CHARACTERIZATION OF THE HCA_2 RECEPTOR IN A MURINE AND A FELINE MODEL OF HUMAN OBESITY

by

Emily Catherine Graff

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 12, 2015

Approved by

Robert L. Judd, Associate Professor, Anatomy, Physiology and Pharmacology
Robert J. Kemppainen, Professor Anatomy, Physiology and Pharmacology
Elizabeth A. Spangler, Associate Professor, Pathobiology
Elizabeth G. Welles, Professor, Pathobiology
Ellen Behrend, Professor, Clinical Sciences

ABSTRACT

Background. There is mounting evidence that the hydroxycarboxyilic acid 2 (HCA_2) receptor, which is present on both adipocytes and immune cells, plays an important role in modulating cross-talk between metabolic and immune functions. Previous work from our laboratory demonstrates that in addition to anti-inflammatory effects, niacin, a pharmacologic ligand of HCA_2, increases serum concentrations of the anti-inflammatory adipokine, adiponectin. Therefore, it is unclear whether niacin's anti-inflammatory effects are due to changes in adiponectin, or if they are adiponectin-independent. In addition, our lab has shown that HCA_2 receptor expression is decreased in high fat diet (HFD)-fed rodent models of obesity, suggesting a possible mechanism for obesity-associated alterations in immune regulation. Cats provide an important translational model for human obesity. However, little is known about obesity-associated inflammation in cats and the feline HCA_2 receptor has not been identified. **Objectives.** Therefore, the objectives of this study are to 1) investigate the underlying mechanisms in niacin-mediated activation of HCA_2 in a HFD-fed mouse model of obesity; 2) characterize the HCA_2 receptor in cats; and 3) evaluate obesity-associated inflammation and HCA_2 receptor expression in cats during the development of obesity. **Methods.** To evaluate the relationship between adiponectin and niacin-mediated activation of the HCA_2 receptor, 32 male C57BL/6J mice (WT) and 32 adiponectin null mice ($AdipoQ^{-/-}$) were fed a control (chow) or HFD. After 6 weeks, half of the mice from

each group received either vehicle (water) or niacin (360 mg/kg/day) in their drinking water for 5 weeks. To evaluate HCA_2 receptor distribution and functionality in cats, tissues and primary adipocytes for culture were collected from lean, healthy, female cats. To investigate inflammation and changes in HCA_2 receptor expression in a developmental model of feline obesity, twelve lean, male neutered cats, fed a chow diet, were allowed to gain weight owing to *ad libitum* feeding for 18 months. **Results.** In adipose tissue of HFD fed mice, there was increased gene expression of monocyte chemo-attractant protein -1 (MCP-1) and cluster differentiation 68 (CD68), and niacin treatment attenuated the HFD-induced increase in MCP-1, but had no effect on CD68. Similar findings were observed in $AdipoQ^{-/-}$ mice. HFD fed mice had increased gene expression of M1 and M2 macrophage markers. Niacin decreased expression of M2 markers and tended to decrease expression of M1 markers. Interestingly, in HFD-fed $AdipoQ^{-/-}$ mice, niacin significantly increased expression of both M1 and M2 markers. The *in silico* predicted feline HCA_2 protein sequence exhibited 83.1% and 86.5% amino acid similarity to human and mouse sequences respectively. The feline HCA_2 receptor is predominantly expressed in adipose tissue and spleen. Exposure of feline adipocytes in primary culture to niacin inhibited lipolysis to a similar degree as insulin. After 18 months of ad libitum feeding, lean cats had ~60% increase in bodyweight, body condition score (BCS) and fat mass, and there was evidence of decreased insulin sensitivity and altered lipid metabolism. Feline obesity was associated with a slight shift in the percent distribution of fat mass with a greater increase in the percent of subcutaneous adipose tissue compared to the percent increase in abdominal adipose tissue. However, there were no changes in systemic markers of inflammation, including

inflammatory cytokines and circulating immune cells. Mean adipocyte diameter increased by ~66% in abdominal adipocytes and 79% in subcutaneous adipocytes. Rare adipose tissue macrophages (ATMs) and crown-like structures (CLS) were observed and distribution was independent of time point and location. During the development of feline obesity, HCA_2 receptor expression tended to decrease in abdominal adipose tissue, but was unchanged in subcutaneous adipose tissue.

Conclusion. In conclusion, niacin does not appear to alter macrophage number, but exerts adiponectin-independent effects on macrophage recruitment. Absence of adiponectin alters niacin's effects on macrophage polarity, implicating adiponectin-dependent mechanisms. The feline HCA_2 receptor is similar to rodent and human HCA_2 in sequence, distribution and function, and similar to rodent models of obesity, feline HCA_2 expression tends to decrease in abdominal adipose tissue. Cats have a unique immune response during the development of obesity that appears to protect them from certain obesity-associated sequelae such as atherosclerosis, while sustaining other metabolic and immunological pathologies such as type 2 diabetes mellitus (T2DM).

ACKNOWLEDGMENTS

This dissertation would not be possible without the continual guidance, support and encouragement of my advisor, Dr. Robert L. Judd, and for that I am profoundly grateful. I would also like to thank my committee members, Dr. Kemppainen, Dr. Behrend, Dr. Elizabeth Welles and Dr. Beth Spangler, for their guidance and expertise along the way. In particular I want to thank all of my mentors who helped me to navigate the combined process of residency and graduate school. Thank you also to Dr. Doug White for his careful review of my doctoral dissertation. I owe my deepest appreciation to my husband, Chris, for his unconditional love, stalwart support, and dedication to family. I am grateful to my parents and my brother, Brian, for their endless encouragement. I am overwhelmingly and wholeheartedly beholden to my two children, Arthur and Alice, who came into my life during this process, and gave me unimaginable perspective.

Style manual of journal used: Metabolism, Clinical and Experimental

Computer software used: Microsoft Word, Microsoft Excel, GraphPad Prism 6, EndNote X6

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABCG1	ATP-binding cassette sub-family G member 1
AdipoQ$^{-/-}$	Adiponectin null mouse
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ATM	Adipose tissue macrophage
AU-IACUC	Auburn University-Institutional Animal Care and Use Committee
BCS	Body condition score
BMI	Body mass index
β-OHB	Beta-hydroxybutyrate
CBC	Complete blood count
CCL	Chemokine ligand containing a C-C motif
CD	Cluster of differentiation
C/EBPα	CCAAT/enhancer binding protein α
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CLS	Crown-like structure
COX	Cyclooxygenase
CRP	C-reactive protein

DP-1	Dopamine receptor D1
EC_{50}	Half maximal effective concentration
ELISA	Enzyme linked immunoassay
EWAT	Epididymal white adipose tissue
FAE	Fumaric acid esters
GAPDH	Glycerol aldehyde 3-phosphate dehydrogenase
G_i	G-inhibitory
GPR	G-protein coupled receptor
HCA	Hydroxycarboxylic acid
HDL	High-density lipoprotein
HFD	High-fat diet
HOMA-IR	Homeostatic model assessment of insulin resistance
HMW	High molecular weight
HSL	Hormone sensitive lipase
IBA1	Ionizing calcium-binding adaptor molecule 1
ICAM	Intracellular adhesion molecule
IκBα	NF-κB light polypeptide gene enhancer in B-cells inhibitor, alpha
IL	Interleukin
INF	Interferon
iNOS	Inducible nitric oxide synthase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LMW	Low molecular weight

MCP-1	Monocyte chemoattractant protein 1
MMF	Monomethyl fumarate
MRI	Magnetic resonance imaging
MRC-1	Mannose receptor C-type 1
mRNA	Messenger RNA
NCBI	National Center for Biotechnology
NEFA	Non-esterified fatty acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor family, pyrin domain containing 3
ORF	Open reading frame
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
PPAR	Peroxisome proliferator activated receptor
PUMA-G	Protein upregulated in macrophages by interferon γ
qPCR	Quantitative polymerase chain reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
RT-PCR	Reverse transcribed polymerase chain reaction
SAA	Serum amyloid A
SCFA	Short-chain fatty acid
SEM	Standard error of the mean
SREBP-1c	Sterol regulatory element binding protein-1c

siRNA	Silencing ribonucleic acid
SIRT-1	Silent mating type information regulation 2 homolog 1
SVF	Stromal vascular fraction
T2DM	Type 2 diabetes mellitus
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule one
VLA	Very late antigen
WBC	White blood cell
WT	Wild type

CHAPTER 1

REVIEW OF THE LITERATURE

INTRODUCTION

The hydroxycarboxylic acid receptors (HCA_{1-3}) are a family of G-protein-coupled receptors that recognize endogenous intermediates of metabolism and are critical for nutrient sensing. The endogenous ligand for HCA_1, formerly known as GPR81, is lactate, an energy intermediate produced by cells in states of anaerobic energy metabolism. The endogenous ligand for HCA_2, formerly known as GPR109a, is beta-hydroxybutyrate (β-OHB), a ketone body produced by the liver through β-oxidation when an individual is in a negative energy balance. HCA_3, formerly known as GPR109b, is only found in higher primates and is activated by 3-hydroxy-octanoate, a ketone body intermediate produced by skeletal muscle [1-3]. All three receptors are predominantly expressed on adipocytes, where they function to inhibit lipolysis. However, more recent studies involving the HCA_2 receptor demonstrate that it has profound anti-inflammatory properties as well [4,5]. This review summarizes the physiological and pathological roles of the HCA_2 receptor in inflammation in various tissues and clinical states and species.

THE HCA_2 RECEPTOR

HCA_2 is a G_i protein-coupled receptor predominantly expressed on adipocytes (white and brown) and immune cells, including macrophages, monocytes, neutrophils and dermal dendritic cells, but not lymphocytes [6-12]. HCA_2 is also present in retinal pigmented and colonic epithelial cells, keratinocytes, microglia, and normal mammary tissue [13-16]. Although a number of studies report that the receptor is not expressed in the liver [9-11], a more recent study demonstrated low basal levels of HCA_2 in primary murine hepatocytes with expression being induced after exposure to inflammatory stimuli [17]. When compared across tissues, relative gene expression is highest in abdominal and epididymal white adipose tissue followed closely by subcutaneous white adipose tissue and the spleen, and lesser expression in lung and lymph node [18,19]. In these studies minimal relative gene expression was also noted in the heart, liver, jejunum, kidney, ovary, testicle, pancreas, brain, and embryo [18,19]. Distribution of receptor expression appears to be fairly consistent among species [18].

It is believed that the primary role of activation of HCA_2 on adipocytes is to inhibit lipolysis. Activation of HCA_2 on adipocytes prompts inhibition of adenylate cyclase activity, reduced cAMP levels, reduced protein kinase A (PKA) activity, and a rapid reduction in the activity of the lipolytic enzyme, hormone sensitive lipase (HSL) [10,20,21]. This triggers a rapid reduction in lipolysis and reduced release of non-esterified fatty acids (NEFAs) from the adipocyte.

Endogenous ligands for HCA_2 include the ketone body, β-OHB and butyrate, both of which serve as nutrient sources for cells under various physiological conditions [22,23]. β-OHB is produced in the liver through beta-oxidation of fatty acids and serves as an alternative fuel source for non-neuronal cells during times of starvation or negative energy balance. In 1968, ketones were first described as inhibitors of lipolysis on adipocytes in order to moderate fat breakdown and conserve glucose in times of starvation [24]. Nearly 40 years later, it was determined that this critical survival event was mediated by β-OHB activation of HCA_2 [23]. The EC_{50} for stimulation of HCA_2 by β-OHB is approximately 0.7mM, which is a level obtained in human serum after 2-3 days of fasting or when fed a ketogenic diet [23]. This negative feedback of β-OHB via HCA_2 promotes efficient utilization of fat energy stores and protects from the development of ketocacidosis [25,26].

Butyrate is a short-chain fatty acid (SCFA) produced by bacterial fermentation of dietary fiber in the colon [27]. In addition, butyrate serves as an important fuel source for colonic epithelial cells and is associated with many beneficial metabolic effects, including improved insulin sensitivity and increased energy expenditure in mice fed a high-fat diet [28,29]. These beneficial metabolic effects along with additional anti-inflammatory properties may be due to butyrate-mediated activation of the HCA_2 receptor on colonic epithelial cells [30].

In addition to endogenous ligands, there are several pharmacologic ligands for HCA_2 [26,31]. The longest known and most commonly utilized pharmacologic ligand is the anti-atherogenic drug niacin, also known as vitamin B3 and nicotinic acid. The pharmacologic effects of niacin were first discovered in 1955 by Dr. Rudolf Altschul, when he observed that consuming pharmacologic doses of niacin (3,000 mg/day; compared to 15-20 mg/day necessary for vitamin properties) resulted in profound reductions in serum triglyceride and cholesterol concentrations [32]. However, it was not until 2003 that activation of HCA_2 was identified as being responsible for the anti-lipolytic properties of niacin [9-11]. Unfortunately, high doses of niacin resulted in the unwanted side effect of cutaneous flushing owing to HCA_2 receptor activation on keratinocytes and dermal dendritic cells [6]. Other exogenous ligands for HCA_2 are fumaric acid esters (FAE), including dimethyl fumarate and its metabolite, monomethyl fumarate (MMF). In 2008, a screen of 1500 low molecular weight carboxylic acids revealed that MMF was a potent and selective HCA_2 agonist [8]. Similarly to niacin, treatment with MMF results in the undesirable side effect of cutaneous flushing. The mechanisms responsible for the cutaneous flushing for niacin and MMF are activation of HCA_2 and subsequent formation of prostaglandin D_2 (PGD_2) and E_2 (PGE_2) which result in vasodilation [6].

While there has been much attention given to the anti-lipolytic properties of HCA_2, activation by endogenous and exogenous ligands has also been associated with anti-inflammatory effects in numerous disease states. In

particular, several studies report that niacin has the ability to reduce inflammation in atherosclerosis [5], obesity [4], sepsis [33], diabetic retinopathy [14] and renal disease [34] (Figure 1). In the autoimmune skin condition psoriasis, MMF has been an important part of therapy for many years due to its anti-inflammatory properties [8]. Dimethyl fumarate is used for the immune-modulating treatment of psoriasis and multiple sclerosis [35]. Additional studies demonstrate anti-inflammatory effects of endogenous HCA_2 agonists, including butyrate and β-OHB [29,36,37]. While some of the beneficial effects of these HCA_2 ligands may be independent of receptor activation [37], there are numerous studies in various tissues and clinical conditions that demonstrate a clear receptor-mediated process [4,5,38,39] (Table 1).

THE HCA_2 RECEPTOR IN VARIOUS TISSUES AND DISEASE STATES

Atherosclerosis

Atherosclerosis is a major cause of human morbidity and mortality in developed countries worldwide. This disease is characterized as a slow, progressive accumulation and oxidation of apolipoproteins within the arterial wall [40]. Inflammation has been identified as a common underlying factor during all phases of the atherosclerotic process, including plaque initiation and progression [40]. The main feature of inflammation in atherosclerosis development is the infiltration of circulating monocytes into the arterial intima and subsequent development of foam cells in response to the accumulation of apolipoprotein B-lipoproteins within the intima-media. Infiltration of circulating monocytes into the

intima is facilitated by the increased production of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and increased expression of endothelial surface receptors such as vascular cells adhesion molecule-1 (VCAM-1) [40].

Pharmacologic doses of niacin have been used for decades to treat dyslipidemia that is associated with the development of atherosclerotic coronary heart disease. The anti-atherogenic effect of niacin was initially believed to be due to its anti-lipolytic activity, which resulted in decreased low-density lipoprotein (LDL) cholesterol, and subsequent increase in high-density lipoprotein (HDL) cholesterol owing to a reduced supply of free-fatty acid substrate to the liver [25,41]. However, recent evidence indicates that the HCA_2-mediated anti-lipolytic effects of niacin may not be related to niacin's effects on serum lipids [42]. Therefore, the beneficial effect of niacin in the treatment of atherosclerosis may involve lipoprotein-independent or pleiotropic effects, including the modulation of inflammatory pathways [38].

Anti-inflammatory effects of niacin have been reported in monocytes and macrophages, as well as vascular endothelial cells [39,43,44]. Human monocytes incubated with pharmacologic doses of niacin demonstrated reduced toll-like receptor (TLR)-2 and TLR-4 mediated release of inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and MCP-1 [45]. Cultured human monocytes treated with niacin also exhibited decreased

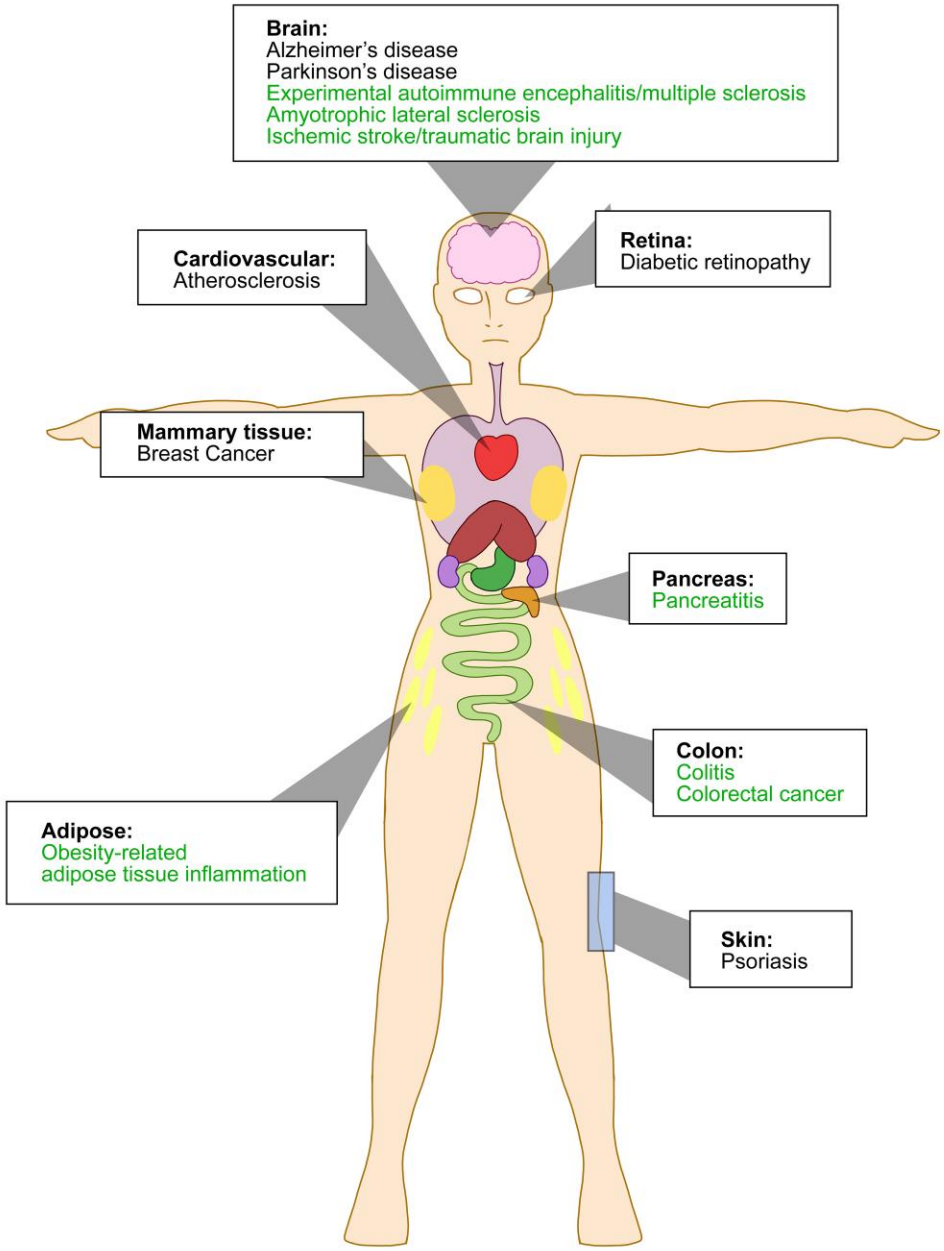


Figure 1. Body systems and various disease states in which the HCA_2 receptor may exert anti-inflammatory effects. Tissues listed in green have been investigated in rodent models only.

adhesion to VCAM due to decreased expression of very late antigen (VLA)-4 on the surface of the monocytes, as well as reduced monocyte chemotaxis towards conditioned media [39]. In 2011, Lukosova *et al.* showed that macrophages localized to atherosclerotic lesions express HCA_2 and that niacin-mediated HCA_2 activation induces the expression of the cholesterol transporter ABCG1 with a subsequent increase in cholesterol efflux from macrophages to HDL3 [5]. These effects were not seen in the absence of HCA_2. To confirm that these beneficial effects were mediated by the HCA_2 receptor, they transplanted bone marrow from the HCA_2-deficient mice into atherosclerotic prone mice and showed that the effects of niacin were abrogated in this model. In addition, they demonstrated that niacin decreases M1-like (classically-activated or pro-inflammatory) macrophage differentiation via the HCA_2 receptor [5]. The presence of HCA_2 on the surface of circulating monocytes suggests a broader and more complex role than specifically alteration of the pathogenesis of atherosclerosis. Most importantly, the data from all of these studies consistently demonstrate that the effects of niacin in the macrophages and monocytes are mediated by HCA_2, and are independent of the prostaglandin pathways and changes in serum lipoproteins [5,39]. More recent work shows that HCA_2 is a regulator of macrophage activation by inhibition of pro-inflammatory cytokine production, LDL cholesterol uptake, and chemotaxis [46].

Disease	Cellular Expression	Ligand	Effect	Ref*
Obesity	Adipocytes Macrophages	Niacin	↓ Lipolysis ↑ Adiponectin ↓ Macrophage migration ↓ M1 polarity	[4] [10] [20] [23] [39] [47] [50]
Neuro-inflammation	Microglial cells Macrophages / immune cells	Niacin βOHB[a] DMF[b]	↓ Cytokine production Improved cognitive function Decreased infarct size ↓ Immune cell infiltration	[15] [96] [98] [100] [101] [103]
Atherosclerosis	Monocytes Infiltrating macrophages Foam cells	Niacin	↓ Macrophage migration ↓ M1 polarity ↓ Endothelial cell adhesion ↓ Cytokine production ↑ Cholesterol efflux (foam cells)	[5] [38] [39] [43] [44] [45]
Colorectal Cancer and Colitis	Colonic epithelium	Butyrate Niacin	↓ Inflammasome-mediated colitis Altered immune regulation ↓ development of colorectal cancer Suspected tumor suppressor	[22] [30] [78]
Diabetic Retinopathy	Retinal pigmented epithelium	Niacin	Suppresses NFκB[c] ↓ Cytokine production ↓ Oxidative stress	[113] [114] [115]
Sepsis	Likely infiltrating immune cells	Niacin	Suppresses NFκB ↓ Lung inflammation ↑ Survival time	[32] [116]
Psoriasis	Keratinocytes Dermal dendritic cells (Langerhan's cells)	Niacin MMF[d]	↑ Vasodilation/flushing	[6] [8] [112] [113]

Table 1: Summary of known and potential HCA_2 mediated anti-inflammatory effects in various tissues and disease states. *reference; [a]Beta-hydroxybutyrate; [b]dimethyl fumarate; [c]nuclear factor Kappa B; [d]monomethylfumarate.

Previous results from our laboratory and others demonstrate that niacin increases serum concentrations of adiponectin and that increased adiponectin secretion from adipocytes is mediated by HCA_2 [47-52]. Along with niacin, adiponectin has been shown to directly improve endothelial dysfunction by stimulation of Nitric oxide production [53], inhibition of vascular smooth muscle cell proliferation [54], and inhibition of the conversion of macrophages to foam cells [55]. Additionally, adiponectin treatment inhibits TNF-α-induced monocyte adhesion to endothelial cells and expression of adhesion molecules on endothelial cells [56]. Adiponectin also directly inhibits pro-inflammatory signaling in macrophages [57]. Thus, the changes in endothelial cells seen with niacin administration may be dependent or independent of changes in adiponectin concentrations. Further work is needed to better understand the relationship and the shared anti-inflammatory effects of niacin and adiponectin.

Adipose Tissue and Obesity

Obesity is a complex medical disorder that involves excess body fat (adiposity), which is associated with a number of medical complications, including type 2 diabetes mellitus (T2DM) and cardiovascular disease [58,59]. In addition to excess adiposity, obesity is now classified as a chronic inflammatory disease process, characterized by 1) increased infiltration of inflammatory cells into adipose tissue, 2) increased circulation of inflammatory markers, and 3) dysregulation of adipose tissue endocrine functions, such as altered adipokine production and secretion [60-62]. In 2003, researchers first described crown like

structures in adipose tissue, which consist of macrophages surrounding dead and dying adipocytes [63]. In adipose tissue of lean individuals, the relatively few macrophages that are present are generally classified as M2, or alternatively activated (anti-inflammatory) macrophages. In contrast, the adipose tissue of obese individuals contains increased numbers of inflammatory cells as well as a change in macrophage polarity to an M1 or classically activated (inflammatory) state [64,65]. Inflammatory cytokines are produced by infiltrating inflammatory cells, as well as adipocytes, with cytokines IL-6, TNF-α, leptin, resistin and MCP-1 increased in the serum and tissues of obese individuals [60-62]. Along with the increases in systemic inflammatory markers, obesity is also associated with decreases in anti-inflammatory adipokines, such as adiponectin [66,67]. Serum adiponectin concentrations are decreased in obese patients as well as individuals with metabolic disease, atherosclerosis, and T2DM, and the reduction in adiponectin appears to precede the development of insulin resistance [61,67]. These findings support the anti-inflammatory properties of adiponectin. In addition, the loss of the protective effects of adiponectin may play a significant role in the pathogenesis of many of these disease processes.

Ligands for HCA_2 have demonstrated anti-inflammatory effects in adipose tissue by acting directly on adipocytes as well as immune cells. As previously mentioned, niacin directly inhibits monocyte chemotaxis in co-culture studies with 3T3-L1 adipocytes [39]. Niacin also inhibits differentiation of macrophages into the pro-inflammatory M1 state in cell culture, which suggests that these

phenotypic changes likely occur *in vivo* as well [5,38]. Activation of HCA_2 by extended release niacin increases serum adiponectin in obese men with metabolic syndrome [47,50]. Therefore, at least part of the anti-inflammatory benefits of this receptor may be attributed to its ability to alter serum adipokine concentrations. The direct and adiponectin-associated effects of HCA_2 activation are present not only in the adipocyte, but may be present in adipose tissue macrophages. Recent work in our lab has shown that niacin reduces high-fat diet-induced expression of M1 macrophage markers, including MCP-1, in adipose tissue in a HCA_2-dependent manner [4]. Others have shown that adiponectin decreases TLR-4 receptors on cultured macrophages and shift macrophages into a M2, anti-inflammatory phenotype [68]. Thus, the anti-inflammatory effects of HCA_2 in adipose tissue may be indirectly related to its ability to locally increase adiponectin concentrations.

Niacin and adiponectin may share similar mechanisms to decrease inflammation. As previously mentioned, niacin decreases monocyte chemotaxis and adhesion, and decreases cytokine expression associated with decreased NF-κB activation [38,39]. In a similar manner to niacin, adiponectin inhibits TNF-α-induced mRNA expression of monocyte adhesion molecules and also suppresses TNF-α-induced IκB-α phosphorylation with subsequent NF-κB activation [69]. In obesity, as well as other inflammatory processes, the anti-inflammatory properties of niacin are dependent on HCA_2 activation [5,14,38,39], which is associated with increases in adiponectin [48,50]. Thus, niacin and adiponectin may be acting

independently, or synergistically to decrease inflammation associated with obesity.

Additional work from our lab demonstrated that gene expression of HCA_1 and HCA_2 are markedly decreased in adipose tissue of mice fed a high-fat diet [70]. The down-regulation of this receptor in response to a high-fat diet suggests that with obesity, there may be a decreased ability of the adipocyte to sense nutrients and metabolites leading to a dysregulation of lipolysis and adiponectin secretion. This potential adipocyte dysfunction may play a role in the increase in adipose tissue inflammation observed in obesity. This would further suggest that the adipose tissue of obese individuals is unable to respond appropriately when fasting, which results in a lack of adiponectin secretion and unregulated lipolysis. Additional work is needed to determine if these changes in gene expression are associated with changes in the adipocytes, immune cells or both. It is likely that these changes in gene expression are found in both adipocytes and macrophages, as others have shown HCA_2 down regulation in human derived foam cells [71]. Interestingly, cell culture studies in 3T3-L1 differentiated adipocytes, RAW 264.7 macrophages, and primary murine peritoneal macrophages indicate that HCA_2 gene expression is actually increased with LPS, TNFα, or IL-1 stimulation [70]. Further *in vivo* studies involving intra-peritoneal injection with LPS also demonstrated increased HCA_2 gene expression [72]. These findings suggest that while acute inflammation is likely associated with an increase in HCA_2 gene expression in adipocytes and immune cells, chronic inflammation associated with obesity results in HCA_2

downregulation. Further work is needed to understand the mechanisms behind these changes and it is also important to investigate whether protein expression will mirror gene expression in obesity and inflammation.

In summary, pharmacologic activation of HCA_2 by niacin improves adipocyte biology by alteration of local and systemic inflammation and reduction of the co-morbidities associated with obesity. These actions may be through either direct effects on adipocytes and macrophages, or indirect effects on systemic pro-inflammatory and anti-inflammatory molecules.

Colonic Inflammation and Cancer

Commensal organisms of the gut have profound effects on human health. The microbiome extracts energy and nutrients and generates numerous important by-products from the diet, which benefit bacterial communities and assure host homeostatic function [73]. Some of the most important by-products of the microbiome are the SCFAs acetate, propionate and butyrate. Among the SCFAs, butyrate (an endogenous HCA_2 ligand) has received the most attention for its effects on colonic health. The functions of butyrate in promotion of colonic health include serving as an energy source for colonocytes and as a key mediator of anti-inflammatory and anti-tumorigenic effects [29,30,74]. Microbial disturbances including inflammatory bowel disease and colorectal cancer change the function of the microbiome, which leads to a depletion of butyrate-producing bacteria [75]. Irrigation of the gastrointestinal tract with butyrate suppresses inflammation

during ulcerative colitis [74]. Although these studies demonstrate that butyrate serves as anti-inflammatory agents in the colon, the underlying molecular mechanisms remain poorly understood.

A number of studies have demonstrated that the effects of butyrate on intestinal inflammation are due in part to its ability to inhibit histone deacetylase [76,77]. However, cell surface receptors for butyrate, including GPR43 and HCA_2, also exist on the colonic epithelium. GPR43 is activated by all three SCFAs, while HCA_2 is only activated by butyrate. Under physiologic conditions within the colonic lumen, butyrate is generated at sufficient quantities (10-20 mM) by gut microbiota to activate HCA_2. It is unclear if butyrate concentrations effect HCA_2 expression in colonic epithelial cells. In 2009, it was observed that the expression of HCA_2 is silenced in human colon cancer, in mouse models of intestinal/colon cancer and in colon cancer cell lines [22]. Further studies indicate that HCA_2 suppresses NF-κB activation and induces apoptosis in normal and colon cancer cell lines, consistent with the notion that HCA_2 mediates the tumor-suppressive effects of butyrate in the colon [22]. Singh *et al.* expanded this work by demonstrating that bacterial-derived butyrate and dietary fiber attenuate intestinal inflammation and colorectal cancer development through HCA_2-mediated differentiation of T regulatory cells and IL-10-producing T cells in colonic macrophages and dendritic cells [30]. They also demonstrated that HCA_2 was essential for butyrate-mediated induction of IL-18 in colonic epithelium [30]. Additional studies from the same group showed that $HCA_2^{-/-}$ mice were more

susceptible to the development of colonic inflammation and cancer, and niacin suppressed colitis and colon cancer in a HCA_2-dependent manner [30]. A potential mechanism by which HCA_2 suppresses colitis may involve the activation of the inflammasome. Recent studies determined that the addition of dietary fiber prevents the development of colitis through NLRP3 activation, resulting in increased IL-18 [78]. It was also shown that both GPR43 and HCA_2 on non-hematopoietic cells are necessary for some of the beneficial effects of a high fiber diet [78]. This effect is the opposite of what is observed other cells, where treatment with HCA_2 ligands inactivates the inflammasome, and blocks inflammation [37]. This may indicate a role for HCA_2 in promotion of the barrier function of the colonic epithelium, rather than direct inhibition of the immune response.

Retinal Pigmented Epithelial Cells and Diabetic Retinopathy

Diabetic retinopathy is the most common diabetic eye disease and the leading cause of blindness in the working-age population [79]. The pathogenesis of diabetic retinopathy is complex and involves multiple cell types including Muller, ganglion, endothelial and retinal pigmented epithelial cells [80]. The main underlying defect is disruption of the blood-retinal barrier. Other confounding factors that contribute to the pathogenesis and progression of diabetic retinopathy include persistent hyperglycemia, hyperlipidemia, altered vitreomacular interface, hypoxia, altered blood flow, retinal ischemia, and inflammation [81]. Persistent hyperglycemia results in the formation of advanced

glycosylated end-products, which in turn activate ICAM-1 in the endothelial cells. ICAM-1 mediates the inflammatory response by increasing transcription factors such as NF-κB and inflammatory cytokines including IL-6 and MCP-1 [82].

HCA_2 is present in retinal pigmented epithelium of rodents and humans and its expression is increased with the development of diabetes [14,83,84]. The retinal pigmented epithelium is responsible for immune regulation and response in the retina by production and secretion of numerous pro- and anti-inflammatory cytokines [85]. Recent studies demonstrated that niacin-mediated activation of HCA_2 suppresses TNF-α-induced increases in NF-κB activation, IL-6 and MCP-1 expression and secretion from retinal pigmented epithelial cells [14]. The authors speculated that increased expression of HCA_2 in diabetic patients along with associated increases in the endogenous ligand β-OHB may be a mechanism by which retinal pigmented epithelium attempts to reduce inflammation associated with diabetic retinopathy [14]. Additionally, the pharmacologic HCA_2 ligand, L-2-oxothiazolidine-4-carboxylic acid, has been shown to attenuate oxidative stress and inflammation in retinal pigmented epithelium, though the mechanisms do not appear to be entirely mediated by HCA_2 activation [86].

Neurodegenerative Diseases and Microglia

Millions of aging Americans suffer from neurodegenerative diseases. While there are limited therapies to treat many of these disease processes, HCA_2 ligands have shown beneficial effects in a variety of neurologic disease conditions

including juvenile epilepsy [87,88], Alzheimer's disease [89,90], amyotrophic lateral sclerosis [91], Huntington's disease [92], Parkinson's disease [93,94], multiple sclerosis [95], ischemic stroke [96] and traumatic brain injury [97]. The mechanisms behind many of these beneficial effects are still unknown. However, in a subset of neurodegenerative diseases, including Parkinson's disease, ischemic stroke, and multiple sclerosis, activation of HCA_2 is associated with certain therapeutic benefits.

Parkinson's disease is characterized by progressive loss of nigrostriatal dopaminergic neurons in the substantia nigra [93]. In rodent models of Parkinson's disease, treatment with either ketogenic diet or β-OHB infusion reduces dopaminergic neurodegeneration, microglial activation, and expression of IL-1β, IL-6, and TNF-α [93,94,98,99]. Recent studies show that HCA_2 is essential in mediating the anti-inflammatory effects of β-OHB in Parkinson's disease models [100]. In studies that utilized rodent primary mesocephalic neuroglial cells, β-OHB attenuated LPS-induced production of iNOS, COX-2, TNF-α, IL-1β and IL-6 and these beneficial effects were abolished by siRNA-mediated knockdown of HCA_2 [100]. Further studies revealed that HCA_2 mediated the inhibitory effect of β-OHB on microglia and this process involved NF-κB signaling [36,100].

Ischemic stroke occurs as a result of obstruction of arterial blood supply to the brain. After the initial damage, monocytes and macrophages infiltrate the

ischemic area. HCA_2 is present on microglia under normal conditions. After induction of ischemic stroke, HCA_2 is expressed on the monocytes/macrophages that infiltrate the ischemic area [15]. When fed a ketogenic diet or treated with either β-OHB or niacin, rodent models of ischemic stroke show reduced infarct size, edema and apoptosis, and improved cognitive function [15,96,101]. In the case of β-OHB treatment, these neuroprotective effects are lost in $HCA_2^{-/-}$ mice [15]. Studies in chimeric mice demonstrated that HCA_2 in bone marrow-derived monocytes/macrophages mediated the neuroprotective effect of niacin in rodent models of ischemic stroke [15]. The authors went on to show that PGD_2 was essential in the neuroprotective effects of niacin [15].

In the autoimmune disease multiple sclerosis, there is infiltration of the central nervous system by immune cells and destruction of myelin sheaths, which impairs signal conduction and damage to axons. Treatment with ketogenic diet improves motor disability and memory, and reduces inflammation in mice with experimental autoimmune encephalomyelitis, a rodent model of multiple sclerosis [95]. The drug dimethyl fumarate, whose active metabolite MMF is a ligand for HCA_2, exhibits protective effects in experimental autoimmune encephalomyelitis [102]. Dimethyl fumarate treatment reduces neurological deficits, immune cell infiltration and demyelination of the spinal nerves in wild-type mice but not in $HCA_2^{-/-}$ mice [102]. A recent study demonstrated that fumarates modulate microglial activation through a novel signaling pathway, which involves activation

of the AMPK-SIRT-1 axis, inhibition of NF-κB and subsequent decreased concentrations of pro-inflammatory molecules [103].

A comprehensive review on the neuroprotective effects of HCA_2 was published recently that summarizes potential mechanisms of HCA_2-mediated neuroprotection [104]. The authors highlight data that show activation of HCA_2 in microglial cells stimulates COX-1 and hematopoietic prostaglandin D synthase-mediated PGD_2 synthesis [6,7]. PGD_2 exerts neuroprotective effects through its receptor DP1 [105,106]. Also, PGD_2 is converted to 15-deoxy-delta-12,14-prostaglandin J2, which inhibits the pro-inflammatory action of the IKK kinase complex and activates the neuroprotective and pro-angiogenic transcription factor peroxisome proliferator-activated receptor gamma [107,108].

Other Conditions

Other disease conditions in which HCA_2 has a clinically significant role include mammary cancer and pancreatitis. Inflammation plays an important role in carcinogenesis [109]. Interestingly, HCA_2 functions as a tumor suppressor in normal mammary tissue but its expression is decreased in human breast tumors, breast cancer cell lines and in tumor tissues of three different mammary tumor models [13]. Transcriptome analysis revealed that HCA_2 activation inhibits genes involved in cell survival and anti-apoptotic pathways in human breast cancer cell lines [13]. Thus, HCA_2 may be a potential target for breast cancer therapy and

further investigation of HCA_2 in mammary cells and tumor associated macrophages is warranted.

In pancreatitis, a recent study demonstrated that β-OHB supplementation suppresses NF-κB activation in tissue macrophages *in vivo* and protects from experimental acute pancreatitis in an HCA_2-dependent manner [110]. Moreover, HCA_2 has a role in limiting sterile inflammation and tissue injury in experimental pancreatitis [110]. The authors suggest that suppression of beta-oxidation may promote pancreatic injury in susceptible individuals by limiting production of β-OHB and insufficiently activating a negative regulatory pathway of sterile inflammatory responses [110]. However, niacin-mediated activation of HCA_2 on pancreatic islets can actually induce pancreatic islet dysfunction by increasing the local production of reactive oxygen species and increasing uncoupling protein 2 expression [111]. While HCA_2 has been identified in mammary and pancreatic tissues, further study is needed to better understand its underlying role.

In contrast to pancreatitis and mammary cancer, there are other disease states where ligands for HCA_2, specifically niacin, are considered therapeutic options but a direct mechanism involving HCA_2 has yet to be established. These conditions include chronic kidney disease, sepsis and psoriasis. The most common causes of chronic kidney disease are diabetes and hypertension [112]. In chronic kidney disease, relatively recent studies using a rat remnant kidney model showed that niacin administration reduced chronic kidney disease-induced increases in renal MCP-1, transforming growth factor-β, and NF-κB activation

[34]. These findings were also associated with improved histologic and clinical findings including decreased proteinuria, ameliorated hypertension, and decreased glomerulosclerosis and tubulointerstitial injury [34,113]. Niacin also improves markers of renal function including lower serum creatinine and phosphorus concentrations and increased creatinine clearance [34,114,115].

In rodent models of sepsis, high doses of niacin attenuated lung inflammation and improved survival time [33]. In another rodent model of acute lung injury, a combination of taurine and niacin suppressed bleomycin-induced activation of NF-κB and subsequent increased production of pro-inflammatory and fibrogenic cytokines such as IL-1α, TNF-α, IL-6 and TGF-β [116]. The decreased activity of NF-κB resulted in decreased mRNA expression of TNF-α and IL-6 in lung tissues, and potentially an overall decrease in systemic inflammation [116]. Therefore, as in chronic kidney disease, the anti-inflammatory effects of niacin observed in sepsis appear to be mediated by decreased NF-κB activation and subsequent decreased inflammatory cytokines [34,116]. These mechanisms are similar to those proposed as HCA_2-mediated anti-inflammatory effects in other tissues and clinical states. While a direct association with HCA_2 has yet to be established in either sepsis or diabetic nephropathy, it will be interesting to determine if the anti-inflammatory effects are directly receptor-mediated and if other anti-inflammatory mediators such as adiponectin play a role.

Psoriasis is an autoimmune condition characterized by red, scaly patches on the skin often associated with itching and pain. The inflammation associated with psoriasis is thought to be due to infiltration of monocytes and hyperproliferation and incomplete differentiation of keratinocytes [117]. FAE have proven to be an effective therapy in patients with psoriasis even though the mechanism of action is not completely understood [117]. Recent evidence suggests that FAE have direct anti-proliferative, pro-differentiative and anti-inflammatory effects on keratinocytes [118]. Therefore, FAE may improve psoriatic lesions by downregulation of the psoriatic cytokine network and restoration of keratinocyte homeostasis [118]. The discovery that MMF is an HCA_2 agonist has raised the question of whether HCA_2 is involved in the anti-psoriatic effects of FAE [8]. There are arguments for and against this possibility. In support, it has been demonstrated that HCA_2 expression is increased in the skin of psoriatic patients [8]. Additionally, activation of HCA_2 by MMF on keratinocytes would increase PGE_2 production, which is a known inducer of COX-2 as well as cellular differentiation [117,119]. However, PGE_2 concentrations are already increased in the psoriatic epidermis prior to FAE treatment [120,121]. Thus, the role of FAE, HCA_2 and PGE_2 in keratinocytes in the context of psoriasis demands further study.

Inflammation is associated with a number of disease processes, including atherosclerosis, obesity, diabetes, multiple sclerosis and cancer. There is mounting evidence that the G-protein coupled receptor HCA_2 plays an important

role in modulating inflammation in multiple clinical situations and tissues **(Table 1)**. Ligands for the HCA_2 receptor, including niacin, MMF and the ketone body β-OHB, activate the receptor, resulting in a variety of inflammation-modulating signaling events. NFK-B, adiponectin, NLRP3 and prostaglandins PGD_2 and PGE_2 have all been implicated as downstream targets of the HCA_2 receptor. Additional HCA_2 targets and mechanisms have yet to be discovered, and they will likely depend on the cellular expression of HCA_2, the tissue type and the physiological state of the individual. In the future, HCA_2 ligands such as niacin will continue to be used in the treatment of dyslipidemia. However, these promising studies suggest that HCA_2 ligands should also be considered in the treatment of inflammatory disease states.

OBESITY IN COMPANION AND FOOD ANIMAL SPECIES

Prevalence and Epidemiology of Companion Animal Obesity

The vast majority of studies in obesity and obesity-related diseases are performed in people and in rodent models of obesity, with fewer studies in non-human primates. While there are relatively fewer studies in companion animal obesity, there has been increased interest in the past decade. The increase in research is especially evident in animals, such as cats, that have the potential to be important translational models [122]. As observed in people, companion animal obesity is associated with many secondary disease processes and alterations in adipokine profiles. There have been some studies into obesity-

associated inflammation in domestic animals and it appears that many of the inflammatory changes may be species specific.

Obesity is reported in dogs, cats, and horses and BCS is closely monitored in food and production animals. Since 1970, incidence of obesity in cats has increased by nearly 30% with a concurrent increase in other disease process such as diabetes and osteoarthritis [123-126]. This mirrors the rise of obesity related disease in people [127]. Similar to cats, the prevalence of obesity in horses is ~31 -35% [128-130], and in dogs the prevalence ranges from ~41 - 44% [131,132].

The causes of obesity are multi-factorial and involve breed, age, sex, gonadal status, and hormonal influences [133]. Seasonal variation also affects the development of equine obesity [130]. In cats, a genetic predisposition is described with polymorphisms in the melanocortin 4 receptor gene [134]. However, as with people, the most important factor associated with the development of obesity in companion animals is the result of energy imbalance; too many calories consumed with too few calories burned.

Companion animal obesity is associated with numerous secondary disease processes as well, some of which are similar to those described in people **(Table 2)**. In dogs, obesity is linked to an increased risk of osteoarthritis, intervertebral disc disease, and cancer [133]. Numerous studies found that food restriction,

with the goal to maintain healthy weight, is directly associated with a marked increase in life-span, improved insulin sensitivity and decreased radiographic evidence of osteoarthritis in dogs [135-142]. In horses, obesity and alterations in glucose metabolism are suspected to the primary predisposing factor in deaths associated with equine hyperlipidemia and equine metabolic syndrome [130,143]. It also has been proposed that obesity and inflammation contribute to laminitis [143].

Relative to other companion animal species, obesity-associated disease processes are extensively studied in cats. This likely is owing to numerous similarities between people and cats in the pathogenesis of some of these disease processes [144-147]. In cats, obesity is associated with hypercoagulability [148], hypertrophic cardiomyopathy [149], impaired pulmonary function [150], hepatic lipidosis [151], insulin resistance [152,153], pancreatitis [154] dyslipidemia [155,156] and T2DM [157]. Obese cats develop a form of T2DM and peripheral insulin resistance that is strikingly similar to what is described in human patients [145,146,157].

Body System	Disease Process
Orthopedic	Osteoarthritis Fractures Cranial cruciate ligament rupture Intervertebral disc disease Joint disorders Laminitis
Endocrine & Metabolic	Hyperadrenocorticism Hypothyroidism Type 2 diabetes mellitus Hypopituitarism Glucose intolerance Hepatic lipidosis Dyslipidemia Pancreatitis
Cardiac & Respiratory	Pickwickian syndrome Hypertrophic cardiomyopathy Tracheal collapse Laryngeal paralysis Brachycephalic airway
Urogenital	Urolithiasis Transitional cell carcinoma Dystocia Idiopathic cystitis
Others	Heat stroke Anesthetic risk Reduced life-span

Table 2. Disorders Associated with Obesity in Domestic Animals. Adapted from [133];

Adipokines in Domestic Animal Species

One of the main characteristics of obesity is adipokine dysregulation. Adipokines are biologically active substances produced in adipose tissue that have an autocrine, paracrine or endocrine function [158,159]. The presence of adipokines appears to be fairly consistent across species [158], and dysregulation of adipokine expression is associated with numerous disease processes that range from diabetes to cancer [61,62,160]. An example of this is feline hepatic lipidosis. While adiponectin concentrations are increased in non-specific liver disease, leptin concentrations are increased, specifically in hepatic lipidosis [161].

In obese humans and non-human primates, dysregulation of adipokine expression is an important contributor to development of insulin resistance [61,162]. The HMW form of adiponectin is the most biologically active and best correlated with insulin resistance [62], thus total adiponectin levels are not considered an adequate representation of biologically active adiponectin [61,62] . A ratio of HMW adiponectin to total serum adiponectin called the serum adiponectin index has been developed in humans to help guide the use of adiponectin as a biomarker of diseases such as diabetes and metabolic syndrome [61].

Adiponectin has been well characterized and sequenced in dogs, with measurement of total and multimeric forms of canine adiponectin [163]. Most

studies indicate that adiponectin levels decrease with obesity in dogs [163,164]. However, a more recent study suggests that obesity has no effect on total adiponectin concentrations [165]. A possible explanation for this discrepancy is that obesity decreases leptin concentrations in intact males, but not neutered dogs [166].

Obese cats have decreased circulating levels of total adiponectin [153,167,168]. Expression and sequencing of feline adiponectin suggests an 86-95% homology with human and canine counterparts, respectively [168,169]. Differences in adiponectin gene expression also have been identified in various fat depots and indicate that unlike other species, cats have higher gene expression of adiponectin in visceral fat depots rather than subcutaneous [168,169]. Work from our lab identified three separate multimers, HMW, LMW and trimers, in cats by use of western blot techniques **(Figure 2)**. Another study identified circulating feline adiponectin multimers by use of a combination of sucrose gradient western blotting and ELISA. In accordance with our results, their findings indicated that cats have a similar circulating adiponectin profile as other species [170].

Two recent studies investigated the correlation between HMW adiponectin and insulin resistance in cats. One study suggested altered serum adiponectin ratios could contribute to decreased insulin sensitivity in cats [171], similar to what is described in humans. The other study, found no correlation between HMW adiponectin concentrations and fat mass, leptin, insulin sensitivity, or

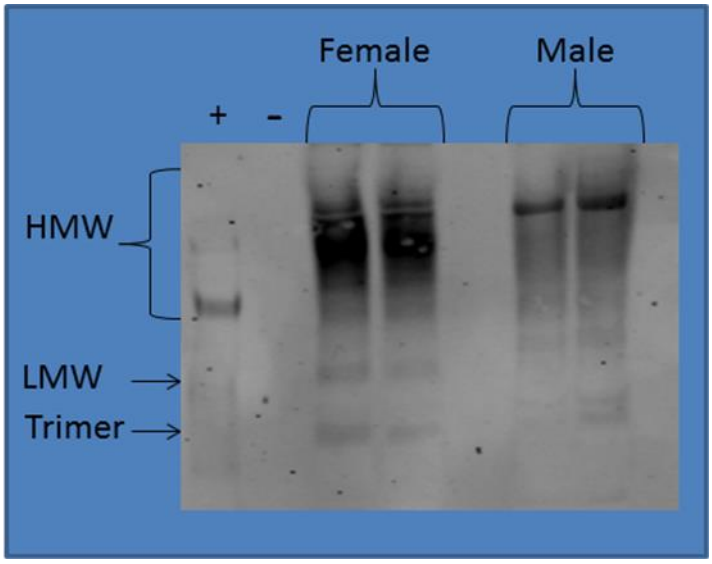


Figure 2. Feline adiponectin multimers in healthy male and female cats (unpublished data). HMW, high molecular weight; LMW, low molecular weight; +positive control, media from 3T3-L1 differentiated adipocytes; −negative control, untreated media in loading buffer.

subcutaneous adipose mRNA expression of adiponectin [172]. The discrepancies between these studies may be explained by variations in methods as well as the populations that were evaluated. In addition, carbohydrate content of the diet can alter adiponectin multimer ratios in cats [170]. Specific details on diet composition were not provided, or not controlled in the previously mentioned studies. At this time, it appears that adiponectin is altered with the development of obesity and insulin resistance in cats. However, unlike other species, the formation and function of adiponectin multimers in cats is unclear.

In horses, ponies and donkeys, adiponectin decreases with increased BCS and fat mass [173,174]. Similar to what is observed in humans and rodent models of obesity, concentrations of equine HMW adiponectin is negatively correlated with BCS and insulin activity [175]. Serum adiponectin is lower in horses previously diagnosed with laminitis, and unlike other markers of inflammation, adiponectin concentrations found in horses in this particular study, did not appear to correlate with BCS based on multivariate analysis [176].

Adiponectin may be an important biomarker for disease, as well as meat quality and milk production in cattle [177-181]. Variations in the adiponectin receptor may be involved in marbling of Hanwoo (Kobe) beef cattle [177] and may regulate mammary gland metabolism [178]. Higher adiponectin concentrations are also associated with increased fertility in Holstein bulls [182]. Adiponectin

has also been identified in other food and production animals, including pigs [183] poultry [184-186] and fish [187-189].

Increased serum leptin concentration is directly associated with obesity and is transiently increased following consumption of food [158]. Circulating leptin levels correlate with fat mass and BCS in dogs, cats, and horses [133,158,190,191]. There are numerous factors that alter leptin expression in domestic species such as breed, age, sex, gonadal status, and hormonal influences [155,158].

In cats, increased leptin concentrations are correlated with many disease processes including hypercoagulability [148] and hepatic lipidosis [161]. Leptin is considered an orexin and is associated with decreased food intake [192]. Two separate studies suggest that the decrease in appetite may be associated with decreased intestinal motility in cats [193,194]. Leptin may also play a role in feline hyperthyroidism, a disease process that is sometimes associated with hyperphagia [195]. In this study, the authors demonstrated increased leptin concentrations with effective treatment for the thyroid-related disease [195].

Expression of leptin in gonadal tissues suggests that it also may play a role in fertility in cats [196,197]. However, it is unclear if leptin has an effect on fertility in other species. One study identified polymorphisms in the genes for leptin and leptin receptor, and suggested an association with altered fertility in cattle [198].

In horses, a recent study demonstrated no effect of leptin on the reproductive performance of Lusitano mares [199].

While there are numerous adipokines, in domestic animal species adiponectin and leptin represent the most extensively studied. While extensive discussion of these adipokines is beyond the scope of this dissertation many of them share profiles and characteristics similar to those described in people [133,145,158,200]. The similarities and differences described between species are important, as companion animals, specifically the cat, are considered important models of human nutrition and disease [144,146,147].

Obesity-associated Systemic and Adipose Tissue Inflammation

One of the main areas in which human and domestic animal obesity differ is in the development of obesity-associated inflammation. While there are studies that suggest obesity-associated adipose tissue inflammation is present in some species [201,202], there is disagreement on the presence or absence of systemic obesity-associated inflammation in all animal species. In addition, the presence of crown-like structures (CLS), a hallmark of human and rodent obesity [63,203], has not been described in the adipose tissue of companion animal species.

A recent comparative study investigated the presence of ATMs in a variety of non-rodent mammals and identified adipose tissue immune cells, specifically, ATMs, in cattle, goat, sheep, dog, cat, horse, wild boar and red fox [202]. The

authors concluded the presence of adipose tissue immune cells is highly conserved amongst mammalian species and thus an important factor in metabolic and immune regulation [202].

In horses, obesity and insulin resistance are associated with increased circulating concentrations of IL-1 and TNF-α [204]. While ATMs have been identified in horse tissue, no studies have been done to determine if they are increased with obesity [202]. Interestingly, there is increased phagocytic burst and increased reactive oxygen species within the neutrophils of obese and hyperinsulinemic horses, yet in this study, circulating cytokines and gene expression of cytokines were decreased [205]. The authors suggest that based on these findings, horses may have different metabolic and pathogenic consequences associated with obesity and hyperinsulinemia than humans [205].

While obesity is not a common problem in cattle, there have been some data to suggest that BCS, lactation and calving status alter both systemic and adipose tissue inflammation [180]. A recent study also demonstrated the presence of ATMs in the SVF of bovine, equine, caprine and ovine adipose tissue [202]. Unfortunately, this study did not investigate changes associated with obesity in these species.

In dogs, a recent study demonstrated an increase in IL-6 and MCP-1 in Labrador retrievers associated with increased BCS [206]. Another study, using a canine

multiplex immunoassay, evaluated serum cytokines and growth factors during weight-loss in dogs and noted that pro-inflammatory cytokines and growth factors were decreased with weight-loss [207], which suggests that obese dogs had increased concentrations of these inflammatory markers. Contrary to these findings, a longitudinal obesity study in dogs did not observe changes in inflammatory cytokines or circulating immune cell function, implicating an absence of systemic inflammation [208]. In a separate study, one of the major acute phase proteins in dogs, CRP was lower in obese dogs compared to lean dog [209]. Little is known about obesity-associated adipose tissue inflammation in dogs. However, one study suggests that the number of ATMs is increased in the SVF of obese dogs compared to lean [202]. Ultimately, more work is needed to clarify if dogs develop obesity-associated inflammation.

Obesity-associated changes in cats is an important area of research as cats and non-human primates are the only naturally occurring model of human T2DM with progressive islet cell amyloidosis [146,157]. While some studies support the development of adipose tissue inflammation based on changes in adipose tissue gene and protein expression [201,202,210,211], it is still unclear to what extent cats develop inflammation in response to obesity. In one study, obese feline adipose tissue had an almost 10 fold increase in TNF-α protein compared to lean [210]. A separate study evaluated cohorts of lean and obese cats and found that obese cats have increased gene expression of inflammatory cytokines such as CCL-5 and MCP-1, as well as an increased number of T-lymphocytes within

adipose tissue [201]. However, neither of the two studies that evaluated adipose tissue in lean and obese cats found an increase in ATM number [201,202]. Taken together, data from these studies suggest that in cats, obesity is associated with low-grade adipose tissue inflammation, but not accumulation of ATMs [201,210,211].

Obesity-associated systemic inflammation in cats is more complicated. In cats, obesity alters inflammatory adipokines such as adiponectin and leptin, but does not appear to alter other markers of systemic inflammation, including SAA, IL-6, and IL-1 [167]. One study evaluated 37 client-owned cats and noted no change in SAA or haptoglobin [212]. This is strikingly different from what is described in humans and rodent models of obesity. While there are no known reports on increased concentrations of circulating cytokines associated with feline obesity, there are studies that demonstrate increased gene expression of pro-inflammatory cytokines in circulating leukocytes of obese cats [201,202]. In addition, experimental models suggest that prolonged hyperlipidemia, a feature of obesity in cats, induces systemic inflammation in cats [213].

HCA2 RECEPTOR IN COMPANION AND FOOD ANIMAL SPECIES

The HCA_2 receptor plays an important role in inflammation, specifically in the cross-talk between metabolic and immune function. There are few studies that have investigated the HCA_2 receptor in companion animals. According to a search in the NCBI (National Center for Biotechnology) managed public

database for nucleotide sequences, GenBank, the only animals with published, known HCA_2 receptor sequence are people, mice, rats, and guinea pigs. We recently submitted the cat sequence, based off work done for this dissertation. Despite the lack of investigation into the presence or absence of HCA_2 in the genome of many animals, there has been some investigation into the proposed gene expression and effects of potential receptor activation by agonists, particularly in cattle.

The majority of the work on HCA_2 in domestic animal species has been done in cattle, where it is believed that cattle have higher expression of HCA_2 in the liver and brain than other species [214]. In this study the authors suggest a possible sequence for the bovine HCA_2 receptor, and indicated that amino acids critical for ligand binding were conserved in cattle and humans [214]. While the increased expression in the liver and brain suggest a unique function of this receptor in cattle, most of the studies in cattle involve the activation of HCA_2 in adipose tissue. Agonists for the HCA_2 receptor, including niacin and β-OHB, have been associated with alterations in rumen fermentation, lactation, heat production and response to heat stress [215-220]. It is also thought that HCA_2 could alter muscle marbling, owing to the profound anti-lipolytic effects of niacin in cattle [221]. A recent study using primary bovine adipose tissue demonstrated that HCA_2 inhibits lipolysis through reduced phosphorylation of serine 563 on hormone sensitive lipase (HSL) [222]. In addition to its effects on lipolysis, niacin

increases adiponectin, in differentiated bovine adipocytes in a receptor dependent manner [223].

Niacin has been used in the management of dairy cattle since 1980, when it was first shown to alter ruminal fermentation [216,218]. A recent study that evaluated various stages of pregnancy in dairy cows suggests that there are changes in the HCA_2 receptor expression and in free fatty acid receptors [224]. Interestingly, β-OHB, the endogenous ligand for HCA_2, inhibited lipolysis in adipose tissue explants from dairy cows with ketosis, which suggests that feedback was not impaired in these animals [225]. There are no known reports of systemic or adipose tissue specific anti-inflammatory effects of niacin or β-OHB in cattle.

There are no published studies on the HCA_2 receptor in horses, goats, pigs, sheep, fish or birds and only a single study in dogs that evaluated the effects of synthetic HCA_2 receptor agonists [226]. The goal of the latter study was to establish animal models that can be used to evaluate therapeutic indices for a variety of potential HCA_2 agonists by means of peripheral vessel dilation and serum NEFA concentrations [226]. The authors concluded that rats and dogs are acceptable models for future agonist evaluation [226]. While there are studies that demonstrate an anti-lipolytic effect of niacin in dogs [227], to our knowledge there are no studies to confirm that dogs express the HCA_2 receptor or that niacin or the synthetic agonists are working through this pathway. In

addition, there are no known studies that have evaluated the anti-inflammatory effects of HCA_2 agonists in dogs.

CONCLUSIONS AND OBJECTIVES

Inflammation is associated with a number of disease processes, including atherosclerosis, obesity, diabetes, multiple sclerosis and cancer. There is mounting evidence that the G-protein coupled receptor, HCA_2, plays an important role in modulating cross-talk between the metabolic and immune systems. This may be especially true in obese individuals. Both macrophages and mature adipocytes express HCA2 [2,12,228] and while activation of this receptor may explain some of niacin's anti-inflammatory effects, the overall mechanisms are not well understood. Previous work from our laboratory and others demonstrate that niacin-mediated activation of the HCA_2 receptor increases serum concentrations of adiponectin in both humans and rodent models [4,39,47-49,51]. Therefore, the anti-inflammatory changes may be due to either direct effects on adipocytes, macrophages and endothelial cells, or indirectly, by alteration of systemic pro- and anti-inflammatory molecules such as adiponectin. Likewise, niacin and adiponectin may be acting synergistically to decrease inflammation associated with obesity. In addition, our lab has shown that HCA_2 receptor expression is decreased in HFD-fed rodent models of obesity [70], which suggests a possible mechanism for obesity-associated alterations in lipolysis and immune regulation. Companion animals develop similar inflammatory disease processes, and provide an important model for the study of human disease. In

particular, feline obesity and subsequent development of T2DM is an important translational model for human medicine [144-147]. This is especially true as cats appear to have a unique immune response during the development of obesity compared to humans and rodents [167].

However, little is known about HCA_2 in cats. Therefore, the goals of this study are to 1) investigate the underlying mechanisms in niacin-mediated activation of HCA_2 in a HFD-fed mouse model of obesity, 2) characterize HCA_2 in cats, a translational model of human obesity and metabolic disease, and 3) evaluate obesity-associated inflammation and HCA2 receptor expression in cats during the development of obesity. Specifically, we determined if the anti-inflammatory effects of niacin are associated with increased adiponectin concentrations in mice and evaluate the effects of niacin on markers of tissue and systemic inflammation in the absence of adiponectin. We will then identify the feline HCA_2 receptor protein sequence, compare this sequence against known human and rodent sequences, determine tissue distribution and relative expression of HCA_2 in lean, healthy cats, and demonstrate *in vitro* functionality of the HCA2 receptor in feline adipose tissue. In addition, we will provide a comprehensive investigation of systemic and adipose tissue inflammation during the development of obesity and insulin resistance in cats, including evaluation of glucose and lipid metabolism, assessment of adipose tissue distribution, cellularity and microanatomy, determination of systemic humoral and cellular markers of inflammation, and evaluation of HCA_2 receptor gene expression in

various adipose tissue depots during the development of obesity in an outbred cat colony.

CHAPTER 2

HIGH-FAT DIET-INDUCED ADIPOSE TISSUE INFLAMMATION IS ATTENUATED BY NIACIN IN BOTH AN ADIPONECTIN-DEPENDENT AND INDEPENDENT MANNER

ABSTRACT

Background. Chronic obesity induces adipose tissue inflammation. Niacin attenuates obesity-induced adipose tissue inflammation and alters adipose tissue macrophage (ATM) polarity away from a classic M1 phenotype. In addition, niacin administration increases tissue and serum adiponectin concentration, an adipokine that promotes an alternative, M2 phenotype. **Objective.** Therefore, the objectives of this study are to determine if the anti-inflammatory effects of niacin are associated with adiponectin and evaluate the effects of niacin on markers of tissue and systemic inflammation in the absence of adiponectin. **Methods.** Male C57BL/6 mice (WT) and adiponectin null mice (AdipoQ$^{-/-}$) were fed a control (chow) or high-fat diet (HFD). After 6 weeks, half of the mice from each group received either vehicle (water) or niacin (360 mg/kg/day) in their drinking water for 5 weeks. **Results.** Niacin increased adiponectin in serum of chow-fed mice only. In adipose tissue of HFD fed mice, there was increased gene expression of MCP-1 and CD68, and niacin treatment attenuated the HFD-induced increase in MCP-1, but had no effect on CD68. Similar findings were observed in AdipoQ$^{-/-}$ mice. HFD fed mice had increased gene expression of M1

and M2 macrophage markers. Niacin decreased expression of M2 markers and tended to decrease expression of M1 markers. Interestingly, in HFD fed AdipoQ$^{-/-}$ mice, niacin significantly increased expression of both M1 and M2 markers. **Conclusion.** In conclusion, niacin does not appear to alter macrophage number, but exerts adiponectin-independent effects on macrophage recruitment. Absence of adiponectin alters niacin's effects on macrophage polarity, which implicates adiponectin-dependent mechanisms.

BACKGROUND

Obesity is a chronic low-grade inflammatory disease characterized by increased circulating inflammatory cytokines, and an increase in immune cells within adipose tissue [62,203,229-233]. Specific findings identified in obese humans and rodent models of obesity include increased numbers of adipose tissue macrophages (ATMs) and a shift in macrophage polarity from an alternatively-activated (M2) to a classically-activated state (M1) [64,65]. Clinically, obesity and chronic adipose tissue inflammation are associated with the development of metabolic syndrome, type 2 diabetes mellitus (T2DM), and low grade systemic inflammation, including increases in serum MCP-1, TNFα, IL-6 [234-236], and C-reactive protein (CRP) [237] and increased numbers of circulating pro-inflammatory mononuclear cells [229,238,239].

Obesity and adipose tissue inflammation also are associated with adipokine dysregulation, specifically decreased adiponectin and increased leptin

concentrations [61,62,67,240,241]. This adipokine dysregulation is a consistent and repeatable finding in obese humans and in rodent models of obesity [242], with decreases in adiponectin concntration preceding the development of insulin resistance [61]. Adiponectin concentrations are inversely correlated to adipose tissue mass, and decreased serum adiponectin is associated with the development of metabolic disease, atherosclerosis, and type 2 diabetes mellitus [61,67,243]. Adiponectin is considered an anti-inflammatory mediator that plays a role in ATM polarization, immune cell migration, and chemokine production [54,66,68,244]. In cell culture, adiponectin inhibits phagocytic activity of mature human macrophages [245] and *in vivo* studies indicate that peritoneal macrophages from adiponectin null mice exhibit an increased M1 profile [244]. Others have shown that treatment with adiponectin decreases TLR-4 receptors on cultured macrophages and shifts macrophages into a M2 phenotype [68]. Overall, maintenance of adequate adiponectin concentrations appear to be tied to improved adipocyte function, metabolic homeostasis and decreased inflammation.

Niacin reduces the co-morbidities associated with obesity by alteration of both local and systemic inflammation [4,5,39,45]. For many years, niacin has been utilized as a treatment for atherogenic dyslipidemia [45]. Recent evidence indicates that the beneficial effects of niacin involve modulation of inflammatory pathways that are independent of its anti-lipolytic effects on adipocytes [4,5,38]. In 2011, Lukosova *et al.* showed that macrophages express the niacin receptor,

$HCA_{2,}$ and that niacin-mediated HCA_2 activation decreases M1 macrophage differentiation [5]. Similar findings are observed in adipose tissue, as chronic administration of niacin attenuates HFD-induced expression of M1 macrophage markers and chemokines, such as MCP-1, in a HCA_2-dependent manner [4]. In addition, treatment with niacin inhibits macrophage recruitment, adhesion and chemotaxis, as well as alters TLR mediated cytokine release [38,39,45,246]. Niacin may be acting either through direct effects on adipocytes, macrophages and endothelial cells or indirectly, by alteration of systemic pro- and anti-inflammatory molecules such as adiponectin. Likewise, niacin and adiponectin may be acting synergistically to decrease inflammation associated with obesity**.**

Based on these findings it is unclear whether the anti-inflammatory effects of niacin are due to increases in serum or tissue adiponectin or if they are adiponectin-independent. Thus, the objective of this study was to evaluate the relationship between the anti-inflammatory effects of niacin and adiponectin. Our goals were to 1) determine if the anti-inflammatory effects of niacin are directly associated with increased serum or tissue adiponectin, 2) evaluate the effects of niacin on markers of adipose tissue inflammation in the absence of adiponectin, and 3) determine if the changes are localized to adipose tissue or present in systemic circulation, specifically by alteration of systemic inflammatory markers.

MATERIALS AND METHODS

Materials

Niacin (nicotinic acid) was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal adiponectin antibodies were from Abcam, Inc. (Cambridge, MA). GAPDH mouse monoclonal antibodies (clone 6C5) were from Millipore (Billerica, MA). Goat anti-mouse IRDye 800CW and donkey anti-rabbit IRDye 680RD secondary antibodies were purchased from LI-COR (Lincoln, NE).

Animal Studies

Thirty-two 3 week old male C57BL/6J mice (Wild type, WT) were purchased from Jackson Laboratories. Mice were either fed a control diet (10% kcal as fat; n = 16) or a high fat diet (HFD; 60% kcal as fat; n = 16) obtained from Research Diets (New Brunswick, NJ) for 11 weeks. After six weeks on the control or high fat diets, half of the mice received niacin (approximately 360 mg/kg/day) dissolved in drinking water or vehicle (water) for five weeks. After five weeks of vehicle or niacin treatments, mice were fasted overnight (12 h) and euthanized by decapitation. Whole blood was collected and processed to isolate serum, and tissues were flash frozen in liquid nitrogen and stored at −80°C until analysis. Parallel studies were conducted in male global adiponectin null mice derived from a breeding pair of B6.129-*Adipoq*^{tm1Chan}/J (AdipoQ$^{-/-}$) mice purchased from Jackson Labs. Thirteen AdipoQ$^{-/-}$ mice were fed the control diet (6 received vehicle; 7 received niacin), and sixteen AdipoQ$^{-/-}$ mice were fed the high fat diet

(8 received vehicle; 8 received niacin). All animal studies were approved by the Auburn University Institutional Animal Care and Use Committee prior to initiation.

Serum Analysis

Serum total adiponectin concentrations were measured using ELISA kits from Millipore (Temecula, CA). Serum triglyceride concentrations were measured using kits from Cayman Chemicals (Ann Arbor, MI). Blood glucose was determined using the AccuChek Active handheld glucometer (Roche, Indianapolis, IN). Serum NEFAs were measured using a kit from Wako Chemicals (Richmond, VA). Serum Insulin, IL-6, MCP-1 and TNF-α were measured using Milliplex multiplex custom kit from Millipore (Temecula, CA) and read on a MAGPix Bio-analyzer from Luminex (Temecula, CA).

Gene expression analysis

RNA was isolated from epididymal white adipose tissue (EWAT) using a Qiagen RNeasy Lipid Tissue Mini Kit with on-column DNA digestion (Qiagen, Valencia, CA). RNA (0.5 or 1 µg) was reverse transcribed into cDNA using iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA). PCR primers used in the real-time-PCR analysis were previously published [4]. Analyses were performed on a Bio-Rad iCycler iQ thermocycler. Samples were analyzed in 30 µl reactions using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA). All expression levels were normalized to the corresponding 36B4 mRNA levels, and analyzed using

the $2^{-\Delta\Delta CT}$ method [247]. 36B4 levels were unchanged in response to HFD or niacin treatment.

Immunoblot analysis

EWAT pads (~100 mg) were homogenized with a handheld homogenizer in Pierce RIPA buffer (Rockford, IL) supplemented with cOmplete Tablet protease inhibitors (Roche, Indianapolis, IN) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO), and the protein fractions were isolated. A DC protein assay (Bio-Rad, Hercules, CA) was then conducted on the samples to determine the protein concentration for each sample. Proteins (20 µg) were separated by SDS–PAGE (10%) and transferred to nitrocellulose membranes. Membranes were blocked in blocking buffer (LI-COR, Lincoln, NE) 1 hour and incubated with primary antibody overnight at 4°C. Membranes were washed with PBS with 0.1% Tween-20 three times and incubated with secondary antibody for one hour at room temperature, then washed with PBS with 0.1% Tween-20 three times. Blots were imaged using the LI-COR Odyssey scanner (LI-COR, Lincoln, NE) and band density was evaluated by use of Image Studio software ver. 2.0 (LI-COR, Lincoln, NE).

Statistical analysis

All data sets were evaluated for normality and data that did not exhibit Gaussian distribution was log transformed. Analyses of serum and tissue protein concentrations and relative gene expression were determined by ANOVA with Tukey's post-hoc analysis. All statistical analyses were performed on Graph Pad

Prism 6 software (La Jolla, CA). All gene expression data are presented as a relative change compared to chow vehicle.

RESULTS

Effects of HFD and niacin on metabolic parameters in mice.

At the start of the study there was no statistical difference in body weight observed in any of the treatment groups, and over the course of the study, no significant difference in food and water intake was observed (data not shown). As expected, WT and AdipoQ$^{-/-}$ mice on a HFD had significantly increased body weight, EWAT weight, liver weight, increased concentrations of blood glucose, serum insulin, and HOMA-IR and decreased NEFA concentrations **(Table 3)**. Niacin tended to increase blood glucose, insulin and HOMA-IR scores. However, none of these values reached statistical significance. Interestingly, the only significant difference owing to niacin treatment was decreased total body weight in WT mice fed a HFD. Under these experimental conditions, HFD fed WT mice treated with niacin weighed ~13% less than HFD fed WT mice treated with vehicle. This finding was not observed in our AdipoQ$^{-/-}$ mice.

Effects of niacin and HFD on tissue and serum adipokines.

WT mice fed a HFD had ~2 fold decrease in adiponectin gene expression **(Figure 3B)**, and a trend towards decreased tissue adiponectin protein concentrations **(Figure 3C)**. However, no change in adiponectin owing to HFD feeding was observed in the serum **(Figure 3A)**. In HFD-fed mice, niacin

	WT Control Diet		WT High Fat Diet		AdipoQ$^{-/-}$ Control Diet		AdipoQ$^{-/-}$ High Fat Diet	
	Vehicle	**Niacin**	**Vehicle**	**Niacin**	**Vehicle**	**Niacin**	**Vehicle**	**Niacin**
Body Weight (g)	26.5 ± 1.2	28 ± 2.9	43.6 ± 7.9[a]	38.14 ± 4.3[a,b]	31.33 ± 2.5	30.57 ± 2.3	42.75 ± 5.1[a]	40.50 ± 2. 6[a]
EWAT Weight (g)	0.56 ± 0.1	0.82 ± 0.3	2.45 ± 0.8[a]	2.48 ± 0.5[a]	0.73 ± 0.4	0.86 ± 0.3	2.17 ± 0.5[a]	1.79 ± 0.3[a]
Liver Weight (g)	0.97 ± 0.1	1.05 ± 0.2	1.65 ± 0.7[a]	1.24 ± 0.3[a]	1.25 ± 0.1	1.21 ± 0.1	1.68 ± 0.6	1.71 ± 0.4
Glucose (mg/dL)	165.13 ± 17	182 ± 22	246.88 ± 24[a]	244.3 ± 28[a]	161.83 ± 28	190.14 ± 18	204 ± 43.4	250.75 ± 49[a]
Insulin (ng/mL)	1.123 ± 0.6	2.151 ± 0.6	4.164 ± 2.5[a]	4.779 ± 1.7[a]	1.787 ± 0.3	3.474 ± 0.8	8.158 ± 4.2	8.372 ± 1.9
HOMA-IR	0.46 ± 0.3	0.97 ± 0.3	2.61 ± 1.7[a]	2.90 ± 1.1[a]	0.73 ± 0.3	1.64 ± 0.5	4.05 ± 2.0	5.24 ± 1.7[a]
NEFA (mMol)	1.05 ± 0.2	1.09 ± 0.1	0.75 ± 0.1[a]	0.81 ± 0.1[a]	0.83 ± 0.1	1.02 ± 0.2	0.76 ± 0.2	0.65 ± 0.2[a]
Adiponectin (ug/mL)	19.48 ± 1.3	22.26 ± 1.5[b]	20.7 ± 3.7	20.59 ± 1.2	ND	ND	ND	ND

Table 3. Effects of HFD and niacin on metabolic parameters in mice. ND=non- detectable; WT=Wild type; [a] $P \leq 0.05$ compared to mice of the same genotype on control diet receiving vehicle (i.e. HFD effect); [b] $P \leq 0.05$ compared to mice of the same genotype and diet receiving vehicle (i.e. drug effect). Values are means ± SEM.

increased adiponectin tissue protein concentrations **(Figure 3C)**, and tended to increase mRNA expression **(Figure 3B)**. In the serum of HFD-fed mice, niacin treatment increased adiponectin by ~8%, but results did not reach statistical significance **(Figure 3A)**. In chow fed WT mice, there was no change in tissue adiponectin protein or mRNA concentrations. However, niacin treatment significantly increased serum adiponectin by ~14% in chow fed mice **(Figure 3A)**. AdipoQ$^{-/-}$ mice did not have measurable serum or tissue **(Figure 3C)** adiponectin concentrations and gene expression was not detected in these mice (data not shown).

Effects of HFD and niacin on markers of macrophage recruitment and number.

Similar to previous studies, HFD resulted in ~35 fold increase in gene expression of the general macrophage marker CD68 and a ~7 fold increase in the pro-inflammatory chemokine MCP-1 in WT mice **(Figures 4A and 4C)**. As seen in previous studies, treatment with niacin did not decrease CD68; however, there was a significant decrease in MCP-1 expression [4]. In AdipoQ$^{-/-}$ mice fed a HFD, there was ~35 fold increase in CD68 gene expression and ~30 fold increase in MCP-1 gene expression. Treatment with niacin elicited similar effects in AdipoQ$^{-/-}$ mice as observed in WT mice, with no change in CD68 and a significant attenuation of MCP-1 **(Figures 4B and 4D)**.

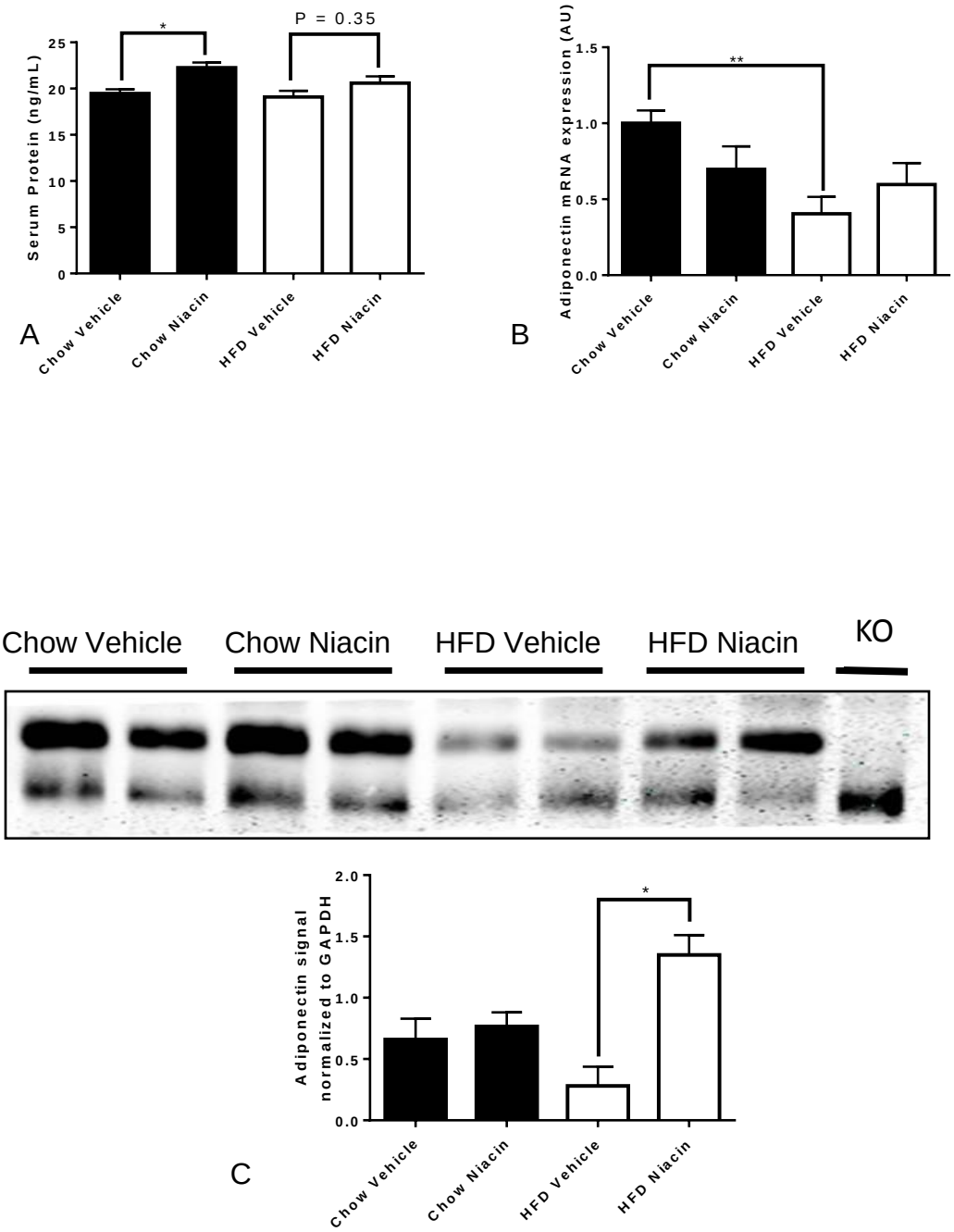


Figure 3: Effects of HFD and niacin on serum and tissue adiponectin concentrations. Effect of HFD and niacin on serum adiponectin protein concentrations (A), relative tissue mRNA expression (B) and tissue protein concentrations (C and D). *P<0.05; **P<0.01; KO = AdipoQ$^{-/-}$ mice

Effects of HFD and niacin on markers of macrophage polarity.

Gene expression for macrophage markers of M1 and M2 polarity were increased in adipose tissue of WT and AdipoQ$^{-/-}$ mice fed a HFD **(Figures 5 and 6)**. In WT mice fed a HFD, CD11c gene expression was significantly increased by ~16 fold, and TNFα gene expression was significantly increased by ~3 fold **(Figures 5A and 5C)**. Treatment with niacin decreased gene expression of both of these markers to levels similar to chow-fed, vehicle treated mice. However, findings did not reach statistical significance. The M2 marker arginase was significantly increased by ~2.5 fold in WT mice fed a HFD, and treatment with niacin normalized the gene expression of this marker **(Figures 6A and 6B)**. There were no gene expression changes observed in the M2 marker MRC-1 owing to either diet or niacin treatment in WT mice **(Figure 6C)**. Interestingly, HFD fed AdipoQ$^{-/-}$ mice treated with niacin had significantly increased gene expression of CD11c, arginase-1 and MRC-1 and trended towards an increase in TNFα in mice fed a HFD **(Figures 5B, 5D, 6B and 6D)**. These findings are the opposite of the effects observed in WT mice.

Effects of niacin and HFD on systemic markers of inflammation.

No significant changes were observed in serum protein concentrations of IL-6 or TNFα for any treatment group **(Figure 7)**. In WT mice there was no effect of either niacin or diet on MCP-1 concentrations **(Figure 8A)**. However, in AdipoQ$^{-/-}$ mice, both niacin and HFD independently increased MCP-1 concentrations by ~4 fold. The effects did not appear to be additive **(Figure 8B)**.

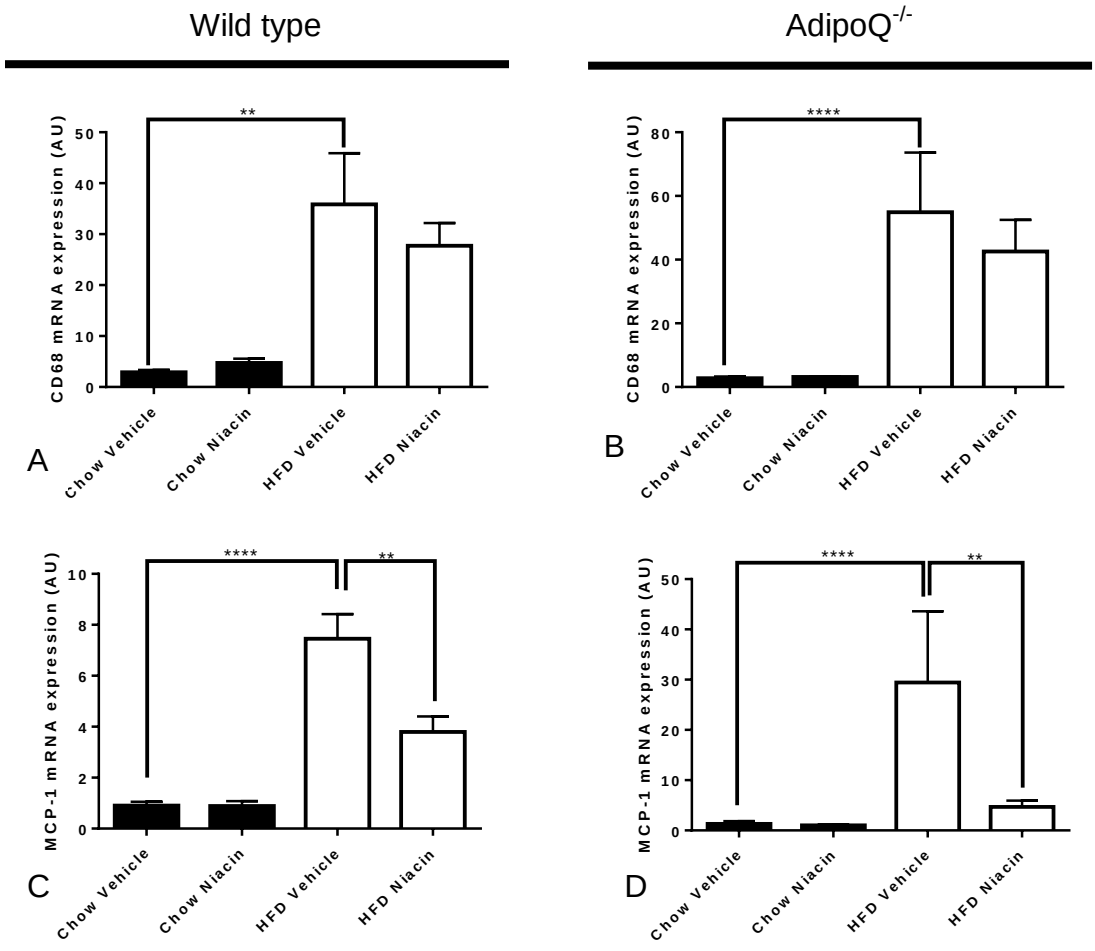


Figure 4: Effects of HFD and niacin on gene expression of markers associated with macrophage number and migration. Effects of HFD and niacin on Wild Type mice (A and C). Effects of HFD and niacin on AdipoQ$^{-/-}$ mice (B and D). **P<0.01; ****P<0.0001

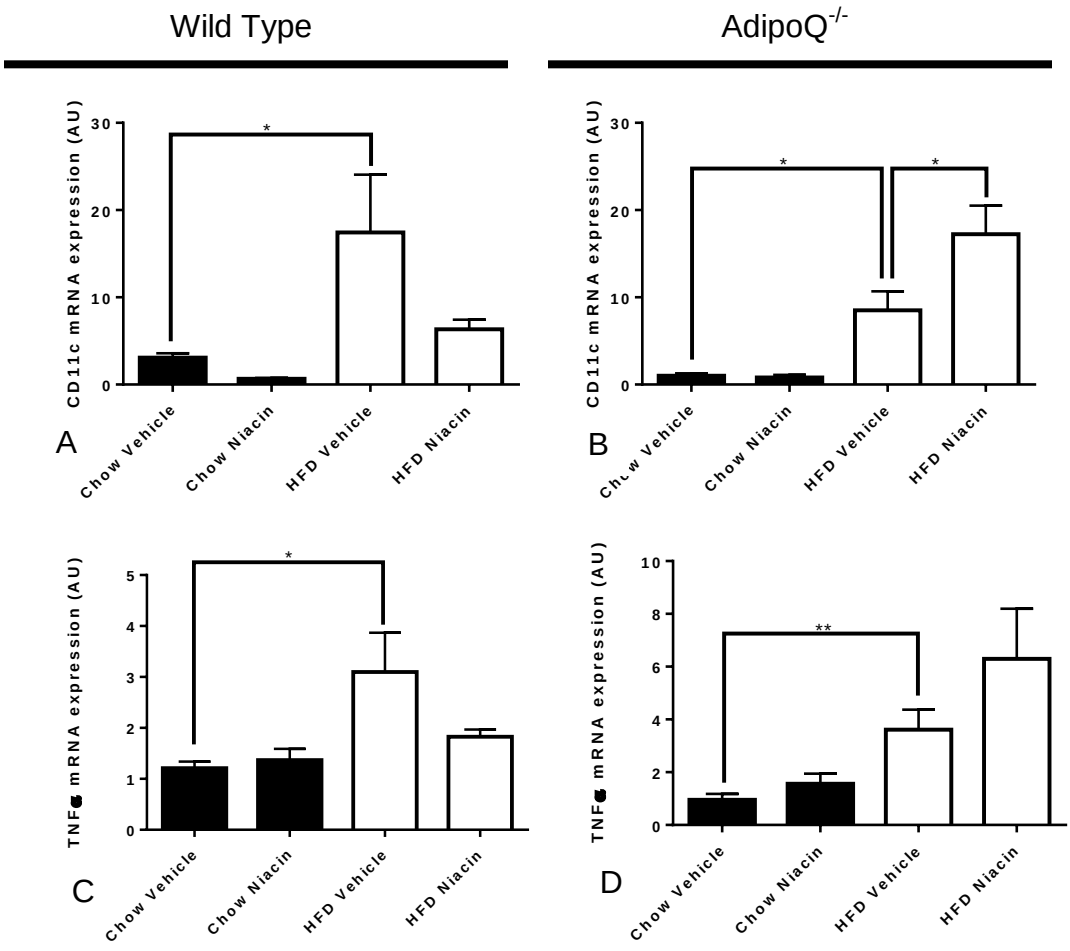


Figure 5. Effects of HFD and niacin on gene expression of M1 markers of macrophage polarity. *P<0.05; **P<0.01

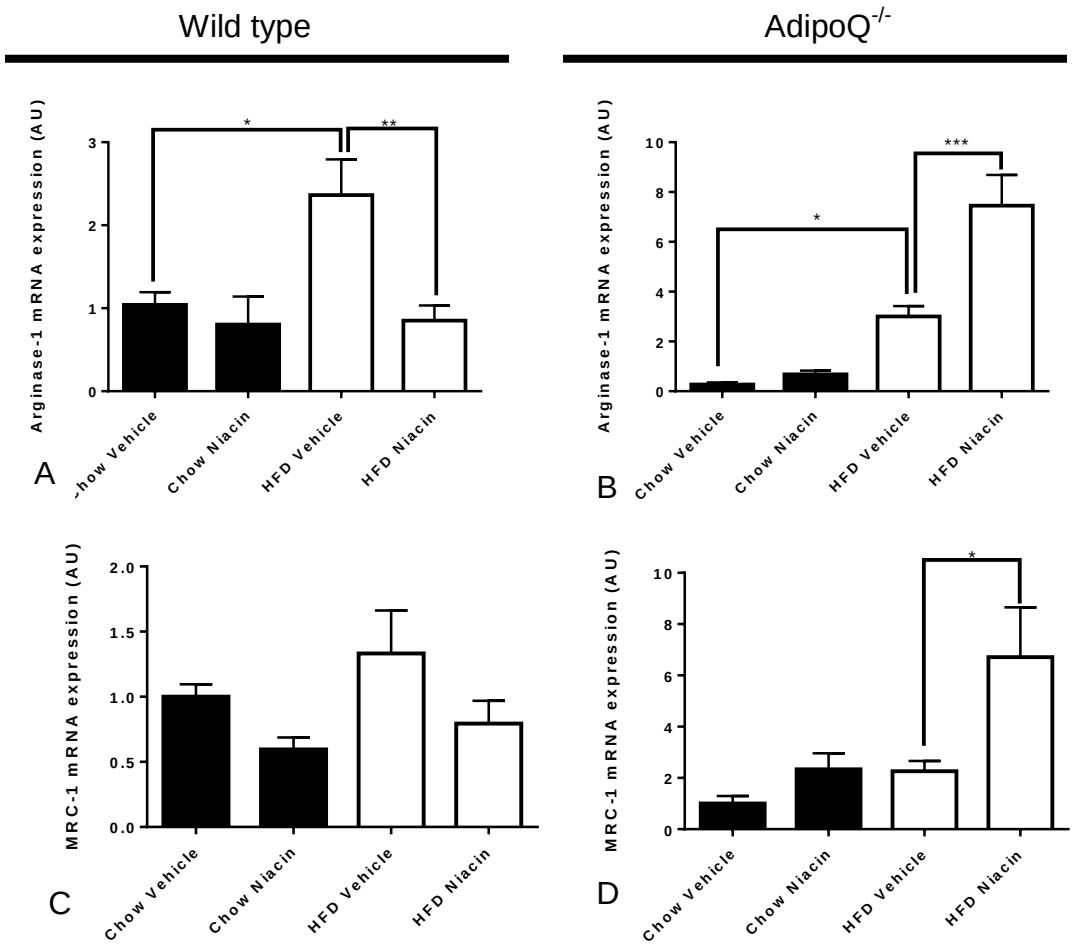


Figure 6. Effects of HFD and niacin on gene expression of M2 markers of macrophage polarity. *P<0.05; **P<0.01; ***P<0.001

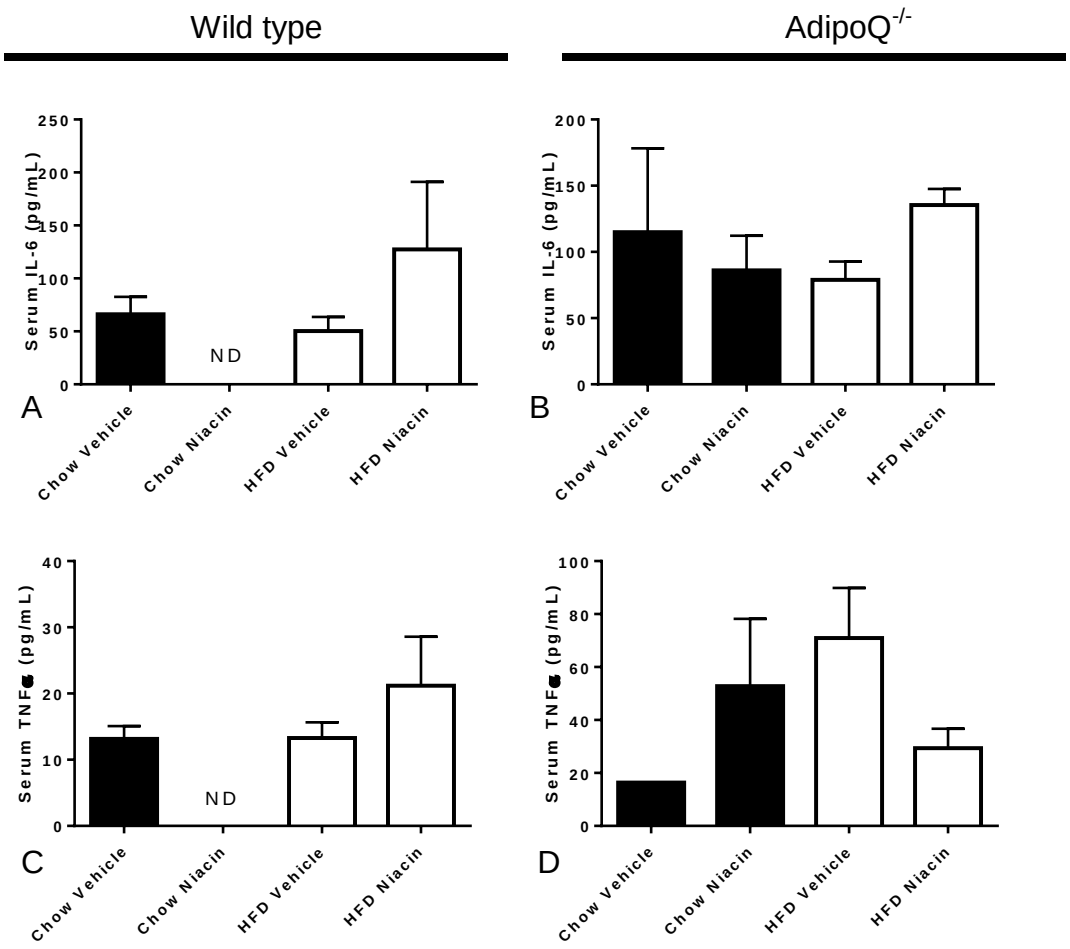


Figure 7. Effects of HFD and niacin on systemic markers of inflammation TNFα and IL-6. ND, not detected.

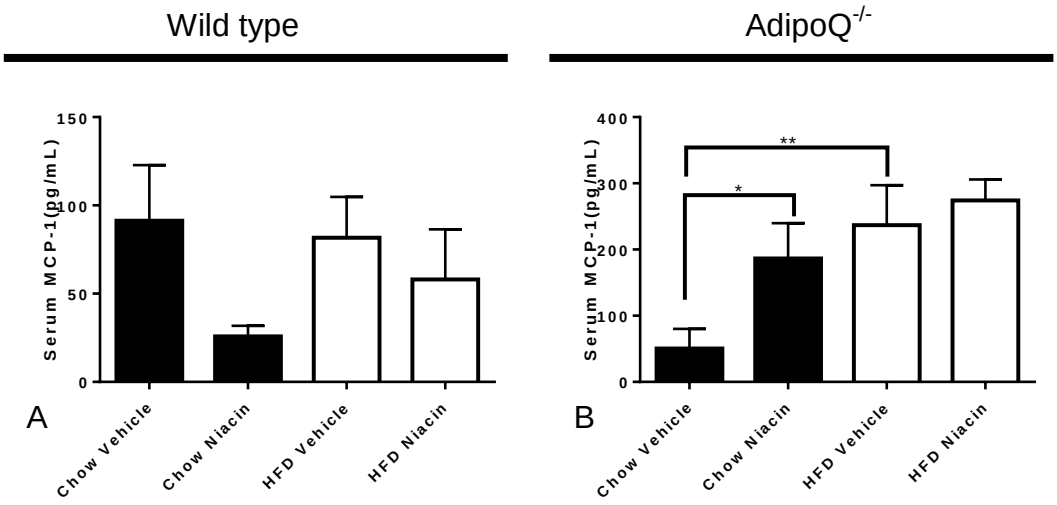


Figure 8. Effects of HFD and niacin on the circulating pro-inflammatory chemokine MCP-1. *P<0.05; **P<0.01

DISCUSSION

There is increasing evidence that the beneficial effects of niacin are independent of its lipid modifying effects on adipocytes [4,5,38,39,246,248]. In addition to its effects on dyslipidemia, niacin has other beneficial properties, including the ability to inhibit inflammation and increase serum and tissue concentrations of the anti-inflammatory adipokine, adiponectin, through activation of the HCA_2 receptor on adipocytes [4,47,49,50,52]. However, the mechanisms underlying niacin's anti-inflammatory effects are not completely understood. Thus, we wanted to determine if the anti-inflammatory properties of niacin were due, at least in part, to its ability to increase local and systemic adiponectin concentrations.

Obesity is associated with decreased tissue and serum adiponectin [243,249]. Consistent with previous work in our lab, we observed a HFD-mediated decrease in tissue adiponectin gene and protein expression, but no change in serum adiponectin concentrations [4]. The lack of systemic changes observed in these mice may reflect the short time frame (11weeks) that the mice were on a HFD. There is some variation on when plasma or serum concentrations of adiponectin decrease in HFD fed mice. One study demonstrated decreased adiponectin concentrations after 4 weeks on a 60% HFD [250]. However, another study, similar to the one we performed, did not observe a decrease in adiponectin after 12 weeks of HFD feeding [251].

Previously, our lab and others demonstrated niacin's ability to increase tissue and circulating adiponectin [4,50,52]. Under the particular experimental conditions of our study, niacin increased adiponectin in HFD fed mice in tissue and tended to increase serum adiponectin concentrations, but the only statistically significant increase in the serum was observed in chow fed mice. Little is known about how niacin mediates increases in adiponectin. Previous work in our lab investigated possible mechanisms, and determined that niacin-mediated increases in adiponectin were independent of changes in gene expression of PPARγ C/EBPα or SREBP-1c, which are key transcription factors known to positively regulate adiponectin gene transcription [4].

WT and AdipoQ$^{-/-}$ mice fed a HFD had significant increases in gene expression for markers of adipose tissue inflammation, which indicates the development of obesity-associated inflammation. Consistent with previous studies, we observed a significant increase in both CD68 and MCP-1 gene expression in the adipose tissue of mice fed a HFD [4,252], and while niacin did not alter CD68, there was a significant decrease in MCP-1 gene expression. These findings suggest that niacin inhibits mechanisms of macrophage recruitment to adipose tissue of mice fed a HFD, but does not appear to alter overall macrophage number. MCP-1 is also secreted by adipocytes, resident ATMs, and endothelial cells [253,254]. Thus, it would be interesting to determine if the changes in MCP-1 concentration are associated with a specific cell type. The similar effect of HFD and niacin in both mouse genotypes suggest that adiponectin does not play a role in niacin-

mediated attenuation of macrophage recruitment to adipose tissue. These findings are important as previous work has shown that these effects are dependent on activation of the HCA_2 receptor, which plays a role in adiponectin secretion [4].

Macrophage polarity is defined by patterns of cell surface receptor expression and cytokine secretion, and often macrophages can be variably activated [255]. Wild-type mice fed a HFD had a significant increase in both M1 and some M2 macrophage markers, which supports the presence of adipose tissue inflammation. In these mice niacin treatment attenuated all HFD-associated increases. These findings are consistent with previous work and suggest that niacin decreases or inhibits the activation of adipose tissue macrophages [4,5,256]. Surprisingly, niacin treatment had the opposite effect in HFD fed $AdipoQ^{-/-}$ mice, with a significant increase in all M2 markers and the M1 marker, CD11c. This increase was significantly greater than even HFD feeding alone. Together, these findings suggest that treatment with niacin in the absence of adiponectin actually may promote rather than abrogate ATM activation, and that adiponectin is necessary for niacin-mediated alterations in macrophage activation and polarity. Additional studies to evaluate protein concentration and cytokine release are needed to confirm the changes in ATM polarity. In addition, it would be interesting to determine if niacin had a similar effect on circulating monocytes and immune cells in $AdipoQ^{-/-}$ mice.

Numerous studies demonstrate that HFD feeding and obesity contribute to increases in circulating cytokines and acute phase proteins, such as CRP, MCP-1, lNFγ, TNFα and IL-6 in humans and rodents [234-237] and MCP-1 and INFγ in mice [257,258]. We wanted to determine if the mice in our study exhibit similar evidence of systemic inflammation. Under these specific experimental conditions, these findings suggest that in WT mice, the adipose tissue inflammation is localized. Based on the lack of significance noted in our serum adiponectin concentrations, these findings are not surprising and may be associated with the short time course of HFD feeding implemented in this study. Interestingly, a lack of adiponectin has a discordant effect on local adipose tissue production of MCP-1 and systemic MCP-1 concentrations. This leads us to believe that the increased serum MCP-1 in AdipoQ$^{-/-}$ mice is associated with changes in inflammation other than in adipose tissue, and requires further investigation. One possible consideration is the effect of HFD induced steatosis in the liver of the AdipoQ$^{-/-}$ mice. When fed a HFD, AdipoQ$^{-/-}$ mice are susceptible to the development of steatohepatitis [259] and adiponectin plays a role in attenuating liver fibrosis by inducing nitric oxide [260]. A recent paper suggests that niacin inhibits oxidative stress and lipid accumulation in hepatocytes [261]. In our study, niacin treated AdipoQ$^{-/-}$ mice on a HFD tended to have higher liver weight than their WT counterparts. This finding may be due to differences in lipid accumulation as well as inflammatory cell infiltration. Further studies are warranted to elucidate other potential causes for increased serum MCP-1 in HFD fed AdipoQ$^{-/-}$ mice. Niacin also has been shown to inhibit

monocyte adhesion to human endothelial cells [248]. Based on these findings, it would be interesting to determine if the discordant MCP-1 changes observed in the tissues and in circulation of AdipoQ$^{-/-}$ mice manifest as a change in circulating monocyte numbers.

CONCLUSIONS

In conclusion, 1) niacin decreases adipose tissue macrophage recruitment in an adiponectin-independent manner, which suggests that the mechanism behind these changes is directly associated with activation of the HCA_2 receptor on adipose tissue macrophages, and 2) niacin alters macrophage polarization in an adiponectin-dependent manner, specifically, by reversing niacin's anti-inflammatory effects on macrophage profiles in adipose tissue of mice fed a HFD, indicating that adiponectin concentrations are critical to niacin's ability to reduce inflammation.

CHAPTER 3

CHARACTERIZATION OF THE HCA_2 RECEPTORS IN CATS

ABSTRACT

Background. The hydroxycarboxylic acid receptor 2 (HCA_2) belongs to a family of nutrient sensing receptors that binds beta-hydroxybutyrate, an alternative fuel source produced during a negative energy balance. The HCA_2 receptor has not been identified or characterized in cats. **Objectives.** Therefore, the objectives of this study were to 1) identify the feline HCA_2 receptor protein sequence and compare against known human and rodent sequences, 2) determine tissue distribution and relative expression in lean, healthy cats, and 3) demonstrate *in vitro* functionality in feline adipose tissue. **Methods.** Tissues (n=6) and primary adipocytes (n=4) were collected from lean, healthy, female cats. The predicted published genomic sequence for cats was used to design primers for PCR isolation of HCA_2. Relative tissue distribution was evaluated using RT-PCR with RNA isolated from nine different tissues (spleen, pancreas, lymph node, jejunum, kidney, liver, heart, and subcutaneous and abdominal adipose tissue). Receptor function was evaluated in primary feline adipocyte culture and changes were compared to basal lipolysis. **Results.** The *in silico* predicted feline HCA_2 protein sequence exhibited 83.1% and 86.5% amino acid similarity to human and mouse

sequences respectively. The feline HCA_2 receptor is predominantly expressed in adipose tissue and spleen. Exposure of feline adipocytes to niacin, a pharmacologic ligand of HCA_2, inhibited lipolysis to a similar degree as insulin, a potent lipolytic inhibitor. **Conclusions.** In conclusion, the feline HCA_2 receptor is similar to human and murine receptors in sequence, distribution, and functionality. By gaining a better understanding of the HCA_2 receptor in cats, we will be able to better manage feline patients.

INTRODUCTION

The hydroxycarboxylic acid receptors (HCA_{1-3}) belong to a family of G-protein coupled receptors that recognize endogenous intermediates of metabolism and are critical for nutrient sensing [1]. All three receptors are expressed in adipose tissue where their primary function is to inhibit lipolysis by coupling to G_i-type proteins [1]. The receptors differ by the endogenous ligands to which they respond. HCA_1 is activated by lactate which is produced in states of anaerobic energy metabolism. HCA_2 is activated by the ketone body beta-hydroxybutyrate (β-OHB), produced by the liver when the body is in a negative energy balance [23]. HCA_3, found only in higher primates, is activated by the ketone intermediate 3-hydroxy-octanoate [1,2].

The primary role of the HCA_2 receptor in adipocytes is to inhibit lipolysis when ketone levels are increased [23]. During times of fasting or starvation, stimulation of the beta-adrenergic receptor in adipocytes results in increased cyclic AMP

levels which drives activation of protein kinase A and stimulation of hormone sensitive lipase (HSL) [1,2,23]. HSL stimulates the release of non-esterified fatty acids (NEFAs) from triglycerides. NEFAs enter circulation, are taken up by the liver, and converted to acetyl-CoA, by beta-oxidation. Acetyl CoA molecules condense to form acetoacetate, which is subsequently converted to β-OHB. These ketones are the primary fuel source for non-neural cells when glucose is not available. When circulating concentrations reach approximately 0.7 mM, β-OHB levels are in sufficient quantity to stimulate the HCA_2 receptor, resulting in a decreased rate of lipolysis which preserves fat stores and prevents the development of ketoacidosis [10,23]. In humans, β-OHB can reach levels capable of stimulating the receptor after approximately a 1-d fast [23].

Tissue distribution of the HCA_2 receptor has been well characterized in mice and humans [2], where it is predominantly expressed on adipocytes (white and brown) and immune cells, including macrophages, monocytes, neutrophils and dermal dendritic cells, but not lymphocytes [6-12]. HCA_2 has also been identified in pigmented retinal epithelial cells, colonic epithelial cells, keratinocytes, microglia, and normal mammary tissue [13-16]. Although a number of studies have reported that the receptor is not expressed in the liver [9-11], a more recent study demonstrated low basal levels of HCA_2 in primary murine hepatocytes with expression being induced after exposure to inflammatory stimuli [17].

Receptor expression appears to be fairly consistent among species. Work done in humans and rodents indicate that the highest receptor gene expression is in fully differentiated adipose tissue with increased expression noted in the spleen and lungs [18]. The increased expression in these secondary tissues is likely associated with high concentrations of immune cells in these organs. Interestingly, a study done in cattle identified a unique distribution of the receptor in this species, with increased expression in the parenchymal cells of the liver suggesting a different physiologic function of the receptor in this species [214]. However, further studies investigating the function of the receptor in cattle suggest a similar physiologic role in adipocytes as seen in humans and rodents, with activation of the receptor resulting in decreased lipolysis and increased adiponectin [222,223].

Cats are a well-established model of human obesity as well as a naturally occurring model of type 2 diabetes mellitus [145,146,201]. Cats exhibit similar changes in adipokine dysregulation associated with obesity that are seen in humans, including decreased plasma adiponectin concentrations [153,262]. During times of negative energy balance, when circulating glucose concentrations are low, cats readily produce ketone bodies as an alternative fuel sources [263]. In addition, β-OHB is significantly increased in ill cats with hepatic lipidosis, diabetes mellitus, and diabetic ketoacidosis and is the primary ketone produced and monitored in the blood of feline diabetic patients [264,265]. However, the HCA_2 receptor has not been identified or characterized in cats. As

cats develop obesity-related diabetes in a manner similar to humans, with increased levels of ketones in circulation, it is important to study the role of the HCA_2 receptor in this species. We hypothesize that the feline HCA_2 receptor will exhibit similarities to the human and murine HCA_2 receptors in protein sequence, tissue distribution, and functionality.

The objectives of this study were to 1) identify feline HCA_2 receptor nucleotide and protein sequence, 2) determine relative gene expression in a variety of tissues, and 3) demonstrate *in vitro* functionality of the feline HCA_2 receptor.

MATERIALS AND METHODS

Tissue distribution and relative gene expression of feline HCA_2 receptor

Nine different tissues (abdominal adipose, subcutaneous adipose, heart, spleen, kidney, lymph node, liver, jejunum, and pancreas) were collected from six lean, healthy, female cats utilized as healthy controls in a separate study approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC). Subjects were determined to be healthy based on physical exam, complete blood count (CBC), chemistry profile and urinalysis. Tissue samples were collected at necropsy within minutes of euthanasia. RNA from adipose tissues and remaining tissues were isolated using RNeasy Lipid Tissue Mini Kit and RNeasy Tissue Mini Kit (Qiagen, Valencia, CA). Aliquots of RNA (0.5 µg) was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Relative tissue mRNA expression was evaluated using quantitative PCR (qPCR)

with Bio-Rad iCycler iQ Thermal Cycler. Samples were analyzed using Bio-Rad SYBR Green PCR Master Mix. Relative changes in gene expression were calculated by the comparative C_T method using the $2^{\Delta\Delta C}{}_T$ equation with results normalized to corresponding GAPDH mRNA levels [247]. Primers used are listed **(Table 4).**

Identification and sequencing of the HCA_2 gene

The feline predicted HCA_2 DNA sequence was retrieved from the National Center for Biotechnology (NCBI) feline database. The predicted feline HCA_2 sequence was 2078 base pairs in length, encoding a protein of 363 amino acids. The primer sets identified in Table 1 were designed in the 5' and 3' untranslated region of this sequence to amplify 1229 base pair amplicon encompassing the entire open reading frame (ORF). Total RNA obtained from feline adipose tissue (described in previous section), was reverse transcribed, and the cDNA obtained was used as the template in a PCR mix with LA Taq DNA polymerase (TaKaRa, Mountain View, CA). Electrophoresis of the PCR products showed a predominant band at approximately 1250 base pairs. This band was excised from the gel, eluted, and ligated into a plasmid using TOPO TA cloning kit (Invitrogen Life Technologies, Grand Island, NY). Bacterial colonies were screened for the presence of the insert and three colonies containing the recombinant plasmid were grown in LB broth to allow amplification of sufficient plasmid for sequencing (Eurofins MWG Operon, Huntsville, AL). The resulting sequence was compared at the nucleotide and predicted amino acid levels with the human and mouse

	Gene	Forward Primer 5'-3'	Reverse Primer 3'-5'
qPCR	HCA_2	ATCTGCCTGCCATTCTTGAC	CCACGGTGAGGAAGATGATG
	GAPDH	ATTCTACCCACGGCAAGTTC	ATACTCAGCACCAGCATCAC
RT-PCR	HCA_2	TCTTTCCTTGGGCATTCGCT	GCTTTCCTCTCTGTTCCCCC

Table 4. Primers.used for PCR. qPCR,quantitative polymerase chain reaction RT-PCR; reverse transcriptase-polymerase chain reaction

HCA_2 sequences using DNASTAR Lasergene (Invitrogen Life Technologies, Madison, WI).

Primary feline adipocytes culture

Primary adipocytes were isolated according to previously published methods with the following specifications [266]. Samples were collected within minutes of euthanasia from four intact, female cats utilized as healthy controls in a separate study approved by the AU-IACUC. Subjects were determined to be healthy based on physical exam, complete blood count, chemistry profile and urinalysis. Four grams of tissue were collected from the subcutaneous (inguinal) and abdominal (falciform) fat pads. Adipose tissue was minced and digested in HEPES buffer containing type I collagenase at 37°C for 30 min with gentle shaking (120 rpm) in a programmable shaker bath. The resulting cell suspension was diluted in HEPES phosphate buffer and the isolated adipocytes were separated from the undigested tissue by filtration through a 175 µm nylon mesh filter. Isolated cells were washed, re-suspended in DMEM with 1% FBS, and incubated for 30 min at 37°C. The isolated adipocytes (200 µL of a 2:1 ratio of packed cells to medium) were plated onto 450 µL of collagen matrix. The cells were allowed to adhere for 24 h and serum starved for 3 h prior to treatment with either 10 µM isoproterenol, 100 µM nicotinic acid, 100 nM bovine insulin, or DMEM with 1% FBS (vehicle control). Media was collected at 24 h and glycerol assay was performed to determine the amount of free glycerol released into the media by the cells. Each plate was prepared from a single animal and results

from each treatment group were analyzed in relation to a control well from the same suspension at three separate time points. The results are expressed as a percent change from the control which was set to 100% and represents basal lipolysis.

Statistical analysis

Analysis of relative gene expression was determined by ANOVA with Bonferroni post-hoc analysis using Graph Pad Prism 6 software (La Jolla, CA). Significance was set at a *P* value of ≤ 0.05.

RESULTS

Identification of the feline HCA_2 receptor

The feline HCA_2 mRNA sequence obtained by PCR cloning using RNA from feline adipose tissue was compared to the predicted feline sequence as well as the known sequences of humans and mice. The sequence cloned using RNA from feline adipose tissue was identical to that in the NCBI database. Feline HCA_2 was 86.1% identical to the human nucleotide sequence and 80.2% identical to the murine nucleotide sequence. The predicted feline HCA_2 protein sequence exhibited 83.1% identity to the human sequence and 86.5% identity to the murine protein sequence **(Figure 9)**. Six amino acids previously determined to be critical for ligand binding and conformation [267] were found to be conserved in the feline HCA_2 amino acid sequence **(Figure 10)**.

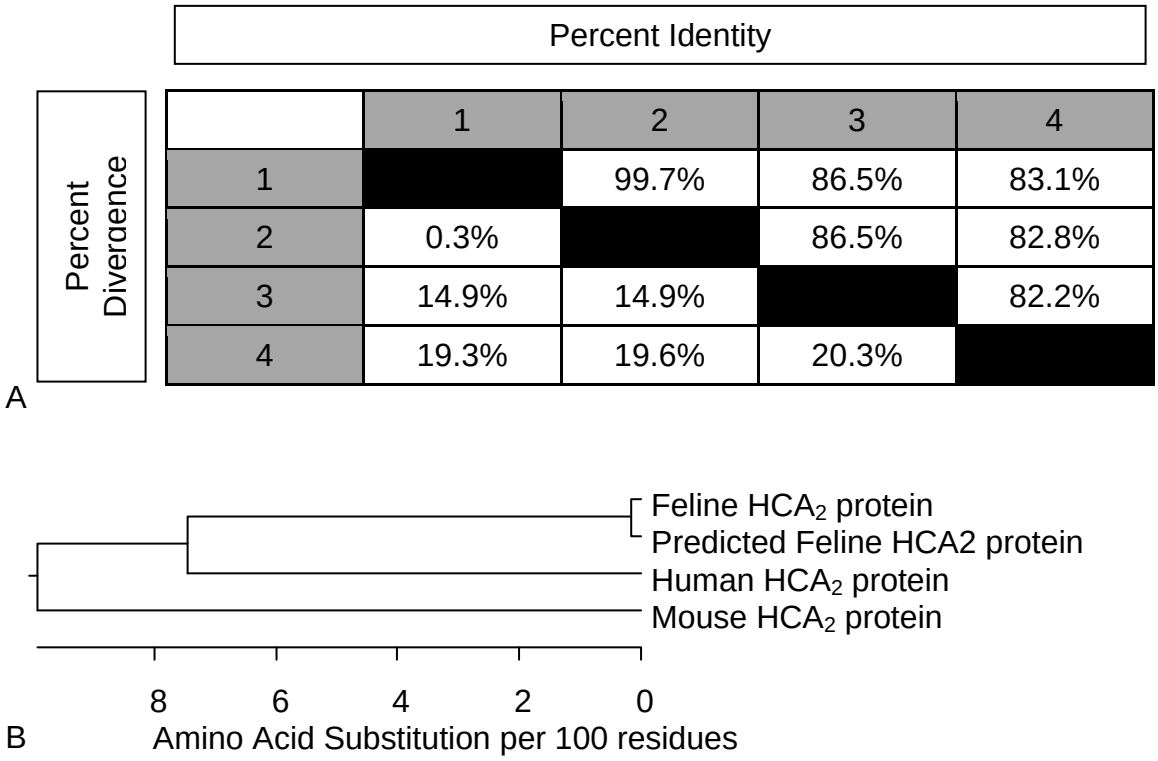


Figure 9. Similarity of the feline HCA_2 protein sequence compared to human and mouse sequences. Identity and divergence diagram (A) and phylogenetic tree (B). 1 submitted feline sequence; 2 predicted feline sequence; 3 Murine sequence; 4 Human sequence.

Figure 10. Feline HCA_2 amino acid sequence compared to predicted feline, human, and murine sequences. Amino acid residues from the mouse and cat sequences that are outlined in black differ from the human HCA_2 sequence. Amino acid residues outlined in red are critical for ligand binding and conformation [267]. These residues are conserved in the predicted HCA_2 sequence obtained in this study.

Tissue distribution and relative gene expression of feline HCA_2

Similar to humans and mice, feline HCA_2 was determined to be predominantly expressed in spleen, subcutaneous adipose, and abdominal adipose tissues **(Figure 11)**. When compared to minimally expressing tissues such as heart, the abundance of HCA_2 mRNA was 700 to 1000-fold in feline adipose tissues ($P < 0.01$) and spleen ($P < 0.0001$).

Primary feline adipocyte culture

Addition of isoproterenol resulted in marked stimulation of lipolysis in both abdominal and subcutaneous adipocytes (~200-300% of control) while addition of insulin, a known inhibitor of adipocyte lipolysis decreased glycerol concentrations by ~25% compared to control cells. After 3 h of stimulation by niacin, a pharmacologic ligand of the HCA_2 receptor, lipolysis was inhibited by ~30% in abdominal and ~17% in subcutaneous feline adipose tissue **(Table 5)**. While the dramatic increase in lipolysis associated with isoproterenol resulted in a statistically significant increase in lipolysis, the changes observed from both insulin and niacin were not statistically significant. However, in each separate feline sample, both niacin and insulin resulted in a decrease in lipolysis at the 3 h time point. By 24 h there was decreased responsiveness of all agonists.

DISCUSSION

Cats are a model of obesity and nutritional disorders in humans [145,146,268]. Obesity is the one of the most common nutritional disorders of cats, with an

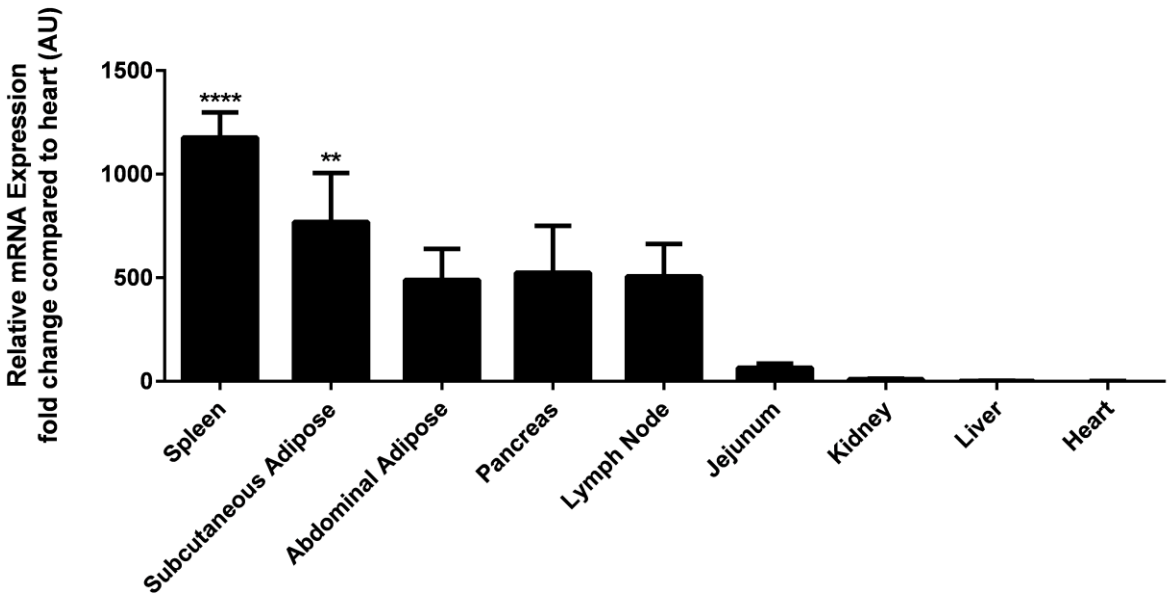


Figure 11. Relative gene expression of the HCA_2 receptor in various tissues of lean healthy cats. ****$P < 0.0001$; **$P < 0.01$

	Abdominal Adipose (% change)			Subcutaneous Adipose (% change)		
	Isoproterenol	Insulin	Niacin	Isoproterenol	Insulin	Niacin
3h	295.7± 8.9***	72.1 ± 4.4	69.2 ± 3.8	287.65 ± 14**	96.95 ± 6.8	82.69 ± 10
6h	273.8 ± 23****	73.8 ± 6.7	77.3 ± 7.1	285.51 ± 17****	94.32 ± 5.2	79.94 ± 2.6
24h	238.1 ± 13***	86.7 ± 11	91.9 ± 1.8	204.12 ± 8.3***	126.97 ± 27	97.69 ± 4.0

Table 5. Effect of isoproterenol, insulin, and niacin as percent change of lipolysis in primary feline adipocytes. Optical density (OD) values were measured and represented as percent change compared to basal lipolysis (set to 100%) for each individual cat at each individual time point. Values are presented as the mean ± SEM (n = 4 cats). ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

estimated 25% to 63% of cats classified as overweight or obese [123-126]. Similarities in physiologic responses to metabolic diseases are likely a critical component of disease progression and may be associated with variation in the regulation of lipolysis in lean versus obese cats. This study cloned and sequenced the feline HCA_2 receptor which likely plays an important role in regulating lipolysis during times of fasting and has marked similarity in both mRNA and protein to known sequences of humans and mice.

In tissues of lean and healthy cats, there is significantly greater HCA_2 receptor expression in abdominal and subcutaneous adipose tissue and spleen compared to other tissues evaluated in this study. This relative gene expression and tissue distribution is similar to patterns previously described in humans and mice and the increased level of expression in adipose tissue of cats is not surprising given the primary role of the receptor as a nutrient sensor of β-OHB [2]. In adipocytes, the HCA_2 receptor binds the ketone body β-OHB, inhibiting lipolysis and regulating the release of free fatty acids [23].

The high expression level in the spleen is similar to rodents and humans and likely reflects the presence of this receptor on immune cells such as macrophages and neutrophils [12]. Currently, the role of the receptor on immune cells is not well known, but it has been suggested that it may mediate a cross-talk between metabolic state and immune function [4,5].

Adipokines are hormones or cytokines produced by adipose tissue, with adiponectin being one of the most prevalent [60]. Adiponectin has insulin sensitizing, anti-atherogenic, and anti-inflammatory effects [5,159,244]. Previous work from our lab and others demonstrated that HCA_2 receptor agonists, such as niacin, increase serum adiponectin concentrations in rodents and humans [47,48,269]. In addition to its impact on adiponectin secretion, stimulation of the HCA_2 receptor has been associated with reduced secretion of pro-inflammatory cytokines and chemokines, inhibition of vascular inflammation, inhibition of monocytes chemotaxis, and a number of other anti-inflammatory effects [4,38,39,45,246]. This is likely important given the presence of the HCA_2 receptor on immune cells.

Chronic inflammation present with obesity is associated with decreased plasma adiponectin concentrations [262]. Previous work from our lab has demonstrated decreased expression of the HCA_2 receptor in obese mice on a high-fat diet [70]. This suggests that with increased obesity there is decreased regulation of lipolysis and indicates a possible mechanisms for decreased adiponectin in obesity. As humans and domestic animals become obese, adipose tissue is unable to respond appropriately, especially during fasting, and there is dysregulation of both lipolysis and a decrease in serum adiponectin [133]. These changes are likely to occur in obese cats as well.

Previous studies of human and murine HCA_2 receptors identified six important amino acids that are critical for ligand binding including arginine 111, serine 178, phenylalanine 276, tyrosine 284, and the transmembrane helix asparagine 86/tryptophan 91 [167,267]. All six of these amino acid residues were found to be conserved in the feline HCA_2 receptor which further supports similar secondary and tertiary protein structure as well as functionality of the receptor among these species **(Figure 10)**.

As suggested by the highly conserved protein sequence, there are likely similarities in receptor function owing to inhibition of lipolysis in primary feline adipocyte culture with niacin. Niacin inhibits lipolysis through direct stimulation of the HCA_2 receptor. Insulin, via the insulin receptor, is a strong physiologic inhibitor of lipolysis and was used as a marker of an intact lipolytic inhibition pathway in this cell culture model. In addition, isoproterenol stimulates lipolysis through the beta-adrenergic receptor and the strong response in these cells throughout the 24-h period indicates a viable adipocyte model capable of undergoing lipolysis. While these findings were not statistically significant owing to the low number of cats in this portion of the study (n = 4), the changes observed by niacin were similar to the effects of insulin, and were consistent in all four outbred cats. It will be interesting to see if niacin exerts similar effects during *in vivo* studies.

In this cell culture model, a modestly greater inhibition of lipolysis by niacin was evident in abdominal adipose tissue compared to subcutaneous adipose suggesting that receptor function differs in various adipose tissue depots. These findings are not surprising as others have shown that different adipose tissue depots have different biologic activity. In cats, abdominal adipose tissue has been shown to have greater amounts of adiponectin gene expression compared to subcutaneous adipose tissue [169]. In humans and rodents, increased serum leptin is associated with increased subcutaneous adipose tissue as opposed to increased abdominal fat [270].

As in humans, obese cats demonstrate peripheral insulin resistance and glucose intolerance [146,153]. In addition, they are susceptible to unregulated lipolysis and development of hepatic lipidosis in times of stress and negative energy balance [265]. Previous studies in cats have shown that body weight is a critical factor in insulin sensitivity and response to glucose with an increase in body weight of 1 kilogram correlated to ~30% loss of insulin sensitivity and glucose effectiveness [152]. Other studies suggest that obese cats have greater difficulty losing weight [153]. Further studies of the HCA_2 receptor and its role in lean versus diseased or obese cats are needed to better understand its roles in both normal and obese individuals.

Important metabolic diseases of cats include hepatic lipidosis and type 2 diabetes mellitus [271,272] . In cats, the pathogeneses of these disease

processes are closely associated with obesity and abnormal regulation of lipolysis. These diseases are challenging to treat, especially with the presence of concurrent illness. By gaining a better understanding of how lipolysis is regulated and controlled in cats we can begin to better understand how to manage these patients.

CONCLUSIONS

In conclusion, our study demonstrates that the HCA_2 receptor has similar structure and function in rodents, humans, and cats and thus indicates that regulation of lipolysis and pathophysiology of obesity-related disorders related to this receptor function are similar across these species. This is important for cats not only as a model of human nutrition and disease, but also in future management of feline patients with metabolic disorders such as hepatic lipidosis and T2DM.

CHAPTER 4

CHARACTERIZATION OF IMMUNOLOGIC RESPONSE TO THE DEVELOPMENT OF OBESITY IN CATS

ABSTRACT

Background. In humans, obesity is classified as a chronic low-grade inflammatory disease characterized by adipose tissue and systemic inflammation. Cats are considered a model for human obesity and are a naturally occurring model of type 2 diabetes mellitus. Recent studies suggest that cats have a unique immune response during the development of obesity that differs from what is observed in humans and rodents. **Objectives.** The goal of this study was to provide a comprehensive investigation of systemic and adipose tissue inflammation during the development of obesity and insulin resistance in cats. **Methods.** Twelve, lean, male neutered, cats gained weight on *ad libitum* chow diet for 18 months. Cats were evaluated for changes in insulin sensitivity, lipid metabolism, adipokine profiles, circulating inflammation, distribution of fat mass and adipose tissue inflammation. **Results.** After 18 months, the cats gained ~ 36%-83% of their initial body weight, with a significant increase in BMI and total fat mass. In this study, cats had greater expansion of their subcutaneous, compared to abdominal adipose tissue depots. Increased adiposity resulted in decreased insulin sensitivity and adiponectin, and increased triglyceride, cholesterol and leptin. Adipocyte size increased in both adipose tissue depots,

with a greater diameter increase in subcutaneous compared to abdominal adipocytes. No changes in circulating inflammatory cells were noted. However, serum concentrations of MCP-1 and TNF-α were significantly increased. While rare crown-like structures were detected in both lean and obese feline adipose tissue, there were no significant changes associated with obesity, and no difference between adipose tissue depots. In addition, gene expression of the nutrient sensing receptor HCA_2 tended to decrease in abdominal, but not subcutaneous adipose tissue. **Conclusions.** With the progression of obesity, cats have some evidence of systemic inflammation, but do not develop cellular adipose tissue inflammation. These findings are important as cats do not develop clinical atherosclerosis or obesity-associated cardiovascular disease. In addition, these findings suggest, that unlike people and rodent model of obesity, cats may rely more heavily on subcutaneous adipose tissue for signals that maintain metabolic and immune homeostasis.

INTRODUCTION

Obesity in both human and domestic animals is established as a major epidemic [124,125,127,273]. In humans, obesity is classified as a chronic low-grade inflammatory disease and is associated with many clinical manifestations such as metabolic syndrome, type 2 diabetes mellitus (T2DM), atherosclerosis, and hypertension [61,62]. Recent studies suggest that in addition to being a major energy storage site, adipose tissue is an important and highly active endocrine organ that produces and secretes a number of molecules including adipokines,

cytokines and hormones [60,62]. Excessive food intake and lack of physical exercise lead to an expansion of adipose tissue in the body, resulting in dysregulation of adipokines, chronic adipose tissue inflammation and the development of systemic inflammation.

A key feature of human obesity and insulin resistance is an altered adipokine profile, specifically decreased adiponectin and increased leptin [61,242,243,270]. Adiponectin is considered a beneficial adipokine that is associated with numerous protective and anti-inflammatory effects in the adipose tissue [57,69,244]. Adiponectin also promotes anti-inflammatory properties in human macrophages [57]. Leptin is a pro-inflammatory and pro-angiogenic adipokine that decreases food intake. Increased leptin concentrations promote the proliferation and activation of circulating monocytes and up-regulate the innate immune receptor TLR-2 on human monocytes [274,275].

Along with changes in adipokines, there are also marked changes in adipose tissue gene expression of inflammatory cytokines [4,276]. During the development of obesity, adipocytes and adipose tissue immune cells, such as macrophages and lymphocytes release pro-inflammatory adipokines, including MCP-1, IL-6 and TNF-α [231,277]. The production of pro-inflammatory molecules further promotes adipose tissue inflammation and insulin resistance. The shift in gene expression likely contributes to the accumulation of adipose tissue immune cells that is observed in obese people.

In humans and rodent models of obesity, there are increased numbers of ATMs [63]. These cells migrate into adipose tissue from bone marrow derived circulating blood monocytes in response to increased MCP-1 [63,203,278,279]. These macrophages aggregate in crown-like structures (CLS) completely surrounding adipocytes and scavenging adipocyte debris [203]. Obesity development is characterized by an increase in the number of ATMs and a shift in the population of macrophages present [65,203,280]. ATMs in lean individuals are primarily in an M2-polarized sate (alternatively activated). Diet-induced obesity is associated with increased numbers of M1-polarized macrophages (classically activated) [203,279,280]. In our experience in the evaluation of high-fat and western diet fed rodent models of obesity, the number of adipose tissue macrophages increases from less than 10 per section in lean and chow fed animals, to greater than 100 per section in diet-induced obese mice (unpublished data).

Human obesity is also associated with systemic inflammation that is believed to contribute to increased morbidity and mortality [281]. It has been suggested that adipose tissue inflammation contributes to the development of systemic inflammation in chronic obesity [282,283]. In obese adults as well as obese children there are increased numbers of circulating pro-inflammatory monocytes and cytokines [229,230,238,239,284,285]. Circulating TNF-α concentrations are implicated in macrophage infiltration and obesity-associated chronic inflammation

[279]. The cytokine IL-6 also may play a role in the development of T2DM, by stimulation of hepatic triglyceride secretion, inhibition of insulin signaling in hepatocytes, and induction of hepatic C-reactive protein synthesis [60,62]. Increased TNF-α and MCP-1 also are correlated with decreased insulin sensitivity [286].

Based on the current understanding of the pathogenesis of obesity, it is not surprising that obesity is associated with the development of metabolic syndrome, T2DM and atherosclerosis. Along with metabolic sequelae, obesity-associated immunologic dysfunction has been correlated with disease such as asthma, hypercoagulability, dementia and cancer [287] and taken together, these findings have proposed that adipose tissue is an immunologic organ [241].

A potential mediator of metabolic-immunologic crosstalk is the nutrient sensing receptor HCA_2. The HCA_2 receptor is present on adipocytes and immune cells, and activation of this receptor alters lipolysis and cytokine secretion [4]. Recent work from our lab demonstrates that with diet-induced obesity, there is decreased HCA_2 gene expression [70]. The down-regulation of this receptor in response to a high-fat diet suggests that with obesity, there may be decreased ability of adipocytes to sense nutrients and metabolites, leading to a dysregulation of lipolysis and adiponectin secretion. This potential adipocyte dysfunction may play a role in the increase in adipose tissue inflammation observed in obesity. In addition, HCA_2 expression on immune cells plays an

important role in the pathogenesis of atherosclerosis and other inflammatory diseases [5].

Companion animal obesity is a significant epidemic that has developed alongside the human obesity epidemic, and cats specifically have been proposed as a model for human obesity and metabolic disease [122,144-147,156,208,288]. Obesity is a common medical condition in cats, and studies suggest that greater than 30% of domestic cats are obese [124,125]. As with human obesity, feline obesity results in chronic complications, such as T2DM and dyslipidemia [133,145,146,156]. However, prevention and treatment of feline obesity-associated diseases is limited by our poor understanding of the systemic and organ level changes that occur during the development of obesity in cats.

As in people, the pathogenesis of feline obesity is characterized by adipose tissue expansion. Cats have similar obesity-associated changes in their adipokine profiles, characterized by decreased adiponectin and increased leptin [145,262]. In one study, obese feline adipose tissue had an almost 10 fold increase in TNF-α protein compared to lean [210]. A separate study evaluated cohorts of lean and obese cats and indicated that obese cats had increased gene expression of inflammatory cytokines such as CCL-5 and MCP-1, as well as increased numbers of T-lymphocytes within adipose tissue [201]. Taken together, data from these studies suggests that obesity in cats, at least at the level of adipose tissue is associated with low-grade inflammation [201,210,211].

However, recent studies suggest that cats may be unique in their immunological response to the development of obesity, in that they lack a systemic inflammatory response [167]. One study evaluated 37 client-owned cats and noted no change in the major acute phase proteins, serum amyloid A or haptoglobin [212]. Recently, results from a longitudinal, developmental model of feline obesity, noted significant changes in the serum adipokine profiles, but no changes in serum IL-1, IL-6 or TNF-α [167]. These findings differ from what is consistently reported in people who gain weight [230,237,239].

In addition to a potential absence of changes in inflammatory cytokines, multiple studies indicate that obese cats do not have increased numbers of ATMs compared to lean cats [201,202]. An investigation that utilized flow cytometry to evaluate the adipose tissue stromal vascular fraction (SVF) showed that macrophage number was unaltered in obese cats compared to lean, and that these findings were independent of changes in cytokine gene expression [202]. A separate study demonstrated changes in MCP-1 gene expression, but did not observe an increase in ATMs based on histopathologic evaluation [201]. Taken together, these results suggest that cats likely develop a unique form of obesity-associated adipose tissue inflammation, and unlike people they do not develop a strong systemic inflammatory immune response. These findings also imply discordance between what is observed in obese people and rodents, and obesity in cats. However, a longitudinal study that directly evaluates changes in

systemic and tissue immune response during the development of obesity and insulin resistance in cats does not exist.

Therefore, the goal of this study is to provide a comprehensive investigation of systemic and adipose tissue inflammation during the development of obesity and insulin resistance in cats. Specific objectives include 1) evaluation glucose and lipid metabolism; 2) assessment of adipose tissue distribution, cellularity and microanatomy; and 3) determination of systemic humoral (cytokine and adipokine concentrations) and cellular (CBC parameters) markers of inflammation during the development of obesity in an outbred cat colony. Recently, our lab has characterized the feline HCA_2 receptor [19]. This receptor is present on adipocytes and immune cells and likely plays a role in cross-talk between the metabolic and immune systems. An additional goal of this study is to evaluate gene expression of the HCA_2 receptor in various adipose tissue depots during the development of obesity in cats.

MATERIALS AND METHODS

Feline Obesity Model

Twelve adult, lean, male, specific pathogen-free (SPF), domestic short-hair cats were purchased at 7 months of age from Liberty Research (Waverly, NY) and housed in an AAALAC-accredited animal facility. At ~8 months old the cats were neutered and fed a chow diet (Laboratory Feline Diet 5003, Constant Nutrition) and remained on that diet throughout the study. The cats were housed and fed

individually for 8 hrs during the day and group-housed and group-fed for the remainder of the 24 h period. Water was provided *ad libitum*. Prior to initiation of the study the cats were fed once daily to meet their estimated resting energy requirements with the appropriate adjustment factor for neutered males. The cats were weighed weekly in order to monitor changes in weight. Each cat's health status was determined through a physical exam and evaluation of clinical laboratory data and all cats were accustomed to being handled and socialized daily. All procedures were approved by the Auburn University Institutional Animal Care and Use Committee.

Study design

The cats were maintained at a lean body weight for ~ 8 months owing to controlled food intake (Figure 12A). Initiation of the study (time point 0) was defined as baseline when all the cats were lean, and the study was concluded at 18 months, which was defined as end point (time point 18). Upon initiation of the study, the cats were allowed *ad libitum* access to food throughout the day. There was no change in diet. A feline body mass index (BMI) reflected as kg/m^2 was calculated as previously described, based on the following equation [146]:

$$BMI = body\ weight\ (kg) / [body\ length\ (m) \times height\ (m)].$$

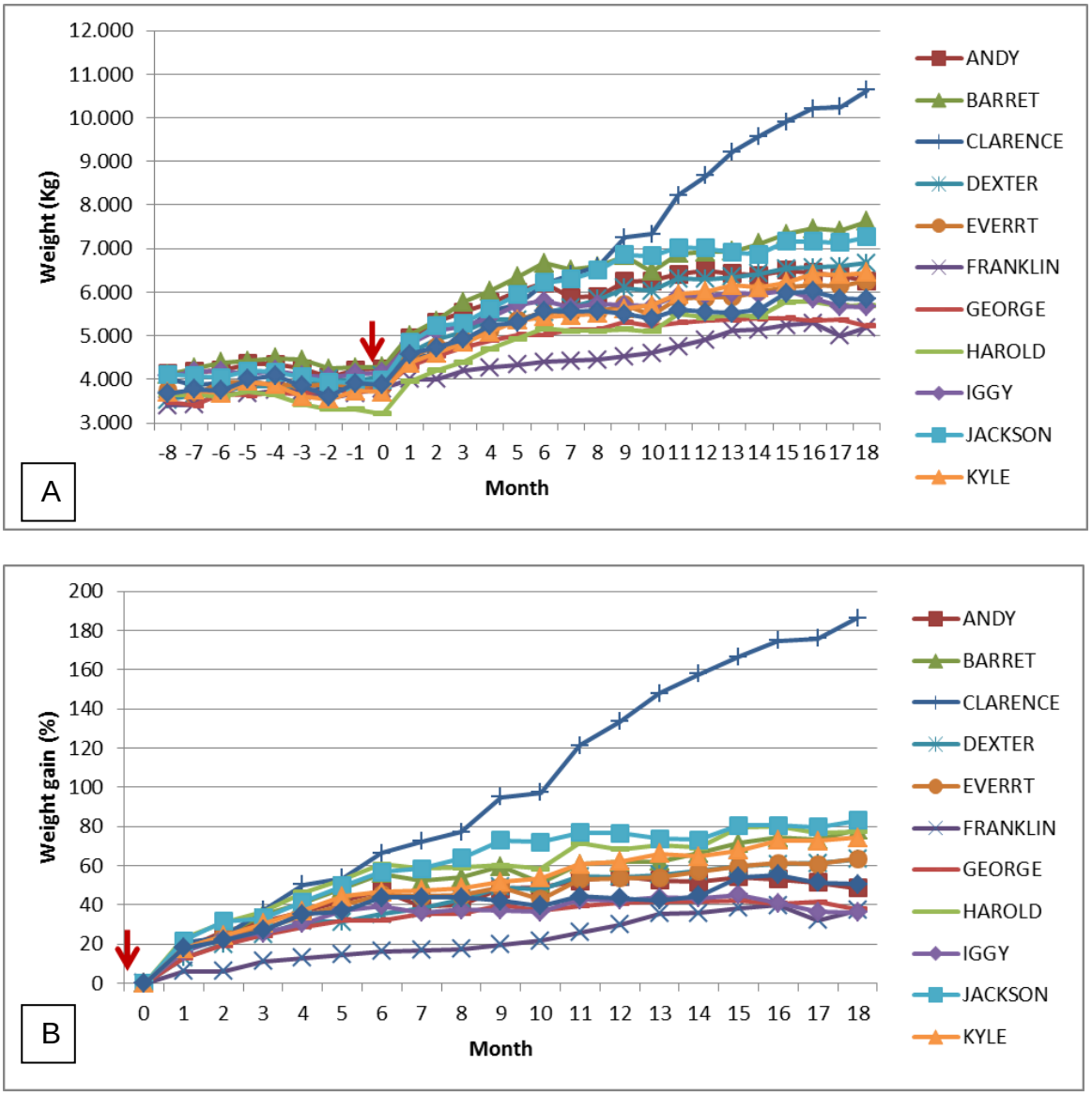


Figure 12. Individual weight gain and percent body weight increase in cats before and during an *ad libitum* diet regimen. Individual weight gain (A) and percent body weight increase (B). Red arrow indicates baseline (time point 0). The cat marked with a blue line and crosshairs (+) indicates Clarence who was excluded from further data analysis based on a divergent rate of weight gain.

Blood samples were collected at each increment of 10% weight gain until the end of the study. Subcutaneous and intra-abdominal adipose tissue samples were collected at the beginning and end of the study. Magnetic resonance imaging (MRI) analysis was performed at baseline and end point to evaluate fat mass and estimate fat distribution. Weight gain and time points for data collection were based on previous published work [167].

Blood samples and Processing

Approximately 5 - 10 mL of blood were collected from the jugular vein using a 5 mL syringe and a 22 GA needle after each cat was sedated with dexmedetomidine (OrionPharma, Espoo, Finland) at a dose 40 - 80 mcg/kg. Blood was collected into plastic silicone coated tubes for isolation of serum and into tubes containing EDTA for complete blood counts (CBC). The anticoagulated blood was immediately transported to the Auburn University Clinical Pathology Laboratory and a complete blood count (CBC) was performed on an Advia 120 hematology analyzer (Siemans, Tarrytown, NY). Differential white blood cell (WBC) counts were confirmed on examination of a blood smear by a board certified veterinary clinical pathologist (ECG). Blood for serum collection allowed to clot for ~20-30 minutes, then centrifuged at ~400xg for 15 minutes. At which time the serum was separated into aliquots and stored at -80˚C until assays were performed.

Adipose tissue biopsies and evaluation of histologic samples

Baseline and endpoint adipose tissue biopsies were collected from abdominal (falciform) and subcutaneous (inguinal) fat pads. To minimize the potential effects of scar tissue on the subcutaneous adipose tissue samples, baseline samples were collected from the left inguinal fat pad, and end point samples were collected from the right inguinal fat pad. Approximately 2 grams of adipose tissue from each sample were immediately placed in 10% neutral buffered formalin, allowed to fix for ~48 hrs and then paraffin embedded. Five micron thick sections were stained with hematoxylin-eosin, slides were scanned into Aperio ScanScope scanner (Vista, CA) and evaluated using Scanscope software (Lieca Biosystems, Buffalo Grove, IL). Five representative full thickness adipocytes from five separate fields were selected from each slide and measured at their widest point. These measurements were used to determine the average adipocyte diameter per sample. Crown-like structures (CLS) were defined as shrunken adipocytes, completely surrounded by morphologically identified macrophages. This definition was based on previous descriptions of CLS [63,203]. The presence of macrophages was confirmed with immunohistochemical staining for IBA1, which is a marker for macrophages. Two entire sections from each biopsy sample were evaluated for the presence of CLS.

Assessment of insulin sensitivity, biomarkers of lipid metabolism, and adipokines

To assess insulin sensitivity during obesity development, fasting serum insulin and glucose concentrations were measured in order to calculate a homeostatic

model assessment-insulin resistance value (HOMA-IR) that has been validated in cats [289]. Serum insulin was measured by use of a previously validated, commercially available feline insulin ELISA (Mercodia, Uppsala, Sweden) [290]. Serum glucose, cholesterol and triglycerides were determined by enzymatic methods using a Cobas C311 chemistry analyzer with manufacturer reagents (Roche, Indianapolis, IN). Non-esterified fatty acids (NEFAs) were measured using a kit from Wako Diagnostics (Richmond, VA). Serum adiponectin and leptin concentrations were determined using previously validated, commercially available kits [153,291].

Inflammatory cytokines and adipokines

Serum IL-6, MCP-1, IL-1β, RANTES (CCL-5) and TNF-α were measured using a feline specific Milliplex Multiplex Custom Kit from Millipore (Temecula, CA) and read on a MAGPix Bio-analyzer from Luminex (Temecula, CA).

Magnetic resonance imaging

To evaluate fat mass and estimate fat distribution, magnetic resonance imaging (MRI) studies were performed at baseline and at the end of the study. All cats were imaged on a Siemens Verio open-bore 3 Tesla MRI scanner located at the Auburn University MRI Research Center. Just prior to the MRI scan the cats were anesthetized with a combination ananesthetic that included dexmedetomidine (0.14mg/mL), butorphanol (1.8mg/mL) and ketamine (68mg/mL). The cats were placed in sternal recumbency and 3mm image slices

using the following sequence, T2w-COR-0.75x0.75x3.0-SLC32-BW416-AVG6-TA7.8min-R2, were taken starting at the apex of the heart and continuing to the pelvic symphysis. Both transverse (axial) and coronal images were collected. Images containing sections through both kidneys were analyzed with Image J software from the National Institutes of Health (Bethesda, MD) to quantify abdominal and subcutaneous adiposity. Adipose tissue areas were measured in each selected scan section. Signal intensity was visually selected in each scan as previously described [292]. In most cases, the optimal threshold was ~140 on a scale of 256. When possible, errors in adipose tissue segmentation were manually corrected. Selection thresholds were performed separately for subcutaneous and abdominal adipose tissue. Results for adipose tissue volume are presented as a percent of total body area within the scanned image. Abdominal and subcutaneous fat mass were expressed as parts of a whole for the total adipose tissue area identified.

Adipose tissue RNA isolation and relative gene expression of feline HCA_2 receptor

RNA from abdominal and subcutaneous adipose tissue was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). Aliquots of RNA (0.5 µg) were reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Relative tissue mRNA expression was evaluated using quantitative PCR (qPCR) with Bio-Rad iCycler iQ Thermal Cycler. Samples were analyzed using Quanta SYBR Green PCR Fast Mix. Relative changes in gene

expression were calculated by the comparative C_T method using the $2^{\Delta\Delta C}{}_T$ equation with results normalized to corresponding GAPDH mRNA levels. Primers were previously published [19]. GAPDH was not altered with obesity. All gene expression data were presented as a relative change compared to baseline.

Statistical analysis

All statistical analyses were performed on Graph Pad Prism 6 software (La Jolla, CA). The data were expressed as means $\pm$ standard error of the mean. All data sets were evaluated for normality and data that did not exhibit Gaussian distribution were log transformed. If rules for normality were still not met, non-parametric analyses were utilized. Paired t-tests or Wilcoxon matched-pairs signed rank tests were used when comparing baseline to end point samples for all included cats. Repeated measures analysis of variance was used to evaluate changes in weight gain and % body weight gain over time. Values of $P \leq 0.05$ were considered significant.

RESULTS

Body weight, BMI and adipose tissue distribution

At the start of the study there was no difference in age (1.4 years $\pm$ 2 weeks) or weight (3.9 $\pm$ 0.2 kg) between cats, and all cats were considered lean. All cats had a significant increase in body weight by 2 months. After 12 months, one cat achieved a rate of weight gain that significantly outpaced all other cats, and by 18

months, this particular cat weighed 10.6 kg and had a 186% increase in body weight over baseline. Based on these findings this cat was considered an outlier and his data were excluded from further statistical analysis **(Figure 12)**. At the end of the study the remaining cats (n = 11) weighed 6.2 $\pm$ 0.24 kg with ~60 $\pm$ 5.37% (range 36% to 83%) increase in body weight gain **(Figure 13)**. The average feline BMI at baseline was 42 $\pm$ 1 (range 36 - 46), and increased to 67 $\pm$ 2 (range 57 - 76) at end point **(Figure 13C)**. The average increase in body fat mass based on MRI analysis was similar to the percent body weight gain and was ~65% of total body mass **(Figure 14 A-D)**. At baseline approximately 8.5% of the total estimated fat mass was distributed in subcutaneous adipose depots, and 91.5% was intra-abdominal. At end point, there was a slight shift in the adipose tissue distribution with 14.5% located within the subcutaneous depot and 85.5% within the abdominal cavity **(Figure 14E)**.

Metabolic Parameters and Adipokines

Similar to previous reports, there was a significant increase in concentrations of serum cholesterol and triglyceride **(Table 6)**. Significant decreases in serum NEFA concentrations also were noted. While there was no significant change in serum insulin concentrations, the mean end point values increased by ~45% from baseline, and all of the cats except one had greater end point insulin measurement than their respective baseline values. The mean serum glucose and HOMA-IR were significantly increased at end point compared to baseline

(Table 6). Serum adiponectin was significantly decreased by ~27% with obesity, and there was a significant ~6.6 fold increase in serum leptin **(Figure 15)**.

Complete Blood Count Parameters and Circulating Cytokines

The mean total WBC count increased by ~10% over time, characterized by a ~25% increase in small lymphocytes. Despite these trends, there were no statistically significant differences noted in any of the CBC parameters, associated with the development of obesity **(Table 7)**. Obese cats had significantly increased concentrations of serum MCP-1 and TNF-α **(Figure 16C and 16D)**. Concentrations of serum IL-6 increased by ~73% and IL-1β increased by ~64% **(Figure 16B and 16E)**. However, neither reached statistical significance. There was no significant difference in serum RANTES concentrations **(Figure 16A)**.

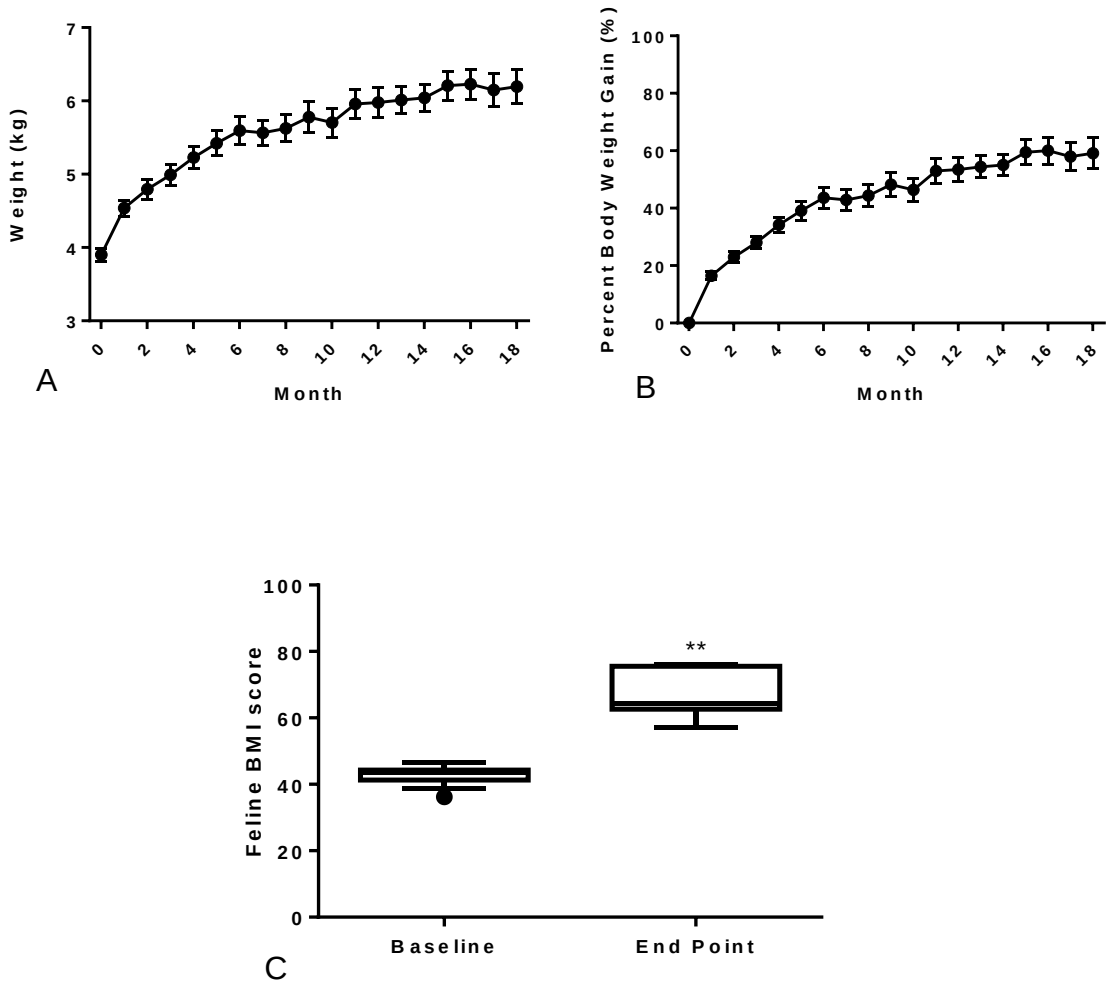


Figure 13. Changes in body weight, percent body weight and feline BMI associated with the development of obesity. Total body weight gain (A), percent body weight gain (B) and change in feline BMI score (C) from baseline to end point. ** P≤ 0.01.

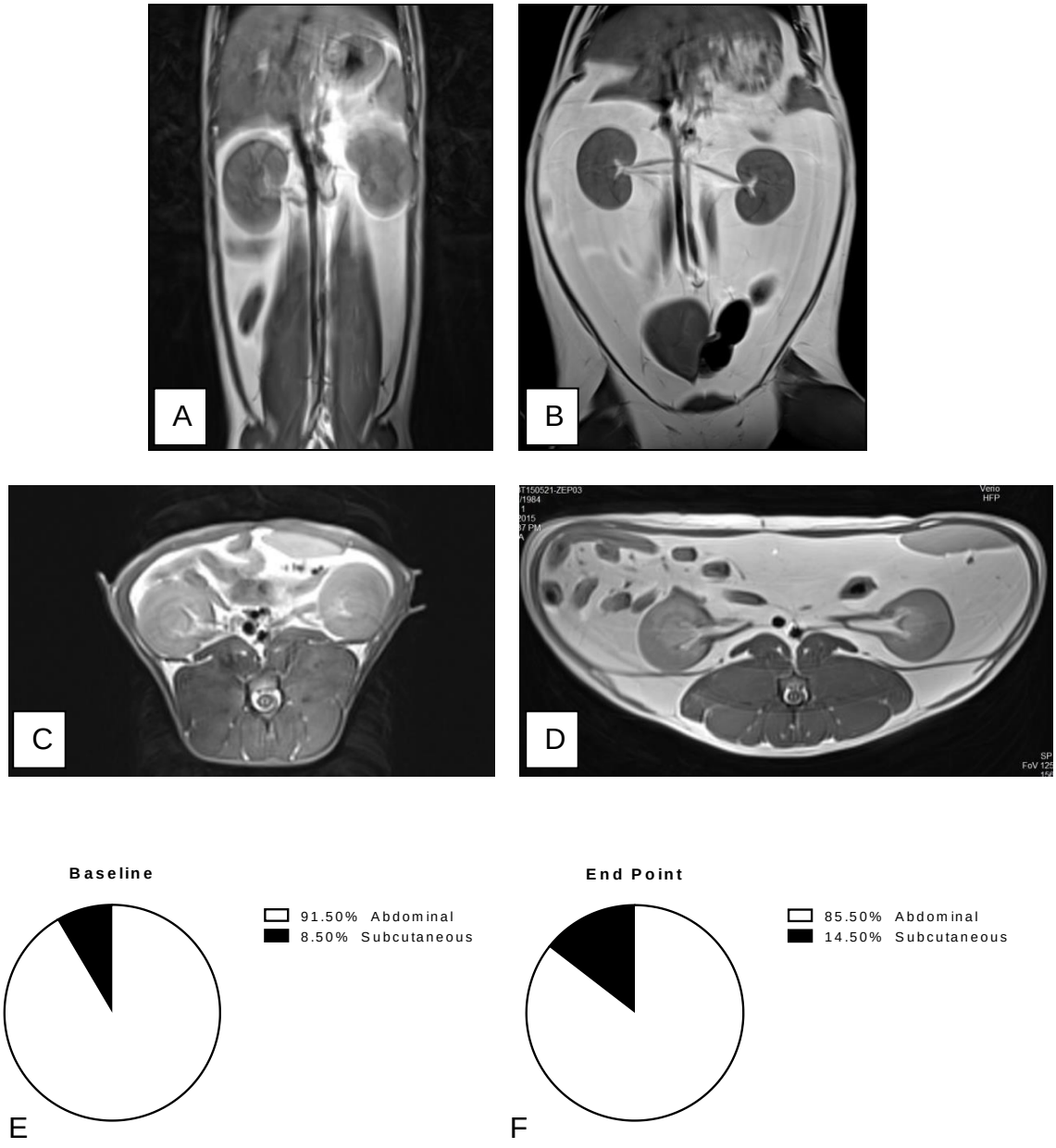
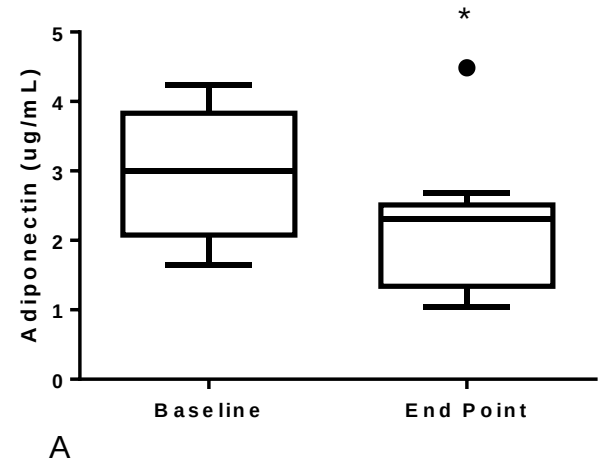


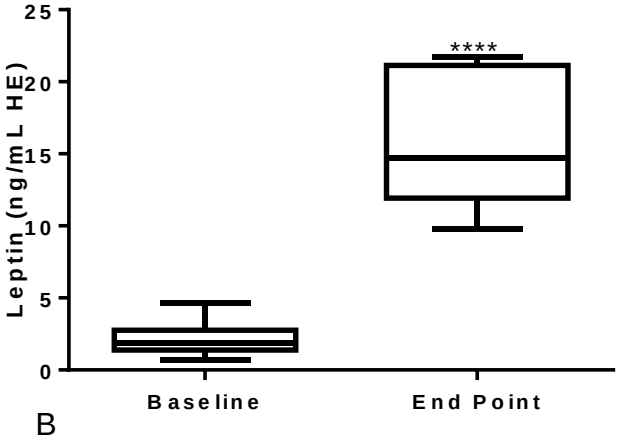
Figure 14. Changes in fat mass and adipose tissue distribution during the development of feline obesity. Representative sections from baseline (A and C) and end point (B and D) of MRI scans and percent distribution of fat mass during the development of obesity (E and F).

Parameter	Baseline	End Point	Significance	P-value
Cholesterol (mg/dL)	124.8 ± 4.97	154.7 ± 7.21	***	0.0006
Triglyceride (mg/dL)	26.36 ± 1.89	65.91 ± 9.09	***	0.0009
NEFA (mg/dL)	39.96 ± 4.55	25.58 ± 2.53	**	0.0027
Glucose (mg/dL)	94.45 ± 4.38	130 ± 12.87	**	0.0098
Insulin (pg/mL)	32.00 ± 5.63	46.36 ± 8.31	ns	0.1197
HOMA-IR	1.06 ± 0.18	1.91 ± 0.24	**	0.0084

Table 6. Metabolic parameters of glucose and lipid metabolism in cats during the development of obesity ** P<0.01; ***P<0.001.



A



B

Figure 15. Changes in serum adipokine concentrations during the development of feline obesity. Changes in concentrations of serum adiponectin (A) and leptin (B) during the development of obesity in cats. •Value exceeds 1.5X the interquartile range from the mean; * P ≤ 0.05; ****P ≤ 0.0001.

Analyte	Baseline	End Point	P-value
Platelets (X10^3/uL)	361.5 ± 19.20	358.5 ± 23.14	0.925
Hematocrit (%)	38.59 ± 0.72	36.65 ± 0.93	0.081
Total WBC (X10^3/uL)	7.56 ± 0.35	8.28 ± 0.48	0.111
Neutrophils (X10^3/uL)	3.53 ± 0.26	3.28 ± 0.25	0.447
Lymphocytes (X10^3/uL)	3.070 ± 0.31	3.82 ± 0.34	0.058
Monocytes (X10^3/uL)	0.302 ± 0.05	0.28 ± 0.03	0.669
Eosinophils (X10^3/uL)	0.67 ± 0.10	0.83 ± 0.13	0.333

Table 7. Complete blood count analytes in cats during the development of obesity.

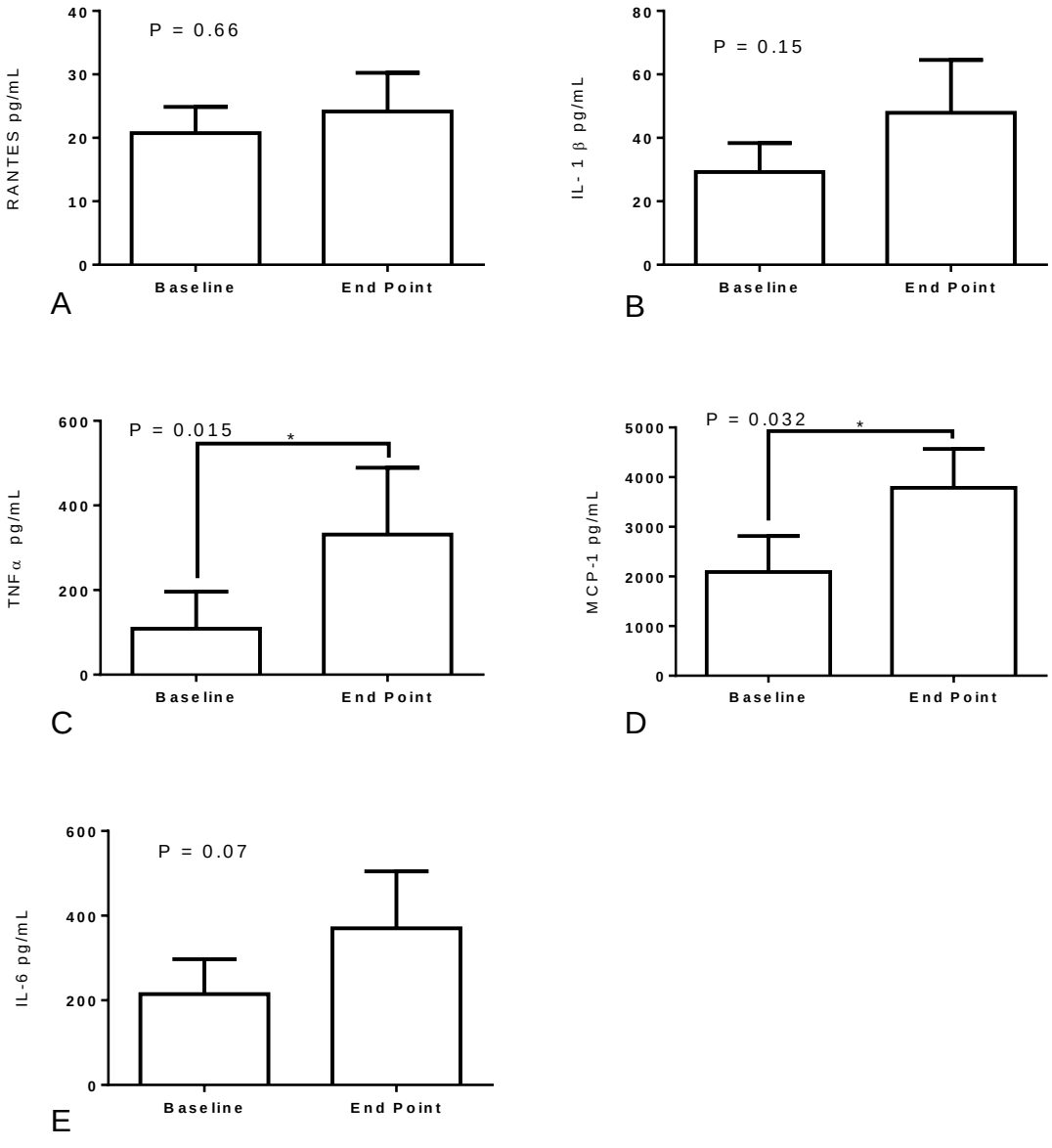


Figure 16. Changes in circulating concentrations of inflammatory markers during the development of feline obesity. RANTES (A), IL-1β (B), TNF-α (C), MCP-1 (D) and IL-6 (E) in cats during the development of obesity. *P ≤ 0.05

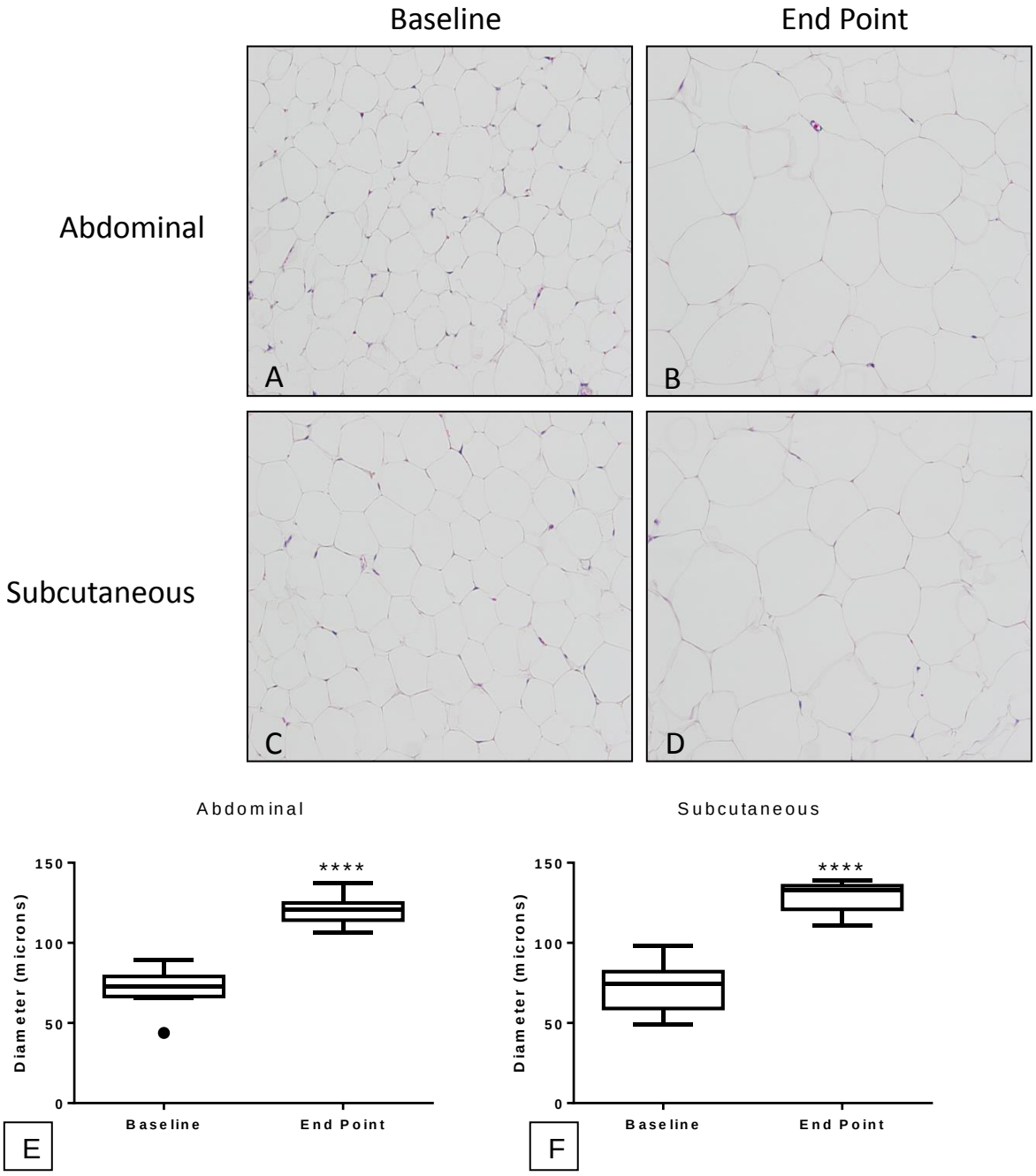


Figure 17. Changes in adipose tissue associated with the development of obesity. Representative images from abdominal (A and B) and subcutaneous (C and D) adipose tissue at baseline (A and C) and end point (B and D), changes in adipocyte diameter in abdominal (E) and subcutaneous (F) adipose tissue. Magnification 200X. *P≤ 0.0001; •Value exceeds 1.5X the interquartile range from the mean.

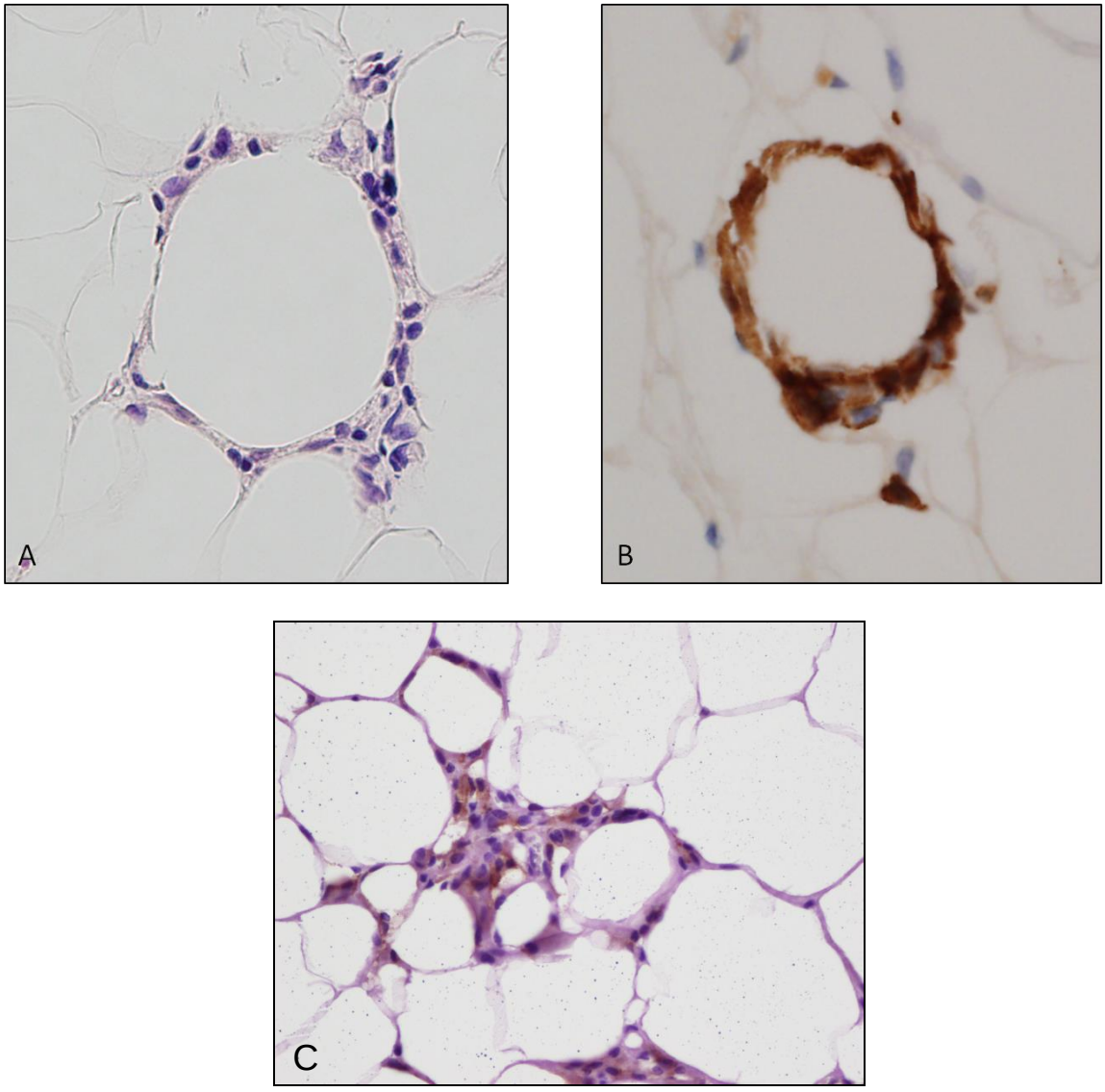


Figure 18. Representative images of crown-like structures (CLS) in feline adipose tissue H&E 400X (A) and confirmatory macrophage marker IBA1 400X (B). Representative image from CLS in adipose tissue from a mouse on a HFD for 12 weeks, F4/80 400X(C).

Adipose tissue

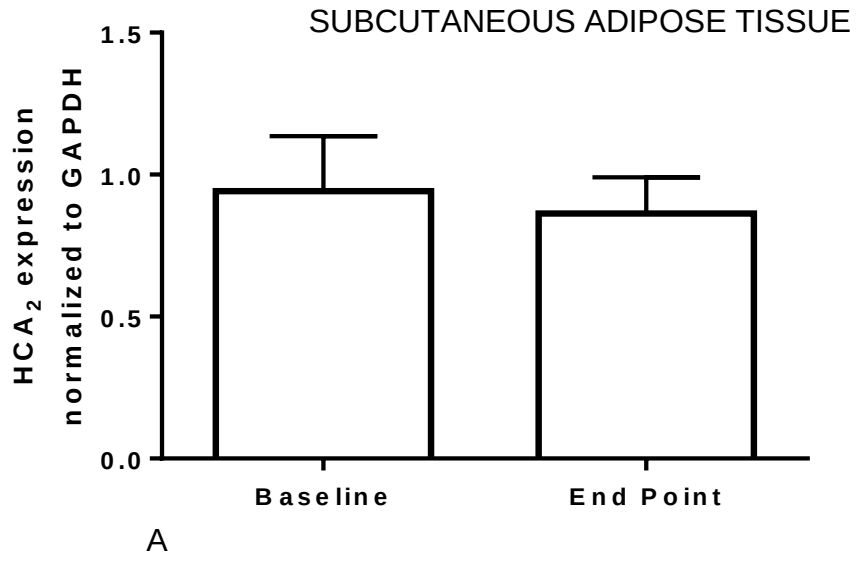
Adipocyte diameter for abdominal and subcutaneous tissue was similar between depots at baseline, with an average diameter of 73 ± 4 µm. At end point, a significant increase in absolute adipocyte diameter in both depots was observed. The mean abdominal adipocyte diameter was 121 ± 2.8 µm (~66% increase from baseline) and the mean subcutaneous diameter was 130 ± 2.6 µm (~79% increase from baseline) **(Figure 17)**. Scattered macrophages were present in low numbers in all adipose tissue samples, and only rare CLS were observed **(Figure 18)**. The majority of samples did not have visually detectable CLS and of those samples where CLS were present only 1 - 5 CLS were seen throughout the entire 2 sections evaluated. Distribution of CLS was independent of time point (baseline and end point) and location (subcutaneous and abdominal).

HCA_2 receptor expression

There was no significant change in HCA_2 gene expression in subcutaneous adipose tissue ($P > 0.999$) from baseline to end point **(Figure 19)**. However, in 8 of the 11 cats evaluated HCA_2 receptor expression in abdominal adipose tissue decreased by approximately one-half from baseline to end point **(Figure 19)**. The remaining three cats had either no change or increased gene expression. Overall, there was a trend toward decreased HCA_2 gene expression in abdominal adipose tissue with increased adiposity ($P = 0.169$), but statistical significance was not observed.

DISCUSSION

There was a dramatic increase in weight in all cats during the 18 month study, as expected. Unlike previous longitudinal studies, our cats did not have restricted activity and were grouped housed for part of each day. While this limited our ability to determine food intake for each individual cat, we feel this model more accurately represents what occurs with people during the development of obesity. Our cats had a broad range of percent body weight gain. However, as seen with other reports of feline obesity, there was dramatic similarity in the overall change in metabolic parameters, with significantly increased leptin, triglycerides, cholesterol and glucose, and decreased insulin sensitivity and adiponectin [167,202]. Metabolic syndrome is defined as increased central obesity, impaired glucose metabolism, hypertension and dyslipidemia [59]. One of the key features of metabolic syndrome, is an altered lipid profile, characterized by increased VLDL and LDL and decreased HDL.



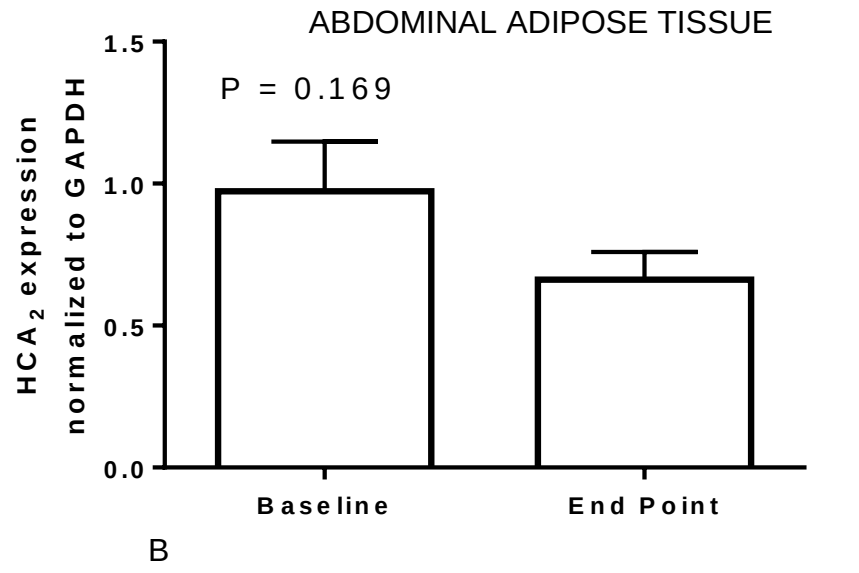


Figure 19. Change in HCA_2 receptor expression during the development of feline obesity. Subcutaneous (A) and abdominal (B) adipose tissue.

Increases in VLDL are directly associated with coronary artery disease, and inversely correlated to insulin sensitivity. Initial work done in cats suggested that obese cats might have increased small particle HDL, and it was thought that this increase in HDL may convey some protective benefits [155]. However, continued evaluation of chronically obese cats showed lipoprotein abnormalities very similar to those described in humans, including changes in small particle HDL concentrations as well as post-prandial lipemia [156]. Other studies indicate that hyperglycemia, not hypertriglyceridemia, plays a more important role in the development of feline insulin resistance and T2DM [293]. Together, these findings suggest that dyslipidemia alone is not sufficient to induce obesity-associated diseases such as hypertension and atherosclerosis. While our study did not investigate changes in lipid profiles, increases in fasting cholesterol and triglyceride concentrations support altered lipid metabolism.

In our study, there was a tendency towards increased fasted insulin concentrations and a significant increase in HOMA-IR. Together, these findings suggest that after 18 months of *ad libitum* feeding there is insulin resistance in our population of cats. This is consistent with other longitudinal studies that observed significant insulin resistance after 12 months [167]. We did not perform glucose tolerance tests in our cats as previous work indicates that HOMA-IR and basal insulin are strong predictors of insulin sensitivity and correlates well with minimal model analysis [289]. In people, an increase in visceral adiposity is highly correlated with decreased insulin sensitivity, dyslipidemia and

atherosclerosis [294]. Unlike previous studies in cats where the distribution of adipose tissue was unaltered during the development of feline obesity [167], our results suggest a shift in adipose tissue distribution. While there was no change in the primary location of overall fat mass within the abdominal cavity, there was an increase in the percentage of total adipose tissue mass within the subcutaneous depot (14.5%) at endpoint as compared to baseline (8.5%). Subsequently, the overall percentage of adipose tissue mass within the abdominal cavity decreased at end point (85.5%) compared to baseline (91.5%). This suggests that during the development of obesity cats preferentially expand their subcutaneous adipose tissue depots compared to their abdominal depots. These findings are supported by the observation that the average diameter of subcutaneous adipocytes increased more than abdominal adipocytes.

Previous studies in depot specific inflammation of feline adipose tissue suggest that increases in inflammatory cytokine gene expression predominates in subcutaneous compared to intra-abdominal adipose tissue [201]. This finding is unique to cats. Proteomic evaluation of human pre-adipocytes indicates that visceral adipose tissue is associated with greater increases in inflammatory cytokines such as IL-6 and MCP-1 [295]. The authors went on to show that visceral adipose tissue also contained increased numbers of ATMs compared to subcutaneous fat [295]. In a separate study, human mesenteric adipose tissue was shown to have an increased inflammatory profile and more closely associated with metabolic complications of obesity than other adipose depots

[296]. Interestingly, human adiponectin gene expression is greater in subcutaneous adipose tissue, and subcutaneous adiponectin gene expression is positively correlated to serum adiponectin and the degree of insulin sensitivity [297]. This is the opposite of what is described in cats, where gene expression of adiponectin is higher in visceral fat depots compared to subcutaneous depots [168,169]. These differences in behavior of the various adipose tissue depots may help explain some of the systemic differences between these species.

Unlike previous feline studies, we observed a significant increase in circulating MCP-1 and TNF-α concentrations in cats during the development of obesity, which suggests that cats develop systemic inflammation associated with obesity. Increases in these circulating markers of inflammation have been consistently reported in obese humans and rodents [230,298]. As previously mentioned, MCP-1 is a chemokine that is associated with increased circulating monocytes and tissue macrophages. Interestingly, we did not observe increased blood monocytes or ATMs in our cats. It is possible that the increased MCP-1 is associated with early inflammation and that with increased time and adiposity, macrophages will increase. However, other studies also indicate that increased ATMs are not a feature of feline obesity [201,202]. In people, increased serum TNF-α, IL-6 and IL-1β are associated with the development of insulin resistance [286]. In our study, there was evidence of decreased insulin sensitivity, increased TNF-α, and a clear trend towards increased concentrations of IL-6 and IL-1β. Together, these findings suggest that the link between increased systemic

inflammatory cytokines and decreased insulin sensitivity is present in cats and similar to what is described in people. The strong trend towards increased IL-6 and IL-1β suggests that with time the differences between obese cats compared to lean in these inflammatory markers may become significant.

Critical components of both innate immunity and the pathogenesis of atherosclerosis are the presence of blood monocytes and circulating adipokines. In our study there were no changes in the concentration of circulating monocytes and neutrophils associated with the development of obesity. This is surprising since we detected increased serum concentrations of MCP-1, and previous results showed that increased gene expression of MCP-1 in obese feline adipose tissue [201]. Adiponectin has been shown to negatively regulate myelomonocytic cell growth and alter macrophage function [245]. Consistent with previous studies, our cats developed decreased serum adiponectin [153,168]. Based on these changes in MCP-1 and adiponectin, we expected to see increased blood monocytes and feline ATMs. A possible explanation for this discrepancy is that MCP-1 may be more critical in other inflammatory and metabolic mechanisms in feline inflammation as compared to humans and rodents. There are also some studies that suggest that MCP-1 may not play as prominent a role in ATM recruitment and insulin sensitivity as previously thought [299]. Further investigation into the role of MCP-1 in macrophage recruitment and decreased insulin sensitivity is warranted.

In this study, serum adipokines were significantly altered with the development of obesity and mirrored what is consistently reported in the literature for cats [153,167-169,201,262]. In people and rodents, adipokines play an important role in immune modulation [57,244,275,300]. Specifically, adiponectin inhibits macrophage function [245] and decreases or alters the production of inflammatory cytokines [66,69,159,244]. Increased leptin is associated with proliferation of monocytes and up-regulation of TLR-2 [274,275]. Given the distinct and consistent change in adipokines and the consistent absence of changes in monocytes and macrophages, it is possible that in cats there discordance between adipokines and their immunologic roles. Closer examination of activation and polarity in circulating monocytes, and evaluation of adipose tissue inflammation in cats is needed.

The number of monocytes and neutrophils remained unchanged in our feline model of obesity, but we did observe a trend towards increased numbers of lymphocytes. This change was not statistically significant, but it is intriguing as T-lymphocytes and gene expression of RANTES appear to be increased in the adipose tissue of obese cats compared to lean [201]. RANTES, also known as CCL5, is a chemokine that plays a role in lymphocyte recruitment. In people, T-lymphocytes precede macrophage infiltration into visceral adipose tissue and contribute to local inflammatory cell activation [301]. RANTES has been implicated in human T2DM [302] and likely plays a role in the development of atherosclerosis [303]. We did not evaluate adipose tissue gene expression of

RANTES in our study, but serum concentrations of this chemokine were unchanged between baseline and endpoint samples. These critical differences suggest that feline obesity-associated inflammation may be mediated by different cell types, and with a different rate of progression than what is described in rodents and people.

One of the most unique aspects of this study is in the evaluation of adipose tissue microanatomy. There is an overall paucity of feline ATMs in both lean and obese cats [201,202]. We evaluated multiple sections of adipose tissue from various depots at baseline and end point during the development of obesity, and only observed rare CLS in samples from both time points, independent of location. This is in stark contrast to what we observe in rodent models (unpublished data) and what is described in the literature [63,203,233]. We did not quantify ATM number in our study, but a recent publication evaluating the SVF of adipose tissue macrophages in various non-rodent species showed that obese cats had a switch in macrophage phenotype that was not associated with an increased number of macrophages [202].

Investigation into ATM macrophages in non-rodent species is critical, as there are key features of mouse and human ATM profiles that are distinctly different [304,305], and there is debate on whether the current rodent models adequately reflect ATM behavior in people [279]. Cats are a particularly intriguing and important species to investigate as they are the only naturally occurring model of

human T2DM with the exception of non-human primates [157]. Another unique and important feature of feline obesity is that despite the similarities to people in insulin resistance, dyslipidemia and metabolic parameters, cats develop a unique obesity-associated inflammatory response and fail to develop atherosclerosis. Nutrient sensing by the immune system may play a key role in regulating obesity-associated inflammation. Previous studies indicate that these pathways are altered with diet-induced obesity [4]. Recent work from our lab characterized the HCA_2 receptor in cats [19], and in this study we show a strong trend towards decreased gene expression of HCA_2 within the abdominal adipose tissue. While we did not observe statistical significance, these changes are consistent with what is observed in rodents [70]. It is possible that with an increased level of adiposity these changes will become significant. This is the first study to explore depot specific changes in the HCA_2 receptor associated with obesity. Interestingly, there was no change in HCA_2 gene expression in the subcutaneous adipose tissue, which may be a more metabolically active than abdominal adipose tissue in cats.

CONCLUSION

This is the first longitudinal study to explore adipose tissue microanatomy along with cellular and humoral markers of inflammation in an insulin resistant feline population. Our findings support the theory that during the development of obesity, cats have a unique immune response that may be important and protect them from certain obesity-associated sequelae such as atherosclerosis, without

altering risks associated with other metabolic and immunological pathologic disorder such as T2DM. The pathologic basis and mechanisms behind the development of T2DM and atherosclerosis are complex and interrelated. The HCA_2 receptor, which has profound anti-inflammatory effects throughout many systems, has been implicated in the development of atherosclerosis. In cats, HCA_2 trends towards decreased gene expression in abdominal adipose tissue during the development of obesity, but is unchanged in subcutaneous adipose tissue. These findings implicate the importance of the cross-talk between the metabolic and immune system as well as depot specific changes in the role of obesity associated sequelae. Cats are an excellent model for the investigation of the relationship between these disease processes and future studies should help us gain a better understanding of underlying mechanisms that drive the development of obesity-associated inflammation.

CHAPTER 5

CONCLUSIONS AND FUTURE OBJECTIVES

Inflammation is associated with a number of diseases processes, including atherosclerosis, obesity, diabetes, multiple sclerosis and cancer. There is mounting evidence that the G-protein coupled receptor, HCA_2 plays an important role in modulating cross-talk between the metabolic and immune systems. This may be especially true in obese individuals. Previous work from our laboratory demonstrates that niacin, a pharmacologic ligand for HCA_2, increases serum concentrations of adiponectin in rodent models of obesity. Thus, the anti-inflammatory adipokine, adiponectin, may play a role in niacin's anti-inflammatory effects. Companion animals provide important models for the study of human disease. In particular, feline obesity and subsequent development of T2DM is an important translational model for human medicine. This is especially true, as cats appear to have a unique immune response during the development of obesity compared to humans and rodents. However, it is unclear if cats develop adipose tissue inflammation similar to what is described in humans and rodents and until now, the HCA_2 receptor had not been identified or characterized in cats.

Our initial studies were directed at using HFD-fed rodent models of obesity to elucidate mechanisms associated with the anti-inflammatory effects of niacin, a

pharmacologic agonist of the HCA_2 receptor, in adipose tissue. Our findings indicate that niacin decreases adipose tissue macrophage recruitment in an adiponectin-independent manner, suggesting that the mechanism behind these changes is directly associated with activation of the HCA_2 receptor on adipose tissue macrophages. In addition, we demonstrated that niacin alters macrophage polarization in an adiponectin-dependent manner, specifically, by reversing niacin's anti-inflammatory effects on macrophage profiles in adipose tissue of mice fed a HFD. This indicates that adiponectin concentrations are critical to niacin's ability to reduce inflammation. Further studies such as histologic evaluation of adipose tissue and flow cytometry of the SVF would allow for more accurate estimation, and further evaluation of the macrophage populations. Additional investigations in cytokine profiles and changes in HCA_2 receptor expression of individual cell types during the development of obesity would also help elucidate the mechanisms underlying the anti-inflammatory effects of HCA2 agonists.

Next, we evaluated the HCA_2 receptor and its potential role during the development of obesity-associated inflammation in cats, a translation model of human obesity. In these studies we demonstrated that the HCA_2 receptor has similar structure and function in rodents, humans, and cats, which indicate that regulation of lipolysis and pathophysiology of obesity-related disorders related to this receptor function in similar manner across these species. We completed the first longitudinal study to explore adipose tissue microanatomy and markers of

systemic inflammation during the development of obesity and insulin resistance in cats. While feline obesity is associated with the development of altered glucose and lipid metabolism, there was 1) an absence of ATMs independent of time and location, 2) variable change in systemic markers of inflammation, with an increase in some serum cytokine concentrations, but no change in circulating immune cells, and 3) a shift in the distribution of adipose tissue, characterized by an increased percentage of total fat mass within the subcutaneous adipose tissue. Previous work from our lab demonstrated decreased HCA_2 gene expression in HFD-fed rodent models of obesity, which suggests a possible mechanism for obesity-associated alterations in lipolysis and immune regulation. In cats, we showed that with the development of obesity the feline HCA_2 receptor gene expression tends to decrease in abdominal tissue, but is unchanged in subcutaneous adipose tissue. Our findings from this study support the idea that during the development of obesity, cats have a unique immune response that appears to protect them from certain obesity-associated sequelae such as atherosclerosis, while sustaining other metabolic and immunological pathologic disorder such as T2DM. The pathologic bases and mechanisms behind the development of T2DM and atherosclerosis are complex and interrelated. The HCA_2 receptor, which has profound anti-inflammatory effects throughout many systems and been implicated in the development of atherosclerosis, does not decrease in feline subcutaneous adipose tissue, which may play a more important role for metabolic-immune cross-talk in cats, than it does in other species. Cats are an excellent model for investigations of the relationship

between these disease processes, and future studies should help us gain a better understanding of underlying mechanisms that drive the development of obesity-associated inflammation.

REFERENCES

[1] Blad CC, Ahmed K, AP IJ, Offermanns S. Biological and pharmacological roles of HCA receptors. Adv Pharmacol. 2011;62:219-50.

[2] Offermanns S, Colletti SL, Lovenberg TW, Semple G, Wise A, AP IJ. International Union of Basic and Clinical Pharmacology. LXXXII: Nomenclature and Classification of Hydroxy-carboxylic Acid Receptors (GPR81, GPR109A, and GPR109B). Pharmacol Rev. 2011;63:269-90.

[3] Ahmed K. Biological roles and therapeutic potential of hydroxy-carboxylic Acid receptors. Front Endocrinol (Lausanne). 2011;2:51.

[4] Wanders D, Graff EC, White BD, Judd RL. Niacin increases adiponectin and decreases adipose tissue inflammation in high fat diet-fed mice. PLoS One. 2013;8:e71285.

[5] Lukasova M, Malaval C, Gille A, Kero J, Offermanns S. Nicotinic acid inhibits progression of atherosclerosis in mice through its receptor GPR109A expressed by immune cells. J Clin Invest. 2011;121:1163-73.

[6] Hanson J, Gille A, Zwykiel S, Lukasova M, Clausen BE, Ahmed K, Tunaru S, Wirth A, Offermanns S. Nicotinic acid- and monomethyl fumarate-induced flushing involves GPR109A expressed by keratinocytes and COX-2-dependent prostanoid formation in mice. J Clin Invest. 2010;120:2910-9.

[7] Maciejewski-Lenoir D, Richman JG, Hakak Y, Gaidarov I, Behan DP, Connolly DT. Langerhans cells release prostaglandin D2 in response to nicotinic acid. J Invest Dermatol. 2006;126:2637-46.

[8] Tang H, Lu JY, Zheng X, Yang Y, Reagan JD. The psoriasis drug monomethylfumarate is a potent nicotinic acid receptor agonist. Biochem Biophys Res Commun. 2008;375:562-5.

[9] Soga T, Kamohara M, Takasaki J, Matsumoto S, Saito T, Ohishi T, Hiyama H, Matsuo A, Matsushime H, Furuichi K. Molecular identification of nicotinic acid receptor. Biochem Biophys Res Commun. 2003;303:364-9.

[10] Tunaru S, Kero J, Schaub A, Wufka C, Blaukat A, Pfeffer K, Offermanns S. PUMA-G and HM74 are receptors for nicotinic acid and mediate its anti-lipolytic effect. Nat Med. 2003;9:352-5.

[11] Wise A, Foord SM, Fraser NJ, Barnes AA, Elshourbagy N, Eilert M, Ignar DM, Murdock PR, Steplewski K, Green A, Brown AJ, Dowell SJ, Szekeres PG,

Hassall DG, Marshall FH, Wilson S, Pike NB. Molecular identification of high and low affinity receptors for nicotinic acid. J Biol Chem. 2003;278:9869-74.

[12] Schaub A, Futterer A, Pfeffer K. PUMA-G, an IFN-gamma-inducible gene in macrophages is a novel member of the seven transmembrane spanning receptor superfamily. Eur J Immunol. 2001;31:3714-25.

[13] Elangovan S, Pathania R, Ramachandran S, Ananth S, Padia RN, Lan L, Singh N, Martin PM, Hawthorn L, Prasad PD, Ganapathy V, Thangaraju M. The niacin/butyrate receptor GPR109A suppresses mammary tumorigenesis by inhibiting cell survival. Cancer Res. 2014;74:1166-78.

[14] Gambhir D, Ananth S, Veeranan-Karmegam R, Elangovan S, Hester S, Jennings E, Offermanns S, Nussbaum JJ, Smith SB, Thangaraju M, Ganapathy V, Martin PM. GPR109A as an anti-inflammatory receptor in retinal pigment epithelial cells and its relevance to diabetic retinopathy. Invest Ophthalmol Vis Sci. 2012;53:2208-17.

[15] Rahman M, Muhammad S, Khan MA, Chen H, Ridder DA, Müller-Fielitz H, Pokorná B, Vollbrandt T, Stölting I, Nadrowitz R, Okun JG, Offermanns S, Schwaninger M. The β-hydroxybutyrate receptor HCA2 activates a neuroprotective subset of macrophages. Nat Commun. 2014;5:1-15.

[16] Kostylina G, Simon D, Fey MF, Yousefi S, Simon HU. Neutrophil apoptosis mediated by nicotinic acid receptors (GPR109A). Cell Death Differ. 2008;15:134-42.

[17] Li X, Millar JS, Brownell N, Briand F, Rader DJ. Modulation of HDL metabolism by the niacin receptor GPR109A in mouse hepatocytes. Biochem Pharmacol. 2010;80:1450-7.

[18] Ge H, Weiszmann J, Reagan JD, Gupte J, Baribault H, Gyuris T, Chen J-L, Tian H, Li Y. Elucidation of signaling and functional activities of an orphan GPCR, GPR81. J Lipid Res. 2008;49:797-803.

[19] Graff EC, Norris OC, Sandey M, Kemppainen RJ, Judd RL. Characterization of the hydroxycarboxylic acid receptor 2 in cats. Domest Anim Endocrinol. 2015;53:88-94.

[20] Carlson LA. Studies on the effect of nicotinic acid on catecholamine stimulated lipolysis in adipose tissue in vitro. Acta Med Scand. 1963;173:719-22.
[21] Carlson LA, Oro L. The effect of nicotinic acid on the plasma free fatty acid; demonstration of a metabolic type of sympathicolysis. Acta Med Scand. 1962;172:641-5.

[22] Thangaraju M, Cresci GA, Liu K, Ananth S, Gnanaprakasam JP, Browning DD, Mellinger JD, Smith SB, Digby GJ, Lambert NA, Prasad PD, Ganapathy V. GPR109A is a G-protein-coupled receptor for the bacterial fermentation product butyrate and functions as a tumor suppressor in colon. Cancer Res. 2009;69:2826-32.

[23] Taggart AK, Kero J, Gan X, Cai TQ, Cheng K, Ippolito M, Ren N, Kaplan R, Wu K, Wu TJ, Jin L, Liaw C, Chen R, Richman J, Connolly D, Offermanns S, Wright SD, Waters MG. (D)-beta-Hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G. J Biol Chem. 2005;280:26649-52.

[24] Senior B, Loridan L. Direct Regulatory Effect of Ketones on Lipolysis and on Glucose Concentrations in Man. Nature. 1968;219:83-4.

[25] Gille A, Bodor ET, Ahmed K, Offermanns S. Nicotinic acid: pharmacological effects and mechanisms of action. Annu Rev Pharmacol Toxicol. 2008;48:79-106.

[26] Offermanns S. The nicotinic acid receptor GPR109A (HM74A or PUMA-G) as a new therapeutic target. Trends Pharmacol Sci. 2006;27:384-90.

[27] Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, Bultman SJ. The Microbiome and Butyrate Regulate Energy Metabolism and Autophagy in the Mammalian Colon. Cell Metab. 2011;13:517-26.

[28] Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, Lefevre M, Cefalu WT, Ye J. Butyrate Improves Insulin Sensitivity and Increases Energy Expenditure in Mice. Diabetes. 2009;58:1509-17.

[29] Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, Calignano A. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol. 2011;17:1519-28.

[30] Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, Thangaraju M, Prasad PD, Manicassamy S, Munn DH, Lee JR, Offermanns S, Ganapathy V. Activation of the receptor (Gpr109a) for niacin and the commensal metabolite butyrate suppresses colonic inflammation and carcinogenesis. Immunity. 2014;40:128-39.

[31] Bobileva O, Bokaldere R, Gailite V, Kaula I, Ikaunieks M, Duburs G, Petrovska R, Mandrika I, Klovins J, Loza E. Synthesis and evaluation of (E)-2-(acrylamido)cyclohex-1-enecarboxylic acid derivatives as HCA1, HCA2, and HCA3 receptor agonists. Bioorg Med Chem. 2014;22:3654-69.

[32] Altschul R, Hoffer A, Stephen JD. Influence of nicotinic acid on serum cholesterol in man. Arch Biochem Biophys. 1955;54:558-9.

[33] Kwon WY, Suh GJ, Kim KS, Kwak YH. Niacin attenuates lung inflammation and improves survival during sepsis by downregulating the nuclear factor-kappaB pathway. Crit Care Med. 2011;39:328-34.

[34] Cho KH, Kim HJ, Rodriguez-Iturbe B, Vaziri ND. Niacin ameliorates oxidative stress, inflammation, proteinuria, and hypertension in rats with chronic renal failure. American journal of physiology Renal physiology. 2009;297:F106-13.

[35] Fox RJ, Miller DH, Phillips JT, Hutchinson M, Havrdova E, Kita M, Yang M, Raghupathi K, Novas M, Sweetser MT, Viglietta V, Dawson KT. Placebo-Controlled Phase 3 Study of Oral BG-12 or Glatiramer in Multiple Sclerosis. N Engl J Med. 2012;367:1087-97.

[36] Fu SP, Li SN, Wang JF, Li Y, Xie SS, Xue WJ, Liu HM, Huang BX, Lv QK, Lei LC, Liu GW, Wang W, Liu JX. BHBA suppresses LPS-induced inflammation in BV-2 cells by inhibiting NF-kappaB activation. Mediators Inflamm. 2014;2014:983401.

[37] Youm YH, Nguyen KY, Grant RW, Goldberg EL, Bodogai M, Kim D, D'Agostino D, Planavsky N, Lupfer C, Kanneganti TD, Kang S, Horvath TL, Fahmy TM, Crawford PA, Biragyn A, Alnemri E, Dixit VD. The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. Nat Med. 2015;21:263-9.

[38] Digby JE, Martinez F, Jefferson A, Ruparelia N, Chai J, Wamil M, Greaves DR, Choudhury RP. Anti-inflammatory effects of nicotinic acid in human monocytes are mediated by GPR109A dependent mechanisms. Arterioscler Thromb Vasc Biol. 2012;32:669-76.

[39] Digby JE, McNeill E, Dyar OJ, Lam V, Greaves DR, Choudhury RP. Anti-inflammatory effects of nicotinic acid in adipocytes demonstrated by suppression of fractalkine, RANTES, and MCP-1 and upregulation of adiponectin. Atherosclerosis. 2010;209:89-95.

[40] Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. Cell. 2011;145:341-55.

[41] van der Hoorn JW, de Haan W, Berbee JF, Havekes LM, Jukema JW, Rensen PC, Princen HM. Niacin increases HDL by reducing hepatic expression and plasma levels of cholesteryl ester transfer protein in APOE*3Leiden.CETP mice. Arterioscler Thromb Vasc Biol. 2008;28:2016-22.

[42] Lauring B, Taggart AK, Tata JR, Dunbar R, Caro L, Cheng K, Chin J, Colletti SL, Cote J, Khalilieh S, Liu J, Luo WL, Maclean AA, Peterson LB, Polis AB, Sirah W, Wu TJ, Liu X, Jin L, Wu K, Boatman PD, Semple G, Behan DP, Connolly DT,

Lai E, Wagner JA, Wright SD, Cuffie C, Mitchel YB, Rader DJ, Paolini JF, Waters MG, Plump A. Niacin lipid efficacy is independent of both the niacin receptor GPR109A and free fatty acid suppression. Sci Transl Med. 2012;4:148ra15.

[43] Wu BJ, Chen K, Barter PJ, Rye KA. Niacin inhibits vascular inflammation via the induction of heme oxygenase-1. Circulation. 2012;125:150-8.

[44] Wu BJ, Yan L, Charlton F, Witting P, Barter PJ, Rye KA. Evidence that niacin inhibits acute vascular inflammation and improves endothelial dysfunction independent of changes in plasma lipids. Arterioscler Thromb Vasc Biol. 2010;30:968-75.

[45] Ruparelia N, Digby JE, Choudhury RP. Effects of niacin on atherosclerosis and vascular function. Curr Opin Cardiol. 2011;26:66-70.

[46] Zandi-Nejad K, Takakura A, Jurewicz M, Chandraker AK, Offermanns S, Mount D, Abdi R. The role of HCA2 (GPR109A) in regulating macrophage function. FASEB J. 2013;27:4366-74.

[47] Plaisance EP, Lukasova M, Offermanns S, Zhang Y, Cao G, Judd RL. Niacin stimulates adiponectin secretion through the GPR109A receptor. Am J Physiol Endocrinol Metab. 2009;296:E549-58.

[48] Fraterrigo G, Fabbrini E, Mittendorfer B, O'Rahilly S, Scherer PE, Patterson BW, Klein S. Relationship between changes in plasma adiponectin concentration and insulin sensitivity after niacin therapy. Cardiorenal Med. 2012;2:211-7.

[49] Hu M, Yang YL, Masuda D, Yamashita S, Tomlinson B. Effect of Extended-Release Niacin/Laropiprant Combination on Plasma Adiponectin and Insulin Resistance in Chinese Patients with Dyslipidaemia. Dis Markers. 2015;2015:154014.

[50] Plaisance EP, Grandjean PW, Brunson BL, Judd RL. Increased total and high-molecular weight adiponectin after extended-release niacin. Metabolism. 2008;57:404-9.

[51] Westphal S, Borucki K, Taneva E, Makarova R, Luley C. Adipokines and treatment with niacin. Metabolism. 2006;55:1283-5.

[52] Westphal S, Luley C. Preferential increase in high-molecular weight adiponectin after niacin. Atherosclerosis. 2008;198:179-83.

[53] Deng G, Long Y, Yu YR, Li MR. Adiponectin directly improves endothelial dysfunction in obese rats through the AMPK-eNOS Pathway. Int J Obes (Lond). 2010;34:165-71.

[54] Arita Y, Kihara S, Ouchi N, Maeda K, Kuriyama H, Okamoto Y, Kumada M, Hotta K, Nishida M, Takahashi M, Nakamura T, Shimomura I, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. Circulation. 2002;105:2893-8.

[55] Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T, Matsuzawa Y. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. Circulation. 2001;103:1057-63.

[56] Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. Circulation. 1999;100:2473-6.

[57] Folco EJ, Rocha VZ, Lopez-Ilasaca M, Libby P. Adiponectin inhibits pro-inflammatory signaling in human macrophages independent of interleukin-10. J Biol Chem. 2009;284:25569-75.

[58] Kahn BB, Flier JS. Obesity and insulin resistance. J Clin Invest. 2000;106:473-81.

[59] Prasad H, Ryan DA, Celzo MF, Stapleton D. Metabolic syndrome: definition and therapeutic implications. Postgrad Med. 2012;124:21-30.

[60] Halberg N, Wernstedt-Asterholm I, Scherer PE. The adipocyte as an endocrine cell. Endocrinol Metab Clin North Am. 2008;37:753-68, x-xi.

[61] Hotta K, Funahashi T, Bodkin NL, Ortmeyer HK, Arita Y, Hansen BC, Matsuzawa Y. Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. Diabetes. 2001;50:1126-33.

[62] Trujillo ME, Scherer PE. Adiponectin--journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. J Intern Med. 2005;257:167-75.

[63] Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003;112:1796-808.

[64] Hill AA, Reid Bolus W, Hasty AH. A decade of progress in adipose tissue macrophage biology. Immunol Rev. 2014;262:134-52.

[65] Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest. 2007;117:175-84.

[66] Ouchi N, Walsh K. Adiponectin as an anti-inflammatory factor. Clin Chim Acta. 2007;380:24-30.

[67] Pyrzak B, Ruminska M, Popko K, Demkow U. Adiponectin as a biomarker of the metabolic syndrome in children and adolescents. Eur J Med Res. 2010;15 Suppl 2:147-51.

[68] Mandal P, Pratt BT, Barnes M, McMullen MR, Nagy LE. Molecular mechanism for adiponectin-dependent M2 macrophage polarization: link between the metabolic and innate immune activity of full-length adiponectin. J Biol Chem. 2011;286:13460-9.

[69] 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. 2000;102:1296-301.

[70] Wanders D, Graff EC, Judd RL. Effects of high fat diet on GPR109A and GPR81 gene expression. Biochem Biophys Res Commun. 2012;425:278-83.

[71] Chai JT, Digby JE, Ruparelia N, Jefferson A, Handa A, Choudhury RP. Nicotinic acid receptor GPR109A is down-regulated in human macrophage-derived foam cells. PLoS One. 2013;8:e62934.

[72] Feingold KR, Moser A, Shigenaga JK, Grunfeld C. Inflammation stimulates niacin receptor (GPR109A/HCA2) expression in adipose tissue and macrophages. J Lipid Res. 2014;55:2501-8.

[73] Jobin C. GPR109a: the missing link between microbiome and good health? Immunity. 2014;40:8-10.

[74] Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther. 2008;27:104-19.

[75] Guzman JR, Conlin VS, Jobin C. Diet, Microbiome, and the Intestinal Epithelium: An Essential Triumvirate? Biomed Res Int. 2013:1-12.

[76] Davie JR. Inhibition of histone deacetylase activity by butyrate. J Nutr. 2003;133:2485S-93S.

[77] Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc Natl Acad Sci U S A. 2014;111:2247-52.

[78] Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, Maruya M, Ian McKenzie C, Hijikata A, Wong C, Binge L, Thorburn AN, Chevalier N, Ang C, Marino E, Robert R, Offermanns S, Teixeira MM, Moore RJ, Flavell RA, Fagarasan S, Mackay CR. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nat Commun. 2015;6:1-15.

[79] Nentwich MM, Ulbig MW. Diabetic retinopathy - ocular complications of diabetes mellitus. World J Diabetes. 2015;6:489-99.

[80] Bandello F, Lattanzio R, Zucchiatti I, Del Turco C. Pathophysiology and treatment of diabetic retinopathy. Acta Diabetol. 2013;50:1-20.

[81] Bhagat N, Grigorian RA, Tutela A, Zarbin MA. Diabetic macular edema: pathogenesis and treatment. Surv Ophthalmol. 2009;54:1-32.

[82] Joussen AM, Murata T, Tsujikawa A, Kirchhof B, Bursell SE, Adamis AP. Leukocyte-mediated endothelial cell injury and death in the diabetic retina. Am J Pathol. 2001;158:147-52.

[83] Martin PM, Ananth S, Cresci G, Roon P, Smith S, Ganapathy V. Expression and localization of GPR109A (PUMA-G/HM74A) mRNA and protein in mammalian retinal pigment epithelium. Mol Vis. 2009;15:362-72.

[84] Yu AL, Birke K, Lorenz RL, Welge-Lussen U. Constitutive expression of HCA(2) in human retina and primary human retinal pigment epithelial cells. Curr Eye Res. 2014;39:487-92.

[85] Detrick B, Hooks JJ. Immune regulation in the retina. Immunol Res. 2010;47:153-61.

[86] Promsote W, Veeranan-Karmegam R, Ananth S, Shen D, Chan CC, Lambert NA, Ganapathy V, Martin PM. L-2-oxothiazolidine-4-carboxylic acid attenuates oxidative stress and inflammation in retinal pigment epithelium. Mol Vis. 2014;20:73-88.

[87] Dupuis N, Curatolo N, Benoist JF, Auvin S. Ketogenic diet exhibits anti-inflammatory properties. Epilepsia. 2015:1-4.

[88] Tolaymat A, Nayak A, Geyer JD, Geyer SK, Carney PR. Diagnosis and Management of Childhood Epilepsy. Current Problems in Pediatric and Adolescent Health Care. 2015;45:3-17.

[89] Kashiwaya Y, Bergman C, Lee J-H, Wan R, King MT, Mughal MR, Okun E, Clarke K, Mattson MP, Veech RL. A ketone ester diet exhibits anxiolytic and cognition-sparing properties, and lessens amyloid and tau pathologies in a mouse model of Alzheimer's disease. Neurobiol Aging. 2013;34:1530-9.

[90] Van der Auwera I, Wera S, Van Leuven F, Henderson ST. A ketogenic diet reduces amyloid beta 40 and 42 in a mouse model of Alzheimer's disease. Nutr Metab (Lond). 2005;2:28-36.

[91] Zhao Z, Lange DJ, Voustianiouk A, MacGrogan D, Ho L, Suh J, Humala N, Thiyagarajan M, Wang J, Pasinetti GM. A ketogenic diet as a potential novel therapeutic intervention in amyotrophic lateral sclerosis. BMC Neurosci. 2006;7:1-10.

[92] Lim S, Chesser AS, Grima JC, Rappold PM, Blum D, Przedborski S, Tieu K. D-β-Hydroxybutyrate Is Protective in Mouse Models of Huntington's Disease. PLoS ONE. 2011;6:e24620.

[93] Tieu K, Perier C, Caspersen C, Teismann P, Wu DC, Yan SD, Naini A, Vila M, Jackson-Lewis V, Ramasamy R, Przedborski S. D-beta-hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease. J Clin Invest. 2003;112:892-901.

[94] Vanitallie TB, Nonas C, Di Rocco A, Boyar K, Hyams K, Heymsfield SB. Treatment of Parkinson disease with diet-induced hyperketonemia: a feasibility study. Neurology. 2005;64:728-30.

[95] Kim do Y, Hao J, Liu R, Turner G, Shi FD, Rho JM. Inflammation-mediated memory dysfunction and effects of a ketogenic diet in a murine model of multiple sclerosis. PLoS One. 2012;7:e35476.

[96] Puchowicz MA, Zechel JL, Valerio J, Emancipator DS, Xu K, Pundik S, LaManna JC, Lust WD. Neuroprotection in diet-induced ketotic rat brain after focal ischemia. J Cereb Blood Flow Metab. 2008;28:1907-16.

[97] Hu ZG, Wang HD, Qiao L, Yan W, Tan QF, Yin HX. The protective effect of the ketogenic diet on traumatic brain injury-induced cell death in juvenile rats. Brain Inj. 2009;23:459-65.

[98] Cheng B, Yang X, An L, Gao B, Liu X, Liu S. Ketogenic diet protects dopaminergic neurons against 6-OHDA neurotoxicity via up-regulating glutathione in a rat model of Parkinson's disease. Brain Res. 2009;1286:25-31.

[99] Yang X, Cheng B. Neuroprotective and anti-inflammatory activities of ketogenic diet on MPTP-induced neurotoxicity. J Mol Neurosci. 2010;42:145-53.

[100] Fu SP, Wang JF, Xue WJ, Liu HM, Liu BR, Zeng YL, Li SN, Huang BX, Lv QK, Wang W, Liu JX. Anti-inflammatory effects of BHBA in both in vivo and in vitro Parkinson disease models are mediated by GPR109A-dependent mechanisms. J Neuroinflammation. 2015;12:1-14.

[101] Suzuki M, Suzuki M, Kitamura Y, Mori S, Sato K, Dohi S, Sato T, Matsuura A, Hiraide A. Beta-hydroxybutyrate, a cerebral function improving agent, protects rat brain against ischemic damage caused by permanent and transient focal cerebral ischemia. Jpn J Pharmacol. 2002;89:36-43.

[102] Chen H, Assmann JC, Krenz A, Rahman M, Grimm M, Karsten CM, Kohl J, Offermanns S, Wettschureck N, Schwaninger M. Hydroxycarboxylic acid receptor 2 mediates dimethyl fumarate's protective effect in EAE. J Clin Invest. 2014;124:2188-92.

[103] Parodi B, Rossi S, Morando S, Cordano C, Bragoni A, Motta C, Usai C, Wipke BT, Scannevin RH, Mancardi GL, Centonze D, Kerlero de Rosbo N, Uccelli A. Fumarates modulate microglia activation through a novel HCAR2 signaling pathway and rescue synaptic dysregulation in inflamed CNS. Acta Neuropathol. 2015.

[104] Offermanns S, Schwaninger M. Nutritional or pharmacological activation of HCA ameliorates neuroinflammation. Trends Mol Med. 2015:245-55.

[105] Masuda Y, Ochi Y, Ochi Y, Karasawa T, Hatano N, Kadokawa T, Shimizu M. Protective effect of prostaglandins D2, E1 and I2 against cerebral hypoxia/anoxia in mice. Naunyn Schmiedebergs Arch Pharmacol. 1986;334:282-9.

[106] Taniguchi H, Mohri I, Okabe-Arahori H, Aritake K, Wada K, Kanekiyo T, Narumiya S, Nakayama M, Ozono K, Urade Y, Taniike M. Prostaglandin D2 protects neonatal mouse brain from hypoxic ischemic injury. J Neurosci. 2007;27:4303-12.

[107] Zhao X, Strong R, Zhang J, Sun G, Tsien JZ, Cui Z, Grotta JC, Aronowski J. Neuronal PPARgamma deficiency increases susceptibility to brain damage after cerebral ischemia. J Neurosci. 2009;29:6186-95.

[108] Kim EH, Surh YJ. The role of 15-deoxy-delta(12,14)-prostaglandin J(2), an endogenous ligand of peroxisome proliferator-activated receptor gamma, in tumor angiogenesis. Biochem Pharmacol. 2008;76:1544-53.

[109] Chai EZ, Siveen KS, Shanmugam MK, Arfuso F, Sethi G. Analysis of the intricate relationship between chronic inflammation and cancer. Biochem J. 2015;468:1-15.

[110] Hoque R, Mehal WZ. Inflammasomes in Pancreatic Physiology and Disease2015.

[111] Chen L, So WY, Li SY, Cheng Q, Boucher BJ, Leung PS. Niacin-induced hyperglycemia is partially mediated via niacin receptor GPR109a in pancreatic islets. Mol Cell Endocrinol. 2015;404:56-66.

[112] Weiner DE. Causes and consequences of chronic kidney disease: implications for managed health care. J Manag Care Pharm. 2007;13:S1-9.

[113] Cho KH, Kim HJ, Kamanna VS, Vaziri ND. Niacin improves renal lipid metabolism and slows progression in chronic kidney disease. Biochim Biophys Acta. 2010;1800:6-15.

[114] Bostom AG, Maclean AA, Maccubbin D, Tipping D, Giezek H, Hanlon WA. Extended-release niacin/laropiprant lowers serum phosphorus concentrations in patients with type 2 diabetes. J Clin Lipidol. 2011;5:281-7.

[115] Maccubbin D, Tipping D, Kuznetsova O, Hanlon WA, Bostom AG. Hypophosphatemic effect of niacin in patients without renal failure: a randomized trial. Clinical journal of the American Society of Nephrology : CJASN. 2010;5:582-9.

[116] Gurujeyalakshmi G, Wang Y, Giri SN. Taurine and niacin block lung injury and fibrosis by down-regulating bleomycin-induced activation of transcription nuclear factor-kappaB in mice. J Pharmacol Exp Ther. 2000;293:82-90.

[117] Hanson J, Gille A, Offermanns S. Role of HCA2 (GPR109A) in nicotinic acid and fumaric acid ester-induced effects on the skin. Pharmacol Ther. 2012;136:1-7.

[118] Helwa I, Patel R, Karempelis P, Kaddour-Djebbar I, Choudhary V, Bollag WB. The antipsoriatic agent monomethylfumarate has antiproliferative, prodifferentiative, and anti-inflammatory effects on keratinocytes. J Pharmacol Exp Ther. 2015;352:90-7.

[119] Nantel F, Denis D, Gordon R, Northey A, Cirino M, Metters KM, Chan CC. Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation. Br J Pharmacol. 1999;128:853-9.

[120] Hammarstrom S, Hamberg M, Samuelsson B, Duell EA, Stawiski M, Voorhees JJ. Increased concentrations of nonesterified arachidonic acid, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid, prostaglandin E2, and prostaglandin F2alpha in epidermis of psoriasis. Proc Natl Acad Sci U S A. 1975;72:5130-4.

[121] Reilly DM, Parslew R, Sharpe GR, Powell S, Green MR. Inflammatory mediators in normal, sensitive and diseased skin types. Acta Derm Venereol. 2000;80:171-4.

[122] Sandoe P, Palmer C, Corr S, Astrup A, Bjornvad CR. Canine and feline obesity: a One Health perspective. Vet Rec. 2014;175:610-6.

[123] Cave NJ, Allan FJ, Schokkenbroek SL, Metekohy CA, Pfeiffer DU. A cross-sectional study to compare changes in the prevalence and risk factors for feline obesity between 1993 and 2007 in New Zealand. Prev Vet Med. 2012;107:121-33.

[124] Colliard L, Paragon BM, Lemuet B, Benet JJ, Blanchard G. Prevalence and risk factors of obesity in an urban population of healthy cats. J Feline Med Surg. 2009;11:135-40.

[125] Courcier EA, O'Higgins R, Mellor DJ, Yam PS. Prevalence and risk factors for feline obesity in a first opinion practice in Glasgow, Scotland. J Feline Med Surg. 2010;12:746-53.

[126] Lund EM, Armstrong P, Kirk CA, Klausner J. Prevalence and risk factors for obesity in adult cats from private US veterinary practices. Intern J Appl Res Vet Med. 2005;3:88-96.

[127] Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity among adults: United States, 2011-2012. NCHS Data Brief. 2013:1-8.

[128] Robin CA, Ireland JL, Wylie CE, Collins SN, Verheyen KL, Newton JR. Prevalence of and risk factors for equine obesity in Great Britain based on owner-reported body condition scores. Equine Vet J. 2015;47:196-201.

[129] Thatcher CD, Pleasant RS, Geor RJ, Elvinger F. Prevalence of overconditioning in mature horses in southwest Virginia during the summer. J Vet Intern Med. 2012;26:1413-8.

[130] Giles SL, Rands SA, Nicol CJ, Harris PA. Obesity prevalence and associated risk factors in outdoor living domestic horses and ponies. PeerJ. 2014;2:e299.

[131] Mao J, Xia Z, Chen J, Yu J. Prevalence and risk factors for canine obesity surveyed in veterinary practices in Beijing, China. Prev Vet Med. 2013;112:438-42.

[132] McGreevy PD, Thomson PC, Pride C, Fawcett A, Grassi T, Jones B. Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. Vet Rec. 2005;156:695-702.

[133] Zoran DL. Obesity in dogs and cats: a metabolic and endocrine disorder. Vet Clin North Am Small Anim Pract. 2010;40:221-39.

[134] Forcada Y, Holder A, Church DB, Catchpole B. A polymorphism in the melanocortin 4 receptor gene (MC4R:c.92C>T) is associated with diabetes mellitus in overweight domestic shorthaired cats. J Vet Intern Med. 2014;28:458-64.

[135] Kealy RD, Lawler DF, Ballam JM, Lust G, Smith GK, Biery DN, Olsson SE. Five-year longitudinal study on limited food consumption and development of osteoarthritis in coxofemoral joints of dogs. J Am Vet Med Assoc. 1997;210:222-5.

[136] Kealy RD, Lawler DF, Ballam JM, Mantz SL, Biery DN, Greeley EH, Lust G, Segre M, Smith GK, Stowe HD. Effects of diet restriction on life span and age-related changes in dogs. J Am Vet Med Assoc. 2002;220:1315-20.

[137] Kealy RD, Lawler DF, Monti KL, Biery D, Helms RW, Lust G, Olsson SE, Smith GK. Effects of dietary electrolyte balance on subluxation of the femoral head in growing dogs. Am J Vet Res. 1993;54:555-62.

[138] Kealy RD, Olsson SE, Monti KL, Lawler DF, Biery DN, Helms RW, Lust G, Smith GK. Effects of limited food consumption on the incidence of hip dysplasia in growing dogs. J Am Vet Med Assoc. 1992;201:857-63.

[139] Larson BT, Lawler DF, Spitznagel EL, Jr., Kealy RD. Improved glucose tolerance with lifetime diet restriction favorably affects disease and survival in dogs. J Nutr. 2003;133:2887-92.

[140] Lawler DF, Ballam JM, Meadows R, Larson BT, Li Q, Stowe HD, Kealy RD. Influence of lifetime food restriction on physiological variables in Labrador retriever dogs. Exp Gerontol. 2007;42:204-14.

[141] Lawler DF, Evans RH, Larson BT, Spitznagel EL, Ellersieck MR, Kealy RD. Influence of lifetime food restriction on causes, time, and predictors of death in dogs. J Am Vet Med Assoc. 2005;226:225-31.

[142] Smith GK, Paster ER, Powers MY, Lawler DF, Biery DN, Shofer FS, McKelvie PJ, Kealy RD. Lifelong diet restriction and radiographic evidence of osteoarthritis of the hip joint in dogs. J Am Vet Med Assoc. 2006;229:690-3.

[143] Geor R, Frank N. Metabolic syndrome-From human organ disease to laminar failure in equids. Vet Immunol Immunopathol. 2009;129:151-4.

[144] Osto M, Lutz TA. Translational value of animal models of obesity-Focus on dogs and cats. Eur J Pharmacol. 2015;759:240-52.

[145] Osto M, Zini E, Reusch CE, Lutz TA. Diabetes from humans to cats. Gen Comp Endocrinol. 2013;182:48-53.

[146] Hoenig M. The cat as a model for human obesity and diabetes. J Diabetes Sci Technol. 2012;6:525-33.

[147] Hoenig M. The cat as a model for human nutrition and disease. Curr Opin Clin Nutr Metab Care. 2006;9:584-8.

[148] Bjornvad CR, Wiinberg B, Kristensen AT. Obesity increases initial rate of fibrin formation during blood coagulation in domestic shorthaired cats. J Anim Physiol Anim Nutr (Berl). 2012;96:834-41.

[149] Freeman LM, Rush JE, Meurs KM, Bulmer BJ, Cunningham SM. Body size and metabolic differences in Maine Coon cats with and without hypertrophic cardiomyopathy. J Feline Med Surg. 2013;15:74-80.

[150] Garcia-Guasch L, Caro-Vadillo A, Manubens-Grau J, Carreton E, Camacho AA, Montoya-Alonso JA. Pulmonary function in obese vs non-obese cats. J Feline Med Surg. 2015;17:494-9.

[151] Fujiwara M, Mori N, Sato T, Tazaki H, Ishikawa S, Yamamoto I, Arai T. Changes in fatty acid composition in tissue and serum of obese cats fed a high fat diet. BMC Vet Res. 2015;11:200.

[152] Hoenig M, Thomaseth K, Brandao J, Waldron M, Ferguson DC. Assessment and mathematical modeling of glucose turnover and insulin sensitivity in lean and obese cats. Domest Anim Endocrinol. 2006;31:373-89.

[153] Hoenig M, Thomaseth K, Waldron M, Ferguson DC. Insulin sensitivity, fat distribution, and adipocytokine response to different diets in lean and obese cats before and after weight loss. Am J Physiol Regul Integr Comp Physiol. 2007;292:R227-34.

[154] Davison LJ. Diabetes mellitus and pancreatitis--cause or effect? J Small Anim Pract. 2015;56:50-9.

[155] Hoenig M, Wilkins C, Holson JC, Ferguson DC. Effects of obesity on lipid profiles in neutered male and female cats. Am J Vet Res. 2003;64:299-303.
[156] Jordan E, Kley S, Le NA, Waldron M, Hoenig M. Dyslipidemia in obese cats. Domest Anim Endocrinol. 2008;35:290-9.

[157] O'Brien TD. Pathogenesis of feline diabetes mellitus. Mol Cell Endocrinol. 2002;197:213-9.

[158] 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. 2009;38:136-56.

[159] Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nat Rev Immunol. 2011;11:85-97.

[160] van Kruijsdijk RC, van der Wall E, Visseren FL. Obesity and cancer: the role of dysfunctional adipose tissue. Cancer Epidemiol Biomarkers Prev. 2009;18:2569-78.

[161] Mazaki-Tovi M, Abood SK, Segev G, Schenck PA. Alterations in adipokines in feline hepatic lipidosis. J Vet Intern Med. 2013;27:242-9.

[162] Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. J Clin Invest. 2006;116:1784-92.

[163] Brunson BL, Zhong Q, Clarke KJ, Bedi D, Braden TD, van Santen E, Judd RL. Serum concentrations of adiponectin and characterization of adiponectin protein complexes in dogs. Am J Vet Res. 2007;68:57-62.

[164] Ishioka K, Omachi A, Sagawa M, Shibata H, Honjoh T, Kimura K, Saito M. Canine adiponectin: cDNA structure, mRNA expression in adipose tissues and reduced plasma levels in obesity. Res Vet Sci. 2006;80:127-32.

[165] Verkest KR, Fleeman LM, Morton JM, Ishioka K, Rand JS. Compensation for obesity-induced insulin resistance in dogs: assessment of the effects of leptin, adiponectin, and glucagon-like peptide-1 using path analysis. Domest Anim Endocrinol. 2011;41:24-34.

[166] Verkest KR, Rose FJ, Fleeman LM, Rand JS, Morton JM, Richards AA, Ishioka K, Whitehead JP. Adiposity and adiponectin in dogs: investigation of causes of discrepant results between two studies. Domest Anim Endocrinol. 2011;41:35-41.

[167] Hoenig M, Pach N, Thomaseth K, Le A, Schaeffer D, Ferguson DC. Cats differ from other species in their cytokine and antioxidant enzyme response when developing obesity. Obesity (Silver Spring). 2013;21:E407-14.

[168] Ishioka K, Omachi A, Sasaki N, Kimura K, Saito M. Feline adiponectin: molecular structures and plasma concentrations in obese cats. J Vet Med Sci. 2009;71:189-94.

[169] Lusby AL, Kirk CA, Bartges JW. The role of key adipokines in obesity and insulin resistance in cats. J Am Vet Med Assoc. 2009;235:518-22.

[170] Tan HY, Rand JS, Morton JM, Fleeman LM, Armstrong PJ, Coradini M, Ishioka K, Verkest KR, Richards AA, Rawlings JM, Rose FJ, Whitehead JP. Adiponectin profiles are affected by chronic and acute changes in carbohydrate intake in healthy cats. Gen Comp Endocrinol. 2011;172:468-74.

[171] Bjornvad CR, Rand JS, Tan HY, Jensen KS, Rose FJ, Armstrong PJ, Whitehead JP. Obesity and sex influence insulin resistance and total and multimer adiponectin levels in adult neutered domestic shorthair client-owned cats. Domest Anim Endocrinol. 2014;47:55-64.

[172] Witzel AL, Kirk CA, Kania SA, Bartges JW, Boston RC, Moyers T, Byrd H, Lauten S. Relationship of adiponectin and its multimers to metabolic indices in cats during weight change. Domest Anim Endocrinol. 2015;53:70-7.

[173] Kearns CF, McKeever KH, Roegner V, Brady SM, Malinowski K. Adiponectin and leptin are related to fat mass in horses. Vet J. 2006;172:460-5.

[174] Mendoza FJ, Estepa JC, Gonzalez-De Cara CA, Aguilera-Aguilera R, Toribio RE, Perez-Ecija A. Energy-related parameters and their association with age, gender, and morphometric measurements in healthy donkeys. Vet J. 2015;204:201-7.

[175] Wooldridge AA, Edwards HG, Plaisance EP, Applegate R, Taylor DR, Taintor J, Zhong Q, Judd RL. Evaluation of high-molecular weight adiponectin in horses. Am J Vet Res. 2012;73:1230-40.

[176] Wray H, Elliott J, Bailey SR, Harris PA, Menzies-Gow NJ. Plasma concentrations of inflammatory markers in previously laminitic ponies. Equine Vet J. 2013;45:546-51.

[177] Choi Y, Davis ME, Chung H. Effects of genetic variants in the promoter region of the bovine adiponectin (ADIPOQ) gene on marbling of Hanwoo beef cattle. Meat Sci. 2015;105:57-62.

[178] Lecchi C, Giudice C, Ugge M, Scarafoni A, Baldi A, Sartorelli P. Characterisation of adiponectin and its receptors in the bovine mammary gland and in milk. Vet J. 2015;203:296-301.

[179] Singh SP, Haussler S, Heinz JF, Akter SH, Saremi B, Muller U, Rehage J, Danicke S, Mielenz M, Sauerwein H. Lactation driven dynamics of adiponectin supply from different fat depots to circulation in cows. Domest Anim Endocrinol. 2014;47:35-46.

[180] Kasimanickam RK, Kasimanickam VR, Olsen JR, Jeffress EJ, Moore DA, Kastelic JP. Associations among serum pro- and anti-inflammatory cytokines, metabolic mediators, body condition, and uterine disease in postpartum dairy cows. Reprod Biol Endocrinol. 2013;11:103.

[181] Kabara E, Sordillo LM, Holcombe S, Contreras GA. Adiponectin links adipose tissue function and monocyte inflammatory responses during bovine metabolic stress. Comp Immunol Microbiol Infect Dis. 2014;37:49-58.

[182] Kasimanickam VR, Kasimanickam RK, Kastelic JP, Stevenson JS. Associations of adiponectin and fertility estimates in Holstein bulls. Theriogenology. 2013;79:766-77 e1-3
.
[183] Jacobi SK, Ajuwon KM, Weber TE, Kuske JL, Dyer CJ, Spurlock ME. Cloning and expression of porcine adiponectin, and its relationship to adiposity, lipogenesis and the acute phase response. J Endocrinol. 2004;182:133-44.

[184] Hendricks GL, 3rd, Hadley JA, Krzysik-Walker SM, Prabhu KS, Vasilatos-Younken R, Ramachandran R. Unique profile of chicken adiponectin, a predominantly heavy molecular weight multimer, and relationship to visceral adiposity. Endocrinology. 2009;150:3092-100.

[185] Ramachandran R, Maddineni S, Ocon-Grove O, Hendricks G, 3rd, Vasilatos-Younken R, Hadley JA. Expression of adiponectin and its receptors in avian species. Gen Comp Endocrinol. 2013;190:88-95.

[186] Tahmoorespur M, Ghazanfari S, Nobari K. Evaluation of adiponectin gene expression in the abdominal adipose tissue of broiler chickens: feed restriction, dietary energy, and protein influences adiponectin messenger ribonucleic acid expression. Poult Sci. 2010;89:2092-100.

[187] Bou M, Todorcevic M, Rodriguez J, Capilla E, Gutierrez J, Navarro I. Interplay of adiponectin, TNFalpha and insulin on gene expression, glucose uptake and PPARgamma, AKT and TOR pathways in rainbow trout cultured adipocytes. Gen Comp Endocrinol. 2014;205:218-25.

[188] Qin C, Wang B, Sun C, Jia J, Li W. Orange-spotted grouper (Epinephelus coioides) adiponectin receptors: molecular characterization, mRNA expression, and subcellular location. Gen Comp Endocrinol. 2014;198:47-58.

[189] Sanchez-Gurmaches J, Cruz-Garcia L, Gutierrez J, Navarro I. Adiponectin effects and gene expression in rainbow trout: an in vivo and in vitro approach. J Exp Biol. 2012;215:1373-83.

[190] Appleton DJ, Rand JS, Sunvold GD. Plasma leptin concentrations in cats: reference range, effect of weight gain and relationship with adiposity as measured by dual energy X-ray absorptiometry. J Feline Med Surg. 2000;2:191-9.

[191] Backus RC, Havel PJ, Gingerich RL, Rogers QR. Relationship between serum leptin immunoreactivity and body fat mass as estimated by use of a novel gas-phase Fourier transform infrared spectroscopy deuterium dilution method in cats. Am J Vet Res. 2000;61:796-801.

[192] 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. 1995;269:540-3.

[193] Gaige S, Abysique A, Bouvier M. Effects of leptin on cat intestinal motility. J Physiol. 2003;546:267-77.

[194] Gaige S, Abysique A, Bouvier M. Effects of leptin on cat intestinal vagal mechanoreceptors. J Physiol. 2002;543:679-89.

[195] Jaillardon L, Burger M, Siliart B. Leptin levels in hyperthyroid cats before and after treatment. Vet Rec. 2012;170:155.

[196] Trisolini C, Albrizio M, Roscino MT, Pantaleo M, Rizzo A, Sciorsci RL. Leptin and queen ovary: new insights about ovulation. Res Vet Sci. 2013;94:707-10.

[197] Dall'Aglio C, Polisca A, Boiti C, Ceccarelli P. Immunolocalization of leptin and its receptor in the placenta of cats. Acta Histochem. 2012;114:719-22.

[198] Trakovicka A, Moravcikova N, Kasarda R. Genetic polymorphisms of leptin and leptin receptor genes in relation with production and reproduction traits in cattle. Acta Biochim Pol. 2013;60:783-7.

[199] Fradinho MJ, Correia MJ, Gracio V, Bliebernicht M, Farrim A, Mateus L, Martin-Rosset W, Bessa RJ, Caldeira RM, Ferreira-Dias G. Effects of body condition and leptin on the reproductive performance of Lusitano mares on extensive systems. Theriogenology. 2014;81:1214-22.

[200] Takashima S, Nishii N, Kato A, Matsubara T, Shibata S, Kitagawa H. Molecular cloning of feline resistin and the expression of resistin, leptin, and adiponectin in the adipose tissue of normal and obese cats. J Vet Med Sci. 2015.

[201] Van de Velde H, Janssens GPJ, de Rooster H, Polis I, Peters I, Ducatelle R, Nguyen P, Buyse J, Rochus K, Xu J, Verbrugghe A, Hesta M. The cat as a

model for human obesity: insights into depot-specific inflammation associated with feline obesity. Br J Nutr. 2013;110:1326-35.

[202] Ampem G, Azegrouz H, Bacsadi A, Balogh L, Schmidt S, Thuroczy J, Roszer T. Adipose tissue macrophages in non-rodent mammals: a comparative study. Cell Tissue Res. 2015.

[203] Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res. 2005;46:2347-55.

[204] Vick MM, Adams AA, Murphy BA, Sessions DR, Horohov DW, Cook RF, Shelton BJ, Fitzgerald BP. Relationships among inflammatory cytokines, obesity, and insulin sensitivity in the horse. J Anim Sci. 2007;85:1144-55.

[205] Holbrook TC, Tipton T, McFarlane D. Neutrophil and cytokine dysregulation in hyperinsulinemic obese horses. Vet Immunol Immunopathol. 2012;145:283-9.

[206] Frank L, Mann S, Levine CB, Cummings BP, Wakshlag JJ. Increasing body condition score is positively associated interleukin-6 and monocyte chemoattractant protein-1 in Labrador retrievers. Vet Immunol Immunopathol. 2015.

[207] Bastien BC, Patil A, Satyaraj E. The impact of weight loss on circulating cytokines in Beagle dogs. Vet Immunol Immunopathol. 2015;163:174-82.

[208] Van de Velde H, Janssens GP, Rochus K, Duchateau L, Scharek-Tedin L, Zentek J, Nguyen P, Cox E, Buyse J, Biourge V, Hesta M. Proliferation capacity of T-lymphocytes is affected transiently after a long-term weight gain in Beagle dogs. Vet Immunol Immunopathol. 2013;152:237-44.

[209] Veiga AP, Price CA, de Oliveira ST, Dos Santos AP, Campos R, Barbosa PR, Gonzalez FH. Association of canine obesity with reduced serum levels of C-reactive protein. J Vet Diagn Invest. 2008;20:224-8.

[210] Miller C, Bartges J, Cornelius L, Norton N, Barton M. Tumor Necrosis Factor-α Levels in Adipose Tissue of Lean and Obese Cats. The Journal of Nutrition. 1998;128:2751S-2S.

[211] Hoenig M, McGoldrick JB, deBeer M, Demacker PN, Ferguson DC. Activity and tissue-specific expression of lipases and tumor-necrosis factor alpha in lean and obese cats. Domest Anim Endocrinol. 2006;30:333-44.

[212] Tvarijonaviciute A, Ceron JJ, Holden SL, Morris PJ, Biourge V, German AJ. Effects of weight loss in obese cats on biochemical analytes related to

inflammation and glucose homeostasis. Domest Anim Endocrinol. 2012;42:129-41.

[213] Zini E, Osto M, Konrad D, Franchini M, Sieber-Ruckstuhl NS, Kaufmann K, Guscetti F, Ackermann M, Lutz TA, Reusch CE. 10-day hyperlipidemic clamp in cats: effects on insulin sensitivity, inflammation, and glucose metabolism-related genes. Horm Metab Res. 2010;42:340-7.

[214] Titgemeyer EC, Mamedova LK, Spivey KS, Farney JK, Bradford BJ. An unusual distribution of the niacin receptor in cattle. J Dairy Sci. 2011;94:4962-7.

[215] Majee DN, Schwab EC, Bertics SJ, Seymour WM, Shaver RD. Lactation performance by dairy cows fed supplemental biotin and a B-vitamin blend. J Dairy Sci. 2003;86:2106-12.

[216] Riddell DO, Bartley EE, Dayton AD. Effect of nicotinic acid on rumen fermentation in vitro and in vivo. J Dairy Sci. 1980;63:1429-36.

[217] Zimbelman RB, Baumgard LH, Collier RJ. Effects of encapsulated niacin on evaporative heat loss and body temperature in moderately heat-stressed lactating Holstein cows. J Dairy Sci. 2010;93:2387-94.

[218] Niehoff ID, Huther L, Lebzien P. Niacin for dairy cattle: a review. Br J Nutr. 2009;101:5-19.

[219] Pires JA, Grummer RR. The use of nicotinic acid to induce sustained low plasma nonesterified fatty acids in feed-restricted Holstein cows. J Dairy Sci. 2007;90:3725-32.

[220] Pires JA, Pescara JB, Grummer RR. Reduction of plasma NEFA concentration by nicotinic acid enhances the response to insulin in feed-restricted Holstein cows. J Dairy Sci. 2007;90:4635-42.

[221] Mielenz M, Kuhla B, Hammon HM. Abundance of adiponectin system and G-protein coupled receptor GPR109A mRNA in adipose tissue and liver of F2 offspring cows of Charolais x German Holstein crosses that differ in body fat accumulation. J Dairy Sci. 2013;96:278-89.

[222] Kenez A, Locher L, Rehage J, Danicke S, Huber K. Agonists of the G protein-coupled receptor 109A-mediated pathway promote antilipolysis by reducing serine residue 563 phosphorylation of hormone-sensitive lipase in bovine adipose tissue explants. J Dairy Sci. 2014;97:3626-34.

[223] Kopp C, Hosseini A, Singh SP, Regenhard P, Khalilvandi-Behroozyar H, Sauerwein H, Mielenz M. Nicotinic acid increases adiponectin secretion from

differentiated bovine preadipocytes through G-protein coupled receptor signaling. Int J Mol Sci. 2014;15:21401-18.

[224] Friedrichs P, Saremi B, Winand S, Rehage J, Danicke S, Sauerwein H, Mielenz M. Energy and metabolic sensing G protein-coupled receptors during lactation-induced changes in energy balance. Domest Anim Endocrinol. 2014;48:33-41.

[225] van der Drift SG, Everts RR, Houweling M, van Leengoed LA, Stegeman JA, Tielens AG, Jorritsma R. Effects of beta-hydroxybutyrate and isoproterenol on lipolysis in isolated adipocytes from periparturient dairy cows and cows with clinical ketosis. Res Vet Sci. 2013;94:433-9.

[226] Carballo-Jane E, Gerckens LS, Luell S, Parlapiano AS, Wolff M, Colletti SL, Tata JR, Taggart AK, Waters MG, Richman JG, McCann ME, Forrest MJ. Comparison of rat and dog models of vasodilatation and lipolysis for the calculation of a therapeutic index for GPR109A agonists. J Pharmacol Toxicol Methods. 2007;56:308-16.

[227] Lipson MJ, Naimi S, Proger S. Effect of a combination of propranolol and nicotinic acid on the inhibition of norepinephrine-induced elevations of serum triglyceride, serum glycerol, and plasma free fatty acids in the dog. Metabolism. 1971;20:580-9.

[228] Ahmed K, Tunaru S, Offermanns S. GPR109A, GPR109B and GPR81, a family of hydroxy-carboxylic acid receptors. Trends Pharmacol Sci. 2009;30:557-62.

[229] Ghanim H, Aljada A, Hofmeyer D, Syed T, Mohanty P, Dandona P. Circulating mononuclear cells in the obese are in a proinflammatory state. Circulation. 2004;110:1564-71.

[230] Breslin WL, Johnston CA, Strohacker K, Carpenter KC, Davidson TR, Moreno JP, Foreyt JP, McFarlin BK. Obese Mexican American children have elevated MCP-1, TNF-alpha, monocyte concentration, and dyslipidemia. Pediatrics. 2012;129:e1180-6.

[231] Tzanavari T, Giannogonas P, Karalis KP. TNF-alpha and obesity. Curr Dir Autoimmun. 2010;11:145-56.

[232] Duffaut C, Galitzky J, Lafontan M, Bouloumié A. Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. Biochem Biophys Res Commun. 2009;384:482-5.

[233] Oh DY, Morinaga H, Talukdar S, Bae EJ, Olefsky JM. Increased macrophage migration into adipose tissue in obese mice. Diabetes. 2012;61:346-54.

[234] Guarner V, Rubio-Ruiz ME. Low-grade systemic inflammation connects aging, metabolic syndrome and cardiovascular disease. Interdiscip Top Gerontol. 2015;40:99-106.

[235] Chang CJ, Jian DY, Lin MW, Zhao JZ, Ho LT, Juan CC. Evidence in obese children: contribution of hyperlipidemia, obesity-inflammation, and insulin sensitivity. PLoS One. 2015;10:e0125935.

[236] Takahashi K, Mizuarai S, Araki H, Mashiko S, Ishihara A, Kanatani A, Itadani H, Kotani H. Adiposity elevates plasma MCP-1 levels leading to the increased CD11b-positive monocytes in mice. J Biol Chem. 2003;278:46654-60.

[237] Choi J, Joseph L, Pilote L. Obesity and C-reactive protein in various populations: a systematic review and meta-analysis. Obes Rev. 2013;14:232-44.

[238] Pang J, Rhodes DH, Pini M, Akasheh RT, Castellanos KJ, Cabay RJ, Cooper D, Perretti M, Fantuzzi G. Increased adiposity, dysregulated glucose metabolism and systemic inflammation in Galectin-3 KO mice. PLoS One. 2013;8:e57915.

[239] Poitou C, Dalmas E, Renovato M, Benhamo V, Hajduch F, Abdennour M, Kahn JF, Veyrie N, Rizkalla S, Fridman WH, Sautes-Fridman C, Clement K, Cremer I. CD14dimCD16+ and CD14+CD16+ monocytes in obesity and during weight loss: relationships with fat mass and subclinical atherosclerosis. Arterioscler Thromb Vasc Biol. 2011;31:2322-30.

[240] Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. The Journal of Clinical Investigation. 2003;112:1821-30.

[241] Grant RW, Dixit VD. Adipose tissue as an immunological organ. Obesity. 2015;23:512-8.

[242] Silha JV, Krsek M, Skrha JV, Sucharda P, Nyomba BL, Murphy LJ. Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. Eur J Endocrinol. 2003;149:331-5.

[243] Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y.

Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun. 1999;257:79-83.

[244] Ohashi K, Parker JL, Ouchi N, Higuchi A, Vita JA, Gokce N, Pedersen AA, Kalthoff C, Tullin S, Sams A, Summer R, Walsh K. Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. J Biol Chem. 2010;285:6153-60.

[245] Yokota T, Oritani K, Takahashi I, Ishikawa J, Matsuyama A, Ouchi N, Kihara S, Funahashi T, Tenner AJ, Tomiyama Y, Matsuzawa Y. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages2000.

[246] Lee JM, Robson MD, Yu LM, Shirodaria CC, Cunnington C, Kylintireas I, Digby JE, Bannister T, Handa A, Wiesmann F, Durrington PN, Channon KM, Neubauer S, Choudhury RP. Effects of high-dose modified-release nicotinic acid on atherosclerosis and vascular function: a randomized, placebo-controlled, magnetic resonance imaging study. J Am Coll Cardiol. 2009;54:1787-94.

[247] Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 2001;29:e45.

[248] Ganji SH, Qin S, Zhang L, Kamanna VS, Kashyap ML. Niacin inhibits vascular oxidative stress, redox-sensitive genes, and monocyte adhesion to human aortic endothelial cells. Atherosclerosis. 2009;202:68-75.

[249] Hu E, Liang P, Spiegelman BM. AdipoQ is a novel adipose-specific gene dysregulated in obesity. J Biol Chem. 1996;271:10697-703.

[250] Chaolu H, Asakawa A, Ushikai M, Li YX, Cheng KC, Li JB, Zoshiki T, Terashi M, Tanaka C, Atsuchi K, Sakoguchi T, Tsai M, Amitani H, Horiuchi M, Takeuchi T, Inui A. Effect of exercise and high-fat diet on plasma adiponectin and nesfatin levels in mice. Exp Ther Med. 2011;2:369-73.

[251] Kentish SJ, Ratcliff K, Li H, Wittert GA, Page AJ. High fat diet induced changes in gastric vagal afferent response to adiponectin. Physiol Behav. 2015.
[252] Stach K, Zaddach F, Nguyen XD, Elmas E, Kralev S, Weiss C, Borggrefe M, Kalsch T. Effects of nicotinic acid on endothelial cells and platelets. Cardiovasc Pathol. 2012;21:89-95.

[253] Hillyer P, Mordelet E, Flynn G, Male D. Chemokines, chemokine receptors and adhesion molecules on different human endothelia: discriminating the tissue-specific functions that affect leucocyte migration. Clin Exp Immunol. 2003;134:431-41.

[254] Christiansen T, Richelsen B, Bruun JM. Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. Int J Obes (Lond). 2005;29:146-50.
[255] Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008;8:958-69.

[256] Lipszyc PS, Cremaschi GA, Zorrilla-Zubilete M, Bertolino ML, Capani F, Genaro AM, Wald MR. Niacin Modulates Pro-inflammatory Cytokine Secretion. A Potential Mechanism Involved in its Anti-atherosclerotic Effect. Open Cardiovasc Med J. 2013;7:90-8.

[257] Terra X, Pallares V, Ardevol A, Blade C, Fernandez-Larrea J, Pujadas G, Salvado J, Arola L, Blay M. Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. J Nutr Biochem. 2011;22:380-7.

[258] Kim DH, Do MS. BAFF knockout improves systemic inflammation via regulating adipose tissue distribution in high-fat diet-induced obesity. Exp Mol Med. 2015;47:e129.

[259] Asano T, Watanabe K, Kubota N, Gunji T, Omata M, Kadowaki T, Ohnishi S. Adiponectin knockout mice on high fat diet develop fibrosing steatohepatitis. J Gastroenterol Hepatol. 2009;24:1669-76.

[260] Dong Z, Su L, Esmaili S, Iseli TJ, Ramezani-Moghadam M, Hu L, Xu A, George J, Wang J. Adiponectin attenuates liver fibrosis by inducing nitric oxide production of hepatic stellate cells. J Mol Med (Berl). 2015.

[261] Ganji SH, Kashyap ML, Kamanna VS. Niacin inhibits fat accumulation, oxidative stress, and inflammatory cytokine IL-8 in cultured hepatocytes: Impact on non-alcoholic fatty liver disease. Metabolism. 2015;64:982-90.

[262] Muranaka S, Mori N, Hatano Y, Saito TR, Lee P, Kojima M, Kigure M, Yagishita M, Arai T. Obesity induced changes to plasma adiponectin concentration and cholesterol lipoprotein composition profile in cats. Res Vet Sci. 2011;91:358-61.

[263] McCue MD. Starvation physiology: Reviewing the different strategies animals use to survive a common challenge. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 2010;156:1-18.
[264] Weingart C, Lotz F, Kohn B. Measurement of β-hydroxybutyrate in cats with nonketotic diabetes mellitus, diabetic ketosis, and diabetic ketoacidosis. J Vet Diagn Invest. 2012;24:295-300.

[265] Aroch I, Shechter-Polak M, Segev G. A retrospective study of serum beta-hydroxybutyric acid in 215 ill cats: clinical signs, laboratory findings and diagnoses. Vet J. 2012;191:240-5.

[266] Carswell KA, Lee MJ, Fried SK. Culture of Isolated Human Adipocytes and Isolated Adipose Tissue. Methods Mol Biol. 2012;806:203-14.

[267] Tunaru S, Lattig J, Kero J, Krause G, Offermanns S. Characterization of determinants of ligand binding to the nicotinic acid receptor GPR109A (HM74A/PUMA-G). Mol Pharmacol. 2005;68:1271-80.

[268] Nelson RW, Reusch CE. Animal models of disease: classification and etiology of diabetes in dogs and cats. J Endocrinol. 2014;222:T1-9.

[269] Wanders D, Plaisance EP, Judd RL. Pharmacological effects of lipid-lowering drugs on circulating adipokines. World J Diabetes. 2010;1:116-28.

[270] Cnop M, Landchild MJ, Vidal J, Havel PJ, Knowles NG, Carr DR, Wang F, Hull RL, Boyko EJ, Retzlaff BM. The concurrent accumulation of intra-abdominal and subcutaneous fat explains the association between insulin resistance and plasma leptin concentrations distinct metabolic effects of two fat compartments. Diabetes. 2002;51:1005-15.

[271] Rand JS. Diabetic ketoacidosis and hyperosmolar hyperglycemic state in cats. Vet Clin North Am Small Anim Pract. 2013;43:367-79.

[272] Armstrong PJ, Blanchard G. Hepatic lipidosis in cats. Vet Clin North Am Small Anim Pract. 2009;39:599-616.

[273] Flegal KM, Carroll MD, Ogden CL, Curtin LR. Prevalence and trends in obesity among US adults, 1999-2008. JAMA. 2010;303:235-41.

[274] Jaedicke KM, Roythorne A, Padget K, Todryk S, Preshaw PM, Taylor JJ. Leptin up-regulates TLR2 in human monocytes. J Leukoc Biol. 2013;93:561-71.

[275] Santos-Alvarez J, Goberna R, Sanchez-Margalet V. Human leptin stimulates proliferation and activation of human circulating monocytes. Cell Immunol. 1999;194:6-11.

[276] Maury E, Brichard SM. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. Mol Cell Endocrinol. 2010;314:1-16.

[277] Li L, Wu LL. Adiponectin and interleukin-6 in inflammation-associated disease. Vitam Horm. 2012;90:375-95.

[278] Aparicio-Vergara M, Shiri-Sverdlov R, Koonen DP, Hofker MH. Bone marrow transplantation as an established approach for understanding the role of macrophages in atherosclerosis and the metabolic syndrome. Curr Opin Lipidol. 2012;23:111-21.

[279] Kosteli A, Sugaru E, Haemmerle G, Martin JF, Lei J, Zechner R, Ferrante AW, Jr. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. J Clin Invest. 2010;120:3466-79.

[280] Zeyda M, Gollinger K, Kriehuber E, Kiefer FW, Neuhofer A, Stulnig TM. Newly identified adipose tissue macrophage populations in obesity with distinct chemokine and chemokine receptor expression. Int J Obes (Lond). 2010;34:1684-94.

[281] Yerrakalva D, Mullis R, Mant J. The associations of "fatness," "fitness," and physical activity with all-cause mortality in older adults: A systematic review. Obesity (Silver Spring). 2015.

[282] Exley MA, Hand L, O'Shea D, Lynch L. Interplay between the immune system and adipose tissue in obesity. J Endocrinol. 2014;223:R41-8.

[283] Mraz M, Haluzik M. The role of adipose tissue immune cells in obesity and low-grade inflammation. J Endocrinol. 2014;222:R113-27.

[284] Meng W, Zhang C, Zhang Q, Song X, Lin H, Zhang D, Zhang Y, Zhu Z, Wu S, Liu Y, Tang F, Yang X, Xue F. Association between leukocyte and metabolic syndrome in urban Han Chinese: a longitudinal cohort study. PLoS One. 2012;7:e49875.

[285] Viardot A, Heilbronn LK, Samocha-Bonet D, Mackay F, Campbell LV, Samaras K. Obesity is associated with activated and insulin resistant immune cells. Diabetes Metab Res Rev. 2012;28:447-54.

[286] Khodabandeloo H, Gorgani-Firuzjaee S, Panahi S, Meshkani R. Molecular and cellular mechanisms linking inflammation to insulin resistance and beta-cell dysfunction. Transl Res. 2015.

[287] Kanneganti T-D, Dixit VD. Immunological complications of obesity. Nat Immunol. 2012;13:707-12.

[288] Klimentidis YC, Beasley TM, Lin HY, Murati G, Glass GE, Guyton M, Newton W, Jorgensen M, Heymsfield SB, Kemnitz J, Fairbanks L, Allison DB. Canaries in the coal mine: a cross-species analysis of the plurality of obesity epidemics. Proc Biol Sci. 2011;278:1626-32.

[289] Appleton D, Rand J, Sunvold G. Basal plasma insulin and homeostasis model assessment (HOMA) are indicators of insulin sensitivity in cats. Journal of Feline Medicine and Surgery. 2005;7:183-93.

[290] Strage EM, Holst BS, Nilsson G, Jones B, Lilliehook I. Validation of an enzyme-linked immunosorbent assay for measurement of feline serum insulin. Vet Clin Pathol. 2012;41:518-28.

[291] Tvarijonaviciute A, German AJ, Martinez-Subiela S, Tecles F, Ceron JJ. Analytical performance of commercially-available assays for feline insulin-like growth factor 1 (IGF-1), adiponectin and ghrelin measurements. J Feline Med Surg. 2012;14:138-46.

[292] Busetto L, Tregnaghi A, Bussolotto M, Sergi G, Beninca P, Ceccon A, Giantin V, Fiore D, Enzi G. Visceral fat loss evaluated by total body magnetic resonance imaging in obese women operated with laparascopic adjustable silicone gastric banding. Int J Obes Relat Metab Disord. 2000;24:60-9.

[293] Zini E, Osto M, Franchini M, Guscetti F, Donath MY, Perren A, Heller RS, Linscheid P, Bouwman M, Ackermann M, Lutz TA, Reusch CE. Hyperglycaemia but not hyperlipidaemia causes beta cell dysfunction and beta cell loss in the domestic cat. Diabetologia. 2009;52:336-46.

[294] Hamdy O, Porramatikul S, Al-Ozairi E. Metabolic obesity: the paradox between visceral and subcutaneous fat. Curr Diabetes Rev. 2006;2:367-73.

[295] Zhu Y, Tchkonia T, Stout MB, Giorgadze N, Wang L, Li PW, Heppelmann CJ, Bouloumie A, Jensen MD, Bergen HR, 3rd, Kirkland JL. Inflammation and the depot-specific secretome of human preadipocytes. Obesity (Silver Spring). 2015;23:989-99.

[296] Kranendonk ME, van Herwaarden JA, Stupkova T, de Jager W, Vink A, Moll FL, Kalkhoven E, Visseren FL. Inflammatory characteristics of distinct abdominal adipose tissue depots relate differently to metabolic risk factors for cardiovascular disease: distinct fat depots and vascular risk factors. Atherosclerosis. 2015;239:419-27.

[297] Sitticharoon C, Nway NC, Chatree S, Churintaraphan M, Boonpuan P, Maikaew P. Interactions between adiponectin, visfatin, and omentin in subcutaneous and visceral adipose tissues and serum, and correlations with clinical and peripheral metabolic factors. Peptides. 2014;62:164-75.

[298] Lasselin J, Magne E, Beau C, Ledaguenel P, Dexpert S, Aubert A, Laye S, Capuron L. Adipose inflammation in obesity: relationship with circulating levels of inflammatory markers and association with surgery-induced weight loss. J Clin Endocrinol Metab. 2014;99:E53-61.

[299] Kirk EA, Sagawa ZK, McDonald TO, O'Brien KD, Heinecke JW. Monocyte chemoattractant protein deficiency fails to restrain macrophage infiltration into adipose tissue [corrected]. Diabetes. 2008;57:1254-61.

[300] Konter JM, Parker JL, Baez E, Li SZ, Ranscht B, Denzel M, Little FF, Nakamura K, Ouchi N, Fine A, Walsh K, Summer RS. Adiponectin attenuates lipopolysaccharide-induced acute lung injury through suppression of endothelial cell activation. J Immunol. 2012;188:854-63.

[301] Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M, Fischer-Posovszky P, Barth TF, Dragun D, Skurk T, Hauner H, Bluher M, Unger T, Wolf AM, Knippschild U, Hombach V, Marx N. T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. Arterioscler Thromb Vasc Biol. 2008;28:1304-10.

[302] Dworacka M, Krzyzagorska E, Iskakova S, Bekmukhambetov Y, Urazayev O, Dworacki G. Increased circulating RANTES in type 2 diabetes. Eur Cytokine Netw. 2014;25:46-51.

[303] Zhang Z, Dong J, Lobe CG, Gong P, Liu J, Liao L. CCR5 facilitates endothelial progenitor cell recruitment and promotes the stabilization of atherosclerotic plaques in ApoE-/- mice. Stem Cell Res Ther. 2015;6:36.
[304] Shaul ME, Bennett G, Strissel KJ, Greenberg AS, Obin MS. Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet--induced obesity in mice. Diabetes. 2010;59:1171-81.

[305] West M. Dead adipocytes and metabolic dysfunction: recent progress. Curr Opin Endocrinol Diabetes Obes. 2009;16:178-82.