that administration of recombinant leptin to leptin-deficient humans also reversed both metabolic and reproductive

dysfunctions, as in the ob/ob mouse [190, 191]. The caveat of this discovery is most obese people are leptin resistant with high circulating levels of leptin compared to normal weight individuals [112, 189]. However, the ob/ob mouse turned out to be a good model to study obesity [192]. They are characterized by extreme hyperphagia, morbid obesity, high levels of lipogenesis, hyperglycemia as well as hyperinsulinemia [193]. Further, Johnson et al demonstrated that their obesity is due to an adipoctye hyperplasia rather than hypertrophy [194]. Given that mutations in the ob gene were also discovered in humans, these models are very relevant to those who are genetically leptin-deficient. Further, cloning of leptin and its receptors have significantly contributed to understanding energy balance and other neuroendocrine regulations.

7.2.2 db/db Mouse

The db/db mouse is another leptin related obesity model; however, it lacks the leptin receptor activity rather than having leptin itself [195, 196]. The db/db mouse is primarily characterized by morbidly obesity (though less than the ob/ob mouse) hyperphagia, insulin resistantance, and diabetes [193, 197]. These mice have a bit more clinical relevance in that they secrete very high amounts of leptin, a trait that is more physiologically relevant to many obese individuals.

7.2.3 fa/fa Zucker Rat

A rodent alternative to using mice as a human model is using rats. They are advantageous because they are larger (a particular advantage if adipose tissue collection is an endpoint). The fa/fa rat is a rat model of obesity characterized by insulin resistance, hyperphagia, hyperinsulinemia, hypertension, and hyperlipidemia [198]. This phenotype is result of a mutation in the leptin receptor rendering the rat leptin resistant [184].

7.2.5 Agouti Mouse

The agouti gene, the first cloned obesity gene, was originally described as a gene regulating coat color in mice [49, 199]. However, it was found that mutations in this gene led to an obese phenotype (along with a yellow coat color) [200]. These mice are hyperphagic, hyperglycemic, insulin resistant, obese and are susceptible to certain cancers. The agouti gene signals through melanocortin receptors but studies have also found a related protein (agouti-related protein, agrp) interferes with leptin signaling and modulates appetite regulation which ultimately leads to obesity [200, 201].

7.2.4 Diet Induced Obesity

The C57 BL/6J (Black 6 or B6) mouse is a commonly used mouse model for diet-induced obesity. The black 6 mouse is lean when fed a low fat, chow diet; obesity is induced induced into obesity through high fat feeding [202]. This type of obesity has a great deal of clinical relevance because it allows for studying the onset of obesity mimicking many of the environmental conditions present in the obesogenic environment we live in today. This is particular advantage because many scientists believe that the environment (rather than or in addition to genetics) is primarily responsible for the staggering rise in obesity we are currently experiencing [14]. However, not all obesity related disease processes are represented in this mouse. For example, studying the cardiovascular effects seen in high fat feeding requires a genetically modified mouse such as an Apo E or LDL receptor knockout[203], [204], a difference in physiology that may lead to a decreases it overall clinical relevance to studying human cardiovascular disease obesity relationship.

7.3 Porcine Models

Overall, the rodent model is good for initial studies, but there are differences in their physiology that make direct application to some aspects of human physiology difficult [205]. Additionally, the rodent models listed above (with the exception of B6) are single gene mutations, and obesity is hypothesized to be a polygenic abnormality, something that is not

adequately represented in many rodent obesity models [184]. Additionally, some genetic and physiological properties of mice and rats, are quite dissimilar to humans and sometimes result in an inability to directly correlate study data to human treatment [206]. Therefore it is important to have an intermediate model to study human disease in general and obesity specifically.

The pig has been an important means of elucidating physiology for hundreds of years, and was one of the first means by which the mechanisms of circulation were expounded [207]. Moreover, the physiology of pigs is similar to that of humans allowing for a more direct and relevant physiological comparison; transgenic and knockout species are available for study as well [206, 208]. The physiologic similarities of the gastrointestinal and cardiovascular systems as well as lipid metabolism and clearance are specific advantages of using pigs to study obesity and adipose tissue biology [209-211]. Further, pigs are omnivores as are humans resulting in similar nutrient digestion and absorption characteristics; a substantial benefit in a nutritional study. [212]. There are, numerous breeds of pigs available to choose from for human studies.

7.3.1 Domestic Pig

The domestic pig, reared primarily for consumption, has historically been the model of choice for human applied medical studies [213, 214]. Their large size paired with the physiological similarities mentioned above allowed for elegant macro scale studies. This was especially important before the development of microscopy and other techniques that allowed for examination of nutrient metabolism microscopically [207]. However, their size and genetic selection for preferential deposition of lean mass (due to changing consumer preferences over time) have made them less than ideal for obesity studies [213].

7.3.2 Miniature (mini) pigs

Minipigs are classified into two weight groups (by weight) 35kg- 55kg and 70kg- 90kg [215]. One major advantage to using minipigs is their retention of feral qualities. This is a distinct advantage due to their retention of what is termed a “thrifty genotype”[216]. In other words, they are able to deposit fat in times of plenty, and be efficient with energy expenditure and

nutrient storage in times of food scarcity [217]. This genotype, once advantageous, may now predispose an organism, human or porcine, to obesity [216, 217]. Therefore, using the minipig allows for a more accurate representation of the human genotype. There are multiple breeds of minipigs, but the Gottingen, Yucatan, Ossabaw, and Sinclair are the four most relevant for obesity and adipose tissue studies. Here we will focus on the Sinclair minipig, as it was used for our studies [205, 218].

Section 8: The Sinclair Minipig as a Model for Human Obesity

8.1 Introduction to Porcine Models

When selecting a swine model, it is important to consider the type of study being performed, the amount of handling required, and the facilities available. It is our contention that the Sinclair minipig is preferable to the domestic pig as an experimental model for adipose tissue and obesity studies because of its small size, docile nature, and propensity to gain weight rapidly.

8.2 Logistical Considerations

There are several advantages to using the Sinclair minipig, including its size, ease of manipulation, and cost of feed. It is approximately 35 kg when it is full grown (compared with the domestic pig's 200-350kg) [215]. The size of the pig has especially important bearing when considering an obesity study which necessitates feeding more that the maintenance amount of food daily. Additionally, corn and soybean prices must be taken into consideration as they are the basis of the pig diet. In 2008, corn was at record high prices; therefore it was particularly advantageous at this time especially to use pigs that were smaller [219]. Additionally, use of the Sinclair minipig, bred for docility and tolerance of handling, requires less chemical restraint,

especially for minor procedures [215]. Finally, the ease of handling may permit a greater number of tests to be performed.

8.3 Genetics and Physiology

The Sinclair minipig is a hybrid of 4 feral breeds, the Guinea Hog from Alabama, wild boars from Catalina Island, California, the Piney Woods pig from Louisiana, and the Ras-n-Lansa pig from Guam, and a Yorkshire boar[220].

The breed is propagated using a least related breeding program, and are an inbred strain. This is beneficial for 2 reasons; first, the retention of the feral genotype, and second, a decrease in genetic variability and a resultant increase in reproducibility between replicates and experiments.

In contrast, domestic pigs are not inbred to promote hybrid vigor and have therefore been selected to deposit lean mass in preference to fat to accommodate a change in consumer preferences [221]. This confers another advantage to the use of the Sinclair minipig due to inbreeding preferential deposition of fat.

Section 9: Conclusion

Obesity is a multifaceted and multidimensional problem, requiring a multidimensional approach for a solution. The physiological and genetic basis for obesity must continue to be studied in order to dissect environment-gene interactions and to better determine the optimal treatment strategies for the obese population, human and animal. Moreover, the porcine model of obesity, specifically the minipig model may offer an intermediate model of study between rodents and humans. A combination between policy changes, lifestyle changes and elucidation of mechanisms is the only way to combat this plague of our modern society.

Chapter II: The Sinclair Minipig Study

Introduction

Obesity has become a burgeoning heath problem in the last fifteen to twenty years, with rates in America above 30% [222]. Dysfunction of white adipose tissue (WAT) leads to the metabolic consequences associated with obesity [15, 93]. There are two major WAT depot visceral (VIF) and subcutaneous (SQF) which correspondingly alter their metabolism to accommodate a change in nutritional status [76]. Evidence has suggested that VIF is more metabolically active in the chronically overfed, obese person [223].

This study was conducted to elucidate and characterize the basic biology of the VIF and SQF in 8-month old Sinclair minipigs. The minipigs included in this study were fed standard porcine diets; however these pigs were part of a more comprehensive nutritional study that is included in the appendix. Following a two week acclimation, we measured changes in metabolic markers including HDL, LDL, VLDL, triglycerides, insulin, and immune cell populations over time. In addition, we obtained gene expression data from VIF and SQF using quantitative real time PCR and Affymetrix microarray. Further, we determined histological and immunological differences between the two depots via adipocyte and macrophage cell counts. Immunofluorescence (IF) studies were employed to determine the differences in macrophage subtype (CD 68 or CD 163 positive) infiltration, and to compare macrophage subtype and infiltration rates of the domestic pig and Sinclair minipig. We compared the number of adipocytes and macrophages between visceral and subcutaneous adipose depots. Finally, we determined differences in protein expression differences via western blot in an attempt to both validate the gene expression data and to determine if up or down-regulation in genes translated to higher or lower levels of protein synthesis.

We expected to see differences in metabolic markers, gene expression, cell number and macrophage phenotype and infiltration between VIF and SQF.

This study sought to add further evidence that adipose tissue is a multifunctional endocrine organ, and that is function is highly conserved. Further, our intent was also to establish the Sinclair minipigs as a model for human obesity and adipose tissue studies. Limited studies have

used this model to dissect adipose tissue biology. Finally, we hope to add evidence to support the theory that the composition of fat as well as its location in the body has a direct relation to the health and well being of the individual as well as the prevention or onset of multiple diseases.

Section 1: Materials and Methods

1.1 In Vivo Data and Tissue Collection

1.1.1 Animals

Twelve 6 month old Sinclair minipigs boars were purchased for Sinclair Bio Resources, LLC (Columbia, MO). Upon arrival, they were randomly placed in individual pens in two separate climate and light controlled rooms. The pigs were allowed to acclimate to their surrounding for 2 weeks, and were then fed approximately 350g of a standard diet (50% above maintenance) twice daily for 8 weeks (see Table 2.1 a and b). The pigs were weighed every 2 weeks for the duration of the study. At the end of the 8 week stud, the pigs were sacrificed using barbiturate overdose, and SQF, VIF, liver, spleen, thymus, hypothalamus, and semi tendonous muscle were collected.

It should be noted that the pigs discussed were control pigs for a larger study aimed at determining the effects of dietary supplements on acute immune challenges (for detailed materials, methods, and results see appendix 2). Only pigs from the control cohort (non-supplemented or immune challenged pigs) were used in the experiments for this thesis. All protocols were approved by the University Animal Care and Use Committee.

1.1.2 Blood Collection

A fasted blood sample was drawn from each pig at the beginning and the end of the study to assess circulating levels of insulin, glucose, triglycerides, LDL, HDL, VLDL, and to determine the number of circulating white blood cells (WBC) as well as neutrophil and lymphocyte percentages.

Table 2.1: Sinclair minipig diet composition

(a)

Ingredient	Percentage in Diet
Corn	66.5%
Soybean Meal	31%
Vitamin Mix	2.5%

(b)

Water	11.64%
Crude Protein	18.19%
Crude Fiber	3.36%
Ether Extract (crude fat)	2.48%
Ash	3.06%
Carbohydrate	58.76%

Table 2.1 a illustrates the general diet composition [205] of the minipig diet. The bulk of the diet is composed of carbohydrates in the form of corn, and the soybean meal is used as a protein source.

Table 2.1 b illustrates a breakdown of the specific macronutrient composition of the diet (water, protein, fiber, fat, ash, and carbohydrate).

1.2 Ex-Vivo Data Analysis

1.2.1 Blood Analysis

The minipig blood was analyzed using the Coulter method as well as a spun hematocrit and manual differential cell count at VetPath laboratories (Tulsa, OK). The metabolic parameters

were analyzed as a completely randomized design with repeated measures (the beginning and end of the study), then analyzed using SAS 9.2.

1.2.2 Multiplex Cytokine Analysis

Circulating levels of cytokines in plasma samples were measured using SearchLight® Proteome Arrays (Aushon BioSystems, Billerica, MA), a quantitative multiplexed sandwich ELISA containing 6 different capture antibodies spotted on the bottom of a 96-well polystyrene microtiter plate. Each antibody captures a specific protein present in the standards and samples added to the plate.

Briefly, samples were incubated for three hours on the array plates that were pre-spotted with capture antibodies specific for each protein biomarker. Plates were decanted and washed three times before adding a cocktail of biotinylated detection antibodies to each well. After incubating with detection antibodies for 30 minutes, plates were washed three times and incubated for 30 minutes with streptavidin-horseradish peroxidase. All incubations were done at room temperature with shaking at 200 rpm. Plates were again washed before adding a chemiluminescent substrate. The luminescent signals produced from the HRP-catalyzed oxidation of the substrate were measured by imaging the plate using the SearchLight Imaging System which is a cooled charge-coupled device (CCD) camera. The data was then analyzed using SearchLight Array Analyst software. The amount of luminescent signal produced is proportional to the amount of each protein present in the original standard or sample. Cytokine concentrations were extrapolated from the standard curve.

1.2.3 Tissue Homogenization

Sections of SQF or VIF adipose tissue were collected and cut into 1cm^3 pieces within 15 minutes after sacrificed by barbiturate overdose. The tissue was then placed in 5mL RNA later (Qiagen, Valencia, CA) and stored in -20°C until processing. The tissue sections were homogenized with the tissuemiser homogenizer (Fisher, Pittsburg, PA) in 5 mL tri reagent

(Invitrogen Carlsbad,CA) at 25-35 Hz, then aliquoted into five 1mL aliquots and stored in -80°C for further processing.

1.2.4 RNA Extraction

Adipose tissue homogenate was thawed on ice, and then incubated at room temperature for 5 minutes. Following the incubation, 200µL chloroform was added to the 1mL of homogenate, vortexed for 10 seconds, incubated at room temperature for 2-3 minutes, and then centrifuged for 15 minutes at 12,000g and 4°C. The aqueous phase generated from centrifuging was transferred to another tube where an equal volume 70% of ethanol was added. The sample was inverted 5 times, added to a column from the qiagen minilipid kit (Qiagen, Valencia, CA), incubated for 4 minutes at room temperature, and centrifuged for 15 seconds at 8,000g and 4°C. This process was repeated until all the aqueous phase/ethanol combination was processed through the column. Once all of the aqueous phase/ethanol was through the colulum, 700µL of Buffer RW1 was added to the column, and then centrifuged for 15 seconds at 8,000g and 10-15°C. Next, 500µL Buffer RPE was then added to column, centrifuged 15 seconds at 8,000g and 10-15°C. An additional 500µL Buffer RPE was added to the column, centrifuged 1 minute at 8,000g and 10-15°C. Finally, 30µL RNAse free water was added to column, incubated for 1 minute, then centrifuged for 1 minute at 8,000g and 10-15°C.

It is noteworthy to mention the difficulty in extracting RNA from the minipig SQF and VIF. After numerous attempts at modifying a few different techniques, using a modified qiagen lipid mini kit was the most successful. Different constancies of the adipose tissue due in part to degree of marbling may have hindered and hence added variation to the success in extracting RNA from the adipose tissue depots among minipigs. One future suggestion would be to flash freeze the tissue in liquid nitrogen after sacrifice, powder it, then extract the RNA from the powdered tissue using the previously described method.

1.2.5 RNA Repurification (for microarray analysis only)

The RNA sample volume was brought up to a final volume of 100µL use 70µL RNAse free water, then 2.5 volumes of 100% molecular grade ethanol and 0.1 volumes of 3M Sodium Acetate (pH 5.5) were added to the sample, then the sample was placed in -80°C. The sample was removed from the freezer, centrifuged for 20 minutes at 4°C and 13,000 rpm. The supernatant was carefully removed to avoid disturbing the pellet which was then washed with 75% molecular grade ethanol, then centrifuged for 5 min at 4°C at 13,000 rpm, and the ethanol was removed. The pellet was then dried with a speed vac for 2 minute, and then the pellet was resuspended in 15µL RNAse free water and stored at -20°C for 1hr. Prior to using the RNA for microarray analysis, total RNA concentration was quantified via nanodrop (Thermo Scientific, Wilming, DE).

1.2.6 Microarray Analysis (Affymetrix)

For microarray analysis RNA from 3 of the 4 minipigs from the study was submitted. One hundred nanograms of repurified RNA was amplified, then cDNA was generated using TargetAmp 1 round Biotin RNA amplification kit 105 (Epicentre, Madison, WI). The RNA was then processed using the Affymetrix microarray system. Briefly, the cDNA was in vitro transcribed with biotinylated UTPs and CTPs. The biotin – labeled RNA was fragmented with heat and $Mg2^{+}$ and hybridized on the Affymetrix porcine GeneChip for 16 hours at 45°C and 60 rpm. The chips were washed with low and high stringency buffers on the Affymetrix GeneChip fluidics station 450 (Affymetrix, Santa Clara, CA). The chips were then stained with streptavidin/phycoerythrin, scanned, and quantified.

The data was normalized using guanine cytosine robust multi-array analysis (GCRMA). The transcripts of significance were determined using the false discovery rate (FDR) with a step-up p value of 0.05 or less.

1.2.7 Microarray Annotation

A limited list of the annotated probe set ID's were publically available; [224]that list was imported into the unannotated list for further analysis. The remainder of the probe set IDs were annotated by hand using the Affymetrix or NCBI website as detailed in appendix 1.

1.2.8 Gene Ontology and Pathway Analysis

Analysis of the transcriptome was performed using database for annotation, visualization and integrated discovery (DAVID) bioinformatic resources 6.7 (NIH, Bethesda, MD) on a human background; a porcine background is currently unavailable. Significance of the pathway was determined using the FDR with an expression analysis systematic explorer (EASE) level of 0.05.

1.2.9 Quantitative Real-Time PCR

In order to prepare the RNA sample for PCR analysis, the DNAses were removed from the RNA using the Turbo Free DNAse kit (Ambion, Carlsbad, CA). Briefly, DNAse enzyme and 10x buffer were added to the RNA incubated at 37^{o}C while shaking for 30 minutes, then inactivated with an activating agent. Next, cDNA was synthesized using high capacity reverse transcriptase kit (Applied Biosystems, Fosters City, CA). Briefly, a mastermix was generated combining 10X buffer, 100mM dNTP Mix, 10X random primers, reverse transcriptase, and added to the RNA. The mixture was run with the following settings: 25°C for 10 minutes, 37° C for 120 minutes, 85° C for 5 minutes, 4° C indefinitely. Finally, the cDNA was combined with taqman (Applied Biosystems Carlsbad, CA) master mix, primers, probes, and water. The samples were then run on the I-Cycler thermal cycler (Biorad, Hercules, CA).

1.2.10 qRT – PCR Analysis

The relative gene expression of the SQF and VIF was determined using the delta delta CT method [225]. The data was statistically analyzed using a randomized block design blocking on pig with a split plot treatment design to compare SQF and VIF. Analysis was performed using SAS 9.2.

1.2.11 Histological Slide Preparation –Frozen Tissue Sectioning

Freshly harvested tissue was immediately submerged in liquid nitrogen, and then placed in -80°C for storage. The tissue was removed from -80°C storage, and placed on dry ice until it was ready for processing. A layer of tissue tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA) was placed on the cryostat base and allowed to freeze (turning white). Once the tissue tek was nearly frozen, the flash frozen tissue was placed in the center of the tissue tek on the base. Another layer of tissue tek was added on top, slightly smaller in circumference than the previous layer, and allowed to freeze. This process was repeated until the tissue was completely covered. The tissue was then cut on the cryostat micron HM 505 N into 20 micron sections. Each section was placed on a positively charged slide (AmLabs, Bradenton, FL), labeled, and placed in -80°C until further processing. The remaining tissue mounted in tissue tek was stored in -80°C.

1.2.12 Immunofluorescence

Permeability Protocol

The frozen tissue slides were removed from the -80°C and were brought to room temperature. A circle was drawn around the sample with a diamond pen. The tissue was then rinsed 6 times with 1mL 1 x PBS. The tissue was incubated for 30 minutes in 1mL of 4% Paraformaldehyde, rinsed 6 times with 1X PBS (5 minute incubation and 2mL each wash). The tissue was blocked in 200 µL blocking solution, 0.25% Triton X 100 and 1% goat serum (Sigma St. Louis, MO) in PBS, for 1 hour at room temperature. The tissue was incubated overnight in a humidified chamber with 500µL of 1:500 dilution of primary antibody made with the block

solution from above (CD 163, Santa Cruz sc-33715 Santa Cruz, CA or CD 68, Abcam ab955). The tissue was then rinsed 6 times with 1X PBS (5 minute incubation and 2mL each wash), then incubated for 1 hour in the dark with a 1:1000 dilution of the secondary antibody (Alexa Fluorfor 488 goat anti - mouse; Invitrogen Eugene, OR). Once all rinses were complete, excess moisture around the tissue was dried with a kim wipe, and tissue was covered with a coverslip containing 1 drop of prolong gold with DAPI (Invitrogen, Carlsbad, CA), and stored at 4°C overnight. The coverslip edges were then sealed using clear nail polish, allowed to sit at 4°C for 30 minutes, and then imaged using the Nikon Eclipse Ti microscope with the PF 20X magnification (oil setting).

General Protocol

The frozen tissue slides were removed from the -80°C and were brought to room temperature. A circle was drawn around the sample with a diamond pen. The tissue was then rinsed 4 times with 1mL 1X PBS (1mL each wash). The tissue was incubated for 15 minutes in 1mL of 4% Paraformaldehyde. The slides were rinsed 5 times with 1mL 1X PBS. The tissue was incubated for 45 minutes with 1mL of a 1:500 dilution primary antibody (mouse macrophage (Calprotectin), Santa Cruz sc-71533 Santa Cruz, CA). The tissue was then rinsed 5 times with 1X PBS (1mL each wash), then incubated in the dark for 30 minutes with a 1:1000 dilution secondary antibody (Alexa Fluorfor 488 goat anti - mouse; Invitrogen Eugene, OR). After the incubation, the tissue was rinsed 5 times with 1X PBS (1mL each wash). After the incubation, excess moisture around the tissue was dried with a kim wipe, and tissue was covered with a coverslip containing 1 drop of prolong gold (Invitrogen, Carlsbad, CA) and stored at 4°C overnight. The coverslip edges were then sealed using clear nail polish, allowed to sit at 4°C for 30 minutes, and then imaged using the Nikon Eclipse Ti microscope with the PF 20X magnification (oil setting).

1.2.13 Histological Slide Preparation -Paraffin Embedding

Sections of SQF or VIF adipose tissue were collected and cut into $1cm^3$ pieces within 15 minutes after euthanasia and placed in 10% formalin for 24 hours, then into 70% ethanol until processing. The paraffin embedding slide preparation was performed at Neuroscience Associates (Knoxville,TN). Briefly, the tissue was dehydrated from 70% Ethanol, and 95% was added and once again the tissue was dehydrated. Finally, absolute ethanol was added and the tissue was dehydrated once again. The tissue was cleared in xylene, infiltrated with paraffin, embedded in Paraplast embedding medium, sliced into 3 micron sections, routinely stained with hematoxylin and eosin (H &E) and mounted, or mounted without stain, then coverslipped.

1.2.13 Adipocyte Number

In order to compare the number of adipocytes in VIF and SQF, representative photos were taken of H & E stained slide, and 8x10 printouts were generated for each pig and each depot. Three 3"x3" boxes were drawn, and all cells that were at least 90% included in the box were counted. The average number of adipocytes of the 3 boxes was averaged for each pig, and then all values from each depot were averaged with their corresponding treatment group. The data was analyzed using a completely randomized design with a split plot treatment design. Statistical analysis was performed using SAS 9.2.

1.2.14 Macrophage Number

The same representative images used in the adipocyte hand count were uploaded into Nikon NIS-Elements AR software. An algorithm was applied to enhance the contrast of the macrophages in relation to the surrounding adipocytes. Statistical analysis was performed using SAS 9.2 for a completely randomized design with a split plot treatment design.

1.2.15 Protein Extraction

Initially after harvest, the tissues were placed in RNA later, and subsequently homogenized in TRI reagent. The advantage of using TRI reagent is the ability to extract RNA, DNA, and proteins from the same sample. Therefore, our initial attempt to extract proteins was from the TRI reagent homogenate. However, these attempts were unsuccessful due to either low or contaminated and protein yield. We then tried to extract the protein by homogenizing the tissue in RIPA buffer (see appendix 1 for details), and then extracting the protein. The protein yield was better than the TRI reagent extract, but still low. The final attempt to extract the protein once again used the RIPA buffer as the homogenizing medium, but this time the tissue was powdered in liquid nitrogen before homogenizing. This variation yielded acceptable levels of quality protein. Powdering the tissue before homogenization and extraction may allow for a greater penetration of the lysis buffer as well as disruption of the connective tissue from the adipose tissue, allowing for a greater ability to extract the proteins.

Tissue was flash frozen in liquid nitrogen then pulverized by hand in liquid nitrogen using a ceramic mortar and pestle. The samples were collected and stored at - 80°C until further processing. The tissue samples were removed from - 80°C, and then 600mg-1g of tissue was weighed out and used for protein extraction. Next, 300-500µL of modified RIPA buffer was added to the powdered tissue (based on weight), and homogenized at 5 rpm for 30-45 seconds. The tissue was then homogenized between 21,200-25,600 rpm with Powergen 125 (Fisher Pittsburg, PA) homogenizer until all the tissue was uniformly broken down (30-45 seconds). The tissue was incubated for 15 minutes on ice and was then, centrifuged for 15 minutes at 4^{o}C and 10,000g. The supernatant was extracted, and the sample was again centrifuged for 15 minutes at 4^{o}C and 10,000g, the supernatant extracted, and stored at -80^{o}C until further processing using western blot.

1.2.15 Protein Quantification

The amount of total protein was quantified using the Pierce Coomassie Assay Kit (Pierce, Rockford, IL) Briefly, 5μL of albumin standard (Pierce, Rockford, IL) or sample was added to a 96 well plate, the 250 μL of Coomassie assay buffer (Pierce, Rockford, IL) was added to each well. The plate was mixed for 30 seconds, incubated for 10 minutes in the dark, and absorbance was measured at 595 nm. The total protein concentration was extrapolated from the standard curve.

1.2.16 Western Blot

Equal quantities of 1X sample buffer were combined with 40 μg of protein (Biorad, Hercules, CA), boiled for 5 minutes, and placed on ice to cool. The samples were loaded into a 10% Tris HCl premade gel (Biorad, Hercules, CA), placed in the running container containing an appropriate amount running buffer. The gel was run for 30 minutes at 100V and 1 hour at 115 volts. The gel was then transferred to a nitrocellulose membrane (Biorad, Hercules, CA) containing an appropriate amount of transfer buffer, and run at 80 V for 1.5 hours. To ensure proper transfer, the membrane was stained with 5mL ponceau stain (Sigma, St. Louis, MO) for 5 minutes, and then rinsed with tris buffered saline (Biorad, Hercules, CA) with 0.01% tween (Fisher Scientific Fair Lawn, NJ) (TBST). The gel was stained with coomassie blue (Thermo Scientific/Pierce, Rockford, IL) for 5 minutes and destained for 1 to 2 hours using coomassie destain (Thermo Scientific/Pierce, Rockford, IL). The membrane was then blocked overnight (approximately 18 hours) in a 5% solution of nonfat dry milk (Great Value, Bentonville AR) and TBST. The following day, the membrane was washed 3 times, 5 minutes each time with TBST and then incubated with the primary antibody (see appendix 1) in a 3% solution of nonfat dry milk for 1 hour. The membrane was washed 1 time in TBST for 5 minutes, and then incubated for 1 hour with the secondary antibody (see appendix 1). The membrane was then washed 3 times with TBST, then tris buffered saline (Biorad, Hercules, CA) (TBS), 5 minutes each time. The proteins were stained by incubating with DAB metal substrate kit (Thermo Scientific/Pierce,

Rockford, IL) for 15 minutes, ECL (GE Healthcare, Buckinghamshire, UK) or supersignal west pico (Thermo Scientific/Pierce, Rockford, IL) chemiluminescent substrate for 5 minutes. The membranes incubated with the DAB metal substrate protocol were imaged via a commercial scanner. The membranes incubated using either the ECL or supersignal west pico protocols were imaged using the versadoc (Biorad, Hercules, CA).

Section 2: Results

2.1 Body Weight

The minipigs gained weight rapidly for the first 6 weeks of the study ($p<0.0001$), but lost weight between weeks 6 to 8 (not significant) as demonstrated by Figure 2.1, indicating a plateau of weight gain before the completion of the study.

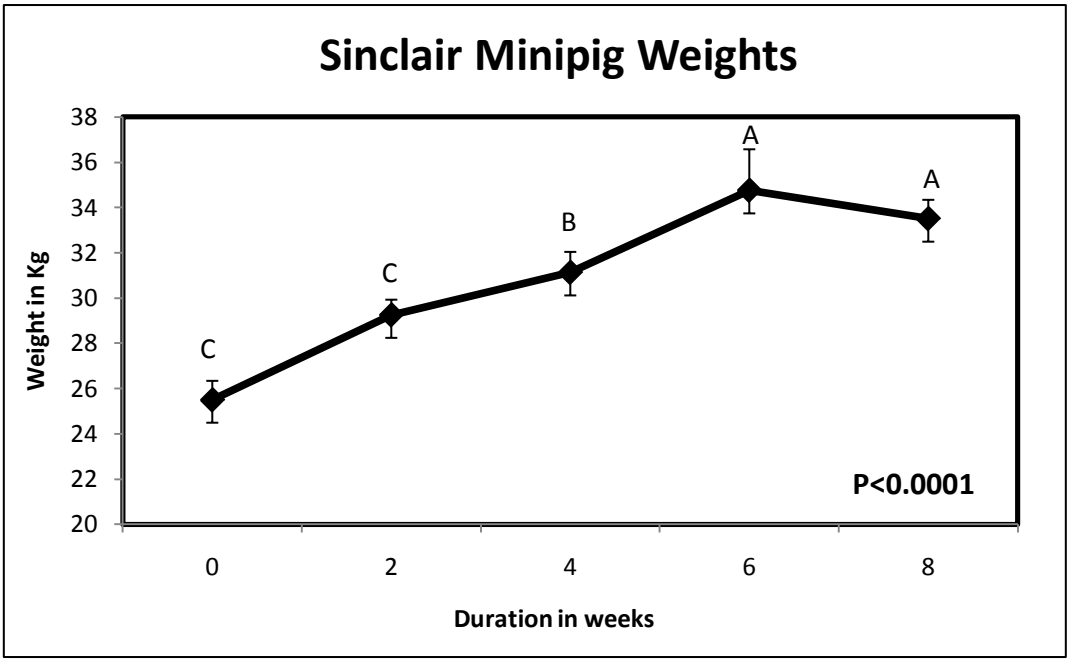


Figure 2.1: The change in Sinclair minipig weights during the course of the 8 week study

2.2 Metabolic Parameters

2.2.1 Plasma Lipids

Figure 2.2 illustrates the lipoprotein component of the lipid profile of the minipigs from the beginning to the end of the study. The triglycerides increased and the HDL decreased during the course of the study, but these changes did not reach statistical significance (p=0.4671 and 0.5074 respectively). Although the VLDL did not change significantly during the course of the study (p=0.8005), there was a statistically significant decrease in LDL from the beginning to the end of the study (p<0.001).

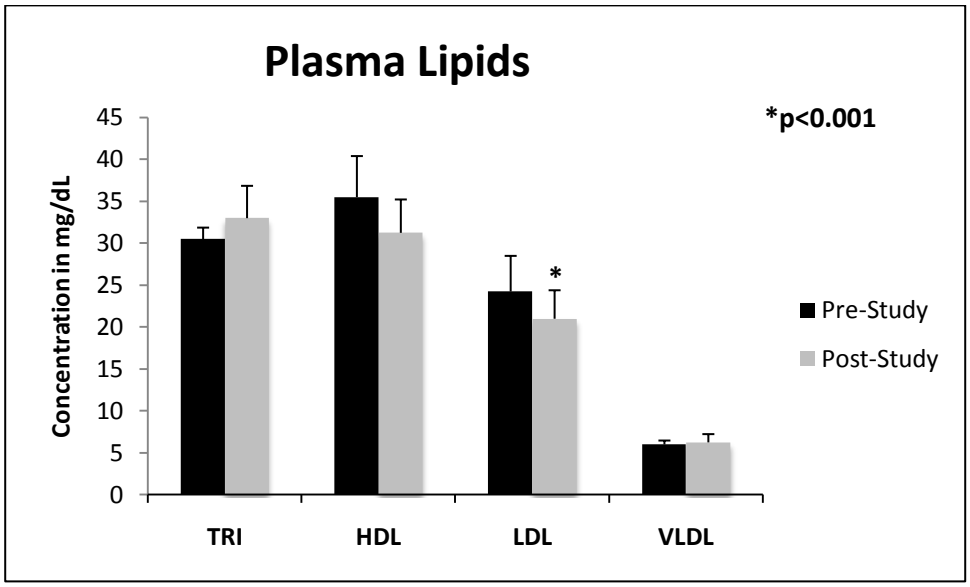


Figure 2.2: Plasma Lipids; Circulating triglycerides (TRI), High Density Lipoprotein (HDL), Low Density Lipoproteins (LDL), and Very Low Density Lipoproteins (VLDL)

2.2.2 Plasma Insulin

The insulin levels in the minipigs showed a trend of increasing, though not significantly (p=0.0722), from the beginning to the end of the study as demonstrated by Figure 2.3.

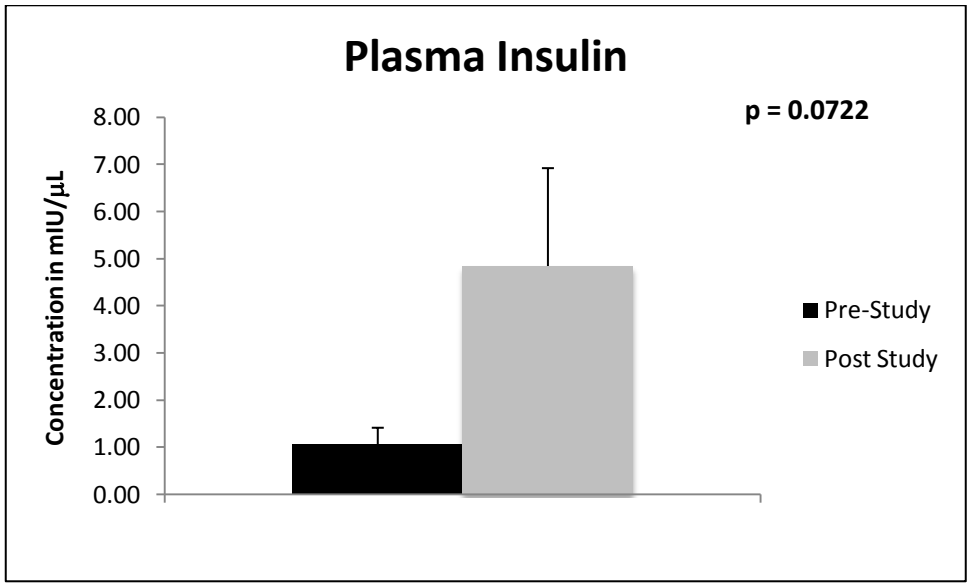


Figure 2.3: Plasma Insulin levels from the beginning to the end of the study in the Sinclair minipig

2.2.3 Total White Blood Cell Count

Figure 2.4 highlights a significant drop in the total numbers of white blood cells that occurred from the beginning of the study to the end (p=0.0046). It should be noted that some of the WBC counts at the beginning of the study were above the normal range.

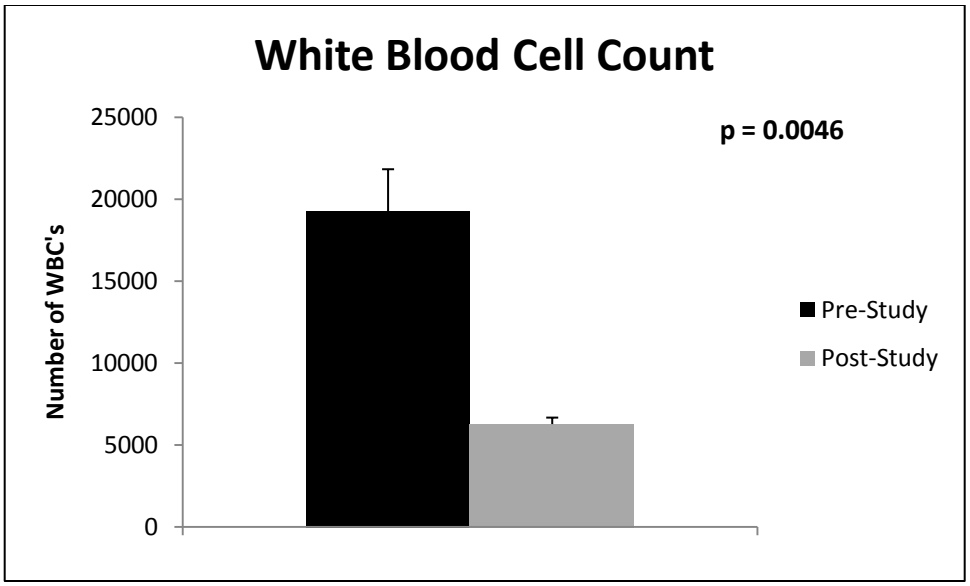


Figure 2.4: Total white blood cell count from the beginning to the end of the study in the Sinclair minipig

2.2.4 White Blood Cell Populations

Figure 2.5 highlights the percentages of segmented neutrophils and lymphocytes in the total white blood cell populations during the course of the study. The segmented neutrophil population did not have a statistical change in the duration of the study (p=0.3809), but the lymphocyte population showed a decrease during the study (p=0.0165).

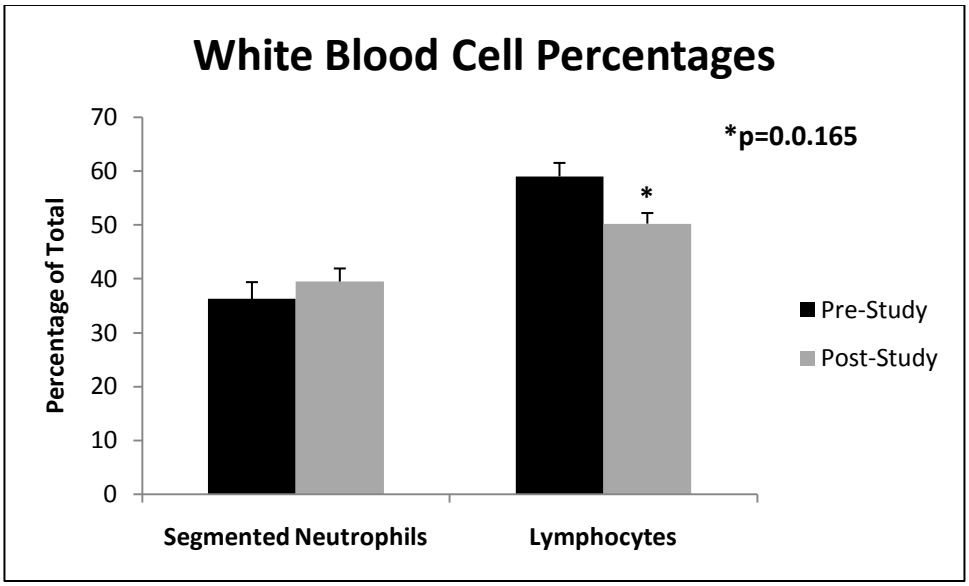


Figure 2.5: Change in percentages of white blood cell populations from the beginning to the end of the study in the Sinclair minipig

2.3 Serum Cytokines

Table 2.2 shows levels of circulating cytokines in the plasma. There is little data on the circulating levels of cytokines in the Sinclair minipig, so these parameters were important to measure for future studies.

Table 2.2: Levels of circulating cytokines at the end of the study

Cytokine	Concentration (pg/mL)	Function
IL-1 β	89.8 +/- 60.2	Pro-Inflammatory
IL-8	2.4 +/- 0.79	Pro-Inflammatory
IFN γ	38.0 +/- 29.2	Pro-Inflammatory
TNF α	23.9 +/- 15.4	Pro-Inflammatory
IL-4	20.2 +/- 11.2	Anti-Inflammatory
IL-10	24.1 +/- 16.7	Anti-Inflammatory

2.4 Microarray Results

2.4.1 Transcriptome Details

In advance of pathway analysis, the details of both the transcriptome are provided for reference. The Affymextrix porcine gene chip had 23,937 total probesets coding for 20,201 genes. It should be noted that the design of the chip has multiple probesets for the same gene to ensure the maximum chance of gene identification. When comparing VIF to SQF we found that 788 total transcripts changed, 240 were up-regulated and 548 were down-regulated (Table 2.3 and Figure 2.6). When SQF was compared to VIF, 598 transcripts changed, 471 were up-regulated and 127 were down regulated (Table 2.4 and Figure 2.6).

Table 2.3: Transcriptome details for the VIF vs. SQF comparison

Total Probesets	23,937
Total Transcriptome	23,256
Total Genes	20,201
Transcripts Changed	788
Up-Regulated	240
Down-Regulated	548

Table 2.4: Transcriptome details for the SQF vs. VIF comparison

Total Probesets	23,937
Total Transcriptome	23,256
Total Genes	20,201
Transcripts Changed	598
Up-Regulated	471
Down-Regulated	127

In total, 508 transcripts between the two comparisons were the same. However, there were 212 differentially expressed transcripts when VIF was compared with SQF compared to only 32 when SQF was compared to VIF. It should be noted that the number of similarly and differentially expressed transcripts does not take into account differences in regulation (up or down). In other words, the transcripts may change in both depots, but it may be up-regulated in one and down-regulated in the other, which was the case (appendix 1 tables A1 and A2.

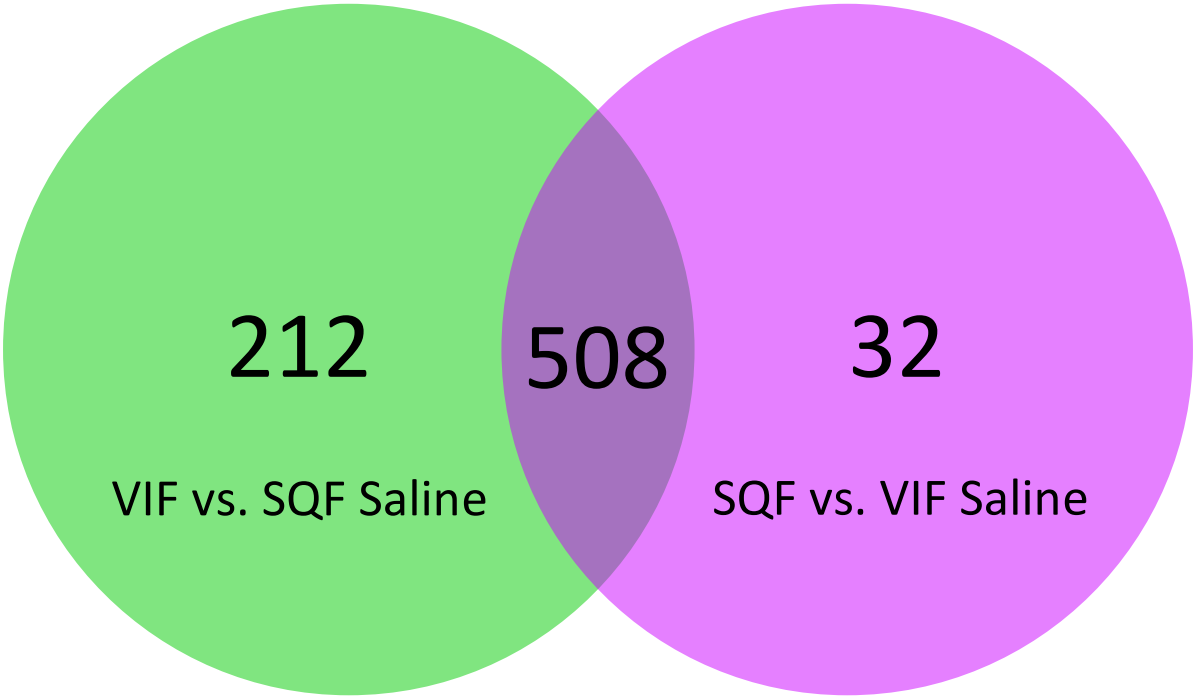


Figure 2.6: Venn diagram illustrating the transcripts distribution between the 2 microarray comparisons made: VIF compared to SQF and SQF compared to VIF

2.4.2 Cluster Analysis

In a microarray analysis, a cluster analysis is an important tool in determining the global changes in the tissue that is being analyzed. Figure 2.7 is a hierarchical cluster analysis of the individual pigs by depot. The lines on the left side of the figure shows the relationships between the individual probeset IDs, the numbers on the bottom of the figure show the individual minipig identification numbers, and the specific depot is indicated at the top of the figure. The blue color pattern indicates a down-regulation, the red color pattern indicates an up-regulation, and the grey color pattern indicates no change in SQF compared to VIF. Individual pigs, as identified by number, are indicated at the bottom of the figure. The cluster analysis demonstrates that, with relatively few exceptions, there is great similarity between minipigs lending confidence to the validity of the microarray analysis. Moreover, there are regions of similarity, but also distinct differences in the clustering patterns of the two depots. Figure 2.8 is hierarchical cluster analysis averaging the clustering of the 3 pigs used for the microarray analysis. Once again, the cluster analysis demonstrates there are distinctly different patterns of gene expression between VIF and SQF. There are, however, many regions of similarity as well. This pattern of similar expression between VIF and SQF is to be expected because the depots are of similar composition.

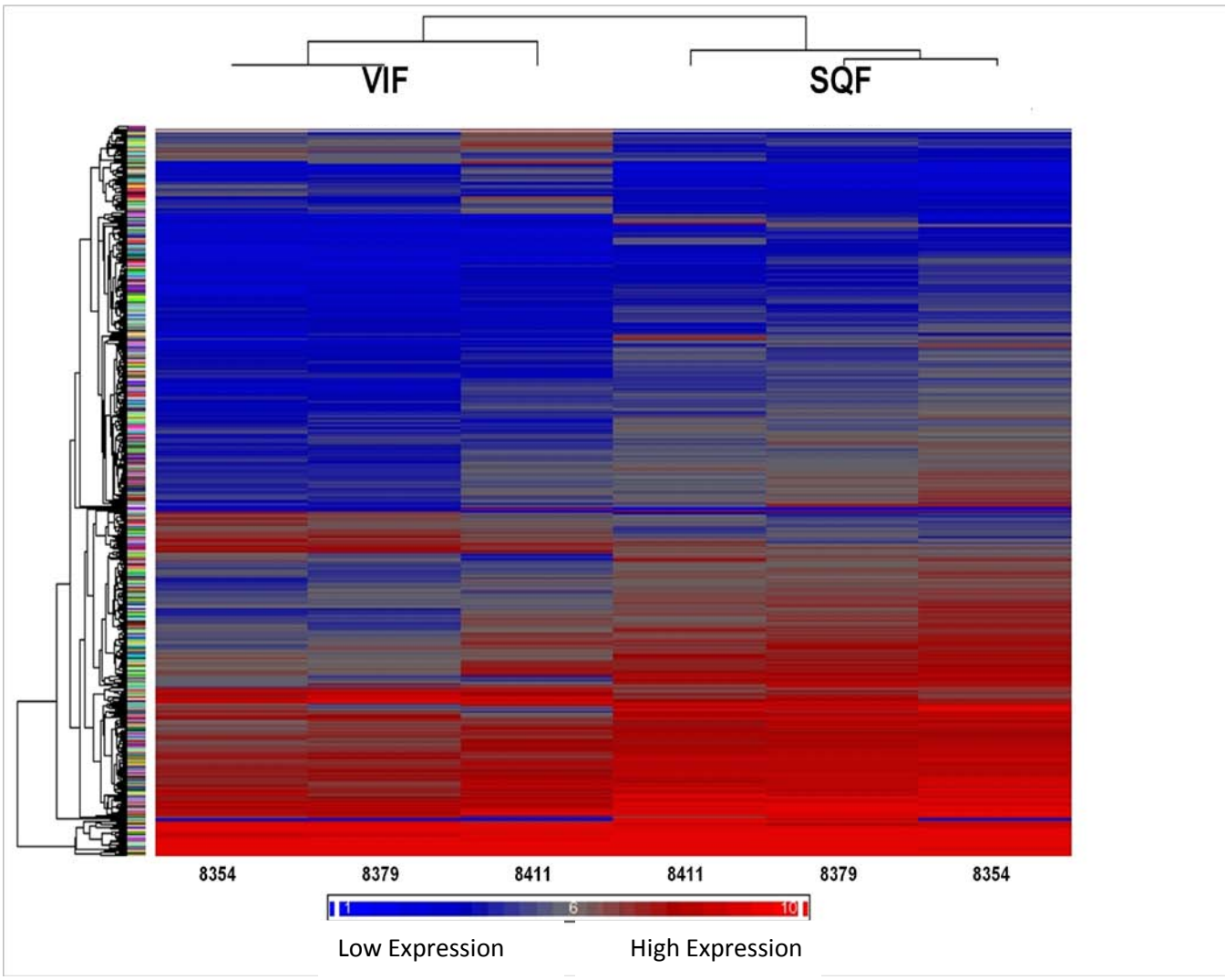


Figure 2.7: Hierarchical cluster analysis evaluating individual minipigs and both adipose tissue depots

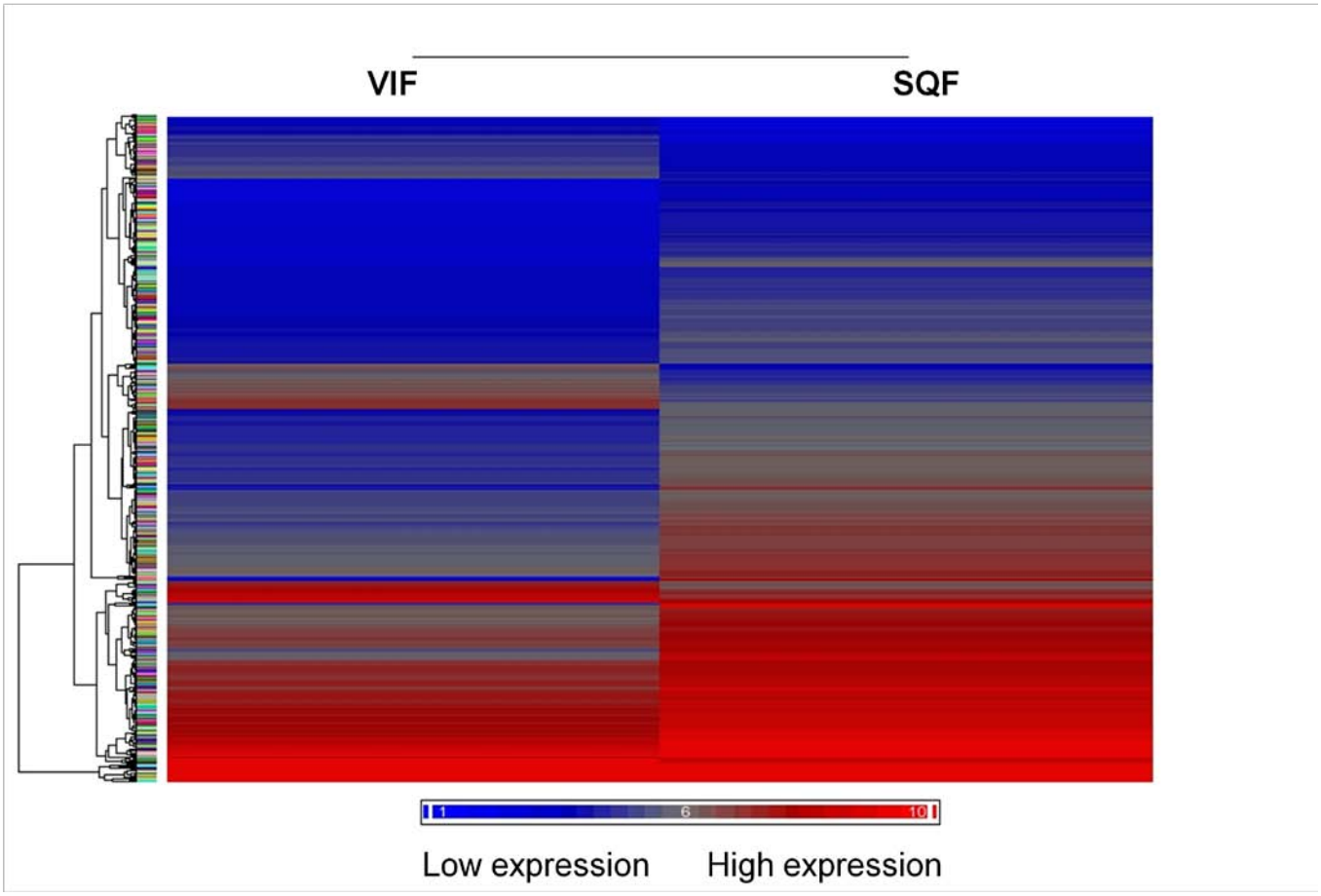


Figure 2.8: Hierarchical cluster analysis evaluating the two adipose tissue depots

2.4.3 Pathway Analysis and Gene Ontology

Tables 2.5 and 2.6 highlight pathways of interest with the number of genes in both microarray comparisons, VIF vs. SQF and SQF vs. VIF respectively. The specific genes and pathways represented by these two tables can be found in appendix 1. It was interesting to note that similar pathways changed in both comparisons (response to hypoxia and lymphocyte mediated immunity) but the genes in that pathway were up-regulated in one comparison and down-regulated in another. The VIF vs. SQF comparison showed the genes in pathways involved in lipid biosynthesis, leukocyte chemotaxis, and response to hypoxia to be almost universally up-regulated. The SQF vs. VIF comparison showed pathways involved in the acute phase response, cholesterol storage, and cell differentiation to be altered. Unlike, the VIF vs. SQF comparison, gene in this comparison were both up and down-regulated.

Table 2.5: Pathways of Interest in the VIF vs. SQF comparison

Pathway	Number of Genes
Lipid Biosynthetic Process	16
Positive Regulation of Inflammatory Response to Antigenic Stimulus	3
Response to Hypoxia	8
Immune Response	17
Response to Steroid Hormone Stimulus	8
Lymphocyte Mediated Immunity	
Regulation of Cell Death	18
Response to Corticosteroid Stimulus	5
Regulation of Cytokine Production	7
Wnt Receptor Signaling Pathway	6
Hormone Metabolic Process	5
Substrate Adhesion-Dependent Cell Spreading	3
Regulation of Insulin-Like Growth Factor Receptor Signaling Pathway	3
Positive Regulation of Leukocyte Chemotaxis	3
Estrogen Metabolic Process	3
Tissue Development	19
Lipid Metabolic Process	19

Table 2.6: Pathways of Interest in the SQF vs. VIF comparison

Pathway	Number of Genes
Protein Processing	10
Response to Hypoxia	12
Protein Maturation	11
Posttranscriptional Regulation of Gene Expression	12
Lipoprotein Transport	3
Regulation of Cholesterol Storage	3
Vasculature Development	20
B Cell Mediated Immunity	9
Adaptive Immune Response	10
Acute Inflammatory Response	11
Blood Vessel Development	18
Lymphocyte Mediated Immunity	9
Response to Hypoxia	12
Cell Morphogenesis Involved in Differentiation	42
Blood Vessel Morphogenesis	12
Retinoic Acid Metabolism Process	3
Oxidation Reduction	24
Positive Regulation of Foam Cell Differentiation	3

2.4.4 Genes of Interest

After evaluating the genes in the pathways in tables 2.5 and 2.6, we further investigated specific target genes of interest. Tables 2.7 and 2.8 highlight the metabolic differences between the two depots. Estrogen receptors 1, the gene that codes for ERα, and platelet derived growth factor D, an angiogenic gene, were down-regulated in VIF but up-regulated in SQF. A noteworthy gene change in VIF was the down regulation of insulin-like growth factor binding protein 5, a marker of insulin sensitivity. In SQF the mechanism to decrease the pro-inflammatory prostaglandin F2α, prostaglandin F2 receptor negative regulator was up-regulated.

Table 2.7: Genes of interest down-regulated in VIF compared to SQF

Gene Name	Gene Abbreviation	Fold Change	Gene Function
Estrogen Receptor 1	ESR 1	-2.2	ER α
Insulin-Like Growth Factor Binding Protein 5	IGFBP5	-3.4	Binds IGF-1
Platelet Derived Growth Factor D	PDGFD	-2.5	Blood Vessel Generation

Table 2.8: Genes of interest up-regulated in SQF compared to VIF

Gene Name	Gene Abbreviation	Fold Change	Gene Function
Estrogen Receptor 1	ESR 1	2	ER α
Prostaglandin F2 Receptor Negative Regulator	PTGFNR	2	Decrease PGF2α
Platelet Derived Growth Factor D	PDGFD	2	Blood Vessel Generation

Tables 2.9 and 2.10 further highlight the metabolic differences between the two adipose tissue depots. Genes coding for fatty acid synthase (lipogenic), interleukin 18, and platelet factor 4 (pro-inflammatory) were up-regulated in VIF but down-regulated in SQF. Moreover, hydroxysteroid 11-beta dehydrogenase 2, an important regulator in glucocorticoid synthesis, was up-regulated in VIF. The pro-inflammatory prostaglandin endoperoxidase synthathe 1, otherwise known as COX 1, was down-regulated in SQF.

Table 2.9: Genes of interest up-regulated in VIF compared to SQF

Gene Name	Gene Abbreviation	Fold Change	Gene Function
Hydroxysteroid (11-beta) Dehydrogenase 2	HSD11B2	3.4	Converts cortisone to cortisol
Fatty Acid Synthase	FASN	3.4	Lipogenesis
Interleukin 18	IL18	4.5	Th1 and IFNγ stimulation
Platelet Factor 4	PF4	6.5	Neutrophil chemo-attractant, inflammation

Table 2.10: Genes of interest down-regulated in SQF compared to VIF

Gene Name	Gene Abbreviation	Fold Change	Gene Function
Prostaglandin Endoperoxidase Synthase 1	COX 1	-2	Eicosanoid synthesis from AA
Fatty Acid Synthase	FASN	-8	Lipogenesis
Interleukin 18	IL18	-4.5	Th1 and IFNγ stimulation
Platelet Factor 4	PF4	-6.5	Neutrophil chemo-attractant, inflammation

2.5 Quantitative Real Time PCR

The gene expression of adiponectin, TNFα, and TLR 4 in the minipigs (Figure 2.9) did not yield any statistically significant differences (p=0.06, 0.8264, and 0.4599 respectively) due to large variations in the pigs. However, adiponectin did show a trend of lower levels in the SQF vs VIF.

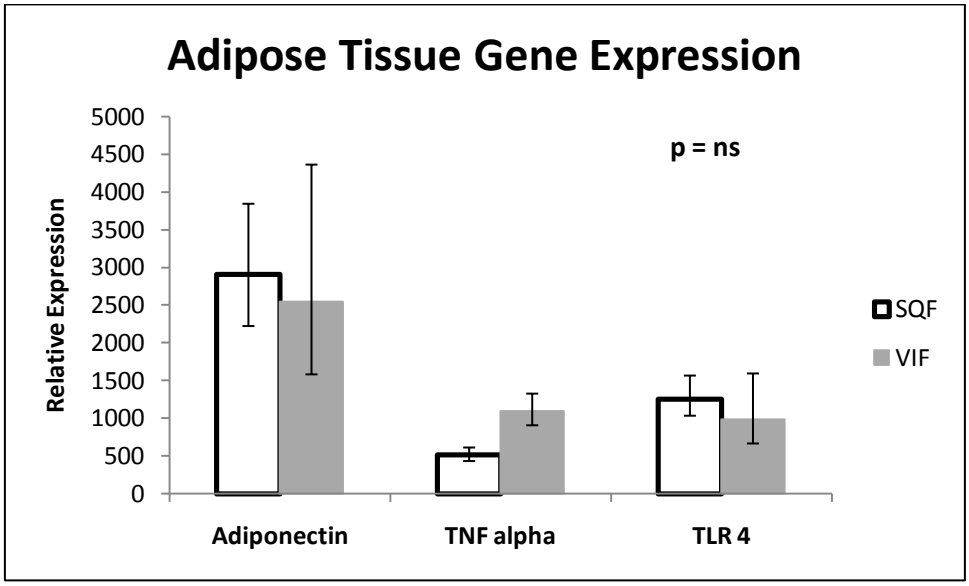


Figure 2.9: Relative gene expression between SQF and VIF

2.6 Immunofluorescence

2.6.1 Calprotectin

The calprotectin molecule was the initial protein selected for immunostaing in the Sinclair minipig using both deparafinized (see appendix 1 for deparaffinization protocol) and frozen sections, based upon previous studies demonstrating its efficacy in the domestic pig. We found, however, that the molecule did not show consistent positive staining in the minipig (MP) VIF, and no staining in the SQF (Figure 2.11). In order to determine if the inconsistent staining was due to procedural error or a lack of antibody specificity, we used frozen sections of SQF, VIF, liver and spleen collected from a domestic pig (DP), and positive staining was detected (Figure 2.12). We assessed protein binding through western blot using the same antibody and determined that the calprotectin was indeed detectable in minipig protein using the same antibody (Figure 2.10). This led us to the conclusion that the there were specificity problems with this antibody in the minipig.

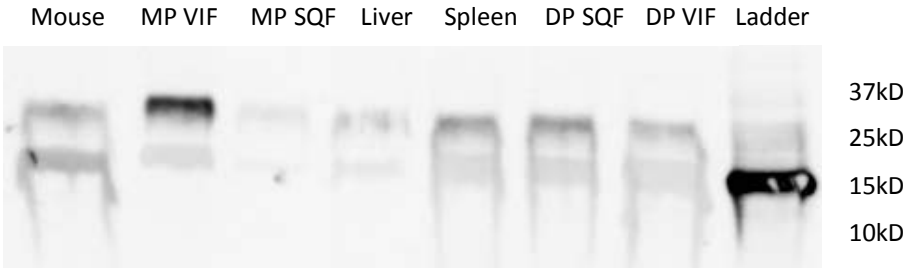
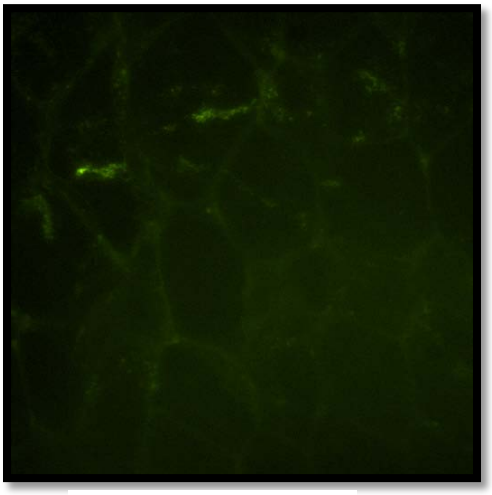
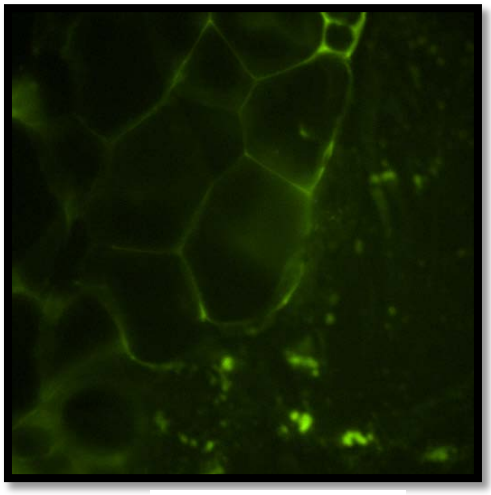


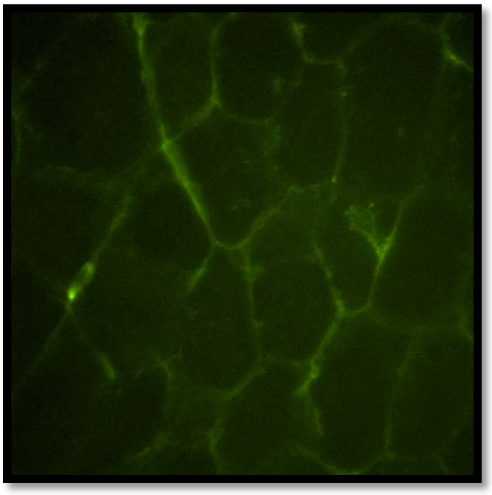
Figure 2.10: Western blot of calprotectin heterodimer heavy (24 kD) and light (12 kD) bands



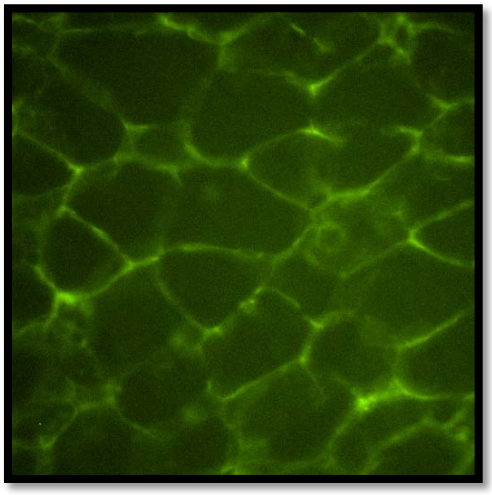
(a) MP SQF



(b) MP VIF

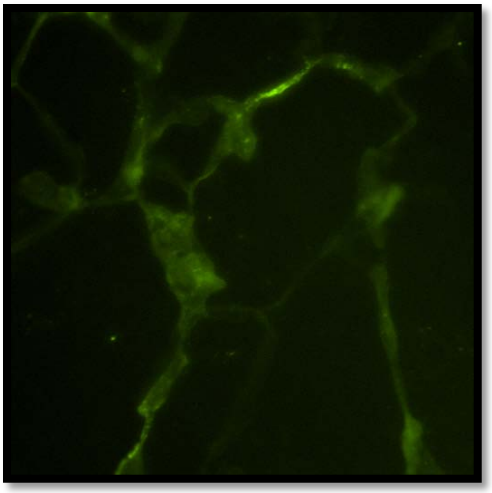


(c) Isotypic control (DP VIF)

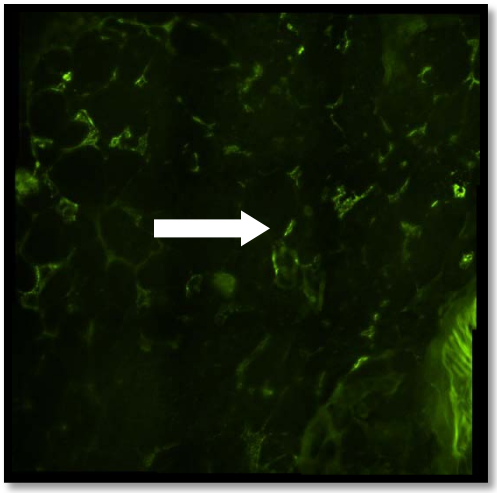


(d) Negative control (DP VIF)

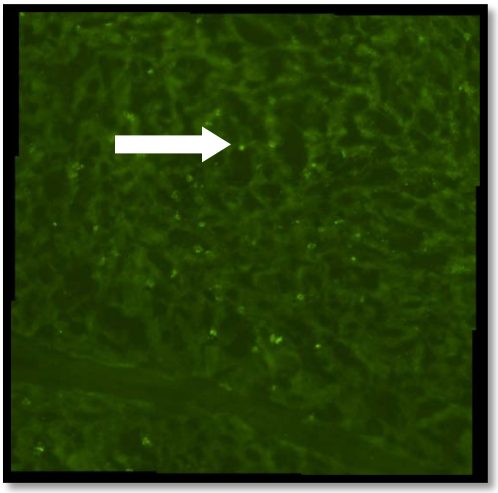
Figure 2.11: Immunofluorescence using calprotectin as a marker of immune cell infiltration in the (a) SQF and (b) VIF of the Sinclair minipig with a (c) isotypic positive control (IC) and a (d) negative control (NC)



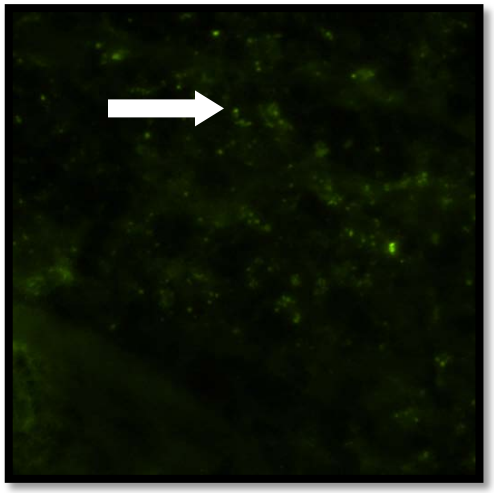
(a) DP SQF



(b) DP VIF



(c) Liver



(d) Spleen

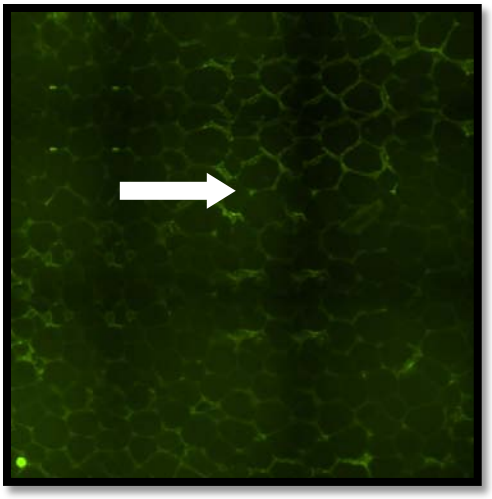
Figure 2.12: Immunofluorescence using calprotectin as a marker of immune cell infiltration in domestic pig (a) SQF, (b) VIF, (c) liver, and (d) spleen

2.6.2 CD 163

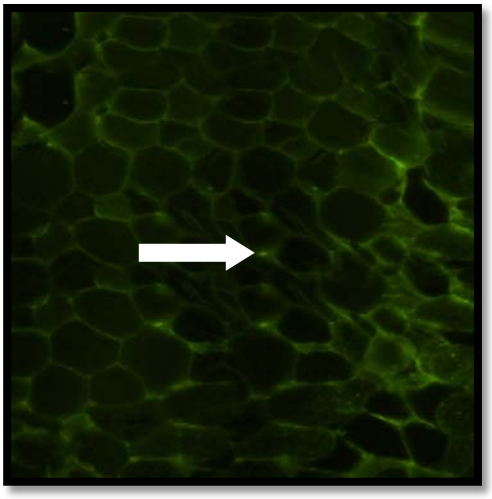
Due to the inconclusive finding from calprotencin staining, we conducted immunofluorescence staining using CD 163, a maker of alternatively activated (M2) macrophages. From this staining we determined that SQF in both the minipig and domestic pig had a greater abundance of the alternatively activated macrophages than VIF (Figures 2.13 and 2.14). Additionally, alternatively activated macrophages were found to be present in the liver, spleen, and lymph node of the domestic pig (Figure 2.15).

2.6.3 CD 68

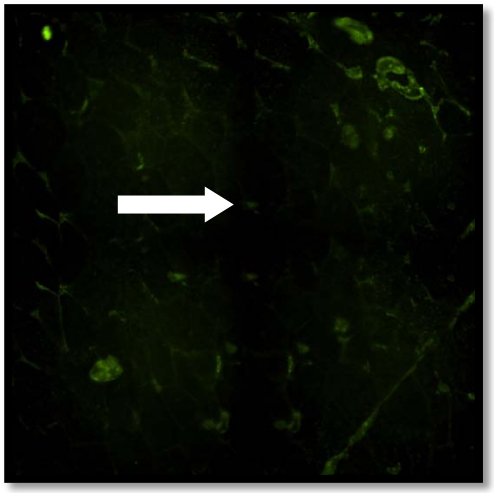
CD 68 was used to determine the population of classically activated (M1) macrophages. VIF of both the minipig and the domestic pig had a greater abundance of classically activated macrophages than SQF (Figures 2.16 and 2.17). Macrophages with this phenotype were found in the liver, spleen, and lymph node of the domestic pig as well (Figure 2.18).



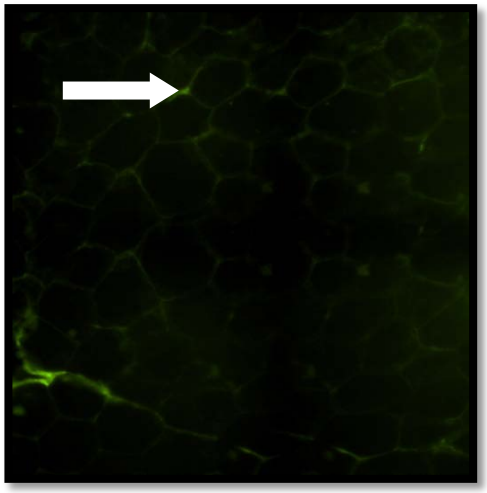
(a) MP SQF



(b) MP VIF

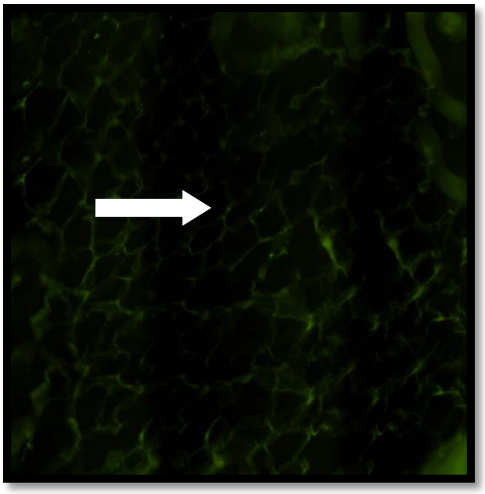


(c) MP SQF

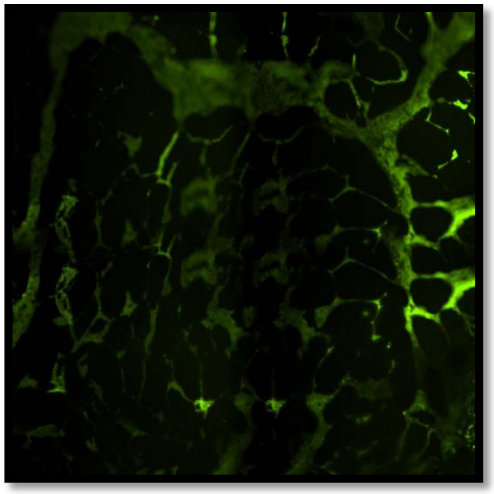


(d) MP VIF

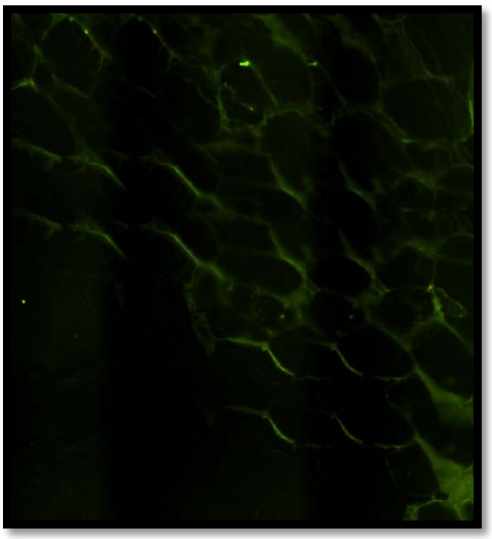
Figure 2.13: Immunofluorescence using CD 163 as a marker for alternatively activated macrophages in the Sinclair minipig (a,c) SQF and (b,d) VIF.



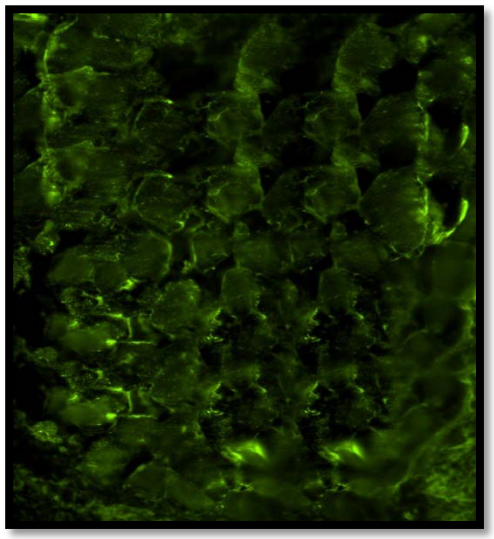
(a) DP SQF



(b) DP VIF

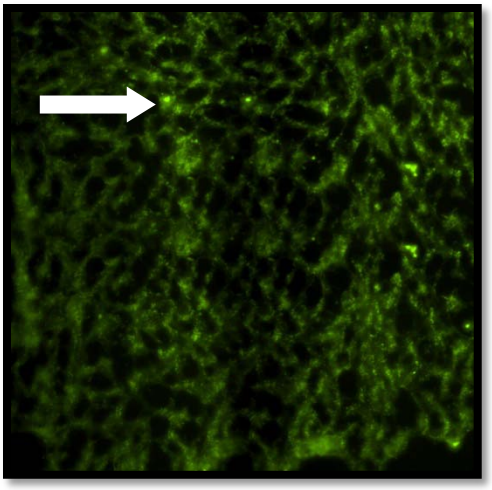


(c) IC (DP VIF)

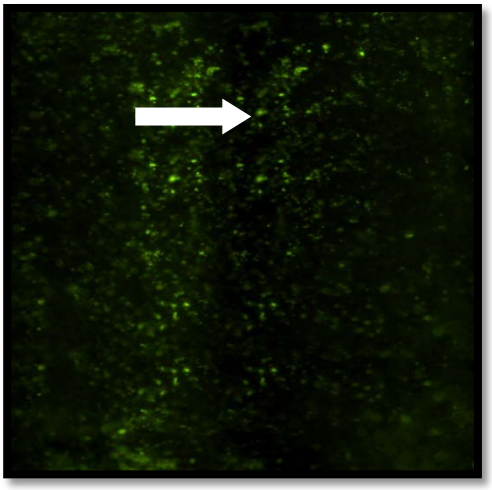


(d) NC (DP VIF)

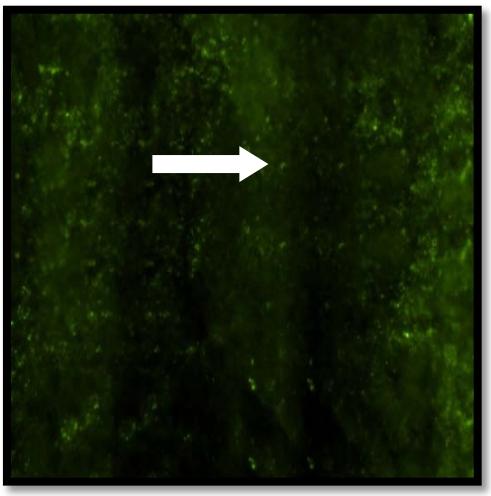
Figure 2.14: Immunofluorescence using CD 163 as a marker of alternatively activated macrophages in domestic pig (a) SQF and (b) VIF with a (c) isotypic positive control (IC) and a (d) negative control (NC)



(a) Liver

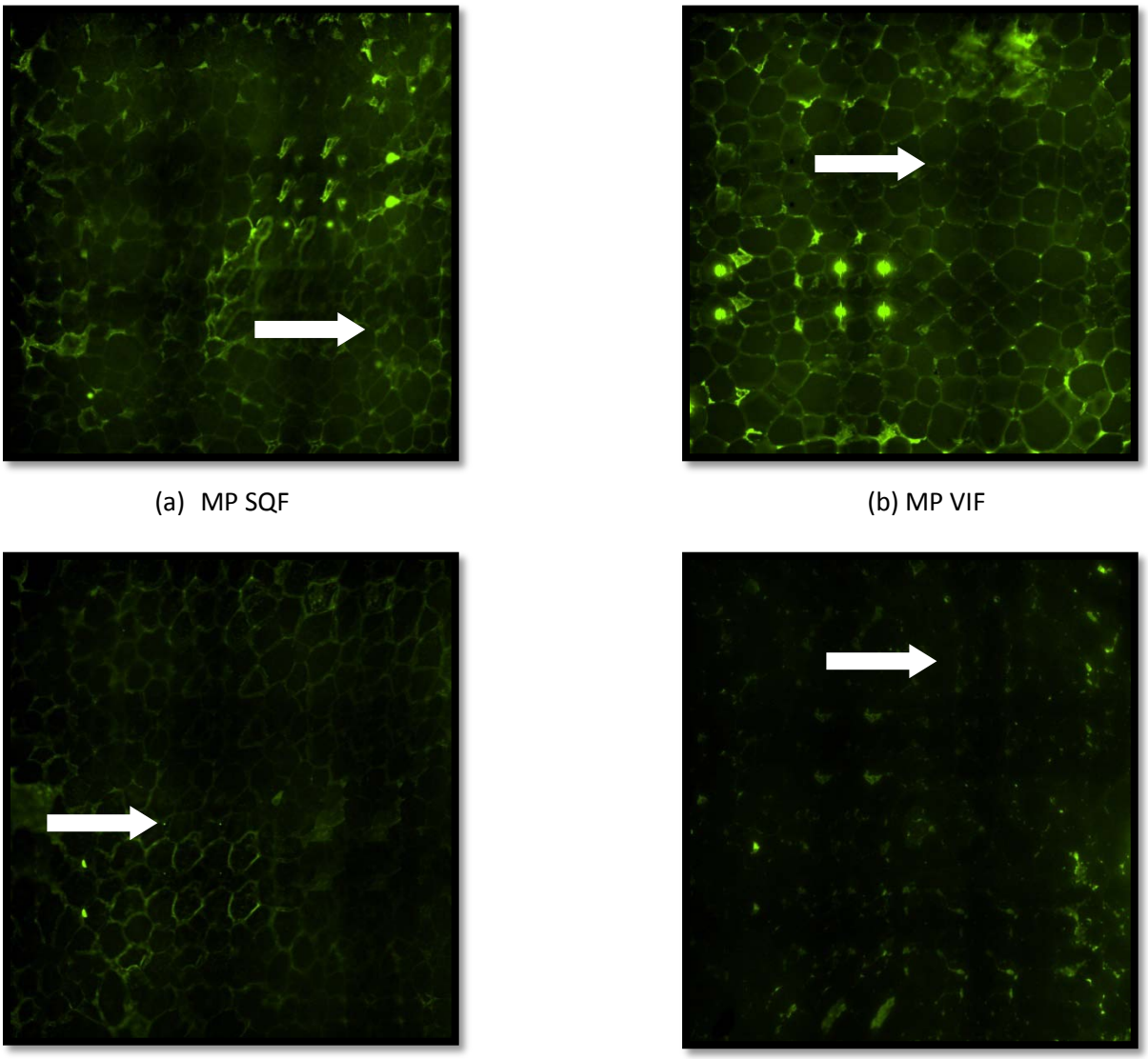


(b) Lymph Node



(c) Spleen

Figure 2.15: Immunofluorescence using CD 163 as a marker for alternatively activated macrophages in domestic pig (a) liver, (b) lymph node, and (c) spleen



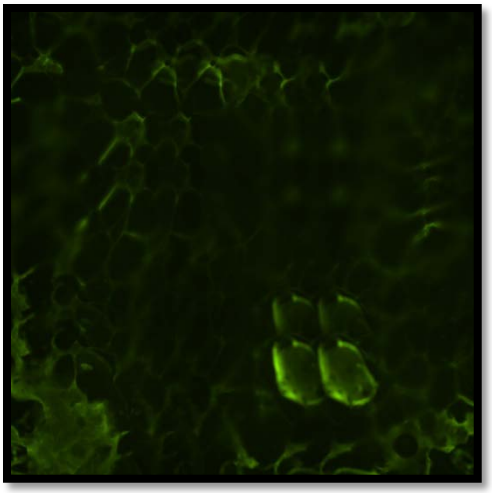
(a) MP SQF

(b) MP VIF

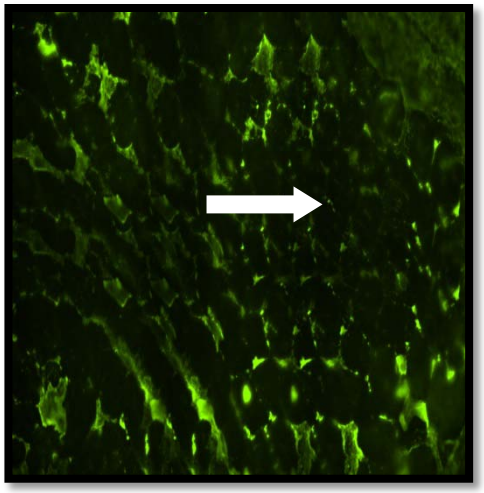
(c) MP SQF

(d) MP VIF

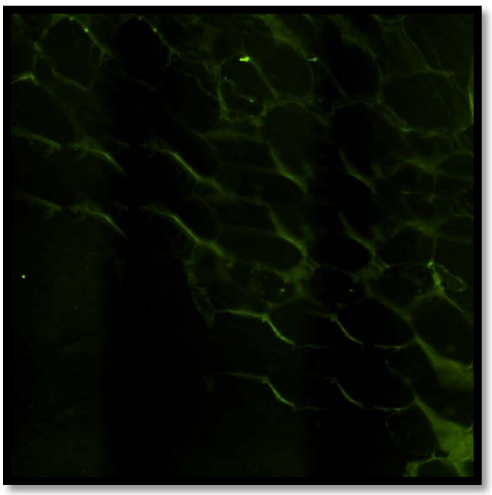
Figure 2.16: Immunofluorescence using CD 68 as a marker of classically activated macrophages in the Sinclair minipig (a,c) SQF and (b,d) VIF



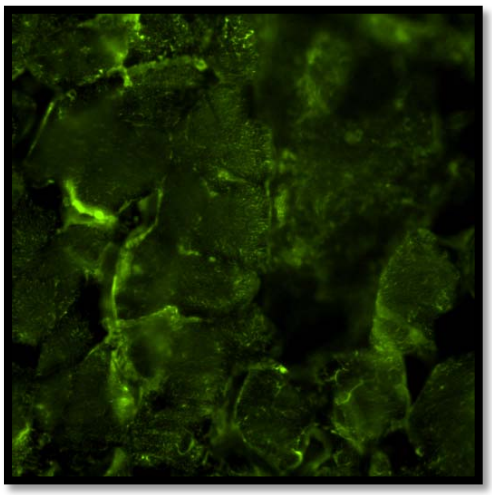
(a) DP SQF



(b) DP VIF

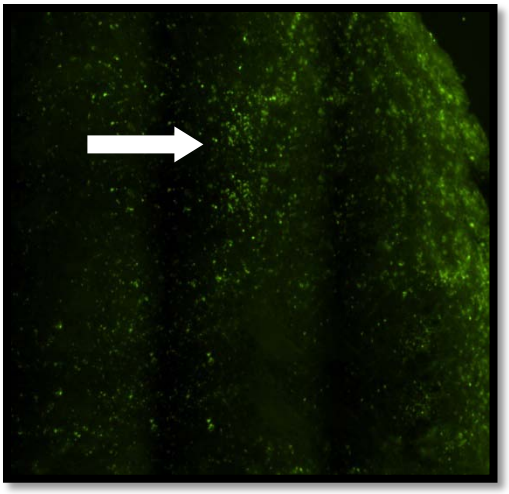


(c) IC (DP VIF)

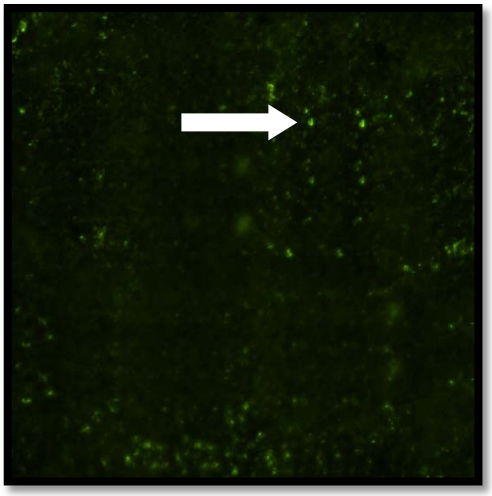


(d) NC (DP VIF)

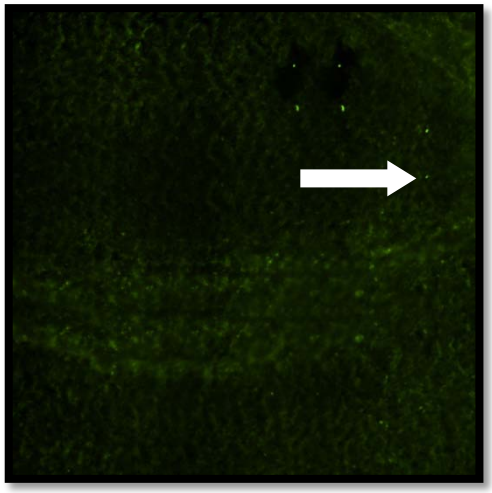
Figure 2.17: Immunofluorescence using CD 68 as a marker of classically activated macrophages in domestic minipig (a) SQF and (b) VIF with a(c) isotypic positive control (IC) and a (d) negative control (NC)



(a) Liver



(b) Lymph Node



(c) Spleen

Figure 2.18: Immunofluorescence using CD 68 as a marker for classically activated macrophages in domestic pig (a) liver, (b) lymph node, and (c) spleen

2.7 Histology

2.7.1 H and E Stained Slides

Figure 2.19 is a representative image of the histological section used for the adipocyte and macrophage cell counts. The adipocytes are white with a dark border, the small dark dots are macrophages, and the long dark streaks are connective tissue.

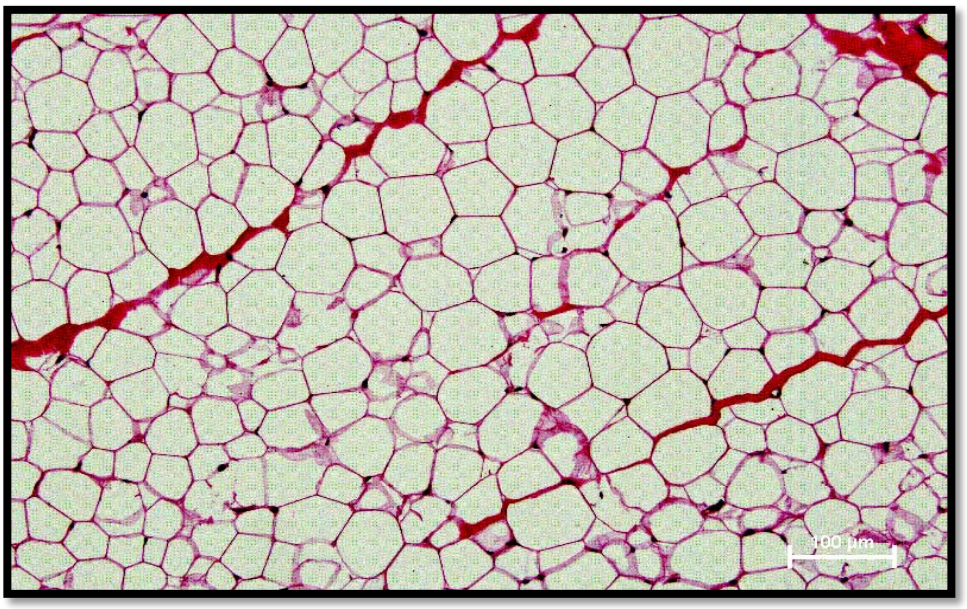


Figure 2.19: H and E stained VIF in the Sinclair minipig

2.7.2 Adipocyte Count

A hand count of the adipocytes (Figure 2.20) revealed that SQF had a greater number of adipocytes than VIF (p= 0.0024).

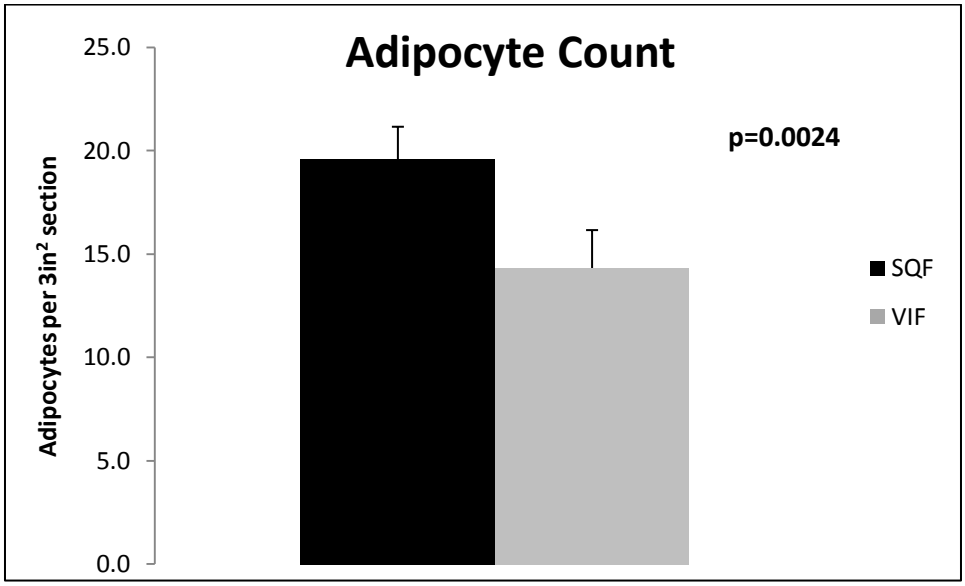


Figure 2.20: Average number of adipocytes in each depot in the Sinclair minipig

2.7.3 Macrophage Count

Figure 2.21 demonstrates that there was not a statistically significant difference in the number of macrophages present between SQF and VIF.

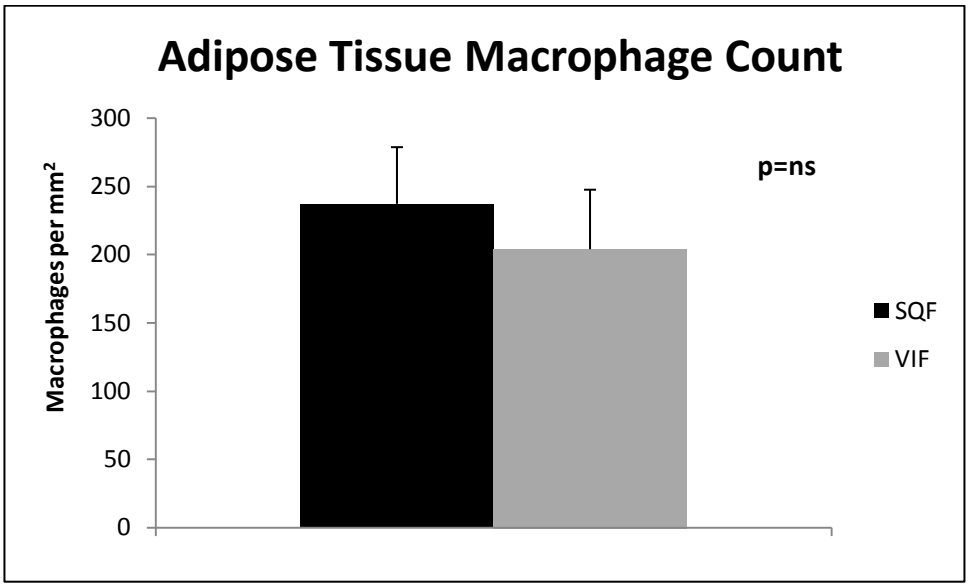


Figure 2.21: Average macrophages per mm^2 in both depots in the Sinclair minipig

2.8 Western Blot

The western blots (Figure 2.22) revealed that there were some specificity differences between domestic pigs, minipigs, and mice. Fatty acid synthase (FAS) was the only protein that showed binding in the domestic and minipig. The antibodies used were all mouse derived and had previously been validated for use in the mouse in our lab. For complete antibody details see appendix 1.

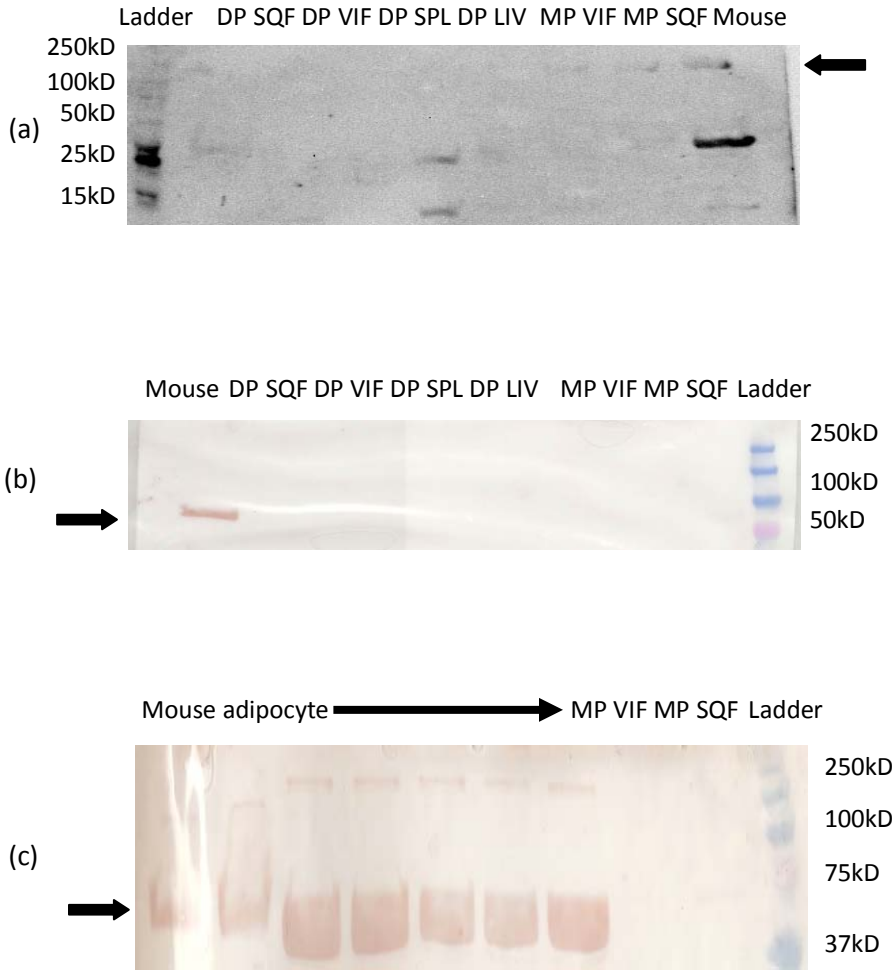


Figure 2.22: Western blot of (a) fatty acid synthase, (b) PPARγ, and (c) vitronectin,

Section 3: Discussion

3.1 Bodyweight

It is necessary to reiterate that the study presented here was part of a larger study that sought to look at the effects of PUFA and LPS on the obese Sinclair minipig.

The study was initially designed with the intention to feed the minipigs 150% of their daily maintenance based on the average weight of the minipigs. However, the amount of diet fed was not re-assed throughout the study, based on the bi-weekly weight change, and was therefore not 150% of daily intake. Given the minipigs rapid weight gain from weeks 0-6 of and plateau from weeks 6-8, it is likely that the minipigs were not overfed after the 6th week of the study. It is important to note, however, that the final weight of the minipigs was consistent with the average species weight information supplied by Sinclair Bio Resources [226].

There may be one other explanation week 8 weight plateaus, the end of juvenile exponential growth phase. The intent at the beginning of the study was to use boars that had already reached sexual maturity to be more physiologically similar to humans. There is anecdotal and metabolic evidence to suggest that the minipigs were not sexually mature upon arrival, and did not reach sexual maturity until week 4. This may have resulted in a decrease in weight at the end of the study.

The average weights of the minipigs were beginning to achieve an overweight to moderately obese state by the 6th week of the study. Therefore the subsequent comparisons of the VIF and SQF should reflect some deviation from normal adipose tissue biology and physiology.

Although the 8th week of study reflected the restrictive nature of the diet, every data point preceding it indicated that the pigs were overweight to moderately obese. Therefore the results, though not reflecting overt obesity, provide important baseline are an interesting comparisons of the VIF and SQF depot differences in the Sinclair minipig (a comparison that has yet to be published).

3.2 Circulating Metabolic Parameters

The lipid data gave a contradictory profile of the minipigs. The decrease in triglycerides from the beginning to the end of the study is consistent with data in young pigs suggesting that there is a preference to allocate dietary recourses to growth and not fat [210]. The decrease in LDL was contradictory to our hypothesis in which we expected it to increase. This may once again be due more to the age of our minipigs and the fact they were not overfed to obesity [227]. The decrease in HDL that occurred by the end of the study, thought not significant, is consistent with the literature which states that in a component of the metabolic syndrome, dyslipidemia, is characterized in part by a decrease in circulating HDL [175].

The total WBC count yielded different than expected results as well. At the beginning of the study the minipigs had comparatively high levels of WBC than at the end of the study. In fact, some of the minipigs were higher than normal. This may partially be an age effect as well as the WBC values in younger animals may higher than those in older animal [228].

This phenomena be due to the continuous assault of novel pathogens and the need to generate a tremendous amount of antibodies in a relatively short amount of time [228]. This study suggests that the Sinclair minipig follows this pattern also. The percentages of segments and lymphocytes follow this same pattern.

Serum cytokines were measured via multiplex in order to gain a global understanding of the pro-inflammatory and anti-inflammatory cytokine profile in the minipigs at the end of the study. The anti-inflammatory cytokines IL-4 and IL-10 as well as the pro-inflammatory cytokines Interferon gamma (IFNγ), Interleukin 1 beta (IL-1β), and TNFα were measured using multiplex technology. Interleukin-4 is considered to be anti-inflammatory because it leads to the Th2 phenotype as well as being responsible for the regulation of M2 macrophages [60, 229]. This phenotype is considered to be anti-inflammatory because the Th2 cells are primarily responsible for B-cell and hence antibody response as well. Additionally, M2 macrophages secrete IL-4 [229]. Therefore, we expected our minipigs to have relatively lower circulating levels IL-4 and IL-10 as well as higher circulating IFNγ, IL-1β, and TNFα. Measurement of these

cytokine parameters have not been measured in the minipigs that are metabolically normal, and warrant further investigation.

3.3 Microarray

Microarray is a very useful technique to determine whole changes in the genome and allows for investigators to see global changes in gene expression, a great benefit if one seeks to elucidate new mechanisms of change or characterize something unknown. Most, if not all models of used for scientific study have been characterized by microarray in some capacity. However, the Sinclair minipig, a very important model of many diseases has been woefully under characterized in general, and more specifically by microarray. Moreover, there is scant data on porcine adipose tissue in general, and comparing VIF and SQF then SQF and VIF specifically [230, 231] .

The VIF was compared to SQF to determine the pathways associated with it. This was done by comparing the results to the VIF against the SQF. Genes associated with lipid biosynthesis, hypoxic response, immune response, wnt signaling pathway, regulation of insulin-like growth factor receptor signaling, and estrogen metabolic process. Specifically, there was an up-regulation in inflammatory and lipogenic genes such as FASN, IL-18 and hydroxysteroid 11-beta dehydrogenase 2(HSD11B2), as well as a down-regulation in genes associated with insulin sensitivity and low adiposity such as ESR1 and insulin like growth factor binding protein 5 (IGFBP5). This metabolic profile suggests that VIF is more pro-inflammatory and lipogenic in nature when compared with SQF due to the up-regulation of pro-inflammatory and lipogenic genes such as IL-18 and FASN respectively as well as a down regulation of anti-inflammatory genes such as ESR1 [144, 232].

As reviewed earlier, SQF may be more metabolically favorable than VIF, but deep SQF, especially abdominal, has been documented to have a metabolic profile similar to VIF, whereas superficial SQF has been demonstrated to posses the protective metabolic profile associated with SQF. The microarray data generally supports the literature suggesting that SQF may be protective of the metabolic consequences of obesity [233]. We found that genes associated with low adiposity like ESR1 (coding for ERα) were up-regulated in SQF compared with VIF. As

reviewed earlier, higher levels of ERα are directly correlated with lower adiposity and insulin sensitivity [143]. Additionally, anti-inflammatory genes such as Prostaglandin F2 receptor negative regulator (PTGFNR) are increased in SQF compared with VIF indicating a lower capacity for inflammation due to its ability to down-regulate PGF2α, a well characterized pro-inflammatory cytokine, and inhibitor of adipocyte differentiation [234]. Additionally, lipogenic genes such as FASN were down regulated. Finally, pro-inflammatory genes such as PF4 and IL-18 were down-regulated. Taken together, the differential expression of these genes in SQF compared with VIF offer a picture of the protective effect of SQF as compared with VIF.

In addition to the specific genes that changed, there were many pathways that changed in SQF compared with VIF. These included genes associated with the pathways of hypoxia, cholesterol transport and metabolism, innate immunity, acute inflammation, B-cell differentiation. The majority of the genes in these pathways were down-regulated; a potentially significant finding with regards to the hypoxia, cholesterol transport and metabolism pathways because metabolic processes associated with these pathways is hypothesized to lead to some of the metabolic consequences associated with obesity [42, 235].

The changes in gene expression in these numerous pathways reiterate the far reaching and diverse effects of adipose tissue and its secreted factors. The microarray data further demonstrates how far reaching the consequences of overweight and obesity may be, and that researchers may have only begun to scratch the surface of the mechanism involved in adipose tissue dysfunction.

3.4 Gene Expression

Unfortunately, the gene expression data were not statistically significant due to large variations between the individual minipigs. The trends of the gene expression indicate SQF expressed a more metabolically favorable expression pattern (higher adiponectin, and lower TNFα), and although not all reached statistical significance, trends are in line with reported expression of TNFα and TLR4, which are both expressed at higher levels in the VIF when compared to the SQF [223, 236].

3.5 Immunofluorescence

Immunofluorescence is a good way to visualize changes in cellularity and protein concentrations in the adipose tissue [237, 238] The immunofluorescence was initially undertaken using paraffin embedded tissue mounted on slides using the macrophage marker calprotectin. The tissue was deparaffinized (see appendix 1 for protocol), which was performed on minipig samples only (liver, SQF, and VIF), yielded inconsistent results. Though the minipig SQF and VIF showed inconsistent positive staining, a potential positive staining was obtained in the liver, but without the expected amount of macrophage (Kupffer cell) population of 3-5%. However, expert pathologist analysis did indicate that the presumed Kupffer cells were in the anatomical location (on the periphery of the hepatic blood vessels). This led to two conclusions, either the antibody was not effective (either due to concentration or specificity), or the protocol to deparaffinize the adipose tissue was not effective.

In order to differentiate between these two hypotheses, two alterations to the protocol were made. First, we systematically varied the dilution and incubation times of the antibody (antibody dilution ranged from 1:200 to 1:500 and incubation times ranged from 1 to 2.5 hours). The best results used the same dilution factor and incubation time; 1:500 and 1 hour respectively. All other combinations resulted in higher background, but still inconsistent staining. Second, we used flash frozen rather than paraffin embedded tissue mounted on slides. However, there was no flash frozen minipig liver to analyze, and therefore from this point on, we were working with minipig SQF and VIF only. In addition, because our first immunostaining assays had limited success in the minipig, we began staining domestic pig liver, spleen, SQF, and VIF obtained from Wampler's Sausage, a local slaughter house and sausage factory to assess potential differences in antibody cross-reactivity between the minipig and domestic pig. We chose to do this because some experiments in our lab comparing the domestic and Sinclair minipig indicated some lack of homology, this seemed to be confirmed with the calprotectin IF. Differences in homology is seen in rodents as well [239]. There was positive staining in the domestic pig VIF, liver, and spleen. The results from the minipig SQF and VIF as well as the domestic pig SQF were not different from the negative control.

Fortunately, this antibody was indicated for western blotting as well as IF, and our western blot analysis confirmed the literature's reports of calprotectin being a heterodimer or tetramer [240]. The western blot showed 2 bands, one at 24 kD the other at 12 kD (Figure 2.10) for the mouse, minipig, and domestic pig, however, the minipig showed the faintest bands. This led us to conclude that the issues with calprotectin binding in the minipig fat were the result of one of three possibilities; failure of one of the isoforms to bind (one that fluoresces), low affinity of the antibody for the minipig, or physiologic differences in the minipig that result in less neutrophil or monocyte infiltration, the two immune cells types calprotectin binds [240]. Therefore we chose to use different antibodies, CD 68 (Figures 2.16 -2.18) and CD 163 (Figures 2.13-2.15) which had the added benefit of being able to differentiate between classically and alternatively activated macrophages respectively.

Experimental evidence has shown that SQF and VIF have different levels of macrophage infiltration, and the macrophages that do infiltrate the fat have different phenotypes[59]. VIF has been show to have more classically activated M1 macrophages and SQF has more alternatively activated M2 macrophages, and our IF supported this. The visceral fat showed very few if any M2 macrophages, but a comparatively greater amount of M1 macrophages. Conversely, SQF showed a number of M2 macrophages, but showed the presence of a small population of M1 macrophages as well. However, the number of M1 macrophages in SQF was far less than the number of M2 macrophages [241].

Interestingly, the domestic pig VIF and SQF showed the same pattern of M1 and M2 expression as the minipig, indicating that this feature of adipose tissue may be conserved. There were more M1 and fewer M2 macrophages present in VIF compared to SQF which is consistent with the literature. The liver, spleen, and lymph node of the domestic pig showed the presence of M1 and M2 macrophages as well, with the concentration of M1 macrophages particularly in the spleen and lymph node.

3.6 Adipocyte and Macrophage Count

We counted average number of adipocytes in order to classify the cellular characteristics between SQF and VIF. The first method employed to count adipocytes was a hand count. The cells were counted using the average count of three 3"x3" squares drawn on a photograph of a representative section of the H&E stained slides. The results from this count are in line with reported findings in the literature that the SQF had a greater number of adipocytes that the VIF [242]. We were not able to quantitatively and accurately assess adipocyte size. The greater number of adipocytes was present despite a general trend of significant connective tissue in the SQF.

The macrophage count illustrated that the SQF had more immune cells than the VIF. This initially seemed contradictory to the literature, but IF subsequently revealed the SQF macrophage population to be primarily composed of M2 macrophages which are generally considered to be more anti-inflammatory in nature, and mentioned in chapter 1. Therefore, the higher immune cell count may indicate an anti-inflammatory and potentially protective phenotype.

3.7 Proteins

We tested several antibodies for proteins we expected to be expressed in minipig and domestic pig adipose tissues. Several of these including COX1, COX2, PPARγ, vitronectin and GAPDH (see appendix 1 for details) could not be detected in the domestic pig or minipig. This was surprising as many of these proteins, especially PPARγ and GAPDH, are relatively conserved between species [243, 244]. Western blots for PPARγ and FAS were run using mouse adipose tissue as a positive control which was positive each time. However, FAS was expressed in adipose tissue from domestic and minipigs. The only consistently reactive antibody was calprotectin (mentioned in the IF section).

Section 4: Conclusion

Overall, the results from the study indicate that the Sinclair minipig is a good model for human adipose tissue and obesity studies. The SQF demonstrated an anti- inflammatory and anti-adipogenic genetic profile. Additionally, VIF demonstrated a general trend of altering gene expression to promote the recruitment of immune cells (neutrophils and Th1 cells), lipogenesis, and insulin resistance. Finally, SQF was found to have a greater immune cell population when compared to VIF; however, the majority of the macrophages present were of the M2 anti-inflammatory phenotype. Based on the data, the adipose tissue of the Sinclair minipig is a good model for human studies.

Chapter III: Summary of Data

Section 1: Conclusion

Overall, the data demonstrated that the Sinclair minipig is a good model for human adipose tissue studies. The gene expression data showed the general trend of the SQF showing a generally protective and in some instances beneficial metabolic profile when compared with VIF. Although the qRT-PCR data for the selected genes we tested did not result in significant differences in gene expression between SQF and VIF, several additional candidate genes remain to be tested. Additionally, SQF had a greater population of the M2, alternatively activated and anti-inflammatory macrophages compared with VIF. Incidentally, the trend of greater population of M2 macrophages in the MP SQF was seen in the domestic pig SQF as well. Finally, the protein data demonstrates that some proteins, even those considered to be fairly evolutionarily conserved may have differences in the minipig. It is possible that different antibodies may need to be tested or developed.

The characterization of a species, or in this case sub-species, is critical before its use as a model. Therefore, the metabolic, gene expression, microarray, immunofluorescence, and protein data, is a good foundation in understanding the adipose tissue biology of the Sinclair minipig. Additionally, learning about the Sinclair minipig's adipose tissue biology has demonstrated that even among sub-species of pigs, there are many similarities, yet there are also distinct differences. This trend is seen in other models including rodents.

Section 2: Limitations

A major limitation of the study was unintentional restriction of the diet in the last 2 weeks of the study. The intent was to feed the minipigs 150% of daily caloric needs for 8 weeks however, the amount of diet was not increased in proportion to the bi-weekly weighing. Additionally, the diet fed was a standard pig diet, and not high fat or sugar. This study was a

pilot study, with 4 pigs per treatment group. Therefore, results showed trends, but not statistical significance, and power calculations suggest that 6 or more pigs per treatment group would be the ideal number. Finally, the minipigs should be 8 months of age when beginning (not towards the end of) the study. Though Sinclair bioresources assured us sexual maturity was at 6 months of age, we have reason to believe that the minipigs reached puberty after we received them, around 7 months of age.

Section 3: Future Studies

The next step in the Sinclair minipig adipose tissue/obesity studies is to feed a high fat, high sugar ad-libitum diet. This would allow for the development of obesity, the metabolic syndrome, and atherosclerosis while simultaneously eliminating the problem of feed restriction or feed recalculation/pair feeding based on weight gain. Further, detailed studies on adipose tissue cellularity (both cell size and number) should be conducted. Finally, assessment of body compostion using imaging techniques (such as DXA) would confirm adiposity as well as allow better characterization of these animals.

Literature Cited

1. Kuczmarski RJ, Flegal KM. Criteria for definition of overweight in transition: background and recommendations for the United States. *Am J Clin Nutr.* Nov 2000;72(5):1074-1081.
2. Christou NV, Sampalis JS, Liberman M, Look D, Auger S, McLean AP, MacLean LD. Surgery decreases long-term mortality, morbidity, and health care use in morbidly obese patients. *Ann Surg.* Sep 2004;240(3):416-423; discussion 423-414.
3. Ellis KJ. Selected body composition methods can be used in field studies. *J Nutr.* May 2001;131(5):1589S-1595S.
4. Chan DC, Watts GF, Barrett PH, Burke V. Waist circumference, waist-to-hip ratio and body mass index as predictors of adipose tissue compartments in men. *QJM.* Jun 2003;96(6):441-447.
5. Archenti A, Pasqualinotto L. Childhood obesity: the epidemic of the third millenium. *Acta Biomed.* Aug 2008;79(2):151-155.
6. Caballero B. The global epidemic of obesity: an overview. *Epidemiol Rev.* 2007;29:1-5.
7. Breslow L. Public health aspects of weight control. *Am J Public Health Nations Health.* Sep 1952;42(9):1116-1120.
8. Statistics NCfH. Prevalence of Overweight, Obesity, and Extreme Obesity among Adults: Untied States, Trends 1976-80 through 2005-2006. December 2008; http://www.cdc.gov/nchs/products/pubs/pubd/hestats/overweight/overweight_adult.htm
9. Awad AB, Bradford PG. *Adipose tissue and inflammation*. Boca Raton, FL: Taylor & Francis; 2010.
10. Fontaine KR, Redden DT, Wang C, Westfall AO, Allison DB. Years of life lost due to obesity. *JAMA.* Jan 8 2003;289(2):187-193.
11. Mukesh M, Bionaz M, Graugnard DE, Drackley JK, Loor JJ. Adipose tissue depots of Holstein cows are immune responsive: inflammatory gene expression in vitro. *Domest Anim Endocrinol.* Apr 2010;38(3):168-178.
12. Rowlands I, Graves N, de Jersey S, McIntyre HD, Callaway L. Obesity in pregnancy: outcomes and economics. *Semin Fetal Neonatal Med.* Oct 9 2009.
13. Walley AJ, Blakemore AI, Froguel P. Genetics of obesity and the prediction of risk for health. *Hum Mol Genet.* Oct 15 2006;15 Spec No 2:R124-130.
14. Hill JO, Peters JC. Environmental contributions to the obesity epidemic. *Science.* May 29 1998;280(5368):1371-1374.
15. Stratigopoulos G, Padilla SL, LeDuc CA, Watson E, Hattersley AT, McCarthy MI, Zeltser LM, Chung WK, Leibel RL. Regulation of Fto/Ftm gene expression in mice and humans. *Am J Physiol Regul Integr Comp Physiol.* Apr 2008;294(4):R1185-1196.
16. Schulz LO, Bennett PH, Ravussin E, Kidd JR, Kidd KK, Esparza J, Valencia ME. Effects of traditional and western environments on prevalence of type 2 diabetes in Pima Indians in Mexico and the U.S. *Diabetes Care.* Aug 2006;29(8):1866-1871.
17. Frayling TM, Timpson NJ, Weedon MN, Zeggini E, Freathy RM, Lindgren CM, Perry JR, Elliott KS, Lango H, Rayner NW, Shields B, Harries LW, Barrett JC, Ellard S, Groves CJ, Knight B, Patch AM, Ness AR, Ebrahim S, Lawlor DA, Ring SM, Ben-Shlomo Y, Jarvelin MR, Sovio U, Bennett AJ, Melzer D, Ferrucci L, Loos RJ, Barroso I, Wareham NJ, Karpe F, Owen KR, Cardon LR, Walker M, Hitman GA, Palmer CN, Doney AS, Morris AD, Smith GD, Hattersley AT, McCarthy MI. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science.* May 11 2007;316(5826):889-894.
18. Fredriksson R, Hagglund M, Olszewski PK, Stephansson O, Jacobsson JA, Olszewska AM, Levine AS, Lindblom J, Schioth HB. The obesity gene, FTO, is of ancient origin, up-regulated during food deprivation and expressed in neurons of feeding-related nuclei of the brain. *Endocrinology.* May 2008;149(5):2062-2071.

19. Andreasen CH, Stender-Petersen KL, Mogensen MS, Torekov SS, Wegner L, Andersen G, Nielsen AL, Albrechtsen A, Borch-Johnsen K, Rasmussen SS, Clausen JO, Sandbaek A, Lauritzen T, Hansen L, Jorgensen T, Pedersen O, Hansen T. Low physical activity accentuates the effect of the FTO rs9939609 polymorphism on body fat accumulation. *Diabetes.* Jan 2008;57(1):95-101.

20. Elinder LS, Jansson M. Obesogenic environments--aspects on measurement and indicators. *Public Health Nutr.* Mar 2009;12(3):307-315.

21. Kregel KC, Zhang HJ. An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. *Am J Physiol Regul Integr Comp Physiol.* Jan 2007;292(1):R18-36.

22. Kaeberlein M. Longevity genomics across species. *Curr Genomics.* Apr 2007;8(2):73-78.

23. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet.* Mar 2003;33 Suppl:245-254.

24. McAllister EJ, Dhurandhar NV, Keith SW, Aronne LJ, Barger J, Baskin M, Benca RM, Biggio J, Boggiano MM, Eisenmann JC, Elobeid M, Fontaine KR, Gluckman P, Hanlon EC, Katzmarzyk P, Pietrobelli A, Redden DT, Ruden DM, Wang C, Waterland RA, Wright SM, Allison DB. Ten putative contributors to the obesity epidemic. *Crit Rev Food Sci Nutr.* Nov 2009;49(10):868-913.

25. Kushner RF, Bessesen DH. *Treatment of the obese patient*. Totowa, N.J.: Humana Press; 2007.

26. Tallett AJ, Blundell JE, Rodgers RJ. Night and day: diurnal differences in the behavioural satiety sequence in male rats. *Physiol Behav.* Apr 20 2009;97(1):125-130.

27. Swoap SJ. The pharmacology and molecular mechanisms underlying temperature regulation and torpor. *Biochem Pharmacol.* Oct 1 2008;76(7):817-824.

28. Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF. Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides.* Nov-Dec 1986;7(6):1189-1192.

29. Kuo LE, Kitlinska JB, Tilan JU, Li L, Baker SB, Johnson MD, Lee EW, Burnett MS, Fricke ST, Kvetnansky R, Herzog H, Zukowska Z. Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome. *Nat Med.* Jul 2007;13(7):803-811.

30. Schwartz MW, Baskin DG, Bukowski TR, Kuijper JL, Foster D, Lasser G, Prunkard DE, Porte D, Jr., Woods SC, Seeley RJ, Weigle DS. Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. *Diabetes.* Apr 1996;45(4):531-535.

31. Leibel RL. Is obesity due to a heritable difference in 'set point' for adiposity? *West J Med.* Oct 1990;153(4):429-431.

32. Leibowitz SF. Brain neuropeptide Y: an integrator of endocrine, metabolic and behavioral processes. *Brain Res Bull.* Sep-Oct 1991;27(3-4):333-337.

33. Pais R, Silaghi H, Silaghi AC, Rusu ML, Dumitrascu DL. Metabolic syndrome and risk of subsequent colorectal cancer. *World J Gastroenterol.* Nov 7 2009;15(41):5141-5148.

34. Pi-Sunyer FX. Health implications of obesity. *Am J Clin Nutr.* Jun 1991;53(6 Suppl):1595S-1603S.

35. Moayyedi P. The epidemiology of obesity and gastrointestinal and other diseases: an overview. *Dig Dis Sci.* Sep 2008;53(9):2293-2299.

36. Bender R, Jockel KH, Trautner C, Spraul M, Berger M. Effect of age on excess mortality in obesity. *JAMA.* Apr 28 1999;281(16):1498-1504.

37. Pingitore R, Dugoni BL, Tindale RS, Spring B. Bias against overweight job applicants in a simulated employment interview. *J Appl Psychol.* Dec 1994;79(6):909-917.

38. Burton WN, Chen CY, Schultz AB, Edington DW. The economic costs associated with body mass index in a workplace. *J Occup Environ Med.* Sep 1998;40(9):786-792.

39. Trogdon JG, Finkelstein EA, Hylands T, Dellea PS, Kamal-Bahl SJ. Indirect costs of obesity: a review of the current literature. *Obes Rev.* Sep 2008;9(5):489-500.

40. Baum CL, 2nd, Ford WF. The wage effects of obesity: a longitudinal study. *Health Econ.* Sep 2004;13(9):885-899.
41. Wang Y, Beydoun MA, Liang L, Caballero B, Kumanyika SK. Will all Americans become overweight or obese? estimating the progression and cost of the US obesity epidemic. *Obesity (Silver Spring).* Oct 2008;16(10):2323-2330.
42. Trayhurn P, Wang B, Wood IS. Hypoxia in adipose tissue: a basis for the dysregulation of tissue function in obesity? *Br J Nutr.* Aug 2008;100(2):227-235.
43. Finkelstein EA, Ruhm CJ, Kosa KM. Economic causes and consequences of obesity. *Annu Rev Public Health.* 2005;26:239-257.
44. Penick SB, Filion R, Fox S, Stunkard AJ. Behavior modification in the treatment of obesity. *Psychosom Med.* Jan-Feb 1971;33(1):49-55.
45. Eckel RH. Clinical practice. Nonsurgical management of obesity in adults. *N Engl J Med.* May 1 2008;358(18):1941-1950.
46. Kopecky J, Rossmeisl M, Flachs P, Kuda O, Brauner P, Jilkova Z, Stankova B, Tvrzicka E, Bryhn M. n-3 PUFA: bioavailability and modulation of adipose tissue function. *Proc Nutr Soc.* Nov 2009;68(4):361-369.
47. Sattar N, Greer IA. Pregnancy complications and maternal cardiovascular risk: opportunities for intervention and screening? *BMJ.* Jul 20 2002;325(7356):157-160.
48. Claesson IM, Sydsjo G, Brynhildsen J, Cedergren M, Jeppsson A, Nystrom F, Sydsjo A, Josefsson A. Weight gain restriction for obese pregnant women: a case-control intervention study. *BJOG.* Jan 2008;115(1):44-50.
49. Kim S, Moustaid-Moussa N. Secretory, endocrine and autocrine/paracrine function of the adipocyte. *J Nutr.* Dec 2000;130(12):3110S-3115S.
50. Rajala MW, Scherer PE. Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology.* Sep 2003;144(9):3765-3773.
51. Lefterova MI, Lazar MA. New developments in adipogenesis. *Trends Endocrinol Metab.* Apr 2009;20(3):107-114.
52. Mariman EC, Wang P. Adipocyte extracellular matrix composition, dynamics and role in obesity. *Cell Mol Life Sci.* Apr 2010;67(8):1277-1292.
53. Halberg N, Wernstedt-Asterholm I, Scherer PE. The adipocyte as an endocrine cell. *Endocrinol Metab Clin North Am.* Sep 2008;37(3):753-768, x-xi.
54. Kennedy A, Martinez K, Chuang CC, LaPoint K, McIntosh M. Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: mechanisms of action and implications. *J Nutr.* Jan 2009;139(1):1-4.
55. Taura D, Noguchi M, Sone M, Hosoda K, Mori E, Okada Y, Takahashi K, Homma K, Oyamada N, Inuzuka M, Sonoyama T, Ebihara K, Tamura N, Itoh H, Suemori H, Nakatsuji N, Okano H, Yamanaka S, Nakao K. Adipogenic differentiation of human induced pluripotent stem cells: comparison with that of human embryonic stem cells. *FEBS Lett.* Mar 18 2009;583(6):1029-1033.
56. Baglioni S, Francalanci M, Squecco R, Lombardi A, Cantini G, Angeli R, Gelmini S, Guasti D, Benvenuti S, Annunziato F, Bani D, Liotta F, Francini F, Perigli G, Serio M, Luconi M. Characterization of human adult stem-cell populations isolated from visceral and subcutaneous adipose tissue. *FASEB J.* Oct 2009;23(10):3494-3505.
57. Molgat AS, Gagnon A, Sorisky A. Preadipocyte apoptosis is prevented by macrophage-conditioned medium in a PDGF-dependent manner. *Am J Physiol Cell Physiol.* Apr 2009;296(4):C757-765.

58. Gustafson B, Gogg S, Hedjazifar S, Jenndahl L, Hammarstedt A, Smith U. Inflammation and impaired adipogenesis in hypertrophic obesity in man. *Am J Physiol Endocrinol Metab.* Jul 21 2009.
59. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest.* Jan 2007;117(1):175-184.
60. Odegaard JI, Chawla A. Mechanisms of macrophage activation in obesity-induced insulin resistance. *Nat Clin Pract Endocrinol Metab.* Nov 2008;4(11):619-626.
61. Zeyda M, Farmer D, Todoric J, Aszmann O, Speiser M, Gyori G, Zlabinger GJ, Stulnig TM. Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. *Int J Obes (Lond).* Sep 2007;31(9):1420-1428.
62. Herrero L, Shapiro H, Nayer A, Lee J, Shoelson SE. Inflammation and adipose tissue macrophages in lipodystrophic mice. *Proc Natl Acad Sci U S A.* Jan 5 2010;107(1):240-245.
63. Tordjman J, Guerre-Millo M, Clement K. Adipose tissue inflammation and liver pathology in human obesity. *Diabetes Metab.* Dec 2008;34(6 Pt 2):658-663.
64. Apovian CM, Bigornia S, Mott M, Meyers MR, Ulloor J, Gagua M, McDonnell M, Hess D, Joseph L, Gokce N. Adipose macrophage infiltration is associated with insulin resistance and vascular endothelial dysfunction in obese subjects. *Arterioscler Thromb Vasc Biol.* Sep 2008;28(9):1654-1659.
65. Murano I, Barbatelli G, Parisani V, Latini C, Muzzonigro G, Castellucci M, Cinti S. Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. *J Lipid Res.* Jul 2008;49(7):1562-1568.
66. Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, 2nd, DeFuria J, Jick Z, Greenberg AS, Obin MS. Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes.* Dec 2007;56(12):2910-2918.
67. Kang K, Reilly SM, Karabacak V, Gangl MR, Fitzgerald K, Hatano B, Lee CH. Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. *Cell Metab.* Jun 2008;7(6):485-495.
68. Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P. A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell.* Jan 2001;7(1):161-171.
69. Kim JA, Park HS. White blood cell count and abdominal fat distribution in female obese adolescents. *Metabolism.* Oct 2008;57(10):1375-1379.
70. Surmi BK, Hasty AH. The role of chemokines in recruitment of immune cells to the artery wall and adipose tissue. *Vascul Pharmacol.* Jan-Feb 2010;52(1-2):27-36.
71. Nijhuis J, Rensen SS, Slaats Y, van Dielen FM, Buurman WA, Greve JW. Neutrophil activation in morbid obesity, chronic activation of acute inflammation. *Obesity (Silver Spring).* Nov 2009;17(11):2014-2018.
72. Elgazar-Carmon V, Rudich A, Hadad N, Levy R. Neutrophils transiently infiltrate intra-abdominal fat early in the course of high-fat feeding. *J Lipid Res.* Sep 2008;49(9):1894-1903.
73. Ahima RS. Adipose tissue as an endocrine organ. *Obesity (Silver Spring).* Aug 2006;14 Suppl 5:242S-249S.
74. Matarasso A, Kim RW, Kral JG. The impact of liposuction on body fat. *Plast Reconstr Surg.* Oct 1998;102(5):1686-1689.
75. Reue K, Phan J. Metabolic consequences of lipodystrophy in mouse models. *Curr Opin Clin Nutr Metab Care.* Jul 2006;9(4):436-441.
76. Kissebah AH, Krakower GR. Regional adiposity and morbidity. *Physiol Rev.* Oct 1994;74(4):761-811.

77. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab.* Jun 2004;89(6):2548-2556.
78. Mohamed-Ali V, Pinkney JH, Coppack SW. Adipose tissue as an endocrine and paracrine organ. *Int J Obes Relat Metab Disord.* Dec 1998;22(12):1145-1158.
79. Coppack SW. Pro-inflammatory cytokines and adipose tissue. *Proc Nutr Soc.* Aug 2001;60(3):349-356.
80. Giusti V, Suter M, Verdumo C, Gaillard RC, Burckhardt P, Pralong FP. Molecular determinants of human adipose tissue: differences between visceral and subcutaneous compartments in obese women. *J Clin Endocrinol Metab.* Mar 2004;89(3):1379-1384.
81. Holloway BR. Reactivation of brown adipose tissue. *Proc Nutr Soc.* Jul 1989;48(2):225-230.
82. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scime A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki MA, Beier DR, Spiegelman BM. PRDM16 controls a brown fat/skeletal muscle switch. *Nature.* Aug 21 2008;454(7207):961-967.
83. Haas B, Mayer P, Jennissen K, Scholz D, Diaz MB, Bloch W, Herzig S, Fassler R, Pfeifer A. Protein kinase G controls brown fat cell differentiation and mitochondrial biogenesis. *Sci Signal.* 2009;2(99):ra78.
84. James WP, Trayhurn P. Thermogenesis and obesity. *Br Med Bull.* Jan 1981;37(1):43-48.
85. Parker N, Crichton PG, Vidal-Puig AJ, Brand MD. Uncoupling protein-1 (UCP1) contributes to the basal proton conductance of brown adipose tissue mitochondria. *J Bioenerg Biomembr.* Aug 2009;41(4):335-342.
86. Farmer SR. Obesity: Be cool, lose weight. *Nature.* Apr 16 2009;458(7240):839-840.
87. Berg F, Gustafson U, Andersson L. The uncoupling protein 1 gene (UCP1) is disrupted in the pig lineage: a genetic explanation for poor thermoregulation in piglets. *PLoS Genet.* Aug 18 2006;2(8):e129.
88. Samaras K, Botelho NK, Chisholm DJ, Lord RV. Subcutaneous and Visceral Adipose Tissue Gene Expression of Serum Adipokines That Predict Type 2 Diabetes. *Obesity (Silver Spring).* Dec 17 2009.
89. Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *Eur Heart J.* Dec 2008;29(24):2959-2971.
90. Janssen I, Powell LH, Kazlauskaite R, Dugan SA. Testosterone and visceral fat in midlife women: the Study of Women's Health Across the Nation (SWAN) fat patterning study. *Obesity (Silver Spring).* Mar 2010;18(3):604-610.
91. Huffman DM, Barzilai N. Role of visceral adipose tissue in aging. *Biochim Biophys Acta.* Oct 2009;1790(10):1117-1123.
92. Pedersen SB, Kristensen K, Hermann PA, Katzenellenbogen JA, Richelsen B. Estrogen controls lipolysis by up-regulating alpha2A-adrenergic receptors directly in human adipose tissue through the estrogen receptor alpha. Implications for the female fat distribution. *J Clin Endocrinol Metab.* Apr 2004;89(4):1869-1878.
93. Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev.* Dec 2000;21(6):697-738.
94. Yamamoto Y, Gesta S, Lee KY, Tran TT, Saadatirad P, Ronald Kahn C. Adipose Depots Possess Unique Developmental Gene Signatures. *Obesity (Silver Spring).* Jan 28 2010.
95. Kelley DE, Thaete FL, Troost F, Huwe T, Goodpaster BH. Subdivisions of subcutaneous abdominal adipose tissue and insulin resistance. *Am J Physiol Endocrinol Metab.* May 2000;278(5):E941-948.
96. Smith SR, Lovejoy JC, Greenway F, Ryan D, deJonge L, de la Bretonne J, Volafova J, Bray GA. Contributions of total body fat, abdominal subcutaneous adipose tissue compartments, and

visceral adipose tissue to the metabolic complications of obesity. *Metabolism.* Apr 2001;50(4):425-435.

97. Walker GE, Marzullo P, Verti B, Guzzaloni G, Maestrini S, Zurleni F, Liuzzi A, Di Blasio AM. Subcutaneous Abdominal Adipose Tissue Subcompartments: Potential Role in Rosiglitazone Effects. *Obesity (Silver Spring).* Jun 19 2008.

98. Arner P. Regional differences in protein production by human adipose tissue. *Biochem Soc Trans.* May 2001;29(Pt 2):72-75.

99. Wulan SN, Westerterp KR, Plasqui G. Ethnic differences in body composition and the associated metabolic profile: a comparative study between Asians and Caucasians. *Maturitas.* Apr 2010;65(4):315-319.

100. Ritchie SA, Connell JM. The link between abdominal obesity, metabolic syndrome and cardiovascular disease. *Nutr Metab Cardiovasc Dis.* May 2007;17(4):319-326.

101. Gaidhu MP, Anthony NM, Patel P, Hawke TJ, Ceddia RB. Dysregulation of lipolysis and lipid metabolism in visceral and subcutaneous adipocytes by high-fat diet: The role of ATGL, HSL and AMPK. *Am J Physiol Cell Physiol.* Jan 27 2010.

102. Schenk S, Saberi M, Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. *J Clin Invest.* Sep 2008;118(9):2992-3002.

103. Fain JN, Madan AK. Regulation of monocyte chemoattractant protein 1 (MCP-1) release by explants of human visceral adipose tissue. *Int J Obes (Lond).* Nov 2005;29(11):1299-1307.

104. Radin MJ, Sharkey LC, Holycross BJ. Adipokines: a review of biological and analytical principles and an update in dogs, cats, and horses. *Vet Clin Pathol.* Jun 2009;38(2):136-156.

105. Saltiel AR. You are what you secrete. *Nat Med.* Aug 2001;7(8):887-888.

106. Inadera H. The usefulness of circulating adipokine levels for the assessment of obesity-related health problems. *Int J Med Sci.* 2008;5(5):248-262.

107. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation.* Sep 12 2000;102(11):1296-1301.

108. Lapointe A, Tchernof A, Lamarche B, Piche ME, Weisnagel J, Bergeron J, Lemieux S. Plasma adiponectin concentration is strongly associated with VLDL-TG catabolism in postmenopausal women. *Nutr Metab Cardiovasc Dis.* Feb 11 2010.

109. Kennedy GC. The role of depot fat in the hypothalamic control of food intake in the rat. *Proc R Soc Lond B Biol Sci.* Jan 15 1953;140(901):578-596.

110. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science.* Jul 28 1995;269(5223):543-546.

111. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature.* Dec 1 1994;372(6505):425-432.

112. Farooqi IS, O'Rahilly S. Leptin: a pivotal regulator of human energy homeostasis. *Am J Clin Nutr.* Mar 2009;89(3):980S-984S.

113. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science.* Jul 28 1995;269(5223):546-549.

114. Houseknecht KL, Baile CA, Matteri RL, Spurlock ME. The biology of leptin: a review. *J Anim Sci.* May 1998;76(5):1405-1420.

115. Lago F, Gomez R, Gomez-Reino JJ, Dieguez C, Gualillo O. Adipokines as novel modulators of lipid metabolism. *Trends Biochem Sci.* Oct 2009;34(10):500-510.

116. Dyck DJ. Adipokines as regulators of muscle metabolism and insulin sensitivity. *Appl Physiol Nutr Metab.* Jun 2009;34(3):396-402.
117. Cancello R, Clement K. Is obesity an inflammatory illness? Role of low-grade inflammation and macrophage infiltration in human white adipose tissue. *BJOG.* Oct 2006;113(10):1141-1147.
118. Vanden Berghe W, Vermeulen L, De Wilde G, De Bosscher K, Boone E, Haegeman G. Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. *Biochem Pharmacol.* Oct 15 2000;60(8):1185-1195.
119. Coley WB. Treatment of Inoperable Sarcoma by Bacterial Toxins(the Mixed Toxins of the Streptococcus erysipelas and the Bacillus prodigiosus. *Proceedings of the Royal Society of Medicine, Surgical Sections.* 1909-1910
1909;3:1-48.
120. Old LJ. Tumor necrosis factor (TNF). *Science.* Nov 8 1985;230(4726):630-632.
121. Ruan H, Pownall HJ, Lodish HF. Troglitazone antagonizes tumor necrosis factor-alpha-induced reprogramming of adipocyte gene expression by inhibiting the transcriptional regulatory functions of NF-kappaB. *J Biol Chem.* Jul 25 2003;278(30):28181-28192.
122. Nieto-Vazquez I, Fernandez-Veledo S, Kramer DK, Vila-Bedmar R, Garcia-Guerra L, Lorenzo M. Insulin resistance associated to obesity: the link TNF-alpha. *Arch Physiol Biochem.* Jul 2008;114(3):183-194.
123. Fasshauer M, Klein J, Neumann S, Eszlinger M, Paschke R. Hormonal regulation of adiponectin gene expression in 3T3-L1 adipocytes. *Biochem Biophys Res Commun.* Jan 25 2002;290(3):1084-1089.
124. Cartier A, Cote M, Bergeron J, Almeras N, Tremblay A, Lemieux I, Despres JP. Plasma soluble tumour necrosis factor-alpha receptor 2 is elevated in obesity: specific contribution of visceral adiposity. *Clin Endocrinol (Oxf).* Mar 2010;72(3):349-357.
125. Vural P, Degirmencioglu S, Saral NY, Demirkan A, Akgul C, Yildirim G, Issever H, Eroglu H. Tumor necrosis factor alpha, interleukin-6 and interleukin-10 polymorphisms in preeclampsia. *J Obstet Gynaecol Res.* Feb 2010;36(1):64-71.
126. de Mello VD, Lankinen M, Schwab U, Kolehmainen M, Lehto S, Seppanen-Laakso T, Oresic M, Pulkkinen L, Uusitupa M, Erkkila AT. Link between plasma ceramides, inflammation and insulin resistance: association with serum IL-6 concentration in patients with coronary heart disease. *Diabetologia.* Dec 2009;52(12):2612-2615.
127. Martin-Cordero L, Garcia JJ, Giraldo E, De la Fuente M, Manso R, Ortega E. Influence of exercise on the circulating levels and macrophage production of IL-1beta and IFNgamma affected by metabolic syndrome: an obese Zucker rat experimental animal model. *Eur J Appl Physiol.* Nov 2009;107(5):535-543.
128. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR, Grabstein K, et al. Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature.* Jun 20-26 1985;315(6021):641-647.
129. Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. *Nature.* Dec 14 2006;444(7121):875-880.
130. Vitseva OI, Tanriverdi K, Tchkonia TT, Kirkland JL, McDonnell ME, Apovian CM, Freedman J, Gokce N. Inducible Toll-like receptor and NF-kappaB regulatory pathway expression in human adipose tissue. *Obesity (Silver Spring).* May 2008;16(5):932-937.
131. Zhang G, Ghosh S. Toll-like receptor-mediated NF-kappaB activation: a phylogenetically conserved paradigm in innate immunity. *J Clin Invest.* Jan 2001;107(1):13-19.
132. Zoico E, Garbin U, Olioso D, Mazzali G, Fratta Pasini AM, Di Francesco V, Sepe A, Cominacini L, Zamboni M. The effects of adiponectin on interleukin-6 and MCP-1 secretion in

lipopolysaccharide-treated 3T3-L1 adipocytes: role of the NF-kappaB pathway. *Int J Mol Med.* Dec 2009;24(6):847-851.

133. Wang C, Li L, Zhang ZG, Fan D, Zhu Y, Wu LL. Globular adiponectin inhibits angiotensin II-induced nuclear factor kappaB activation through AMP-activated protein kinase in cardiac hypertrophy. *J Cell Physiol.* Jan 2010;222(1):149-155.

134. Schaeffler A, Gross P, Buettner R, Bollheimer C, Buechler C, Neumeier M, Kopp A, Schoelmerich J, Falk W. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity. *Immunology.* Feb 2009;126(2):233-245.

135. Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. *Curr Pharm Des.* 2008;14(12):1225-1230.

136. Ajuwon KM, Spurlock ME. Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases PPARgamma2 expression in adipocytes. *Am J Physiol Regul Integr Comp Physiol.* May 2005;288(5):R1220-1225.

137. Beuther DA. Obesity and asthma. *Clin Chest Med.* Sep 2009;30(3):479-488, viii.

138. Tilg H, Moschen AR. Role of adiponectin and PBEF/visfatin as regulators of inflammation: involvement in obesity-associated diseases. *Clin Sci (Lond).* Feb 2008;114(4):275-288.

139. Cleland WH, Mendelson CR, Simpson ER. Effects of aging and obesity on aromatase activity of human adipose cells. *J Clin Endocrinol Metab.* Jan 1985;60(1):174-177.

140. Bruun JM, Lihn AS, Pedersen SB, Richelsen B. Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. *J Clin Endocrinol Metab.* Apr 2005;90(4):2282-2289.

141. Straub RH. The complex role of estrogens in inflammation. *Endocr Rev.* Aug 2007;28(5):521-574.

142. Foryst-Ludwig A, Clemenz M, Hohmann S, Hartge M, Sprang C, Frost N, Krikov M, Bhanot S, Barros R, Morani A, Gustafsson JA, Unger T, Kintscher U. Metabolic actions of estrogen receptor beta (ERbeta) are mediated by a negative cross-talk with PPARgamma. *PLoS Genet.* Jun 2008;4(6):e1000108.

143. Naaz A, Zakroczymski M, Heine P, Taylor J, Saunders P, Lubahn D, Cooke PS. Effect of ovariectomy on adipose tissue of mice in the absence of estrogen receptor alpha (ERalpha): a potential role for estrogen receptor beta (ERbeta). *Horm Metab Res.* Nov-Dec 2002;34(11-12):758-763.

144. Shin JH, Hur JY, Seo HS, Jeong YA, Lee JK, Oh MJ, Kim T, Saw HS, Kim SH. The ratio of estrogen receptor alpha to estrogen receptor beta in adipose tissue is associated with leptin production and obesity. *Steroids.* Jun 2007;72(6-7):592-599.

145. Riant E, Waget A, Cogo H, Arnal JF, Burcelin R, Gourdy P. Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice. *Endocrinology.* May 2009;150(5):2109-2117.

146. Kliewer SA, Xu HE, Lambert MH, Willson TM. Peroxisome proliferator-activated receptors: from genes to physiology. *Recent Prog Horm Res.* 2001;56:239-263.

147. Semple RK, Chatterjee VK, O'Rahilly S. PPAR gamma and human metabolic disease. *J Clin Invest.* Mar 2006;116(3):581-589.

148. Lehrke M, Lazar MA. The many faces of PPARgamma. *Cell.* Dec 16 2005;123(6):993-999.

149. Amin RH, Mathews ST, Camp HS, Ding L, Leff T. Selective Activation of PPAR{gamma} in Skeletal Muscle * Induces Endogenous Production of Adiponectin and Protects Mice From Diet-Induced Insulin Resistance. *Am J Physiol Endocrinol Metab.* Oct 20 2009.

150. Murphy GJ, Holder JC. PPAR-gamma agonists: therapeutic role in diabetes, inflammation and cancer. *Trends Pharmacol Sci.* Dec 2000;21(12):469-474.

151. de Souza CJ, Eckhardt M, Gagen K, Dong M, Chen W, Laurent D, Burkey BF. Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. *Diabetes.* Aug 2001;50(8):1863-1871.
152. Altshuler D, Hirschhorn JN, Klannemark M, Lindgren CM, Vohl MC, Nemesh J, Lane CR, Schaffner SF, Bolk S, Brewer C, Tuomi T, Gaudet D, Hudson TJ, Daly M, Groop L, Lander ES. The common PPARgamma Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. *Nat Genet.* Sep 2000;26(1):76-80.
153. Tan CY, Vidal-Puig A. Adipose tissue expandability: the metabolic problems of obesity may arise from the inability to become more obese. *Biochem Soc Trans.* Oct 2008;36(Pt 5):935-940.
154. Stefan N, Kantartzis K, Machann J, Schick F, Thamer C, Rittig K, Balletshofer B, Machicao F, Fritsche A, Haring HU. Identification and characterization of metabolically benign obesity in humans. *Arch Intern Med.* Aug 11 2008;168(15):1609-1616.
155. Cheatham WW. Peroxisome proliferator-activated receptor translational research and clinical experience. *Am J Clin Nutr.* Jan 2010;91(1):262S-266S.
156. Leulier F, Lemaitre B. Toll-like receptors--taking an evolutionary approach. *Nat Rev Genet.* Mar 2008;9(3):165-178.
157. Kopp A, Buechler C, Neumeier M, Weigert J, Aslanidis C, Scholmerich J, Schaffler A. Innate immunity and adipocyte function: ligand-specific activation of multiple Toll-like receptors modulates cytokine, adipokine, and chemokine secretion in adipocytes. *Obesity (Silver Spring).* Apr 2009;17(4):648-656.
158. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes.* Jul 2007;56(7):1761-1772.
159. Baumann H, Gauldie J. The acute phase response. *Immunol Today.* Feb 1994;15(2):74-80.
160. Goldsby RA. *Immunology*. 5th ed. New York: W.H. Freeman; 2003.
161. Bone RC. The pathogenesis of sepsis. *Ann Intern Med.* Sep 15 1991;115(6):457-469.
162. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol.* Dec 2005;6(12):1182-1190.
163. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med.* Feb 11 1999;340(6):448-454.
164. Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol.* Apr 2006;6(4):318-328.
165. Fantuzzi G. Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol.* May 2005;115(5):911-919; quiz 920.
166. Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, Gallimore JR, Pepys MB. Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses. *BMJ.* Jul 22 2000;321(7255):199-204.
167. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med.* Apr 10 1997;336(15):1066-1071.
168. Navab M, Fogelman AM, Berliner JA, Territo MC, Demer LL, Frank JS, Watson AD, Edwards PA, Lusis AJ. Pathogenesis of atherosclerosis. *Am J Cardiol.* Sep 28 1995;76(9):18C-23C.
169. Linton MF, Fazio S. Macrophages, inflammation, and atherosclerosis. *Int J Obes Relat Metab Disord.* Dec 2003;27 Suppl 3:S35-40.
170. Alexander RW. Inflammation and coronary artery disease. *N Engl J Med.* Aug 18 1994;331(7):468-469.

171. Haskell WL. Cardiovascular disease prevention and lifestyle interventions: effectiveness and efficacy. *J Cardiovasc Nurs.* Sep-Oct 2003;18(4):245-255.
172. Strong JP, McGill HC, Jr. The pediatric aspects of atherosclerosis. *J Atheroscler Res.* May-Jun 1969;9(3):251-265.
173. Grundy SM. Obesity, metabolic syndrome, and coronary atherosclerosis. *Circulation.* Jun 11 2002;105(23):2696-2698.
174. McGill HC, Jr., McMahan CA, Herderick EE, Zieske AW, Malcom GT, Tracy RE, Strong JP. Obesity accelerates the progression of coronary atherosclerosis in young men. *Circulation.* Jun 11 2002;105(23):2712-2718.
175. Mauras N, Delgiorno C, Kollman C, Bird K, Morgan M, Sweeten S, Balagopal P, Damaso L. Obesity without established comorbidities of the metabolic syndrome is associated with a proinflammatory and prothrombotic state, even before the onset of puberty in children. *J Clin Endocrinol Metab.* Mar 2010;95(3):1060-1068.
176. Guz Y, Montminy MR, Stein R, Leonard J, Gamer LW, Wright CV, Teitelman G. Expression of murine STF-1, a putative insulin gene transcription factor, in beta cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. *Development.* Jan 1995;121(1):11-18.
177. McCarty MF. A paradox resolved: the postprandial model of insulin resistance explains why gynoid adiposity appears to be protective. *Med Hypotheses.* Aug 2003;61(2):173-176.
178. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med.* Aug 2001;7(8):941-946.
179. Black PH. The inflammatory consequences of psychologic stress: relationship to insulin resistance, obesity, atherosclerosis and diabetes mellitus, type II. *Med Hypotheses.* 2006;67(4):879-891.
180. Pitombo C, Araujo EP, De Souza CT, Pareja JC, Geloneze B, Velloso LA. Amelioration of diet-induced diabetes mellitus by removal of visceral fat. *J Endocrinol.* Dec 2006;191(3):699-706.
181. Kaplan NM. The deadly quartet. Upper-body obesity, glucose intolerance, hypertriglyceridemia, and hypertension. *Arch Intern Med.* Jul 1989;149(7):1514-1520.
182. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes.* Dec 1988;37(12):1595-1607.
183. Swanson WF. Research in nondomestic species: experiences in reproductive physiology research for conservation of endangered felids. *ILAR J.* 2003;44(4):307-316.
184. Speakman J, Hambly C, Mitchell S, Krol E. The contribution of animal models to the study of obesity. *Lab Anim.* Oct 2008;42(4):413-432.
185. Aitman TJ, Critser JK, Cuppen E, Dominiczak A, Fernandez-Suarez XM, Flint J, Gauguier D, Geurts AM, Gould M, Harris PC, Holmdahl R, Hubner N, Izsvak Z, Jacob HJ, Kuramoto T, Kwitek AE, Marrone A, Mashimo T, Moreno C, Mullins J, Mullins L, Olsson T, Pravenec M, Riley L, Saar K, Serikawa T, Shull JD, Szpirer C, Twigger SN, Voigt B, Worley K. Progress and prospects in rat genetics: a community view. *Nat Genet.* May 2008;40(5):516-522.
186. Ingalls AM, Dickie MM, Snell GD. Obese, a new mutation in the house mouse. *J Hered.* Dec 1950;41(12):317-318.
187. Weigle DS, Bukowski TR, Foster DC, Holderman S, Kramer JM, Lasser G, Lofton-Day CE, Prunkard DE, Raymond C, Kuijper JL. Recombinant ob protein reduces feeding and body weight in the ob/ob mouse. *J Clin Invest.* Oct 1995;96(4):2065-2070.

188. Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science.* Jul 28 1995;269(5223):540-543.
189. Enriori PJ, Evans AE, Sinnayah P, Cowley MA. Leptin resistance and obesity. *Obesity (Silver Spring).* Aug 2006;14 Suppl 5:254S-258S.
190. Farooqi IS, Jebb SA, Langmack G, Lawrence E, Cheetham CH, Prentice AM, Hughes IA, McCamish MA, O'Rahilly S. Effects of recombinant leptin therapy in a child with congenital leptin deficiency. *N Engl J Med.* Sep 16 1999;341(12):879-884.
191. Farooqi IS, Keogh JM, Kamath S, Jones S, Gibson WT, Trussell R, Jebb SA, Lip GY, O'Rahilly S. Partial leptin deficiency and human adiposity. *Nature.* Nov 1 2001;414(6859):34-35.
192. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature.* Dec 21 2006;444(7122):1027-1031.
193. Coleman DL. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. *Diabetologia.* Mar 1978;14(3):141-148.
194. Johnson PR, Hirsch J. Cellularity of adipose depots in six strains of genetically obese mice. *J Lipid Res.* Jan 1972;13(1):2-11.
195. Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM. Abnormal splicing of the leptin receptor in diabetic mice. *Nature.* Feb 15 1996;379(6566):632-635.
196. Hummel KP, Dickie MM, Coleman DL. Diabetes, a new mutation in the mouse. *Science.* Sep 2 1966;153(740):1127-1128.
197. Staats J. Diabetes in the mouse due to two mutant genes - a bibliography. *Diabetologia.* Aug 1975;11(4):325-327.
198. King PA, Horton ED, Hirshman MF, Horton ES. Insulin resistance in obese Zucker rat (fa/fa) skeletal muscle is associated with a failure of glucose transporter translocation. *J Clin Invest.* Oct 1992;90(4):1568-1575.
199. Bultman SJ, Michaud EJ, Woychik RP. Molecular characterization of the mouse agouti locus. *Cell.* Dec 24 1992;71(7):1195-1204.
200. Claycombe KJ, Xue BZ, Mynatt RL, Zemel MB, Moustaid-Moussa N. Regulation of leptin by agouti. *Physiol Genomics.* Apr 27 2000;2(3):101-105.
201. Moussa NM, Claycombe KJ. The yellow mouse obesity syndrome and mechanisms of agouti-induced obesity. *Obes Res.* Sep 1999;7(5):506-514.
202. El-Haschimi K, Pierroz DD, Hileman SM, Bjorbaek C, Flier JS. Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. *J Clin Invest.* Jun 2000;105(12):1827-1832.
203. Jackson CL, Bennett MR, Biessen EA, Johnson JL, Krams R. Assessment of unstable atherosclerosis in mice. *Arterioscler Thromb Vasc Biol.* Apr 2007;27(4):714-720.
204. Nicoletti A, Kaveri S, Caligiuri G, Bariety J, Hansson GK. Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice. *J Clin Invest.* Sep 1 1998;102(5):910-918.
205. Spurlock ME, Gabler NK. The development of porcine models of obesity and the metabolic syndrome. *J Nutr.* Feb 2008;138(2):397-402.
206. Vodicka P, Smetana K, Jr., Dvorankova B, Emerick T, Xu YZ, Ourednik J, Ourednik V, Motlik J. The miniature pig as an animal model in biomedical research. *Ann N Y Acad Sci.* May 2005;1049:161-171.
207. Harvey W, Keynes G. *Exercitatio anatomica De motu cordis et sanguinis in animalibus. Being a facsimile of the 1628 Francofurti edition, together with The Keynes English translation of 1928*. Birmingham, AL: The Classics of Medicine Library; 1978.

208. Carey GB. The swine as a model for studying exercise-induced changes in lipid metabolism. *Med Sci Sports Exerc.* Nov 1997;29(11):1437-1443.
209. Casper RC, Sullivan EL, Tecott L. Relevance of animal models to human eating disorders and obesity. *Psychopharmacology (Berl).* Aug 2008;199(3):313-329.
210. Dixon JL, Stoops JD, Parker JL, Laughlin MH, Weisman GA, Sturek M. Dyslipidemia and vascular dysfunction in diabetic pigs fed an atherogenic diet. *Arterioscler Thromb Vasc Biol.* Dec 1999;19(12):2981-2992.
211. Li Y, Lu RH, Luo GF, Pang WJ, Yang GS. Effects of different cryoprotectants on the viability and biological characteristics of porcine preadipocyte. *Cryobiology.* Oct 2006;53(2):240-247.
212. Coelho JC, Solhaug JH, Moody FG, Li YF. Experimental evaluation of gastric banding for treatment of morbid obesity in pigs. *Am J Surg.* Feb 1985;149(2):228-231.
213. Miller ER, Ullrey DE. The pig as a model for human nutrition. *Annu Rev Nutr.* 1987;7:361-382.
214. Douglas WR. Of pigs and men and research: a review of applications and analogies of the pig, sus scrofa, in human medical research. *Space Life Sci.* Jun 1972;3(3):226-234.
215. Bollen PJA, Hansen AK, Rasmussen HJ. *The laboratory swine*. Boca Raton, FL: CRC Press; 2000.
216. Russell JC, Proctor SD. Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis. *Cardiovasc Pathol.* Nov-Dec 2006;15(6):318-330.
217. Hausman GJ, Poulos S. Recruitment and differentiation of intramuscular preadipocytes in stromal-vascular cell cultures derived from neonatal pig semitendinosus muscles. *J Anim Sci.* Feb 2004;82(2):429-437.
218. Liang Y, Zhu H, Friedman MH. The correspondence between coronary arterial wall strain and histology in a porcine model of atherosclerosis. *Phys Med Biol.* Sep 21 2009;54(18):5625-5641.
219. Trostle R, United States. Dept. of Agriculture. Economic Research Service. Global agricultural supply and demand factors contributing to the recent increase in food commodity prices. [Washington, D.C.]: U.S. Dept. of Agriculture, Economic Research Service; 2008: http://hdl.handle.net/10113/41689.
220. Tumbleson ME, Schook LB. *Advances in swine in biomedical research*. New York: Plenum Press; 1996.
221. Ngapo TM, Martin JF, Dransfield E. International preferences for pork appearance: I. Consumer choices. *Food Quality and Preference.* Jan 2007;18(1):26-36.
222. Main ML, Rao SC, O'Keefe JH. Trends in obesity and extreme obesity among US adults. *JAMA.* May 5 2010;303(17):1695; author reply 1695-1696.
223. Alvehus M, Buren J, Sjostrom M, Goedecke J, Olsson T. The Human Visceral Fat Depot Has a Unique Inflammatory Profile. *Obesity (Silver Spring).* Feb 25 2010.
224. Lkhagvadorj S, Qu L, Cai W, Couture OP, Barb CR, Hausman GJ, Nettleton D, Anderson LL, Dekkers JC, Tuggle CK. Microarray gene expression profiles of fasting induced changes in liver and adipose tissues of pigs expressing the melanocortin-4 receptor D298N variant. *Physiol Genomics.* Jun 10 2009;38(1):98-111.
225. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* Dec 2001;25(4):402-408.
226. Bioresources S. Growth Charts. 2008; http://www.sinclairbioresources.com/Products/Sinclair.aspx

227. Verstegen M, Vanderhel, W, Pijls, FJM. Growth-Rate and Growth Compositon of Artifically Reared Piglets from Specific Pathogen Free Sows. *Animal Production.* April 1993 1993;56:217-223.

228. Caramihai E, Karayalcin G, Aballi AJ, Lanzkowsky P. Leukocyte count differences in healthy white and black children 1 to 5 years of age. *J Pediatr.* Feb 1975;86(2):252-254.
229. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol.* Jan 2003;3(1):23-35.
230. Hornshoj H, Conley LN, Hedegaard J, Sorensen P, Panitz F, Bendixen C. Microarray expression profiles of 20.000 genes across 23 healthy porcine tissues. *PLoS One.* 2007;2(11):e1203.
231. Hausman GJ, Poulos SP, Richardson RL, Barb CR, Andacht T, Kirk HC, Mynatt RL. Secreted proteins and genes in fetal and neonatal pig adipose tissue and stromal-vascular cells. *J Anim Sci.* Jul 2006;84(7):1666-1681.
232. Troseid M, Seljeflot I, Arnesen H. The role of interleukin-18 in the metabolic syndrome. *Cardiovasc Diabetol.* 2010;9:11.
233. Nesto RW. Obesity: a major component of the metabolic syndrome. *Tex Heart Inst J.* 2005;32(3):387-389.
234. Liu L, Clipstone NA. Prostaglandin F2alpha induces the normoxic activation of the hypoxia-inducible factor-1 transcription factor in differentiating 3T3-L1 preadipocytes: Potential role in the regulation of adipogenesis. *J Cell Biochem.* Sep 1 2008;105(1):89-98.
235. Fryirs M, Barter PJ, Rye KA. Cholesterol metabolism and pancreatic beta-cell function. *Curr Opin Lipidol.* Jun 2009;20(3):159-164.
236. Zha JM, Di WJ, Zhu T, Xie Y, Yu J, Liu J, Chen P, Ding G. Comparison of gene transcription between subcutaneous and visceral adipose tissue in Chinese adults. *Endocr J.* 2009;56(8):935-944.
237. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerback S, Nuutila P. Functional brown adipose tissue in healthy adults. *N Engl J Med.* Apr 9 2009;360(15):1518-1525.
238. Wu H, Ghosh S, Perrard XD, Feng L, Garcia GE, Perrard JL, Sweeney JF, Peterson LE, Chan L, Smith CW, Ballantyne CM. T-cell accumulation and regulated on activation, normal T cell expressed and secreted upregulation in adipose tissue in obesity. *Circulation.* Feb 27 2007;115(8):1029-1038.
239. Velica P, Davies NJ, Rocha PP, Schrewe H, Ride JP, Bunce CM. Lack of functional and expression homology between human and mouse aldo-keto reductase 1C enzymes: implications for modelling human cancers. *Mol Cancer.* 2009;8:121.
240. Striz I, Trebichavsky I. Calprotectin - a pleiotropic molecule in acute and chronic inflammation. *Physiol Res.* 2004;53(3):245-253.
241. Ortega Martinez de Victoria E, Xu X, Koska J, Francisco AM, Scalise M, Ferrante AW, Jr., Krakoff J. Macrophage content in subcutaneous adipose tissue: associations with adiposity, age, inflammatory markers, and whole-body insulin action in healthy Pima Indians. *Diabetes.* Feb 2009;58(2):385-393.
242. Ibrahim MM. Subcutaneous and visceral adipose tissue: structural and functional differences. *Obes Rev.* Jul 28 2009.
243. Moyes CD. Controlling muscle mitochondrial content. *J Exp Biol.* Dec 2003;206(Pt 24):4385-4391.
244. Garcia-Meunier P, Etienne-Julan M, Fort P, Piechaczyk M, Bonhomme F. Concerted evolution in the GAPDH family of retrotransposed pseudogenes. *Mamm Genome.* Dec 1993;4(12):695-703.
245. Moustaid N, Jones BH, Taylor JW. Insulin increases lipogenic enzyme activity in human adipocytes in primary culture. *J Nutr.* Apr 1996;126(4):865-870.
246. Jones BH, Standridge MK, Moustaid N. Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. *Endocrinology.* Apr 1997;138(4):1512-1519.

247. Kim S, Whelan J, Claycombe K, Reath DB, Moustaid-Moussa N. Angiotensin II increases leptin secretion by 3T3-L1 and human adipocytes via a prostaglandin-independent mechanism. *J Nutr.* Jun 2002;132(6):1135-1140.

248. Wortman P, Miyazaki Y, Kalupahana NS, Kim S, Hansen-Petrik M, Saxton AM, Claycombe KJ, Voy BH, Whelan J, Moustaid-Moussa N. n3 and n6 polyunsaturated fatty acids differentially modulate prostaglandin E secretion but not markers of lipogenesis in adipocytes. *Nutr Metab (Lond).* 2009;6:5.

Appendix 1- Supplemental Information from the Main Study

Section A1 : Supplemental Materials and Methods

A1.1 Microarray Annotation

www.affymetrix.com --- Net Affix---Net Affix Query--- "Porcine"GeneChip Array---Refine Query---Enter Affymetrix Probeset ID---Details (below ID)--- BLASTn GenBank NR---View Report---GENE ID---Copy and Paste gene ID and gene name into excel sheet
www.ncbi.nlm.nih.gov/sites/entrez--- search gene---enter gene number (NP_xxxxxx)--- click on gene abbreviation--- Copy and Paste gen ID and gene name into excel sheet.

A1.2 Immunofluorescene EZ-Dewax Protocol

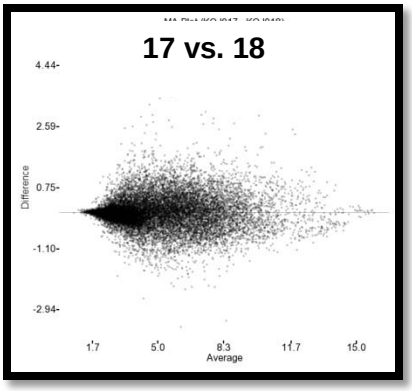
EZ-DeWax Solution (BioGenex San Ramon, CA) (125mL) was poured into two slide baths. The slides were immersed in the first bath for five minutes (agitating constantly). The slides were then transfered to the second bath, and immersed for five minutes (agitating constantly). The slides were then rinsed 2X's with 60 mL de-ionized water. Finally, the slides were rinsed 3X's with OptiMax Wash Buffer (BioGenex San Ramon, CA).

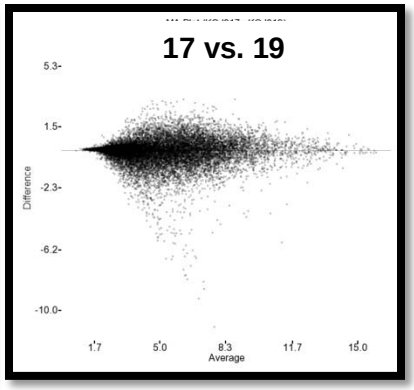
Section A2: Supplemental Results

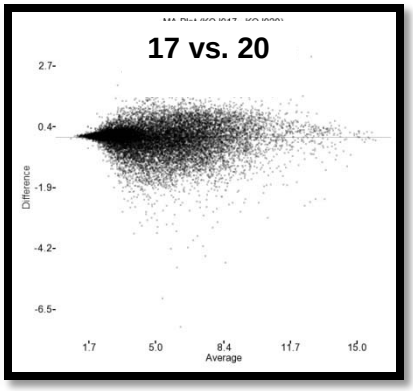
A2.1 Microarray

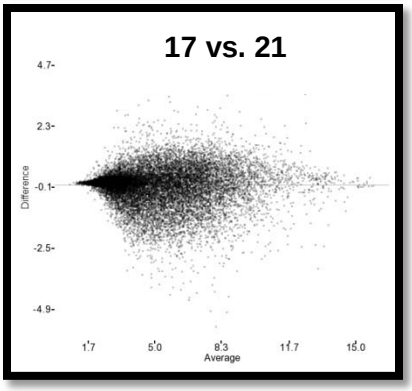
A2.1.1 Intensity Ratio (M) vs. Average Intensity (A) Plots

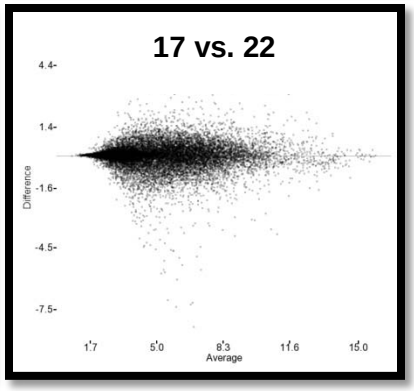
Figure A2.1 shows the distribution of the individual transcripts (represented by a dot) in the microarray comparison using an M (y-axis) vs. A (x-axis) plot. A normal distribution has the majority of the dots should be concentrated in the center around zero.

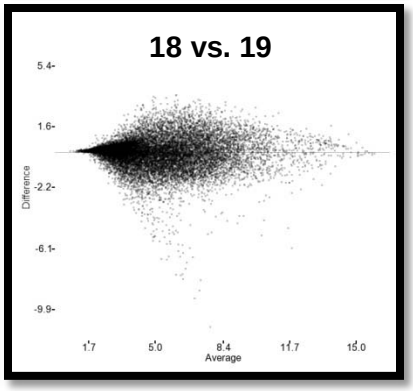


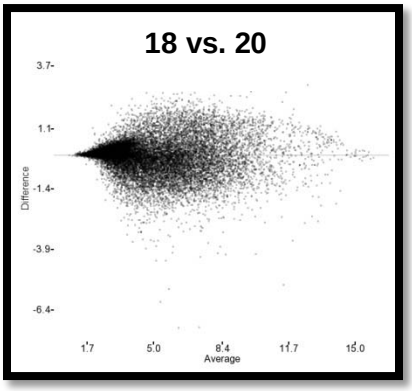


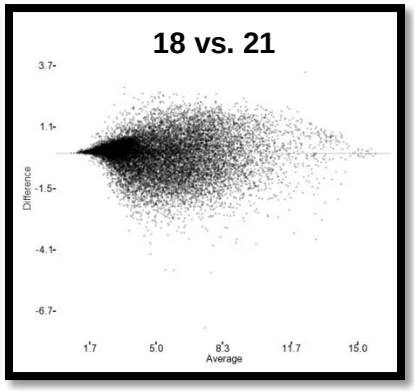


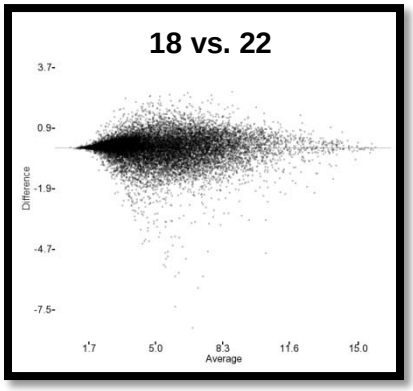


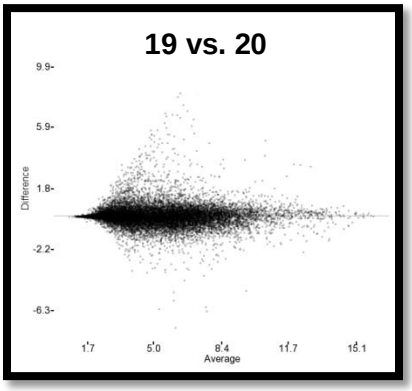


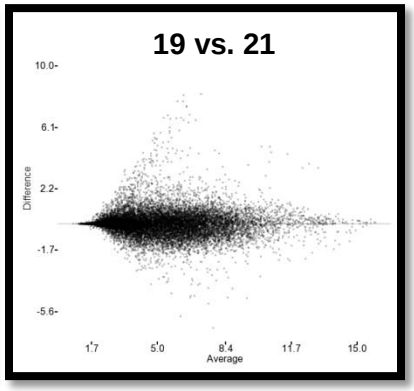


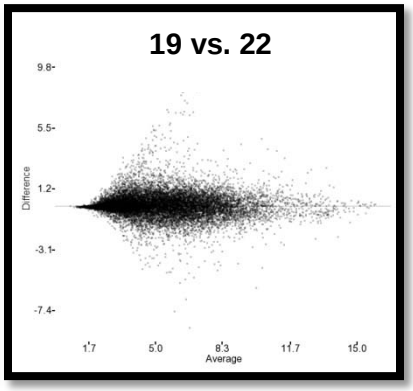












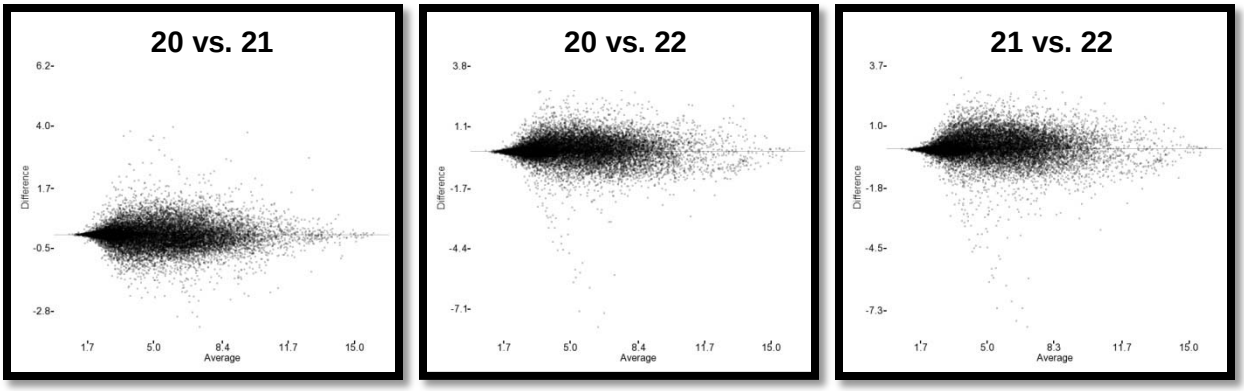


Figure A2.1: M vs. A plots demonstrating the individual pig comparisons of transcript intensity when compared with the entire transcriptome; individual pigs are represented by numbers 17-19 (VIF) and 20-22 (SQF)

A2.1.2 Pathway Analysis and Gene Ontology

Tables A1 and A2 are the genes with corresponding fold changes in the VIF vs. SQF and SQF vs. VIF comparisons respectively.

Table A1: Genes differentially expressed in VIF compared to SQF

Gene ID	Gene Name	Fold Change
Lipid Biosynthetic Process		
ACLY	ATP citrate lyase	3.2
CDS1	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1	3.2
FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	3.4
NANS	N-acetylneuraminic acid synthase	3.4
C9orf3	chromosome 9 open reading frame 3	2.2
CYP39A1	cytochrome P450, family 39, subfamily A, polypeptide 1	2.1
DEGS2	degenerative spermatocyte homolog 2, lipid desaturase (Drosophila)	2.2
DGAT1	diacylglycerol O-acyltransferase homolog 1 (mouse)	2.1
DGAT2	diacylglycerol O-acyltransferase homolog 2 (mouse)	2.3
FASN	fatty acid synthase	3.4
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	3.4

HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12	2.6
HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	4.8
IDI1	isopentenyl-diphosphate delta isomerase 1	2.5
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	2.5
SMPD1	sphingomyelin phosphodiesterase 1, acid lysosomal	2.4
Postitive Regulation of Inflammatory Response to Antigenic Stimulus		
CD24	CD24 molecule; CD24 molecule-like 4	4.3
FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	3.4
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	2.0
Response to Hypoxia		
ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	2.3
CD24	CD24 molecule; CD24 molecule-like 4	4.3
ALAS2	aminolevulinate, delta-, synthase 2	6.9
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	2.5
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	3.4
IL18	interleukin 18 (interferon-gamma-inducing factor)	4.5
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	2.3
RYR2	ryanodine receptor 2 (cardiac)	-2.1
Immune Response		
CD24	CD24 molecule; CD24 molecule-like 4	4.3
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	2.0
FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32); Fc fragment of IgG, low affinity IIc, receptor for (CD32)	2.4
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	2.0
VSIG4	V-set and immunoglobulin domain containing 4	2.4
CTSS	cathepsin S	2.3
CR2	complement component (3d/Epstein Barr virus) receptor 2	2.9
C1QA	complement component 1, q subcomponent, A chain	3.5
C1QB	complement component 1, q subcomponent, B chain	4.0
IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	2.1
IGHA2	immunoglobulin heavy constant alpha 2 (A2m marker)	2.3

IGHM	immunoglobulin heavy constant gamma 1 (G1m marker); immunoglobulin heavy constant mu; immunoglobulin heavy variable 3-7; immunoglobulin heavy constant gamma 3 (G3m marker); immunoglobulin heavy variable 3-11 (gene/pseudogene); immunoglobulin heavy variable 4-31; immunoglobulin heavy locus	2.1
IGLC1	immunoglobulin lambda variable 2-11; immunoglobulin lambda constant 2 (Kern-Oz- marker); immunoglobulin lambda variable 1-44; immunoglobulin lambda constant 1 (Mcg marker); immunoglobulin lambda variable 1-40; immunoglobulin lambda variable 3-21; immunoglobulin lambda locus; immunoglobulin lambda constant 3 (Kern-Oz+ marker)	4.0
IL18	interleukin 18 (interferon-gamma-inducing factor)	4.5
PF4	platelet factor 4	6.5
TRPM4	transient receptor potential cation channel, subfamily M, member 4	2.1
TUBB	tubulin, beta; similar to tubulin, beta 5; tubulin, beta pseudogene 2; tubulin, beta pseudogene 1	2.0
Response to Steroid Hormone Stimulus		
CD24	CD24 molecule; CD24 molecule-like 4	4.3
GATA3	GATA binding protein 3	2.4
COL1A1	collagen, type I, alpha 1	-4.9
C1QB	complement component 1, q subcomponent, B chain	4.0
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	3.4
KRT19	keratin 19	2.1
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	2.5
SDC1	syndecan 1	2.3
Lymphocyte Mediated Immunity		
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	2.0
CR2	complement component (3d/Epstein Barr virus) receptor 2	2.9
C1QA	complement component 1, q subcomponent, A chain	3.5
C1QB	complement component 1, q subcomponent, B chain	4.0
TUBB	tubulin, beta; similar to tubulin, beta 5; tubulin, beta pseudogene 2; tubulin, beta pseudogene 1	2.0

Fatty Acid Biosynthetic Process		
FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	3.4
C9orf3	chromosome 9 open reading frame 3	2.2
DEGS2	degenerative spermatocyte homolog 2, lipid desaturase (Drosophila)	2.2
FASN	fatty acid synthase	3.4
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	2.5
Regulation of Cell Death		
CD24	CD24 molecule; CD24 molecule-like 4	4.3
CD3G	CD3g molecule, gamma (CD3-TCR complex)	2.3
NQO1	NAD(P)H dehydrogenase, quinone 1	2.3
PERP	PERP, TP53 apoptosis effector	4.0
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	-2.2
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	2.5
CYCS	cytochrome c, somatic	2.2
HSPE1	heat shock 10kDa protein 1 (chaperonin 10)	2.1
HSPB1	heat shock 27kDa protein-like 2 pseudogene; heat shock 27kDa protein 1	3.0
IL2RB	interleukin 2 receptor, beta	2.3
PF4	platelet factor 4	6.5
RYR2	ryanodine receptor 2 (cardiac)	-2.1
SCIN	scinderin	2.1
SMPD1	sphingomyelin phosphodiesterase 1, acid lysosomal	2.4
SFN	stratifin	2.4
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	6.8
TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	2.3
TUBB	tubulin, beta; similar to tubulin, beta 5; tubulin, beta pseudogene 2; tubulin, beta pseudogene 1	2.0
Response to Corticosteroid Stimulus		
COL1A1	collagen, type I, alpha 1	-4.9
C1QB	complement component 1, q subcomponent, B chain	4.0
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	3.4
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	2.5
SDC1	syndecan 1	2.3

Regulation of Cytokine Production		
CD24	CD24 molecule; CD24 molecule-like 4	4.3
FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	3.4
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	2.0
VSIG4	V-set and immunoglobulin domain containing 4	2.4
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	2.5
IL18	interleukin 18 (interferon-gamma-inducing factor)	4.5
PF4	platelet factor 4	6.5
Wnt Receptor Signaling Pathway		
CD24	CD24 molecule; CD24 molecule-like 4	4.3
DKK3	dickkopf homolog 3 (Xenopus laevis)	3.1
SLC9A3R1	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	2.6
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	6.8
WNT5A	wingless-type MMTV integration site family, member 5A	2.1
WNT5B	wingless-type MMTV integration site family, member 5B	2.2
Hormone Metabolic Process		
COMT	catechol-O-methyltransferase	2.1
FOXA1	forkhead box A1	3.4
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	3.4
RBP1	retinol binding protein 1, cellular	5.3
SULT1E1	sulfotransferase family 1E, estrogen-preferring, member 1	8.4
Substrate Adhesion-Dependent Cell Spreading		
ANTXR1	anthrax toxin receptor 1	-2.5
FN1	fibronectin 1	-2.4
LAMC1	laminin, gamma 1 (formerly LAMB2)	-2.5
Regulation of Insulin-Like Growth Factor Receptor Signaling Pathway		
ATXN1	ataxin 1	-2.3
CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	-3.1
IGFBP5	insulin-like growth factor binding protein 5	-3.4

Positive Regulation of Leukocyte Chemotaxis

CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	-3.8
F2RL1	coagulation factor II (thrombin) receptor-like 1	2.0
THBS1	thrombospondin 1	-10.0

Estrogen Metabolic Process

COMT	catechol-O-methyltransferase	2.1
HSD3B1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	-2.9
SULT1E1	sulfotransferase family 1E, estrogen-preferring, member 1	8.4

Table A2: Genes differentially expressed in SQF compared to VIF

Gene I D	Gene Name	Fold Change
Vasculature Development		
ANPEP	alanyl (membrane) aminopeptidase	-2.7
AMOT	angiomotin	2.0
CAV1	caveolin 1, caveolae protein, 22kDa	3.6
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	3.8
COL1A1	collagen, type I, alpha 1	4.9
COL1A2	collagen, type I, alpha 2	4.0
COL3A1	collagen, type III, alpha 1	2.1
IL18	interleukin 18 (interferon-gamma-inducing factor)	-4.5
JUN	jun oncogene	3.1
LOX	lysyl oxidase	-2.1
MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	3.6
PRRX1	paired related homeobox 1	5.2
PRRX2	paired related homeobox 2	2.3
PPAP2B	phosphatidic acid phosphatase type 2B	2.7
PDPN	podoplanin	2.7
ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila); similar to roundabout 1 isoform b	2.1
THBS1	thrombospondin 1	5.5
TCF21	transcription factor 21	-2.0
TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	-2.3
ZFP36L1	zinc finger protein 36, C3H type-like 1	2.1
B Cell Mediated Immunity		
FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	-2.0
CR2	complement component (3d/Epstein Barr virus) receptor 2	-2.9
C1QA	complement component 1, q subcomponent, A chain	-3.5
C1QB	complement component 1, q subcomponent, B chain	-4.0
C1S	complement component 1, s subcomponent	2.2
C2	complement component 2	-2.1
C4A	complement component 4A (Rodgers blood group)	7.6
C5	complement component 5	2.1
CFI	complement factor I	-2.4

Adaptive Immune Response

CER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	-2.0
CR2	complement component (3d/Epstein Barr virus) receptor 2	-2.9
C1QA	complement component 1, q subcomponent, A chain	-3.5
C1QB	complement component 1, q subcomponent, B chain	-4.0
C1S	complement component 1, s subcomponent	2.2
C2	complement component 2	-2.1
C4A	complement component 4A (Rodgers blood group)	7.6
C5	complement component 5	2.1
CFI	complement factor I	-2.4
IL18	interleukin 18 (interferon-gamma-inducing factor)	-4.5

Acute Inflammatory Response

SIG4	V-set and immunoglobulin domain containing 4	-2.4
CR2	complement component (3d/Epstein Barr virus) receptor 2	-2.9
C1QA	complement component 1, q subcomponent, A chain	-3.5
C1QB	complement component 1, q subcomponent, B chain	-4.0
C1S	complement component 1, s subcomponent	2.2
C2	complement component 2	-2.1
C4A	complement component 4A (Rodgers blood group)	7.6
C5	complement component 5	2.1
CFI	complement factor I	-2.4
FN1	fibronectin 1	2.4
TF	transferrin	7.9

Blood Vessel Development

ANPEP	alanyl (membrane) aminopeptidase	-2.7
AMOT	angiomotin	2.0
CAV1	caveolin 1, caveolae protein, 22kDa	3.6
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	3.0
COL1A1	collagen, type I, alpha 1	-5.5
COL1A2	collagen, type I, alpha 2	4.0
COL3A1	collagen, type III, alpha 1	2.1
IL18	interleukin 18 (interferon-gamma-inducing factor)	-4.5
JUN	jun oncogene	3.1
LOX	lysyl oxidase	-2.1
MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	3.6
PRRX1	paired related homeobox 1	5.2

PRRX2	paired related homeobox 2	2.3
PPAP2B	phosphatidic acid phosphatase type 2B	2.7
ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila); similar to roundabout 1 isoform b	2.1
THBS1	thrombospondin 1	5.5
TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	-2.3
ZFP36L1	zinc finger protein 36, C3H type-like 1	2.1

Lymphocyte Mediated Immunity

FCER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	-2.0
CR2	complement component (3d/Epstein Barr virus) receptor 2	-2.9
C1QA	complement component 1, q subcomponent, A chain	-3.5
C1QB	complement component 1, q subcomponent, B chain	-4.0
C1S	complement component 1, s subcomponent	2.2
C2	complement component 2	-2.1
C4A	complement component 4A (Rodgers blood group)	7.6
C5	complement component 5	2.1
CFI	complement factor I	-2.4

Response to Hypoxia

ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	-2.3
EP300	E1A binding protein p300	2.2
ALAS2	aminolevulinate, delta-, synthase 2	-6.9
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	-2.5
CAV1	caveolin 1, caveolae protein, 22kDa	3.6
EGLN1	egl nine homolog 1 (C. elegans)	2.1
IL18	interleukin 18 (interferon-gamma-inducing factor)	-4.5
MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	3.6
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	-2.3
RYR2	ryanodine receptor 2 (cardiac)	-3.5
THBS1	thrombospondin 1	5.5
TF	transferrin	7.9

Cell Morphogenesis Involved in Differentiation

GRK1	G protein-coupled receptor kinase 1	-3.0
APP	amyloid beta (A4) precursor protein	2.4
ANTXR1	anthrax toxin receptor 1	2.5
CHST3	carbohydrate (chondroitin 6) sulfotransferase 3	2.3

CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	3.0
CLIC5	chloride intracellular channel 5	2.0
DST	dystonin	2.4
FN1	fibronectin 1	2.4
GAP43	growth associated protein 43	-3.1
LAMC1	laminin, gamma 1 (formerly LAMB2)	2.3
MAP1B	microtubule-associated protein 1B	2.3
MYH10	myosin, heavy chain 10, non-muscle	3.3
OPHN1	oligophrenin 1	2.4
ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila); similar to roundabout 1 isoform b	2.1
UCHL1	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	2.1
CER1G	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	-2.0
FCGR2B	Fc fragment of IgG, low affinity IIb, receptor (CD32); Fc fragment of IgG, low affinity IIc, receptor for (CD32)	-2.4
GEM	GTP binding protein overexpressed in skeletal muscle	2.8
GCH1	GTP cyclohydrolase 1	2.0
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	-2.0
VSIG4	V-set and immunoglobulin domain containing 4	-2.4
CTSS	cathepsin S	-2.6
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	3.0
COL4A3BP	collagen, type IV, alpha 3 (Goodpasture antigen) binding protein	2.1
CR2	complement component (3d/Epstein Barr virus) receptor 2	-2.9
C1QA	complement component 1, q subcomponent, A chain	-3.5
C1QB	complement component 1, q subcomponent, B chain	-4.0
C1S	complement component 1, s subcomponent	2.2
C2	complement component 2	-2.1
C4A	complement component 4A (Rodgers blood group)	7.6
C5	complement component 5	2.1
CFI	complement factor I	-2.4
IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	-2.1
IGHA2	immunoglobulin heavy constant alpha 2 (A2m marker)	-2.3

IGHM	immunoglobulin heavy constant gamma 1 (G1m marker); immunoglobulin heavy constant mu; immunoglobulin heavy variable 3-7; immunoglobulin heavy constant gamma 3 (G3m marker); immunoglobulin heavy variable 3-11 (gene/pseudogene); immunoglobulin heavy variable 4-31; immunoglobulin heavy locus	-4.0
IGLC1	immunoglobulin lambda variable 2-11; immunoglobulin lambda constant 2 (Kern-Oz- marker); immunoglobulin lambda variable 1-44; immunoglobulin lambda constant 1 (Mcg marker); immunoglobulin lambda variable 1-40; immunoglobulin lambda variable 3-21; immunoglobulin lambda locus; immunoglobulin lambda constant 3 (Kern-Oz+ marker)	-4.0
IL1RAPL1	interleukin 1 receptor accessory protein-like 1	5.8
IL18	interleukin 18 (interferon-gamma-inducing factor)	-4.5
MBP	myelin basic protein	25.3
NFIL3	nuclear factor, interleukin 3 regulated	3.4
PF4	platelet factor 4	-6.5
THBS1	thrombospondin 1	5.5
TRPM4	transient receptor potential cation channel, subfamily M, member 4	-2.1
Blood Vessel Morphogenesis		
NPEP	alanyl (membrane) aminopeptidase	-2.7
AMOT	angiomotin	2.0
CAV1	caveolin 1, caveolae protein, 22kDa	3.6
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	3.0
IL18	interleukin 18 (interferon-gamma-inducing factor)	-4.5
JUN	jun oncogene	3.1
PRRX1	paired related homeobox 1	5.2
PRRX2	paired related homeobox 2	2.3
ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila); similar to roundabout 1 isoform b	2.1
THBS1	thrombospondin 1	5.5
TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	-2.3
ZFP36L1	zinc finger protein 36, C3H type-like 1	2.1
Retinoic Acid Metabolism Process		
CRABP2	cellular retinoic acid binding protein 2	2.2
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1	3.2
RBP1	retinol binding protein 1, cellular	-5.6

Oxidation Reduction

COX15	COX15 homolog, cytochrome c oxidase assembly protein (yeast)	2.2
ERO1L	ERO1-like (S. cerevisiae)	2.2
NELL1	NEL-like 1 (chicken)	2.2
CYP2A13	cytochrome P450, family 2, subfamily A, polypeptide 13	5.6
CYP26B1	cytochrome P450, family 26, subfamily B, polypeptide 1	3.2
CYP39A1	cytochrome P450, family 39, subfamily A, polypeptide 1	-2.1
CYBRD1	cytochrome b reductase 1	2.2
CYCS	cytochrome c, somatic	-2.2
DPYD	dihydropyrimidine dehydrogenase	-2.5
EGLN1	egl nine homolog 1 (C. elegans)	2.1
FASN	fatty acid synthase	-8.5
GLUD1	glutamate dehydrogenase 1	2.2
GPX7	glutathione peroxidase 7	2.0
HSD3B1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	2.9
HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	2.8
HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12	-2.6
LOX	lysyl oxidase	-2.1
LOXL1	lysyl oxidase-like 1	-2.1
LOXL4	lysyl oxidase-like 4	2.1
MSRB3	methionine sulfoxide reductase B3	2.5
PHGDH	phosphoglycerate dehydrogenase	2.5
PHYHD1	phytanoyl-CoA dioxygenase domain containing 1	-2.0
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	-2.3
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	-2.5
TXN	thioredoxin	-2.1

Positive Regulation of Foam Cell Differentiation

CD36	CD36 molecule (thrombospondin receptor)	-5.7
MSR1	macrophage scavenger receptor 1	2.3
PF4	platelet factor 4	-6.5

A2.2 Proteins

A2.1.1 Antibodies used for Western Blot and Immunofluorescence

Protein	Animal	Dilution	Incubation	Manufacturer	Cat. Number	Secondary Ab	Dilution	Incubation	Manufacturer	Cat. Number	Use
COX 1	goat	1:200	1 hour	Santa Cruz	sc1754	donkey anti goat	1:500	1h50m	Santa Cruz	sc2020	Western
COX 2	goat	1:200	1 hour	Santa Cruz	sc1747	donkey anti goat	1:500	1h50m	Santa Cruz	sc2020	Western
FAS	rabbit	1:200	1 hour	Santa Cruz	sc20140	goat anti rabbit	1:5000	1h50m	Santa Cruz	sc2004	Western
PPARγ	rabbit	1:100	1 hour	Santa Cruz	sc7196	goat anti rabbit	1:1250	1 hour	Santa Cruz	sc2004	Western
GAPDH	rabbit	1:500	1 hour	Santa Cruz	sc25778	goat anti rabbit	1:500	1h50m	Santa Cruz	sc2004	Western
Vitronectin	mouse	1:500	1 hour	Santa Cruz	sc74485	goat anti mouse	1:500	1 hour	Santa Cruz	sc2005	Western
Calprotectin	mouse	1:100	1 hour	Santa Cruz	sc71533	goat anti mouse	1:500	1 hour	Santa Cruz	sc2005	Western
Calprotectin	mouse	1:500	1 hour	Santa Cruz	sc71533	Alexa Fluor 488	1:1000	1 hour	Invitrogen	A11001	IF
CD 68	mouse	1:500	18 hours	Abcam	ab955	Alexa Fluor 488	1:1000	1 hour	Invitrogen	A11001	IF
CD 163	mouse	1:500	18 hours	Santa Cruz	sc33715	Alexa Fluor 488	1:1000	1 hour	Invitrogen	A11001	IF

Figure A1.2: Primary and secondary antibodies used for western blot and immunofluorescence

A2.2.2 RIPA Buffer Recipie

TRIS-HCl - 50Mm, Ph 7.4
NaCl – 150 mM
IGEPAL(or NP-40) – 1%
SDS - 0.1%
EDTA – 5mM
Na_3VO_4 – 5mM
NaF – 5mM
DTT – 10mM
Protease Inhibitor 10uL/mL

Appendix 2- All Treatment Groups of the Sinclair Minipig Study

Introduction to the Full Study

Once again, the Sinclair minipigs analyzed for this thesis were part of larger study to compare the effects of PUFA supplementation on the effects of obesity and adipose tissue, and then to compare these effects in the face of an LPS challenge. The adipose tissue of the Sinclair minipig has not been well characterized, and we thought it important to do so with the control group analyzing the rest of the data from the study. Additionally, we sought to corroborate some of our in vivo data with some in vitro data using cultured primary porcine adipocytes.

Section A1: Materials and Methods

A1.1 Full Study Materials and Methods

A1.1.1 Additional Notes

All of the materials and methods for the analyses below were the same as those described in chapter 2 unless otherwise mentioned.

A1.1.2 Supplementation Phase

In the supplementation phase (Figure, the minipigs were separated into two feeding groups and fed for 8 weeks after the two week acclamation period. Eight Sinclair minipigs were fed 350g of the control diet twice daily for 8 weeks, and 4 minipigs were fed 350g PUFA supplemented (Gromega 365 (JBS United Sheridan, IN)) diet twice daily for 8 weeks (Table A3). The diets were not isocaloric.

A1.1.3 Challenge Phase

At the end of the 8 week supplementation phase, 4 pigs fed the control diet and the 4 pigs fed the PUFA diet were challenged with 15 μg/kg lipopolysaccharide (LPS) with a total injection volume of 2.5 mL. The 4 designated control pigs were injected with 2.5 mL of saline. Blood samples were drawn every 20 one hour pre and 4 hours post injection to measure the effect of LPS on metabolic parameters (Figures A2.1 and A2.2) The following day, the pigs were challenged at a 5 μg/kg LPS in 2.5mL of saline or 2.5mL of saline alone. Two hours post injection visceral and subcutaneous adipose tissue, liver, spleen, thymus, hypothalamus, and semi tendonous muscle were collected.

Table A3: PUFA Diet Composition

(a)

Ingredient	Percentage in Diet
Corn	60.0%
Soybean Meal	30%
Vitamin Mix	2.5%
PUFA Supplement	7.5%

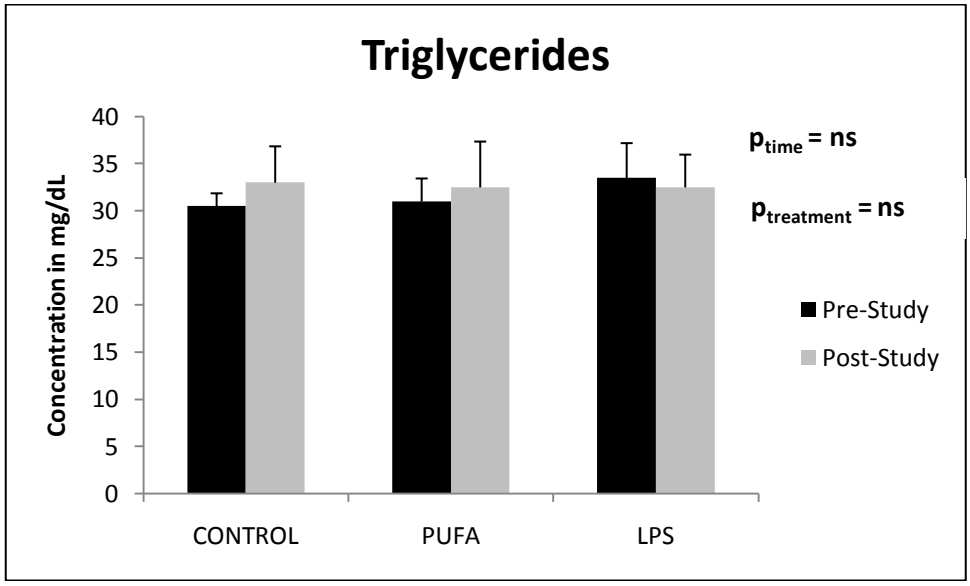
(b)

Water	10.8%
Crude Protein	17.7%
Crude Fiber	3.24%
Ether Extract (crude fat)	2.25%
Ash	2.94%
Carbohydrate	53.07%

Section A2: Sinclair Minipig Results

A2.1 Metabolic Parameters

(a)



(b)

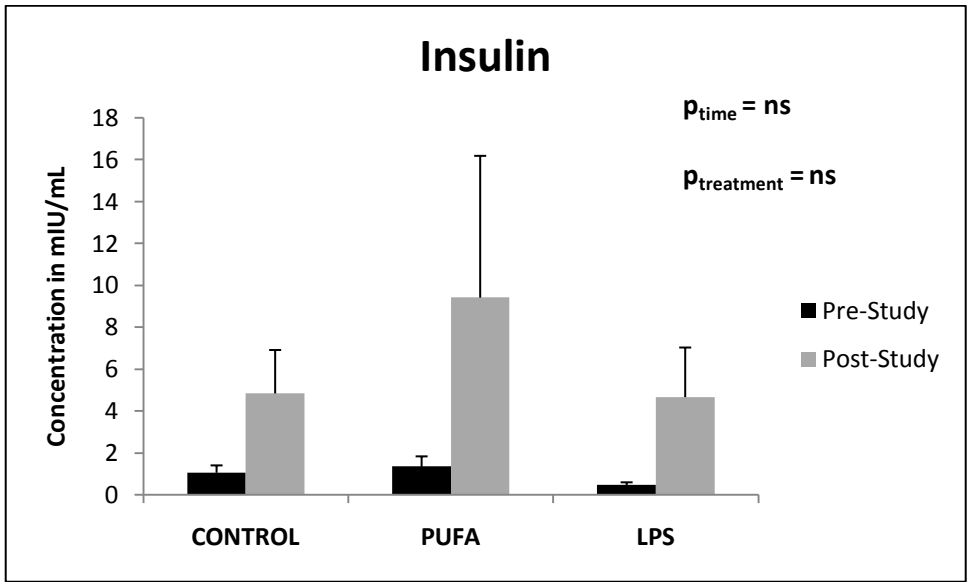
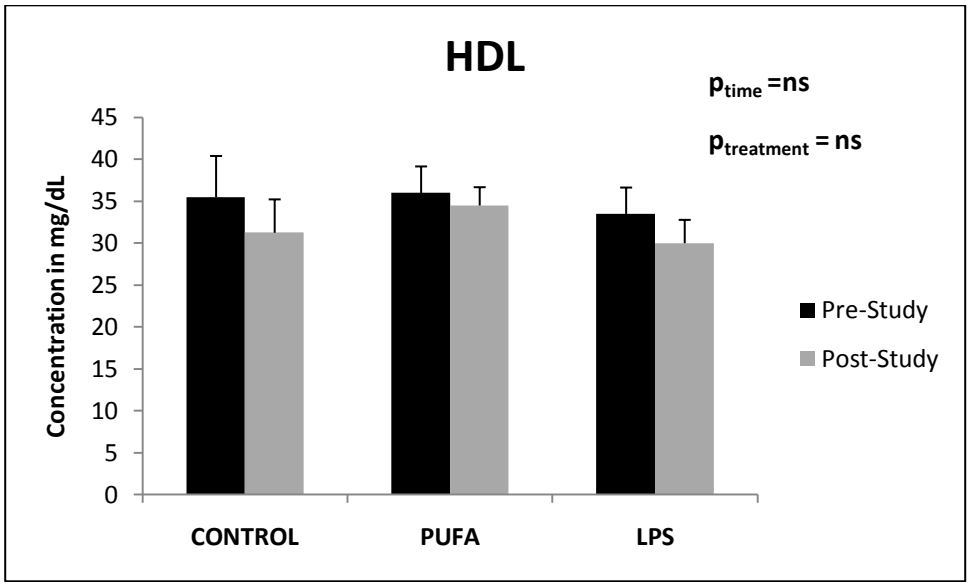
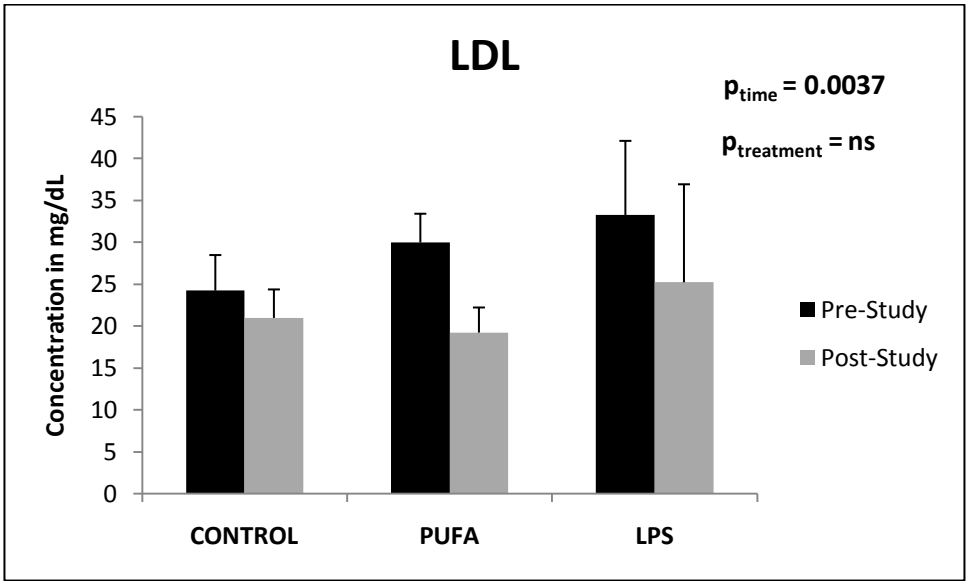


Figure A2.1: Circulating (a) Triglycerides and (b) Insulin at the beginning and the end of the study

(a)



(b)



(c)

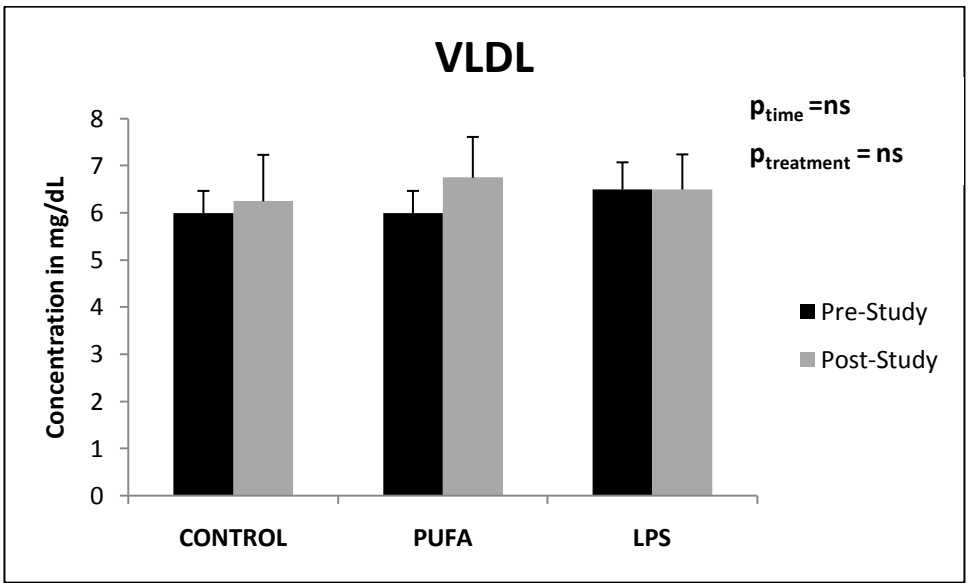


Figure A2.2: This figure illustrates the differences in (a)HDL, (b) LDL, (c) VLDL from the beginning and the end of the study

Table A4: Transcript details for all treatments and all comparisons

Comparison	Total Transcripts Δ	Up-Regulated	Down-Regulated
SQF LPS vs. Saline	1658	1117	541
SQF Saline vs. VIF Saline	598	471	127
VIF Saline vs. SQF Saline	788	240	548
SQF PUFA vs. VIF PUFA	1317	881	436
VIF PUFA vs. SQF PUFA	1461	523	938
SQF LPS vs. VIF LPS	2008	1390	618
VIF LPS vs. SQF LPS	2166	684	1482
SQF PUFA vs. LPS	1993	745	1248
VIF LPS vs. Saline	16	7	9
VIF PUFA vs. LPS	106	71	35

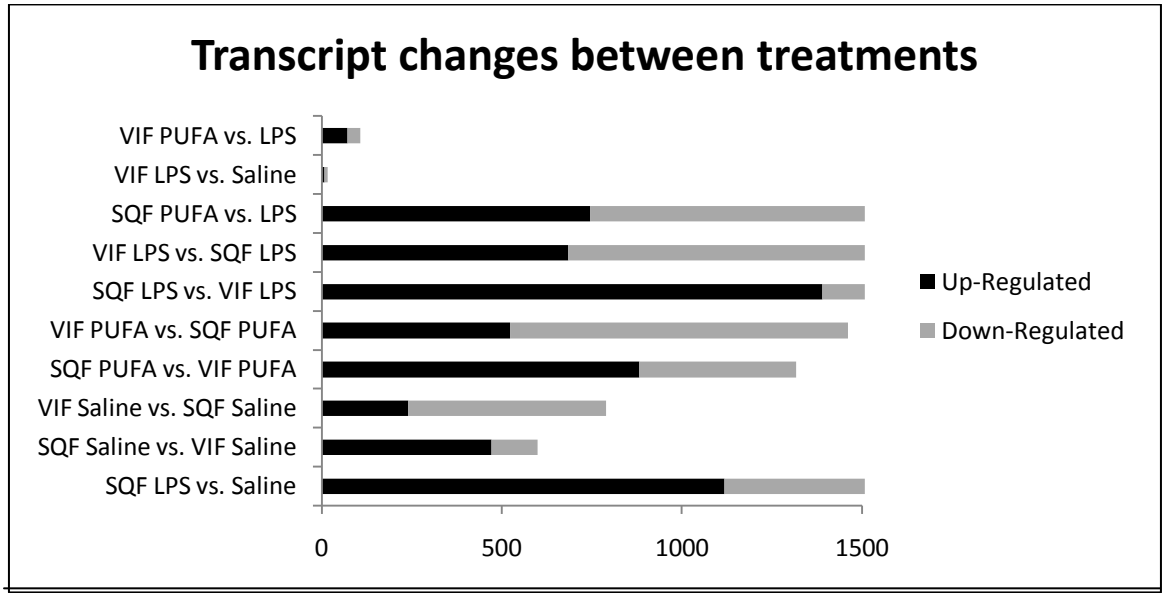


Figure A2.3: Transcript details for all treatments and all comparisons

This figure illustrates the distribution of the transcripts between the three treatment groups LPS (control diet/ LPS challenge), Saline (control diet/no LPS injection) and PUFA (PUFA supplemented diet/LPS challenge). Interestingly, each of the comparisons are comparing the effects of the treatment in SQF and only 108 of the transcripts are differentially expressed between all 3 treatments

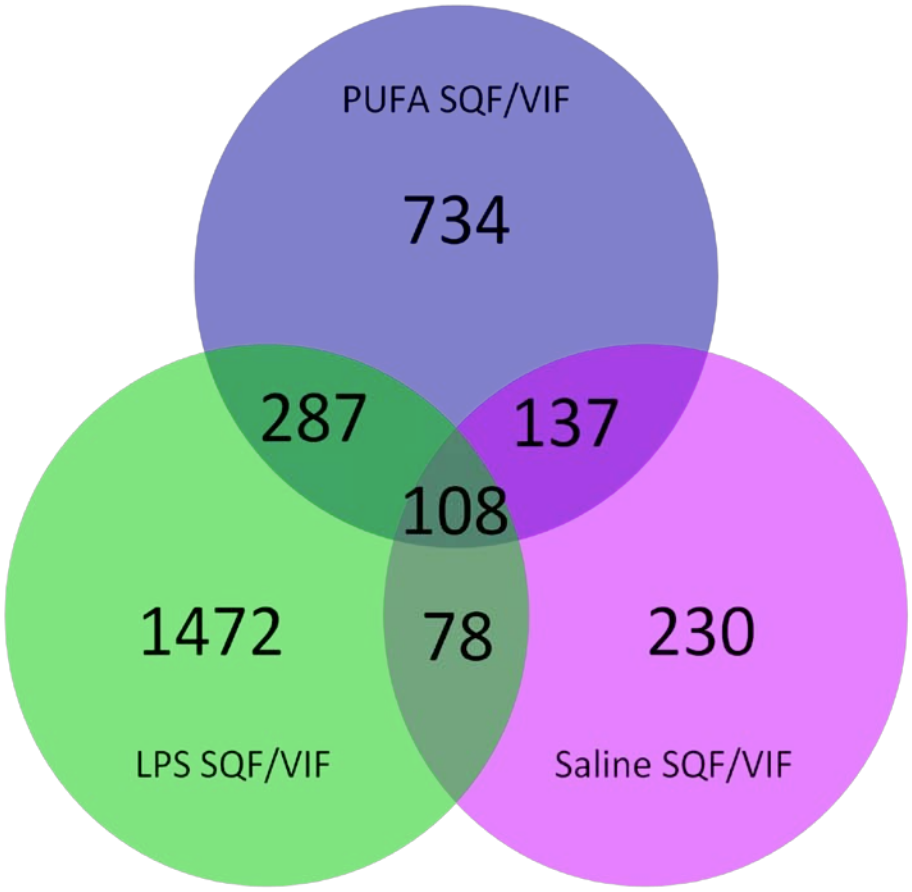
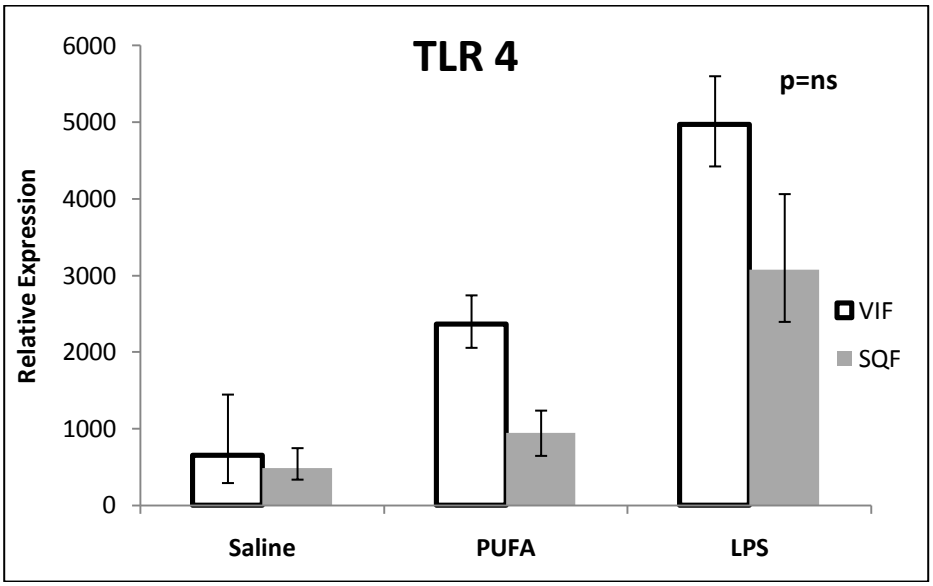


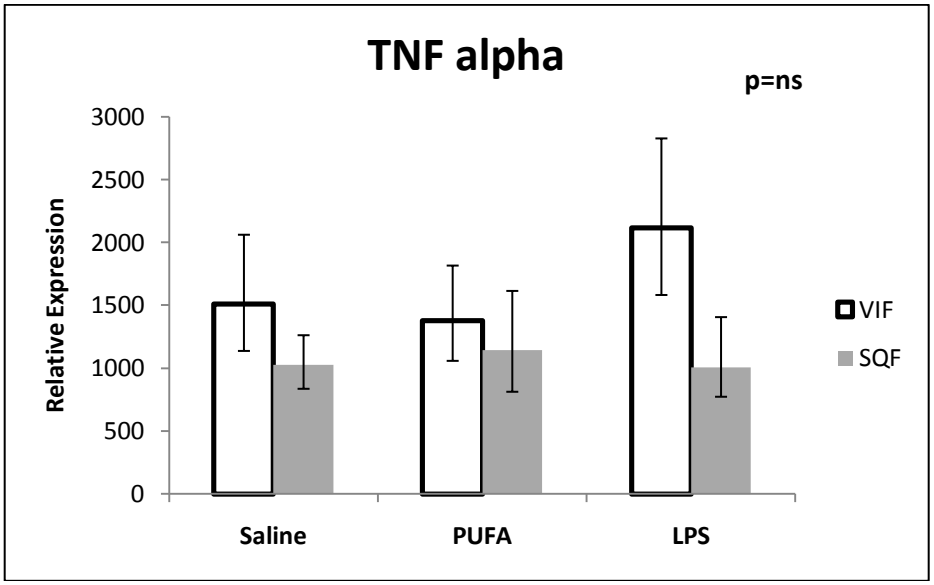
Figure A2.4: Venn diagram illustrating the transcript relationship between SQF and VIF among the three treatment groups: LPS (control diet/ LPS challenge), Saline (control diet/no LPS injection), and PUFA (PUFA supplemented diet/LPS challenge)

A2.1 Gene Expression

(a)



(b)



(c)

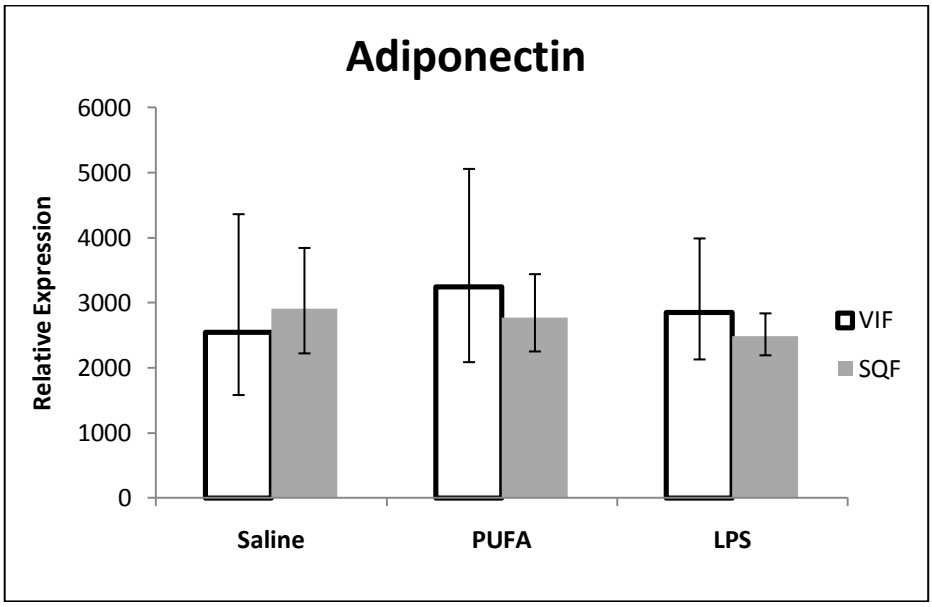


Figure A2.5: Relative gene expression of (a) TLR 4, (b) TNF alpha, and (c) Adiponectin in VIF and SQF

Section A3: Results and Discussion

A3.1 Metabolic Parameters

There was no significant difference between circulating triglycerides and VLDL, between the treatment groups and between the beginning and the end of the study. There was however, a significant difference between circulating insulin from the beginning and the end of the study ($p< 0.01$). This suggests that the minipigs were becoming hyperinsulinemic and possibly insulin resistant. Additionally, the minipigs that were supplemented with PUFA had lower circulating LDL than the minipigs that were only fed the control diet ($p<0.01$), additionally there was a trend for the PUFA supplemented minipigs to have more circulating HDL that the control fed pigs ($p=0.0626$), indicating that PUFA offered protection from dyslipidemia; one defining characteristic of the metabolic syndrome.

A3.2. Gene Expression

There was no significant difference between the treatment group's relative expression of TNF alpha, TLR 4, and adiponectin.

A3.3 Microarray

In general, we found SQF to be more reactive and responsive to LPS, as evidenced by the SQF LPS vs. VIF LPS comparison having the most transcript changes. There are three potential explanations for this. The first is that the SQF is the more reactive depot in the face of any immune challenge because it is more superficial that VIF and therefore more likely to receive an immune challenge first. Second, ample evidence demonstrates VIF to be more metabolically active, and therefore the differences between the basal levels of gene expression and those during an immune challenge may not change. Finally, some of the proteins that the genes code for may be regulated by post translational modifications and there may not have been a need in the VIF to up-regulate the genes.

Appendix 3- Porcine Cell Culture

Section A1: Cell Culture Materials and Methods

A1.1 Tissue Collection

Porcine subcutaneous adipose tissue (SQF) was collected from Wampler's Farm Sausage Company (Lenoir City, TN) from skinless carcasses that had been washed with lactic acid. The SQF was removed with a scalpel blade 4-6 inches below the shoulder blades in 2" x 2" sections, and immediately placed into Hanks balanced salt solution (Hanks) (Gibco/Invitrogen, Grand Island, NY) supplemented with 1% Penicillin/Streptomycin (P/S) (Lonza, Walkersville, MD) and 0.1% Geneticin (Gen) (Gibco/Invitrogen, Grand Island, NY Lot # 507095), and transported back to the lab at room temperature for further processing.

A1.2 Tissue Culture

One 2"x 2" section of SQF was incubated in hanks with 1% (P/S) and 0.1% (Gen) at 37°C for 4 hours. The tissue was cut into 4 equal size pieces, briefly dipped in 70% ethanol (EtOH), and rinsed in hanks (without antibiotic). The pieces were then cut into $5mm^3$ pieces briefly dipped in 70% ethanol (EtOH), and rinsed in hanks (without antibiotic). After the EtOH wash, 2-3 pieces of SQF were placed into each well (with 5mL DMEM solution) of a 6 well plate. The tissue was allowed to rest for 3 days with daily media changes. The tissue was then treated with DMSO (control), EPA (150mM), Genistein (25mM), or EPA(75mM) + Genistein (12.5mM) for 48 hours. After the treatment the tissues were homogenized in either RIPA buffer (for protein extraction) or TRI reagent (for RNA extraction) and stored in -80°C.

A1.3 Collagenase Digestion

One 2"x 2"section was incubated in hanks with 1% (P/S) and 0.1% (Gen) at 37°C for 4 hours. The tissue samples were cut into 4 equal size pieces, briefly dipped in 70% ethanol (EtOH), and rinsed in hanks (without antibiotic). The pieces were then cut into $5mm^3$ pieces briefly dipped in 70% ethanol (EtOH), rinsed in hanks (without antibiotic), then incubated overnight in flasks with 100mL DMEM solution. The following day collagenase was made fresh using a 1mL

hanks/1mg type I collagenase (Sigma, St. Louis, MO) ratio, and filter sterilized with a 0.45µm filter (Millipore, Billerica, MA) The pieces of tissue were emptied into a ceramic flask and washed two times with 25mL 70% EtOH. After washing, 10-15 pieces of SQF were placed into 35mL of collagenase, place incubated for 2 hours and 15 minutes at rotating incubator at 37°C. The digested contents were filtered in a funnel with nylon mesh. The supernatant was centrifuged @ 1700 RPM for 20 min. The stromal vascular fraction (SVF) was placed in a 60mm cell culture dish containing 7mL DMEM solution (total of 8) and placed in an incubator at 37°C, 5% CO2, and 98% relative humidity.

A1.4 Growth and Differentiation

Pre-adipocytes were maintained at 37°C, 5% CO2, and 98% relative humidity. The media was changed 5 times weekly for 14 days at which point the cells reached confluence. The pre-adipocytes were trypsinized with 3mL trypsin (Gibco, Carlsbad, CA) for 3-5 minutes to detach the pre-adipocytes from the plate and placed into media. The pre-adipocytes were then placed in DMEM solution, which was then added to 100mm cell culture dish (8-60mm plates were split into 40-100mm plates. The pre-adipocytes were once again maintained at 37°C, 5% CO2, and 98% relative humidity.

Differentiation media 1 (see below) was initially used to differentiate the pre-adipocytes, however approximately 90% of they died. Once the pre-adipocytes grew back to a plate average of 90% confluence, pre-adipocytes were either differentiated with differentiation media 2 or 3. The adipocytes differentiated in differentiation media 3 were maintained with 10 nM insulin in the DMEM solution.

A1.5 Freezing cells

Pre-adipocytes were frozen at 3 points during the growth phase; before splitting into 100mm dishes, and before the addition of the first and second differentiation media (from plates split at these times). The pre-adipocytes were trypsinized for 3-5 minutes to detach the pre-adipocytes from the plate and placed into media. The pre-adipocytes were then placed in 25mL DMEM solution, which was centrifuged for 5 minutes @ 5000 RPM. The cell pellet was

placed into 1 mL freeze media (see below), placed in -80°C overnight, then was placed in liquid nitrogen for long-term storage.

A1.6 Treatment and Harvest

The plates were divided into 4 treatments groups, and after being maintained with starvation media for 24 hours, were treated with DMSO (control), EPA (150mM), Genistein (25mM), or EPA(75mM) + Genistein (12.5mM) in DMEM solution for 48 hours. After treatment the adipocytes were harvested in either RIPA buffer, for proteins, or 1mL Buffer RLT (Quigen) with 10μL β – Mercaptoethanol (BME)(Sigma, St. Louis, MO) ,for RNA, and stored in -80°C.

A1.7 Media and Buffers

A1.7.1 Dulbecco's Modified Eagle Medium (High Glucose) Solution

DMEM(Thermo Scientific, Logan, UT)

- 25mM HEPES
- +4.5g/L glucose
- 4.00mM L-Glutamine
- no Sodium Pyruvate
- 0.1μm sterile filtered

10% Fetal Bovine Serum (Atlanta Biological Lawrenceville, GA; Lot H0098)
1% Penicillin/Streptomycin (Lonza, Walkersville, MD Lot multiple)
0.1% Geneticin (Gibco/Invitrogen, Grand Island, NY Lot # 507095)

A1.7.2 Differentiation Media 1

DMEM(Thermo Scientific, Logan, UT)

- 25mM HEPES
- +4.5g/L glucose
- 4.00mM L-Glutamine
- no Sodium Pyruvate
- 0.1μm sterile filtered

10% Fetal Bovine Serum
1% Penicillin/Streptomycin
0.1% Geneticin
0.5mM MIX (isobutylmethylxanthine)(Sigma Aldrich St. Louis, MO)
0.25 μMDexamethasone(Sigma Aldrich St. Louis, MO)
10 nM Insulin(Eli Lilly and Company, Indianapolis, IN

A1.7.3 Differentiation Media 2

DMEM(Thermo Scientific, Logan, UT)

- 25mM HEPES
- +4.5g/L glucose
- 4.00mM L-Glutamine
- no Sodium Pyruvate
- 0.1µm sterile filtered

10% Fetal Bovine Serum
1% Penicillin/Streptomycin
0.1% Geneticin
500nM Rosiglitizone
10nM Insulin

A1.7.4 Differentiation Media 3

Zenbio human omental differentiation media (Zenbio, Research Triangle Park, NC Lot#'s: 012610 & 030910)

A1.7.5 Freeze Media

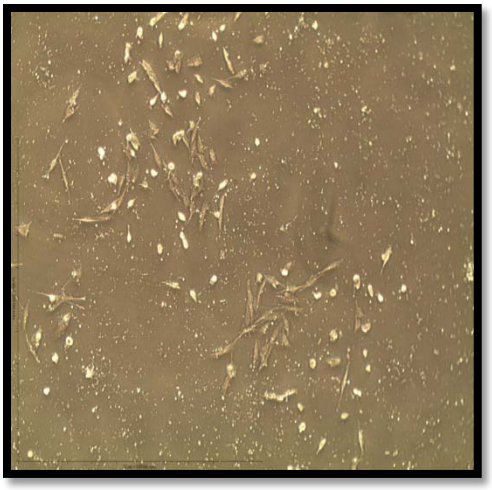
DMEM(Thermo Scientific, Logan, UT)

- 25mM HEPES
- +4.5g/L glucose
- 4.00mM L-Glutamine
- no Sodium Pyruvate
- 0.1µm sterile filtered

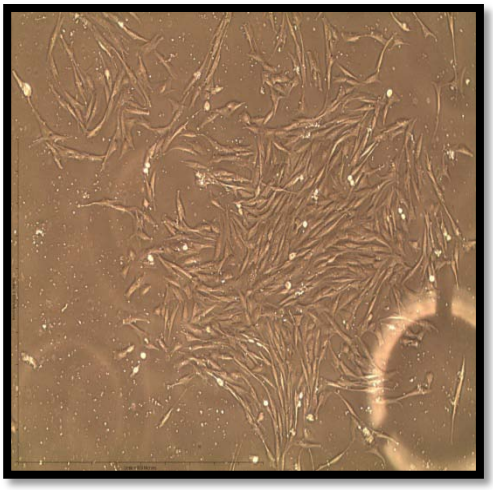
20% Fetal Bovine Serum
1% Penicillin/Streptomycin
0.1% Geneticin
10% DMSO

Section 2: Results

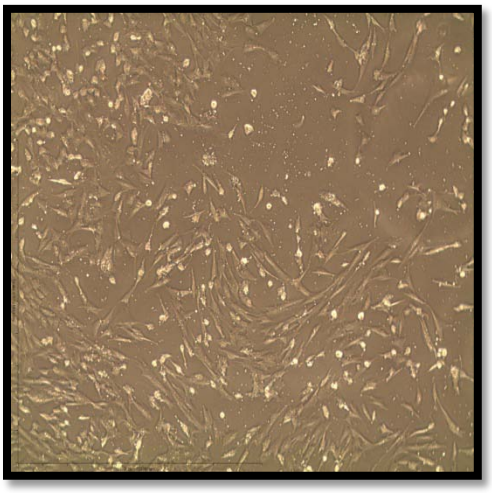
In an attempt to corroborate our in-vivo and ex-vivo data, we sought to establish a porcine cell line. Adipose tissue from domestic pigs was digested using type 1 collagenase and the resultant pre-adipocytes were grown to confluence (Figure A3.1). In an attempt to optimize pre-adipocyte differentiation into adipocytes, 3 different differentiation media were used. Differentiation media 1 was unsuccessful and resulted in the death of most of the pre-adipocytes (Figure A3.2). Differentiation media 2 resulted in approximately 50-75% differentiation, but it took approximately 14 days (Figure A3.3). Differentiation media 3 was the most successful resulting in 80-90% differentiation (Figure A3.4).



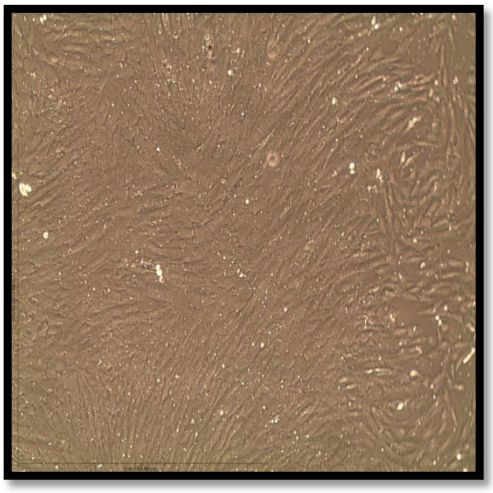
(a)



(b)

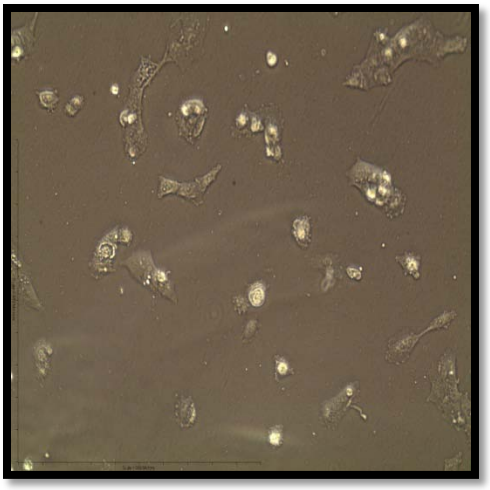


(c)

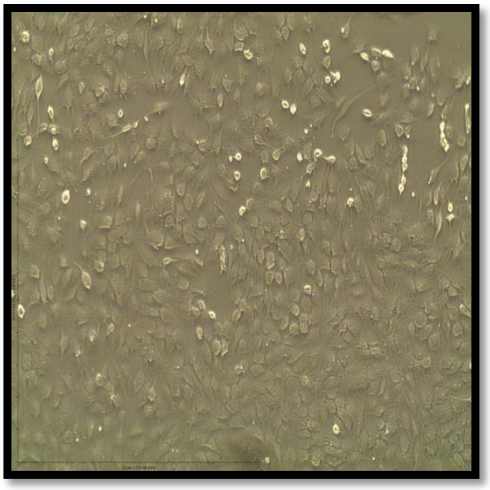


(d) (Confluence)

Figure A3.1: Porcine pre-adipocyte growth days (a) 6, (b) 9, (c) 12, (d) 14 after collagenase digestion



(a)



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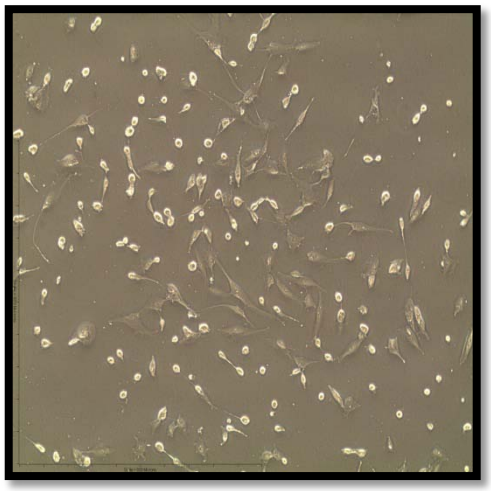
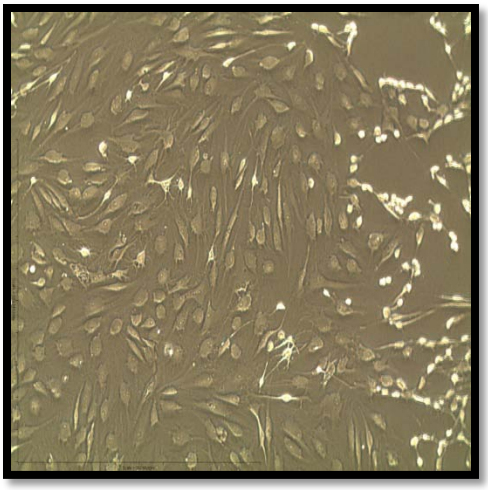
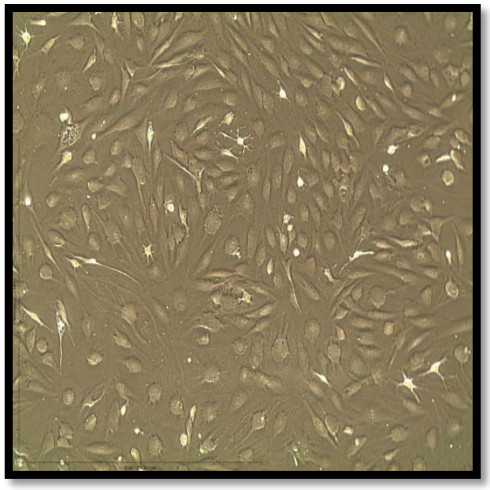


(c)

Figure A3.2: Porcine pre-adipocytes days (a) 3, (b) 5, and (c) 7 post differentiation using 0.5 Mm MIX (isobutylmethylxanthine), 0.25 μM Dexamethasone, and 10 nM Insulin



(a)



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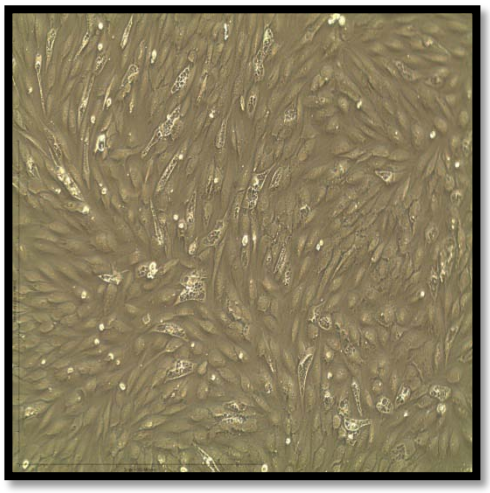
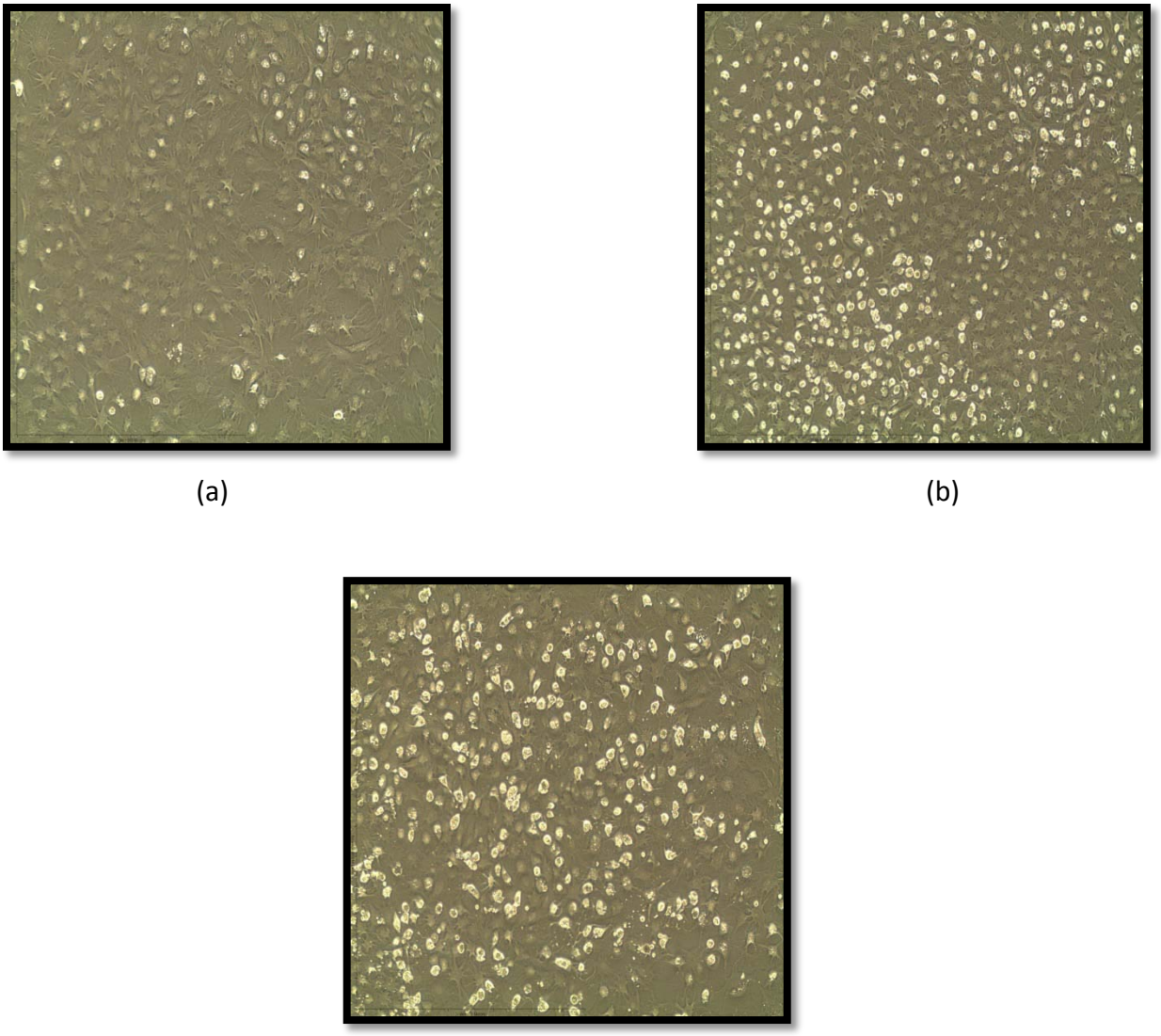


(c)

Figure A3.3: Porcine pre-adipocytes days (a) 2, (b) 4, and (c) 6 post differentiation with 10nM Insulin and 500mM Rosiglitizone



(a) (b)

(c)

Figure A3.4: Porcine pre-adipocytes days (a) 3, (b) 5, and (c) 8 post differentiation Zenbio human omental adipocyte differentiation medium

Section A3: Discussion

The primary porcine pre-adipocytes grew slower than 3T3-L1 mouse, taking approximately 2 weeks to reach confluence. Additionally, there was some sort of cell growth present in the first pre-adipocyte culture after collagenase digestion which gradually went away as the pre-adipocytes grew. These cells were not able to be identified, and did not appear in subsequent generations of cells. As mentioned above, differentiation media 1 caused a massive pre-adipocyte detachment and death which is hypothesized to have been caused by either the MIX or the dexamethasone. This differentiation media was first used because of its success in differentiating 3T3-L1 pre-adipocytes, a major cell line used both in obesity studies and in our lab and others [245-248]. The pre-adipocytes differentiated in differentiation media 2 resulted in better and more rapid differentiation than that those pre-adipocytes that were differentiated in differentiation media 3. Treatment of the adipocytes with their respective treatments resulted in a massive detachment of the adipocytes, especially those treated with EPA. Subsequent investigation of the problems revealed that the EPA may not have been reconstituted properly, and may be one reason for the detachment and death. The protein and RNA data has not been processed to date.

Vita

Suzanne Lauren Booker was born in Delaware, Ohio, on February 26, 1982. She was raised by her mother, and enjoyed the company of a younger brother and sister for the duration of her childhood. She attended preschool and elementary school at Cincinnati Country Day School in Cincinnati, Ohio. Middle school, junior high, and senior high school were completed in the Pickerington school system in Pickerington, Ohio. After graduation, she attended the University of Cincinnati, completing her Bachelor of Science degree in Biology and receiving a business certificate in 2005. After graduation, she began working at a small animal veterinary hospital while continuing to work at the Cincinnati Zoo and Botanical Garden. In 2008, she enrolled at the University of Tennessee, and earned a Master of Science degree in Animal Science in 2010.