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GHRELIN AS A METABOLIC REGULATOR DURING CALORIC RESTRICTION

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Abstract

The actions of caloric restriction (CR) on lifespan are long-known and well-described. Many mechanisms for this relationship have been postulated and studied, however none has emerged as a sole explanation for CR's actions. Characteristics that transcend multiple models of CR actions may or may not be related to a single origin. The purpose of this dissertation was to describe the actions of ghrelin, a protein hormone known to have similar effects as the actions of CR and its levels to be changed during CR, in multiple mouse and human CR-like studies. Mouse models of varying severities of traditional CR, CR mimetics, or potential CR mechanisms provided systems for ghrelin-CR evaluation in the first two studies of this dissertation. In the third study, polymorphic variation in the promoter region of the ghrelin receptor gene and protein levels of ghrelin and other metabolic peptides were evaluated in a human CR condition: Roux-en-Y gastric bypass surgery. Ghrelin was demonstrated to have nuanced effects in models or mimetics of CR. Ghrelin did not increase consistently with increasing levels of CR or generally in CR mimetics. Ghrelin did show differential effects between sexes in mice and secondly, polymorphisms in the promoter region of its receptor gene were associated with different weight loss trajectories one year post-surgery in gastric bypass patients. Therefore, there may be some role for ghrelin or the ghrelin signaling pathway during CR models, and particularly describing how genetic changes affect clinical outcomes in the obese surgical population warrants further attention.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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CHAPTER I: Introduction

The life-extending property of caloric restriction (CR) is long-known and has been identified in many species ranging from yeast to humans (Weindruch and Walford 1988). CR not only extends lifespan, but also healthspan, conferring a wide variety of physiological benefits including increased vigor to reduced incidence of metabolic and malignant disease (Masoro 2000). Animals on long-term moderate CR eventually adapt to the change in energy balance and have an adjusted metabolic rate and food consumption that is, adjusted for body weight, the same if not higher than *ad libitum (AL)* or freely-fed animals. Yet animals undergoing long-term CR retain a sense of hunger leading to, if given the opportunity, overeating and weight regain to nearly the level of controls. How or why this sense of hunger is retained in long-term CR is unknown, as all physiological systems have adjusted to the new caloric level and the animal portrays an overall healthier phenotype. Regardless of an evolutionary or otherwise explanation, we propose the stomach-derived hormone ghrelin as the appetite-stimulating factor conferring hunger during long-term CR. Its coordinating actions in several physiological systems which overlap with those altered in CR, combined with its increased levels during negative energy balance states, make ghrelin a candidate hormone for coordinating the physiological changes accompanying CR. This body of work examines ghrelin's role in several rodent and human models of CR.

CHAPTER II: Background

Introduction to Caloric Restriction

First described in 1935, CR is a process known to affect both the health and longevity of an organism. This first documented restriction paradigm showed that a moderate CR diet in rats led to an average 35% increase in lifespan (McKay, Crowell et al. 1935). Since then, caloric restriction and lifespan have been linked in diverse organisms including yeast, C. elegans, Drosophila melanogaster, rodents, dogs, and primates (Weindruch and Walford 1988). In all classes of animals there have been demonstrated beneficial CR effects. However, as with any manipulation, there is great variability within CR treatments. For instance in mice, some strains exhibit robust extensions in lifespan with a CR diet while other strains are not impacted. Surprisingly, there are also some mouse strains which respond negatively to dietary restriction; their lifespan in response to CR, on average, is decreased (Liao, Rikke et al. 2010).

Although many modified diets have been utilized, the most studied variation on CR is a 30-40% reduction in food intake from the *ad libitum* level. At this caloric level, many species have responded favorably by marked reductions in disease burden and significant extensions in lifespan. A less severe restriction, i.e. 10-20% CR, may also provide some benefits to longevity and health, although not to the same degree as a 30-40% CR. On the other hand, severe restrictions, i.e. 40% CR or greater, typically have high mortality rates just after initiation of the CR diet. Animals that do survive experience enhanced protection from disease and tremendous increases in lifespan (Weindruch and Walford 1988).

Caloric restriction was previously described by the name 'dietary restriction.' It was only in the last decade that the term 'caloric restriction' was readily adopted in response to the finding that the calorie is the primary nutritional factor responsible for CR's effect (Masoro 2000). For best results, adequate amounts of essential nutrients are vital; vitamins, minerals, essential amino and fatty acids, with reduction in calories coming from a combination of fat, protein, and carbohydrates make up the healthiest model of CR. Adherence to a proper CR diet yields physiological benefits that are numerous and robust.

Benefits of Caloric Restriction

The beneficial effects of caloric restriction on internal physiology are varied. Nearly every organ system in the body is in some way altered by the restriction, and most exhibit a net positive result. Systems that are affected include: neural, cardiovascular, respiratory, renal, hepatic, immune, gastrointestinal, and reproductive. In each of these systems, not only are there prolonged physiological adaptations, but onset of common age-related disorders (diabetes, atherosclerosis, cancer, and autoimmune, kidney or respiratory diseases), is delayed or altogether prevented (Weindruch and Walford 1988; Guo, Mitchell-Raymundo et al. 2002; Hursting, Lavigne et al. 2003; Masoro 2005). Changes in various physiological processes are described below.

Caloric restriction has both positive and negative effects in the brain. Diseases such as Alzheimer's, Huntington's, Parkinson's, and stroke improve with CR due to decreased age-associated neurodegeneration and improved neurogenesis in animal

models (Masoro 2000; Mattson 2005). Conversely, in animal models of Amyotropic Lateral Sclerosis (ALS), CR could be harmful (Mattson, Cutler et al. 2007). The implications of CR on mood and memory are less clear. While it is difficult to measure both mood and memory in animals, certain biological markers or measures of performance during learned tasks suggest some mood or memory-related neural changes in CR animals (Vitousek, Manke et al. 2004). Restricted animals have been shown to exhibit enhanced psychological stress: higher circulating levels of the stress hormone corticosterone as well as increased frequency of anxiety-like behaviors during open arm tests (Abbott 2009). Although not directly parallel, these findings are consistent with evidence from restricted non-obese humans who report increased feelings of stress or anxiety, especially regarding thoughts of food (Polivy 1996). Memory may also be affected in CR. Evidence suggests that a modest 10% or 20% reduction in calories improves memory (Deng, Wu et al. 2009). A restriction at the 40% or greater level may be counter-productive (Yanai, Okaichi et al. 2004). Little evidence exists in humans but in restrictive diets for the obese, mood and memory were not impaired (Redman, Martin et al. 2008).

Little research has been done with regard to changes in cardiovascular physiology with CR. This information is especially important for human health since it is the primary cause of death in Americans (CDC 2010). One study of human CR volunteers did find remarkably low risk factors for cardiovascular disease suggesting a relationship, but not enough empirical data exist to confirm this relationship (Fontana, Meyer et al. 2004). In rodents, it has been shown that the presence of atherosclerotic lesions and cardiomyopathies are reduced upon necropsy (Guo, Mitchell-Raymundo et al. 2002).

However, cardiovascular disease is not the primary threat to lifespan in rodents as it is in humans. In fact, a significant proportion of deaths of *ad libitum* fed Fischer 344 rats are attributed to renal pathologies and exhibit cancer-related deaths as a major cause of mortality (Maeda, Gleiser et al. 1985). CR diets reduce frequency of fatal renal pathologies including cancer, and nearly one-third of restricted animals have no discernable cause of death upon necropsy (Shimokawa, Higami et al. 1993).

The effects of CR on the immune system are mixed. In some models, the ability to fight infection suffers. Several studies have shown that CR animals inoculated with various infectious agents present more severe symptoms and are affected longer than non-CR controls. This is thought to be in part mediated by a reduction in Natural Killer (NK) cell activity (Gardner 2005). Yet despite weakened protection against infectious agents, there is enhanced protection against the development of neoplasms, renal pathologies, autoimmune conditions etc (Weindruch and Walford 1988; Jolly 2004). A similar effect may be seen for immune function in aged animals. CR seems to improve age-related decline in immune function, perhaps due to maintaining naïve T-cells and their ability to proliferate (Nikolich-Zugich and Messaoudi 2005).

The link between reproduction and CR has been closely investigated. It is known that animals beginning a moderate CR in early life may experience a delay in reproductive maturity compared to normally-fed controls (Holliday 1989). An opposite effect has been recorded in the human population; the age of menarche has decreased with improvements in nutrition over the past 100 years, although the link between nutrition and reproductive fitness endures (McDowell, Brody et al. 2007). In addition to later onset of reproductive maturity, already mature animals may enter a state of

reproductive dauer, where reproductive organ function pauses until a positive energy state is regained. This concept was first presented as the 'disposable soma theory of aging' and states that in low energy states, an animal will shift its energy investments. Growth and reproductive functions will suffer and energy will be diverted toward homeostasic maintenance and cellular repair (Shanley and Kirchwood 2000). A metabolic trade-off of sorts may exist, where restricted animals enter a state of self-protection. This energy conserving state would not be conducive to the large energy expenditure states of pregnancy, parturition, and lactation. In contrast then, it has been proposed that fertility and reproduction are costly to lifespan.

In addition to changes within these coordinated systems, there are systemic effects on lifespan, metabolic rate, body temperature, and spontaneous physical activity, plasma glucose, insulin, cholesterol, IGF-1, and free fatty acids. Although some facets of lifespan changes in CR have already been described, it is important to reinforce that disparities exist in these effects. Aside from the species and strain differences in lifespan extension, the age of onset for a CR diet can have dramatic effects. As was previously indicated with regard to reproduction and CR, restrictive diets begun early in life provide the greatest benefits (Weindruch and Walford 1988). Further, as a general rule, the more severe the restriction, the greater the benefits. There is a limit however to this strategy, where the ~40% CR level provides the greatest benefit: cost ratio. A restriction greater than this level can result in too many deaths in the short-term and vulnerability to infection in the long-term.

Plasma insulin, glucose, cholesterol, IGF-1, and free fatty acids are all decreased in CR. Low levels of cholesterol and free fatty acids confer beneficial effects for the

prevention of weight-related diseases like cardiovascular disease and steatohepatitis (Fan, Zhong et al. 2003). Low plasma glucose levels reflect the reduced food intake and insulin correspondingly decreases. Although insulin is low in CR, insulin sensitivity increases due to mobilization of Glut4 receptors to cell surfaces (Wilson and Cushman 1994; Wing, Blair et al. 1994). Low plasma levels of IGF-1 have been explored as a mechanism of CR and while its status as such is undetermined, reduced signaling may have protective effects on lifespan and cellular damage due to oxidative stress (Holzenberger, Dupont et al. 2003).

Metabolic rate, physical activity, respiratory quotient (RQ), and body temperature are all markedly changed in CR. Metabolic rate has been repeatedly reported to decrease during the onset of CR, but one study showed that MR is only decreased in short-term CR (McCarter, Masoro et al. 1985). The authors found that MR is only decreased until a new metabolic equilibrium is reached. This equilibrium is characterized by proportional weight loss which results in food intake being the same per kilogram body weight as before CR. During this approximate eight-week transition state, the metabolic rate is clearly suppressed, but thereafter returns to normal. Physical activity, however, is increased. It was first thought that activity should be decreased as if the animal were entering a state of torpor or temporary hibernation (Himms-Hagen 1985). Nevertheless, repeated findings showing that physical activity is indeed increased and remains high throughout the duration of the restriction (McCarter, Shimokawa et al. 1997). RQ, a measure of the type of fuel utilized at a given time, has an altered diurnal pattern in CR. AL-fed animals use a constant mix of carbohydrates and lipids as fuel sources throughout the day. CR animals burn primarily carbohydrates during feeding times and lipids during

fasting times (Masoro, McCarter et al. 1992; McCarter, Shimokawa et al. 1997). This diurnal variation is attributed to efficient fuel use based on substrate availability. Finally, body temperature is also decreased (Blanc, Schoeller et al. 2003). Although this feature has been investigated as a potential mechanism of CR's pervasive actions, no studies have determined it to be the cause. The argument was that by slightly lowering body temperature, cellular turnover may slowed, improving health and prolonging life. Moreover, the decrease in body temperature results in the animals being more sensitive to cold temperatures which can also negatively impact health.

Several other disadvantages to caloric restriction exist. Attention has been given to effects such as decreased wound healing, increased sensitivity to thermal stress, inability to fight infectious agents, increased cortisol levels, decreased body size and decreased fertility. The former three have been identified as requiring a period of hypermetabolism for defense. It has been suggested that CR animals cannot effectively manage a stressor necessitating additional energy expenditure (Masoro 2000). However, if given supplemental food provisions during periods of increased stress, CR animals are then successfully able to survive the stressor (Reed, Penn et al. 1998). While these negative attributes of CR have been repeatedly demonstrated, the lifespan and health benefits sustained from a moderate CR outweigh such threats to mortality.

In sum, it is known that there are potent physiological changes resulting from a calorie-deficient diet. Systems coordinating metabolism, reproduction, and ultimately lifespan may be radically altered, whereas other systems' changes may be less apparent. However, it is still unknown how CR stimulates these changes. There have been many efforts to describe the mechanisms by which CR may provide such robust physiological

benefits. None thus far has emerged as the leading theory. Several actions that certainly play a role in the CR and lifespan process will be highlighted below. In addition, there has been interest in investigating potential mimetics. If feasible, mimetic diets would provide the same physiological advantages as traditional CR without the psychological difficulty of significantly reducing calories from the diet.

Mechanisms of Caloric Restriction

Despite the profound and diverse physiological changes resulting from a CR diet scheme, one mechanism that coordinates all of these effects has not been found. A reduction in global factors like growth hormone, metabolic rate, or systemic inflammation have all been suggested as they would have actions on many organ systems, but none has emerged as the sole modulator of health or lifespan in CR(Straus 1994; Jolly 2004). Cellular mechanisms to delay aging have been lately proposed for their health maintenance actions and systemic distribution. Modulation of stress resistance pathways (via resveratrol), genes in insulin signaling pathways (*age-1, Daf2, Daf16*), proinflammatory processes, collagen cross-linking, cellular waste products (oxidated lipids, proteins, and DNA; advanced glycation endproducts), or global nutrient sensors (*Sir1* and *Sir2* genes) is the current trend to further understanding of the mechanism by which CR extends lifespan (Leeuwenburgh, Wagner et al. 1997; Lawrence, Snape et al. 2002; Sell, Lane et al. 2003; Dhabi, Kim et al. 2004; Perez, Bokov et al. 2009).

As effective as caloric restriction is, its use in humans is impractical due to the degree and duration of the restriction required. In addition to these restraints, CR is most effective when begun at an early age. Most humans are unlikely to begin a restrictive diet from early age, continue it throughout life, and supplement their diets with adequate amounts of all essential nutrients. Therefore, 'mimetics' have begun to be investigated which would serve to provide the physiological benefits of CR without a calorie-deficient diet. As proposed by Ingram et al. (Ingram, Zhu et al. 2006), the major tenets defining a CR mimetic include (1) mimicking hormonal, metabolic, and physiological responses of CR; (2) not limiting caloric intake; (3) targeting the same stress response pathways that are affected by CR and providing protection against diverse stressors and (4) extending lifespan and reducing disease burden similar to CR. Some current mimetics under investigation are: insulin action suppressants (metformin), glycolysis inhibitors (2-deoxyglucose), and modulators of the stress signaling pathway (resveratrol). Similarly, physiological changes that occur with CR can be otherwise induced; low body temperature and increased insulin sensitivity have been proposed as effective CR mimetics (Ingram, Zhu et al. 2006), although evidence for these lacks.

It is widely believed that CR mimetics may exert effects by activating stress pathways at low levels. This idea adheres to the hormetic theory of aging. The hormetic theory parallels the idea that continual small doses of a toxin may condition an animal to have enhanced defenses to stress (Masoro and Austad 1996; Rattan 2001). With CR and its mimetics, stress pathways are activated at low levels over a sustained period of time which provides defense against other stressors like those of disease and aging processes(Masoro and Austad 1996). One mimetic that will be discussed in this

manuscript is that of limiting methionine in the diet. Several studies have found that restricting an essential amino acid like methionine may provide the same life-extending effects as a traditional caloric restriction (Orentreich, Matias et al. 1993).

In summary, much information is known about effects of CR and regimens have been described using mild and severely restrictive diets, diets of short and long duration, diets initiated at various life stages and in various species. Physiological characteristics of CR have been well-described despite little understanding of the underlying mechanisms. Yet there are many questions that remain. One such question surrounds the hunger that a CR animal continues to feel despite entering a new metabolic setpoint. As was described in relation to metabolic rate, body weight after a brief adjustment phase settles at a new level where food consumption is the same per kilogram body weight as the animal was consuming before CR (Masoro, Yu et al. 1982). Even after several months at this new setpoint where all systems have adjusted and where physiological benefits are still prominent, the animal continues to feel hunger. This has been demonstrated by offering extra food to animals that have been restricted for a significant proportion of their lifespan. In such cases, the animals will eat more food and rapidly regain body mass. This continues until the animal is close to, but does not reach the body weight of, animals given access to food *ad libitum* (Yu, Masoro et al. 1985). Why the animal continues to feel hungry during long-term CR is not known. Additionally puzzling is what sort of metabolic signal could be sustaining both the physiological benefits of CR and sense of hunger during CR despite the new setpoint. A coordinating signal of this type would need to be affected by feeding and affect appetite, be relevant to multiple organ systems, and be subject to change with long-term body weight changes.

We propose the hormone ghrelin as one potential regulator of physiological change during caloric restriction, a regulator which sustains the beneficial effects of CR despite the adaptation of body weight to the new, reduced input of food.

Introduction of Ghrelin

Ghrelin's discovery is a story in reverse pharmacology. While the ghrelin protein was yet unidentified, its receptor's actions in the pituitary first suggested this protein to be a major contributor to somatic function. Three years prior to ghrelin's discovery, the ghrelin receptor was isolated from rodent pituitary and hypothalamic tissues. Howard et al. (Howard, Feighner et al. 1996) discovered that, when stimulated, a receptor in the anterior pituitary potently released growth hormone. The receptor, named Growth Hormone Secretagogue Receptor (GHSR), was different from growth hormone releasing hormone receptor (GHRH-R), the known receptor responsible for growth hormone's release. GHSR, on the other hand, had a different conformation despite similar actions and its ligand was unknown. Subsequent work identified a small 28-AA peptide from stomach extracts that was the ligand for the orphan GHSR and this protein was termed ghrelin (Kojima, Hosoda et al. 1999). The prefix 'ghre' is the protoindoeuropean term for 'grow' and the 'ghr' spelling at the beginning of the word aptly reflected its actions: growth hormone release (Young Cruz and Smith 2008). After identification of ghrelin, further characterization delineated some of its important properties. Such properties include its forms, production, alternative actions, and regulation.

The protein form of ghrelin is translated from the ghrelin gene located in humans on the third chromosome and in mice on the sixth (UCSC 2010). Splicing the ghrelin gene first creates a non-active preproghrelin molecule. This protein is then cleaved in the endoplasmic reticulum of an endocrine cell by a signal peptidase complex. The resulting prohormone is then further cleaved by prohormone convertase 1/3 to yield the mature, unacylated ghrelin protein (Reactome 2010). Ghrelin is then octanoylated by a lipid acyl transferase termed Ghrelin *O*-acyltransferase or G*O*AT (Gutierrez, Solenberg et al. 2008; Reactome 2010). The final peptide consists of 28 amino acids and includes a serine at position three. This serine is vital; it attaches to an acyl functional group which increases ghrelin's preference for the GHSR receptor. The acylation is specifically an octanylation, as the acyl group that is added at this site consists of an eight-carbon backbone. The acylated/octanoylated form is known as acyl ghrelin or acylated ghrelin and the protein without the functional group is referred to as unacylated or des-acyl ghrelin. References to just 'ghrelin' within this document will refer to the octanoylated/acylated form in blood (plasma or serum), as most of the biological actions are transmitted through circulating levels of this ghrelin form. Additionally, ghrelin's actions will refer to binding of ghrelin to the GHSR1α receptor subtype, the primary receptor for its known actions, unless otherwise stated.

The dynamics between the presence of the acylated form and the unacylated form are also quite complex. X/A-like and endocrine cells located in mouse and human stomach tissues produce the unacylated form of ghrelin. Most of the circulating plasma ghrelin protein is derived from stomach tissue, although other tissues (e.g. intestine, hypothalamus, placenta, lungs, liver, and fat) are also sites of ghrelin protein synthesis

(Kojima, Hosoda et al. 1999; Gnanapavan, Kola et al. 2002; Tanaka, Minoura et al. 2003; Dixit, Shaffer et al. 2004). However, the contribution to plasma ghrelin from these tissues is minor; at these sites, ghrelin is primarily generated for autocrine regulation. Within the endoplasmic reticulum lumen of ghrelin-producing cells are acetylases or deacetylases which control the octanylation of ghrelin (e.g. ghrelin-*O*-acyl transferase) (Yang, Brown et al. 2008). The half-life for the acylated or bioactive form is ~10 minutes, during which time it has potent effects on the GHSR1α receptor (Nagaya, Kojima et al. 2001). After stimulating GHSR, ghrelin is converted back to its unacylated form via either butyrylcholinesterase or platelet-activating factor acetylhydrolase, both of which circulate in blood (De Vriese, Hacquebard et al. 2007). The unacylated form of ghrelin cannot bind to the canonical shape of the GHSR. However, it is now thought that unacylated ghrelin carries out both similar and unique actions as acylated ghrelin but via a different receptor/mechanism (van der Lely, Tschoep M et al. 2004). For instance, unacylated ghrelin has been linked to insulin secretion in rat insulinoma cells (Gauna, Delhanty et al. 2005), luteinizing hormone secretion in male rats (Martini, Fernandez-Fernandez et al. 2006), and stimulation of food consumption behaviors in rodents (Toshinai, Yamaguchi et al. 2006). The reaction dynamics for converting ghrelin between its two forms are controlled by hormonal and nutrient sensors.

Ghrelin's production is known to be controlled by feeding status and changes in body weight. Ghrelin increases with fasting and negative energy balance and decreases with feeding and positive energy balance states. Ghrelin production in the stomach is thus controlled by features of these states. Although not entirely known, ghrelin's release seems to be impaired by feeding (high levels of plasma insulin, glucose, glucagon and

fatty acids) as well as obesity, gastric bypass, several neuroregulatory factors (GHRH, somatostatin (SS), cholecystokinin (CCK), Peptide YY, melatonin) growth hormone, and leptin (Litwack 2008). Ghrelin's release is stimulated by acetylcholine and muscarinic receptor agonists, testosterone, and features of fasting: low concentrations of plasma insulin, glucose, and ingested nutrients (Litwack 2008).

Growth Hormone Secretagogue Receptor (GHSR)

As previously introduced, the receptor specific for ghrelin is termed the Growth Hormone Secretagogue Receptor. This name reflects that fact it was an orphan receptor, known only for its actions on Growth Hormone before the ghrelin ligand was discovered. Currently it is also referred to as the ghrelin receptor, but the name and acronym GHSR have remained in the literature. The GHSR protein is encoded by the GHSR gene located on the third chromosome in both humans and mice (UCSC 2010). The GHSR gene can splice into two variants: the GHSR-1α subtype and the GHSR-1β subtype. Acylated ghrelin carries out its potent effects via downstream signaling by its preferred receptor type, Growth Hormone Secretagogue Receptor-Type 1α (GHSR1α). Alternately, the GHSR-1β can bind the ghrelin protein but has a truncated intracellular domain incapable of transmitting downstream signals. Therefore none of ghrelin's identified actions are carried out via the GHSR-1β receptor. It is important to note however, that the GHSR1α subtype is an extracellular-residing receptor. The GHSR1β, on the other hand, is a nuclear receptor (Smith, Jiang et al. 2005). Although this receptor form is not known for

stimulating any transcription or genomic effects, its location in the nucleus of diverse somatic cells makes its presence curious.

The primary locations for GHSR1α receptors are in the appetite/satiety centers of the hypothalamus (arcuate nucleus and ventromedial hypothalamus), anterior pituitary, as well as thyroid, pancreas, stomach, heart, lung, adrenal cortex, immune system, adipose tissue and human breast carcinoma. However, a 2002 study tested for ghrelin mRNA and both of its receptor subtypes (GHSR1α and GHSR1β) mRNA in various tissues and revealed that mRNA of each type was present in all tissues examined: adrenal gland, atrium, breast, buccal mucosa, esophagus, Fallopian tube, fat tissue, gall bladder, human lymphocytes, ileum, kidney, left colon, liver, lung, lymph node, muscle, myocardium, ovary, pancreas, pituitary, placenta, prostate, right colon, skin, spleen, testis, thyroid, and vein (Gnanapavan, Kola et al. 2002). The wide distribution of ghrelin and its receptors suggests many physiological roles for ghrelin.

The previous findings establish ghrelin's anatomical presence, but its physiological impact is yet to be evaluated in the context of health and longevity. The following paragraphs will discuss these topics. Discussion of ghrelin in the 'health' context will consist of some of ghrelin's known major actions, specifically in regard to energy metabolism/food consumption behavior, stimulation of GH release, and its emerging role in modulating immune responses. Ghrelin's role in 'longevity' will be discussed in terms of changes that occur during the aging process. A review of the literature indicates it may be premature to suggest that ghrelin alone can affect longevity, but there seems to be compelling evidence that ghrelin can have a significant impact on an organism's physiology as it ages. Finally, physiological actions of ghrelin will be

compared to the known effects of the caloric restriction process and present the case for ghrelin as a metabolic regulator during CR.

Physiological Actions of Ghrelin

The hormone ghrelin has well-known effects on energy metabolism, feeding behavior, and Growth Hormone (GH) release, but investigations in the past few years have provided examples of ghrelin's nuanced actions that were not previously described. For instance, ghrelin is now known to have effects on the following: peripheral hormone secretion, appetite, gastric emptying and acid secretion, gastrointestinal tract motility, pancreatic function, glucose homeostasis, cardiovascular function, immunity and inflammation, cell proliferation and survival, bone metabolism, reproductive organ function, neurogenesis, memory, and sleep, as reviewed in Dixit and Taub (Dixit and Taub 2005).

Appetite and Energy Metabolism

Ghrelin's actions in energy balance are thought to be its primary and most important role. Plasma ghrelin levels peak approximately 30 minutes before ingestion of a meal and hit their nadir approximately one hour after meal termination (Nagaya, Kojima et al. 2001). This result, combined ghrelin's stimulation of feeding behavior in rats (Asakawa, Inui et al. 2001; Tang-Christiansen, Vrang et al. 2004) and decreased time intervals between meals, all suggest ghrelin affects metabolism in the direction of positive energy balance (Cummings, Frayo et al. 2004). Similarly, high plasma ghrelin levels exist in underweight, but otherwise healthy individuals, and increased ghrelin

levels potently stimulate appetite in rodent and human studies (Tschoep, Smiley et al. 2000; Asakawa, Inui et al. 2001; Wren, Seal et al. 2001a). This action, coupled with low ghrelin levels in overweight and obese individuals, suggest that ghrelin attempts to correct for imbalances in body weight and energy intake. High ghrelin levels stimulate appetite and, in many organisms, lead to additional food intake (Tschoep, Smiley et al. 2000; Asakawa, Inui et al. 2001; Wren, Seal et al. 2001a). There's evidence in humans that ingested food is then moved more quickly through the gastrointestinal tract and gastric emptying is also increased (Levin, Edholm et al. 2006). The food bolus is then also exposed to a higher concentration of gastric acid, as was demonstrated in rats given exogenous ghrelin (Masuda, Tanaka et al. 2000). These results suggest the ingested food is processed more quickly with higher levels of ghrelin and, in normal physiological states, ghrelin may regulate consumptive behaviors. For instance, if ghrelin levels are high, appetite is stimulated and more food is consumed. Additionally, if the food is digesting more rapidly, the organism may shorten the interval until the next meal, all acting in the direction of weight gain.

Ghrelin derived from stomach does not just affect gastrointestinal (GI) physiology, but also stimulates vagal afferents that innervate feeding centers in the brain. Stomach-derived ghrelin in plasma travels to and stimulates the Lateral Hypothalamic Area (LHA), the appetite center of the hypothalamus. At this location, ghrelin's actions result in the release of Neuropeptide Y (NPY) and Agouti-related peptide (AGRP). The increase of these orexigenic neuropeptides yield potent appetite-stimulating effects. There is some evidence that without the stimulation of these potent appetite enhancers, and resulting release of nitric oxide, ghrelin would not be able to carry out its orexigenic

role (Nakazato, Murakami et al. 2001; Gaskin, Farr et al. 2003). Simultaneously, ghrelin acts on the Ventromedial Nucleus (VMN) to inhibit the activation of satiety-inducing Pro-opiomelanocortin (POMC) and Cocaine and amphetamine-related transcript (CART) peptides (Tschoep, Statnick et al. 2002). Because of these effects, ghrelin is known to potently stimulate appetite, but its actions on actual consumption behavior are unclear (Cummings 2006; Litwack 2008).

Other peptides work in concert with ghrelin to impact consumptive behaviors. Ghrelin's counterpart leptin is released from adipocytes and circulates in plasma at levels directly reflecting the amount of body fat stored (Margetic, Gazzola et al. 2002). Leptin has opposite effects on neural peptides in the arcuate nucleus. Additionally, high levels of insulin in the bloodstream have similar effects to leptin. Insulin and leptin both circulate at levels in proportion to body fat stores (Friedman and Halaas 1998). These proteins interact with short-term feeding signals from the GI tract (CCK e.g.) to regulate meal size according to the amount of existing fat (Woods and D'Alessio 2008). All of these signals serve as tight homeostatic controls and function to maintain body weight in the long-term. Although ghrelin, leptin, and to some degree, insulin are the primary hormones regulating appetite, other hormones also play a role. Short-term stress hormones (Norepinephrine (NE), Epinephrine) may function to decrease appetite during periods of acute stress. However, increased glucose mobilization required for sympathetic nervous system activation in addition to slower, longer acting stress hormones or chronic low levels of stress hormones may serve to increase appetite later (cortisol, eg). The interplay of these hormones regarding appetite and food consumption is reviewed in von Haehling et al. (von Haeling, Lainscaka et al. 2009).

Further, ghrelin acts on other organs with important metabolic consequences. Ghrelin is known to have autocrine effects in the pancreas, as the pancreas has been identified as a minor source of ghrelin production (Date, Nakazato et al. 2002). The exact role of ghrelin on the pancreas is not well understood, but it seems to stimulate insulin and gastrin production in isolated rodent pancreatic cells (Date, Nakazato et al. 2002; Lee, Wang et al. 2002) but inhibit insulin release in humans, despite a rise in blood glucose (Broglio, Arvat et al. 2001). Further, ghrelin stimulates gluconeogenesis in liver and unacylated ghrelin may antagonize this process (Gauna, Delhanty et al. 2005). In rat adipocytes, ghrelin increases insulin-mediated glucose uptake, promotes fat differentiation and deposition, and conversely antagonizes lipolysis (Muccioli, Pons et al. 2004; Thompson, Gill et al. 2004; Patel, Stanley et al. 2006). In sum, ghrelin's role in energy metabolism is in the favor of positive energy balance and may, in an evolutionary sense, counteract leptin and other anorexigenic actions to protect an animal from starvation.

The evidence discussed in the prior paragraph discusses ghrelin's role in normal physiology. In general, ghrelin levels adjust to compensate for changes that occur under disruptions in energy balance. For instance, ghrelin levels are constitutively higher in men and women who are underweight due to illness-induced cachexia, anorexia nervosa, dieting, or any negative energy balance condition versus normal weight individuals. In contrast, ghrelin levels decrease as organisms enter long-term states of positive energy balance, such as overweight or diet-induced obese conditions. It is only in congenic causes of obesity, like Prader-Willi syndrome, where ghrelin levels are abnormally high versus Body Mass Index (BMI)-matched controls. These high ghrelin levels are thought

to contribute to the hyperphagic behaviors and resulting obesity exhibited by Prader-Willi individuals. In individuals with ghrelin derangements, (underweight, Prader-Willi, obese individuals), only metabolic effects of ghrelin have been examined. The effects of ghrelin on other systems that will be discussed next have not been evaluated in the current scientific literature.

GROWTH HORMONE (GH) RELEASE

The stimulatory effects of ghrelin on Growth Hormone (GH) release are the first identified actions of ghrelin. The strength of ghrelin's effect was similar to that of Growth Hormone Releasing Hormone (GHRH), somatotrophs' natural stimulant. In the last decade, ghrelin's direct stimulation of GH release through its 1α receptor was identified. However, ghrelin also stimulates Growth Hormone Releasing Hormone (GHRH) release from the hypothalamus (also through GHSR1α). GHRH is the primary stimulator of GH release from the anterior pituitary and its effects as such are potent. It has also been suggested that there may be a synergistic effect of ghrelin and GHRH simultaneously stimulating somatotrophs which results in a massive secretion of GH (Wren, Small et al. 2002). Further, ghrelin inhibits SS at the level of the hypothalamus and SS is a potent inhibitor of GH release (Di Vito, Broglio et al. 2002). Therefore, the combination of these three effects allows ghrelin to have a very potent stimulating effect on GH release.

In contrast, GH and its related downstream protein Insulin-like Growth Factor-1 (IGF-1) have feedback effects on ghrelin. While ghrelin stimulates this axis, there is compensatory feedback from both GH and IGF-1 on ghrelin's release from the stomach.

GH decreases ghrelin production and release but does not affect stored ghrelin protein. Qi et al. (Qi, Reed et al. 2003)determined high plasma GH decreased stomach ghrelin mRNA and plasma levels, but had no effect on the amount of protein in stomach endocrine cell vesicles. IGF-1 may also have a negative feedback effect on ghrelin's release, but these effects are thought to be secondary to GH's primary feedback effect.

GHRELIN AND THE IMMUNE SYSTEM

Early tissue scans for ghrelin and its receptor mRNA demonstrated the presence of both on immature and mature human T cells, B cells, and neutrophils, but the degree of expression was highly variable between subjects (Hattori, Saito et al. 2001). This was a somewhat persuasive finding because it suggested that ghrelin with GHSR may have effects outside of GH secretion in the immune system. This was largely due to the simultaneous finding that GH and GH receptor transcripts were not identified on T cells or neutrophils in that same study. Subsequent reports have shown that ghrelin greatly inhibits both artificially-induced T cell differentiation and mRNA expression of cytokines: IL-2, IFN-γ, IL-4, and IL-10 (Xiaa, Panga et al. 2004). This indicates ghrelin may be an inhibitor of T cell growth, proliferation and function since these cytokines are critical mediators in both cell-mediated inflammatory (IL-2, IFN- γ) and humoral (IL-4, IL-10) immune responses (Coffman and Mosmann 1991). Finally, it was shown that ghrelin's suppressive effects on the immune system could actually be very beneficial to the organism when that immune-response is pro-inflammatory and/or leptin-induced. Dixit et al. (Dixit, Shaffer et al. 2004) showed ghrelin inhibited the expression of the detrimental proinflammatory cytokines IL-1β, IL-6, and TNF-α induced by leptin in T lymphocytes and monocytes. These cytokines are known to be anorectic. The finding

that ghrelin also attenuates endotoxin-induced appetite loss in a murine endotoxemia model (Dixit, Shaffer et al. 2004), implies that ghrelin could potentially be an important mediator between immune function and metabolism.

As stated in the introduction, ghrelin has many more actions than just on energy metabolism, GH secretion, and immune function, but the experimental evidence on the other functions is, at best, limited. Briefly, ghrelin has also been shown to have modest effects increasing vasodilation and having positive treatment effects in chronic heart failure (Nagaya, Moriya et al. 2004), relaxing vascular tone in smooth muscle (Wiley and Davenport 2002), increasing anxiety-like behaviors (Carlini, Martini et al. 2008), stimulating osteoblast proliferation and differentiation (Maccarinelli, Silbilia et al. 2005), regulating fat mass in pregnancy and lactation (Caminos, Tena-Sempere et al. 2003), inhibiting the gonadotropic axis in times of energy deficit (Fernandez-Fernandez, Tena-Sempere et al. 2004), and possibly regulating energy balance in some cancers.

Pathological Changes in Ghrelin that May Contribute to the Aging Phenotype

There is interesting interplay between ghrelin and the aging phenotype. First, changes in ghrelin levels in an aging organism will be described. Second, the potential influence of ghrelin on the aging phenotype will be explored. Importantly, levels of plasma ghrelin decrease with increasing age. This is a result of reduced mRNA transcription of ghrelin and subsequently lower plasma protein levels, decreased receptor mRNA and number in critical tissues, and potentially decreased sensitivity to the effects of ghrelin, independent of the decreased receptor number. Overall, ghrelin and its actions

are significantly reduced with age in both rodents and humans. In general, the plasma level of ghrelin decreases with increasing age (Rigamonti, Pincelli et al. 2002). Subsequently, ghrelin could be in part responsible for endocrine and immune changes that occur with aging. In rodents, a high body weight for their species at younger ages is predictive of an earlier death. In humans the same is true for high body weight resulting from high fat mass (Lane and Dickie 1958; Eriksson, Forsen et al. 1999; Fontaine, Redden et al. Years of Life Lost Due to Obesity). However, after a critical older age threshold is reached, ~70 years in humans, higher body mass/body fat levels are correlated with better health and survival (Potter, Schafer et al. 1988; Grabowski and Ellis 2001). There is sound reason then to preserve appetite among the oldest-aged individuals of a species. The age-related attenuation in ghrelin may contribute to the similarly-timed decline in appetite that is experienced by both humans and rodents, as described earlier. Similarly, in some cases there may be a loss of absolute body fat with age, which, as described above, can be deleterious in the oldest-aged people (Newman, Lee et al. 2005). Ghrelin may be in part responsible, since the adipocyte proliferating and differentiating signal fat cells receive via ghrelin, would now be diminished.

Low GH and its downstream mediator of action Insulin-like Growth Factor-1 (IGF-1) is also a characteristic of increasing age. The low levels of these hormones at old ages, but not young ages, are also thought to be deleterious (Corpas, Harman et al. 1993). Since ghrelin is a potent stimulator of GH release, its loss over time may contribute to decreased amplitude and increased interval between pulses of GH release. Restoration of ghrelin and GH to old-aged animals have been shown to have some positive benefits on skeletal muscle, lipid profile, etc. Yet there is really no clear evidence that restoration of

GH to higher levels is beneficial in the frail, and even may have deleterious consequences (Holloway, Butterfield et al. 1994; Lanfranco, Gianotti et al. 2003). The mixed results have prevented its therapeutic use in the elderly.

Finally, it is known that older rodents and humans exhibit reduced capacity to fight noxious agents. The aging process contributes to a slow involution of, and increased adiposity in the thymus, which leads to some specific reductions T-cell function (Steinmann 1986; Aspinall and Andrew 2000). Although there are many specific changes that occur in the immune system during aging, it is widely accepted that the immune system is just not as functional as at younger ages and as a result, may be less capable of fighting off some infections and cancers (for review, see(Miller 1991)). However, older aged individuals exhibit a low-grade inflammation with a presence of proinflammatory cytokines permanently detectable (Franceschi 2000; Bruunsgaard, Pedersen et al. 2001). This low-grade inflammation has harmful effects on health in humans, specifically regarding atherosclerosis, Alzheimer's disease, Type II diabetes, etc (reviewed in (Bruunsgaard, Pedersen et al. 2001)). Ghrelin's ability to suppress these proinflammatory cytokines at young ages may be lost as ghrelin levels are reduced with age (Dixit and Taub 2005).

In conclusion, there is evidence for additional regulatory roles of ghrelin: inhibition of insulin release (Wierup, Yang et al. 2004), protection of reproductive organ function (Fernandez-Fernandez, Tena-Sempere et al. 2004), and maintenance of cognition (Carlini, Martini et al. 2008). The effectiveness of ghrelin in these roles may be impaired as ghrelin levels decrease with age, perhaps contributing to other age-related

conditions like insulin resistance and Type II diabetes, loss of fertility, and decreased performance on cognitive and memory tasks with advancing age.

Does Ghrelin Play a Major Role in CR?

The similarity of actions between caloric restriction and ghrelin is provocative. As caloric restriction has been shown to affect hunger, the GH/IGF-1 axis, insulin and glucose homeostasis, immune system function and proinflammatory responses, anxiety, cognition, cardiovascular pathologies, etc, ghrelin has also shown similar effects in these areas *(Table 1)*. Since ghrelin is high in CR, a comparison of positive associations between ghrelin and CR with each system is warranted. Some processes respond in the same direction with high ghrelin and CR: increased hunger and anxiety, reduced insulin and glucose levels, and beneficial effects on proinflammatory cytokines. Others work in the opposite direction; CR reduces activity of the GH/IGF-1 system while ghrelin stimulates. Still others, like immune function, are mixed. CR both positively and negatively affects immune action; ghrelin similarly has mixed actions. Additionally, both play a role in aging. CR decreases age-related decline. Ghrelin levels typically decline with age, but exogenous restoration of ghrelin improves a variety of age-related immune, appetite, and insulin derangements. Additionally, ghrelin has been shown to be affected in the short-term by changes in feeding and in the long-term by changes in body weight. These properties are important as CR modifies both. The significant overlap between CR and ghrelin processes suggest ghrelin may play a role in the beneficial effects of CR on health and lifespan. This dissertation will begin to characterize ghrelin in various CR

models and mimetics and attempt to lay the foundation for the proposition that ghrelin plays a major role in CR processes.

Table 1. Comparison of the Actions of Ghrelin and CR

Physiological Parameter	CR	↑ Ghrelin
Anxiety	+	+
GH/IGF-1 axis stimulation	-	+
Hunger	+	+
Immune response	+/-	+/-
Insulin release	-	-
Insulin sensitivity	+	+
Lifespan	+	?
Pro-inflammatory cytokines	-	-

CHAPTER III. INTRODUCTION TO STUDIES

Hypothesis and Specific Aims

As outlined previously, CR not only has dramatic effects on lifespan but also on health. The widespread physiological benefits conferred by CR require a systemic mechanism to carry out its effects. This mechanism is yet unknown and is likely a combination of cellular and physiological stimuli. The presence of hunger despite a new body weight setpoint and an equivalent metabolic rate concomitant with widespread health benefits belies assumptions that CR is entirely advantageous.

Ghrelin is a stomach-derived signal which confers a sense of hunger in the absence of food and in negative energy balance states. Its effects on appetite are potent and its role in metabolic processes is varied. The literature supports the case that ghrelin could be a metabolic regulatory signal during caloric restriction. This evidence, as previously narrated, confirms ghrelin's presence in the Growth Hormone/Insulin-like Growth Factor (GH/IGF-1) axis, important role in glucose and insulin homeostasis, and stimulation of appetite and obesigenic effects.

Therefore, the ultimate goal of this work is to investigate if ghrelin is a metabolic regulator which in part confers hunger during CR. This hypothesis will be addressed by four separate CR paradigms and described here in three studies. Both mice and human versions of CR will provide data.

Based on these specific studies, the Specific Aims of this body of work are to

i. characterize the response of plasma and tissue ghrelin to different models/mimetics of caloric restriction

ii. compare ghrelin's actions to known changes resulting from various CR methods

iii. investigate ghrelin's role with regard to caloric restriction and disease states, i.e. during gastric bypass surgery and obesity-related diseases

These aims will be addressed by three studies. Study I will be based on a short-term, severe CR administration in male and female heterogeneous stock mice. A more moderate restriction diet and *ad libitum* diet are included for comparison. Study I is termed the 'Army' Study.

Study II will span three rodent studies of CR. The first substudy explores a potential mechanism of CR. This substudy, termed the 'Sell' study, characterizes the metabolic properties of male and female mice genetically altered for low plasma IGF-1. These animals have been previously found to have some extensions in lifespan in the absence of CR. The second substudy of Study II, or the 'Glenn' study investigates a mimetic CR diet of low methionine initiated in middle age. Middle-aged male and female heterogeneous stock mice on a traditional 40% CR and an *ad libitum* diet are likewise compared. Substudy 3 of Study II is the Army study.

Finally, Study III describes metabolic changes in humans undergoing gastric bypass surgery. Gastric bypass surgery is one of the only human CR paradigms.

Changes in ghrelin levels will be described in all of the above studies and each study will also individually contribute some specific information about ghrelin during CR.

STUDIES AT A GLANCE

STUDY I: The Army Study

Comparison of short-term severe 60% CR to moderate 40% CR and an ad libitum *diet in male and female heterogeneous stock mice.*

STUDY II: Rodent CR Comparison Study

Compilation of three studies investigating CR models/mimetics in mice

Substudy 1: The 'Sell' Study – Characterization of metabolic properties in male and female genetically altered for low IGF-1 and genetically intact control mice

Substudy 2: The 'Glenn' Study- Comparison of physiology and lifespan for middle aged, heterogeneous stock mice initiated on a low-methionine, 40% CR or control diet

Substudy 3: The 'Army' Study

STUDY III: Human Gastric Bypass Study

Investigation of physiological and genetic traits of weight loss disease mitigation success in gastric bypass patients

CHAPTER IV. STUDY I: THE ARMY STUDY

"Investigating ghrelin as a metabolic signal in a severe, short-term mouse model of caloric restriction."

Background and Aims

There is much interest regarding caloric restriction and metabolic changes over the lifespan which lead to reduced disease burden in later life and ultimately, extended lifespan. However, this paradigm does not translate well to human studies as very few people engage in a lifetime of traditional dietary restriction. Further, there is great variability in the diets followed by these restrictors which does not even allow for carefully monitored observational study. Yet there are situations where humans are subjected to types of CR and the consequences of these actions are unknown. For instance, soldiers in war zones dispatched for extended periods of time are required to carry all meals as Meal-Ready-to-Eat (MRE) packs. Under stressful conditions such as extreme heat, dangerous surroundings, rocky terrain, and grueling physical requirements, many soldiers self-select to carry fewer MREs on their backs. Therefore, these soldiers are experiencing a short-term severe CR due to lack of nutrition combined with intense physical activity. This study mimics this type of restriction in a cohort of heterogeneous stock mice, outbred to represent the genetic diversity of the human population. The aims of this study are to:

i. Investigate metabolic consequences of degrees of restriction during short-term CR

ii. Identify any short-term changes in body composition

iii. Measure multiple aspects of ghrelin: changes in plasma levels, tissue mRNA expression and stomach size

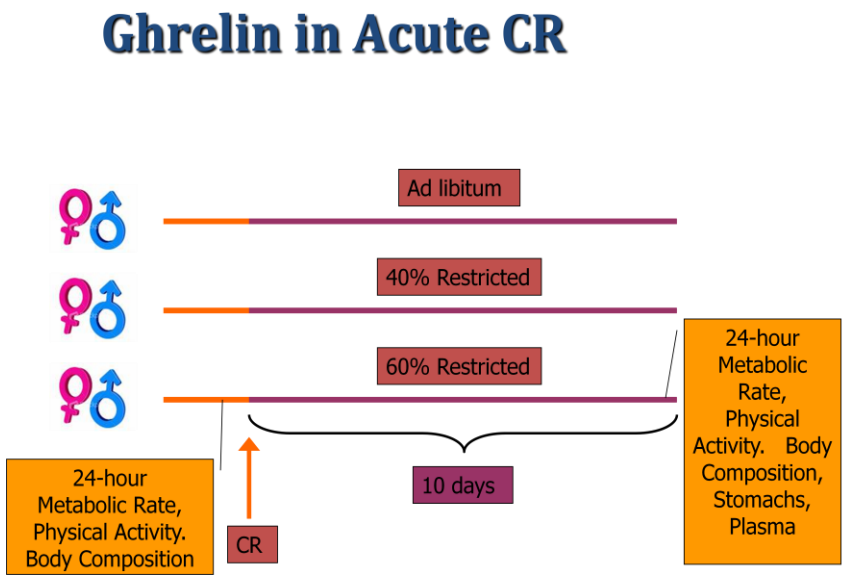
Methods

To address the above aims, a short-term, ten-day food restriction or control diet was administered to ~60 male and female heterogeneous stock mice. The three diet groups included both males and females in a ten-day 60% CR, 40% CR or *ad libitum* diet. Food consumption was measured for three days and averaged; the 40% and 60% restriction numbers were calculated from these *ad libitum* periods. Each diet group was measured just before the start of the diet period for the following: 24-hour continuous metabolic rate, simultaneous 24-hour physical activity, body weight, and body composition. The diets were applied in parallel groups for ten days. At the end of the ten days, a 24-hour metabolic rate and physical activity run was completed and each animal also underwent body weight and composition measurements. Metabolic rate was measured by placing a single mouse in an enclosed cage with a constant flow of air with known concentrations of CO_2 and O_2. Metabolic rate (MR) was calculated from samples of expired air leaving the cage via CO_2 production and O_2 consumption. Using a food energy conversion, MR is then reported as kcal/kg/min. Physical activity was measured by placing the clear, plastic MR cage within a physical activity apparatus. This device emits a series of laser beams in two dimensions which measures two dimensions of lateral activity as well as movement in the vertical direction. Total distance traveled is the sum of distance traveled each second and is reported in centimeters. Hourly sums

were calculated as well as a total 24-hour value. Body weight was measured by a standard balance and body composition by whole animal DEXA. Animals were sedated with a combination of ketamine and xylazine for ~30 minutes while the ~ 10 minute scan is taken. Results include total grams of and percent body fat, totals grams of and percent lean mass, and bone mineral density.

At the end of the ten day diet scheme and final 24-hour MR and physical activity measurements, the animals are sacrificed. Trunk blood is collected with EDTA preservative. Plasma ghrelin, insulin, and glucose were measured. All measures were compared between sexes within diet groups and pooled for diet group analysis when not significantly different. Moreover, stomach weight relative to body weight at time of sacrifice was compared between groups. While most studies of CR animals show a relative decrease in organ size to the restriction, one study has shown stomach tissue may actually be increased in CR (Yu, Masoro et al. 1985; Yang, Youm et al. 2007). Since ghrelin is produced in the stomach, this finding may have clear implications for ghrelin's increased production and circulating plasma levels. Overall, this study will provide an assessment of various ghrelin characteristics during short-term severe CR. A schematic portraying the study protocol and measures is below.

Figure 4-1. Schematic of the Army Study



CHAPTER V. STUDY II: COMPARISON OF THREE CR DIETS IN MICE

"Ghrelin as a metabolic regulator during rodent models and mimetics of caloric restriction."

The primary goal of this research is to compare plasma ghrelin levels in three separate rodent models or mimetics of caloric restriction. The first study, or 'Sell' study, establishes ghrelin's actions in male and female mice genetically modified for low plasma IGF-1 levels. This is a long-term observational study characterizing aspects of metabolism (metabolic rate, physical activity, food intake, plasma ghrelin, glucose, and insulin, ghrelin and GHSR expression in stomach tissue, organ size, etc) in these animals which have been shown to have extended lifespan. The second study examines the effects of a low methionine diet started in middle age in male and female heterogenous stock mice. A specially formulated diet which contains 0.15% concentration of the essential amino acid methionine (vs 0.4% in controls) is compared with a tradition 40% CR and a control, *ad libitum* diet. Animals in this study were measured for metabolic rate, physical activity, food consumption, body weight, ghrelin, glucose and insulin every 60 days until death. This study is referred to as the 'Glenn' study. Finally, plasma ghrelin levels from Study I of this proposal (the Army study) will be compared to plasma ghrelin levels from both the Sell and Glenn studies. The Army study measured plasma ghrelin levels in male and female young adult heterogeneous stock mice after a ten-day 60% CR, 40% CR or *ad libitum* diet. Additional measurements included metabolic rate, physical activity, and body weight and composition before and after the diet period, and

plasma glucose and insulin at time of sacrifice. Comparing ghrelin levels across these multiple animal groups in each of the three studies will help describe ghrelin's responses during disparate variations of CR.

Background for the Sell Study

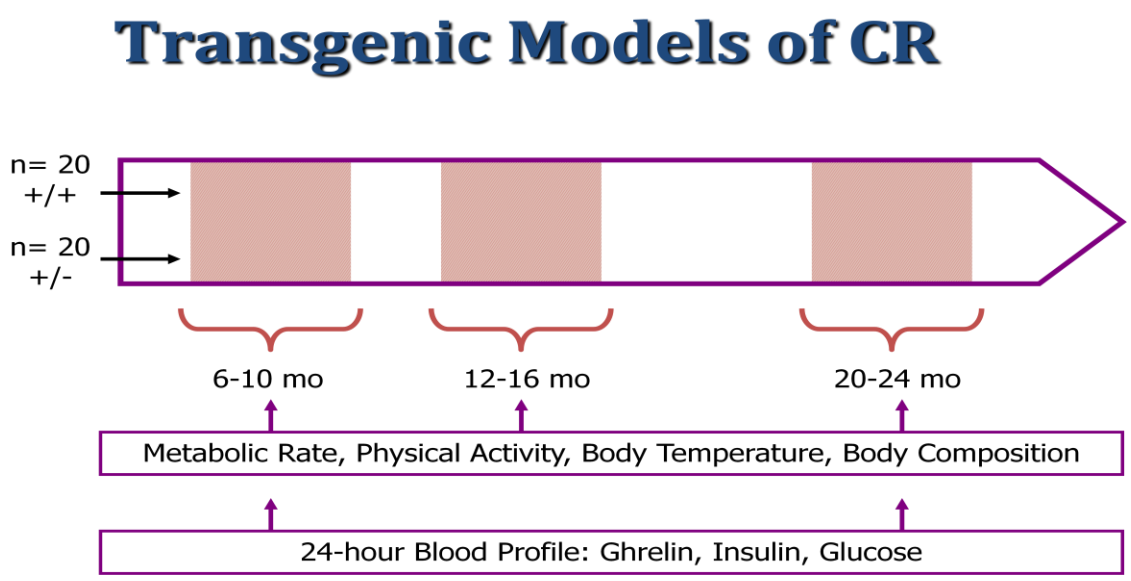
Low plasma insulin like growth factor-1 (IGF-1) is a well known characteristic of the CR response. Suspicion arose that it might be a major mechanism of the CR phenotype as it is both markedly lower in animal models of CR but also because of its diverse roles in the body. IGF-1 is not only a hormone that promotes growth in early life; IGF-1 continuously regulates aspects of development throughout the lifespan. At some point in an organism's development, IGF-1 is in part responsible for a number of diverse processes: bone development, tissue growth, organ structure, reproductive function, and fetus development (for review see (Butler and Roith 2001)). Growth, fertility, and longevity processes are also altered in CR, likewise supporting a role for IGF-1 in CR. Additionally, in response to CR, IGF-1 levels drop markedly and remain low throughout the duration of the restriction. Subsequently, several knockout models of IGF-1, Growth Hormone, or Growth Hormone Receptor have been developed to probe the lifespan effects of reduced signaling along the GH/IGF-1 axis (see (Perez, Bokov et al. 2009) for review). Some studies have shown dramatic extensions in lifespan, including those from Holzenberger et al. (Holzenberger, Dupont et al. 2003), who showed that female mice with null mutations for the IGF-1 receptor lived on average 33% longer than their female genetically-intact counterparts. Males exhibited a non-significant 16% extension in

lifespan, but overall the effect between genotypes was significant (26%, $p<0.02$). Other models in C. elegans and D. melanogaster have shown similar results (Carter, Ramsey et al. 2002; Tatar, Bartke et al. 2003). The sexual dimorphism favoring females has been repeated as well, demonstrating alterations in IGF-1 axis signaling may have unique effects between sexes (Selman, Lingard et al. 2008). Molecular approaches have been applied to investigate how signaling at the physiological level may be affecting longevity genetics. Studies in *C. elegans* have pointed to a downregulation in the insulin signaling pathway genes *age-1* and *daf-2* and animals genetically mutated at these sites have also shown lifespan effects (Dorman, Albinder et al. 1995). Therefore, there is a confirmed contribution of the GH/IGF-1 axis on lifespan. Although molecular mechanisms have been postulated, the full characterization of this process is not completely known.

Other aspects of reduced IGF-1 signaling as a CR mechanism also remain unknown. For instance, while the lifespan effects were apparent in invertebrate models, knocking out IGF-1 in rodents did not yield viable animals. Full deletion of the IGF-1 gene resulted in low birthweight pups with underdeveloped cardiovascular and pulmonary systems resulting in their immediate death. This gene deletion is therefore perinatally lethal (reviewed in (Carter, Ramsey et al. 2002)). Yet one successful knockdown model has been developed. Sell et al (personal communication) have developed a mouse model which combines one allele on the IGF-1 gene which results in full mRNA deletion with one fully functional allele. Combined, these alleles produce approximately 50% reduced concentrations of plasma IGF-1 hormone. Subsequently, cellular signaling is similarly reduced to about half that of controls. This mouse model has demonstrated a slight increase in lifespan but has not been characterized for its

physiological properties. Therefore, the goal of this study is to longitudinally measure the metabolic properties of ~60 male and female genetically altered mice, termed 'midi' mice for their small body size and ~80 male and female I29/C57/CD-1 controls. This paradigm will investigate if the same metabolic alterations seen during CR are apparent in these low IGF-1 animals and possibly support the theory that low IGF-1 is a mechanism of CR. A schematic of this study follows:

Figure 5-1. Schematic of the Sell Study

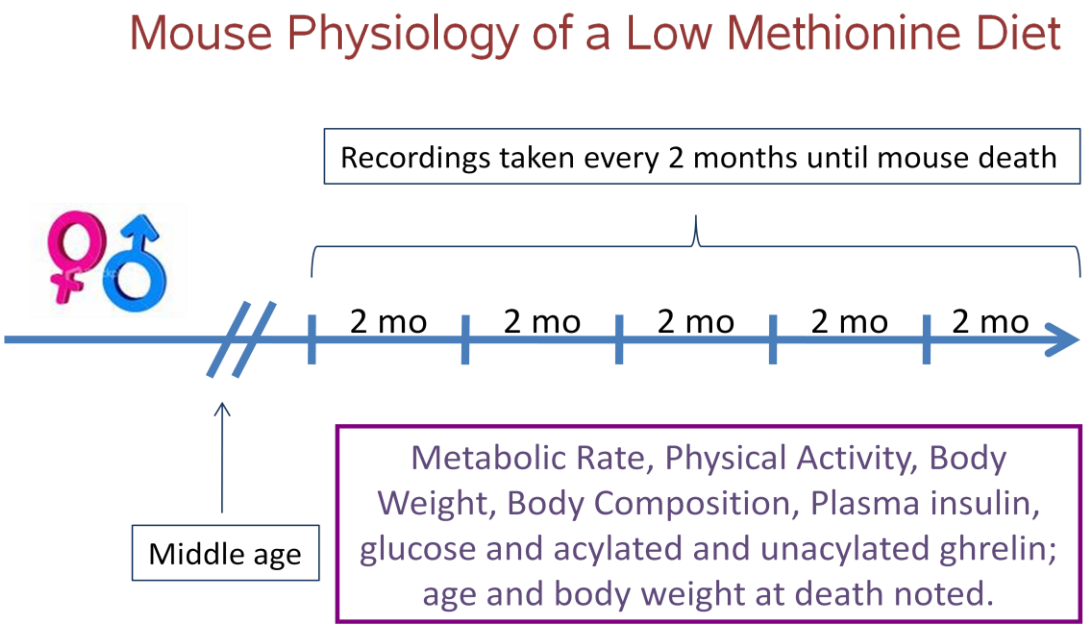


Background for the Glenn Study

The Glenn study examines male and female heterogeneous stock mice undergoing three different diet schemes begun in middle age. The mice are treated with a low methionine diet, a traditional 40% CR, or a normal rodent chow diet and measured for metabolic properties and lifespan. Methionine is an essential amino acid making up .4%

of the protein content in a typical rodent diet. It has been identified that limiting some essential amino acids, like methionine, from the diet has dramatic effects on immune, tissue, cellular stress, and metabolic physiology (Miller, Beuhner et al. 2005). It was also discovered that limiting the specific essential amino acid methionine resulted in increased lifespan in rodents (Miller, Beuhner et al. 2005). This was not an effect attributed to CR; the animals on average ate more food than the normally-fed control group but had reduced body weight. The metabolic alteration that leads to extended lifespan is therefore potentially the same as it is during CR, although this is not a CR effect. The ability to consume more food by volume and still have positive effects on lifespan and potentially disease burden is very promising. What is unknown however, is whether beginning these diets in middle-late adult life produces the same benefits. For that reason, the Glenn study aims to compare the metabolic and lifespan properties of genetically diverse heterogeneous stock mice beginning a low methionine, 40% CR or normal diet in middle age. The metabolic alterations will then be compared among sexes, diet groups, and to the known changes that accompany CR begun earlier in life. MR, physical activity, body weight and composition, plasma insulin, glucose, acylated and unacylated ghrelin levels will be measured at two month intervals. The protocol for the Glenn study is depicted below.

Figure 5-2. Schematic of the Low Methionine Study



A comparison of ghrelin across these three studies will provide two advantages. The first will be characterizing ghrelin in three genetically-intact, *ad libitum* fed models. In two of these groups, the animals are derived from the same heterogeneous stock lineage. Therefore, plasma ghrelin measurements are repeated and can be compared for consistency. Additionally, each substudy provides a unique CR population from which ghrelin levels can also be described.

CHAPTER VI. STUDY III: THE GASTRIC BYPASS STUDY

"Differential actions of ghrelin in aspects of human bariatric surgery"

There are very few models of caloric restriction processes in humans. In animal models, the greatest benefits from CR are derived from starting CR at a young age. In humans, it is generally not until middle or older age when individuals are interested in strict lifestyle modifications which benefit health and longevity. Many people interested in beginning a CR lifestyle are unable to construct a proper diet or sustain it in the long term. Therefore there are only a few CR-like paradigms that are informative to study in humans. One of the major CR-like schemes that has permanent effects on metabolism and reduction of disease is gastric bypass surgery. There are many versions of gastric bypass surgery and all result in the intended effect of limiting volume of food that can be consumed at one time, amount of nutrients absorbed, or a combination of these mechanisms. Currently the most popular technique is called the Roux-en-Y bypass. This procedure resects the jejunum, or middle section of the small intestine, with a small upper section of the stomach, cutting out much of the large-volume lower stomach and nutrient-absorbing duodenum. This combination results in a small stomach pouch connected directly to the jejunum and the rest of the small and large intestines (detailed in(Schauer, Ikramuddin et al. 2000)). Many anatomic and physiological features that contribute to weight loss are thus modified:

1. the small portion of stomach that remains holds a much reduced volume of food resulting in less food consumed in one sitting,

2. the duodenal section of the small intestines that is involved with the most nutrient absorption is bypassed,
3. Plasma ghrelin levels, a known appetite stimulant, are quickly and markedly reduced (Cummings, Weigle et al. 2002).

Additionally, remarkable and effects on insulin resistance and/or Type II Diabetes are seen after the procedure (Schauer, Burgeura et al. 2003). In one study, nearly all (98%) of Type II Diabetics had a reversal of their clinical diagnosis at one year post surgery (Wittgrove and Clark 2000) and other studies show a 75% -97% resolution (reviewed in (Rubino, Moo et al. 2009)). Additionally, in some patients, insulin resistance is nearly abolished in hours or days post-surgery (Wickremesekera, Miller et al. 2005; Laville and Disse 2009). It is unknown how blood glucose and insulin levels drop in the hours post surgery before any weight is lost, but many Type II Diabetics no longer suffer from the condition nor require medicine. One possibility is that stomach or intestinal-derived hormones that regulate glucose and insulin homeostasis are abruptly changed. Ghrelin is one of such hormones and is possibly involved with the dramatic recovery from a diabetic state.

Action of Ghrelin During Roux-en-Y Gastric Bypass

In search of factors that make gastric bypass surgery so effective, ghrelin was identified as one important metabolic player because it is dramatically altered by the surgery (Cummings, Weigle et al. 2002). It is well known that ghrelin is closely linked to obesity. Plasma ghrelin levels are very much reduced in obese individuals in comparison

to normal levels in normal-weight individuals and high levels in underweight individuals. With dietary weight loss, plasma ghrelin levels begin to increase, an increase which is thought to stimulate appetite and help protect the body against loss of body weight (Cummings 2006). Ghrelin receptor antagonists have been investigated as potential therapeutic agents against obesity, and some have been effective in rodent models (Asakawa, Inui et al. 2003; Salome, Hansson et al. 2009). However, these effects have not been demonstrated in humans. Ghrelin gene knockout models have yielded modest information about its involvement in obesity. In rodents lacking a functional ghrelin gene, plasma ghrelin levels were nearly zero, yet had no differences in body weight compared to controls (Wortley, Anderson et al. 2004; Sun, Butte et al. 2008). However, when fed a high-fat diet, these animals were more resistant to dietary-induced weight gain and displayed a healthier metabolic phenotype (ie lower plasma insulin, glucose levels) (Pfluger, Kirchner et al. 2008). Therefore, ghrelin is known to have several actions in obesity, and is important to investigate during the gastric bypass process. In order to best determine ghrelin's contributions in a human bariatric surgery model, we will examine aspects of ghrelin action in three situations. The aims of the study are as follows:

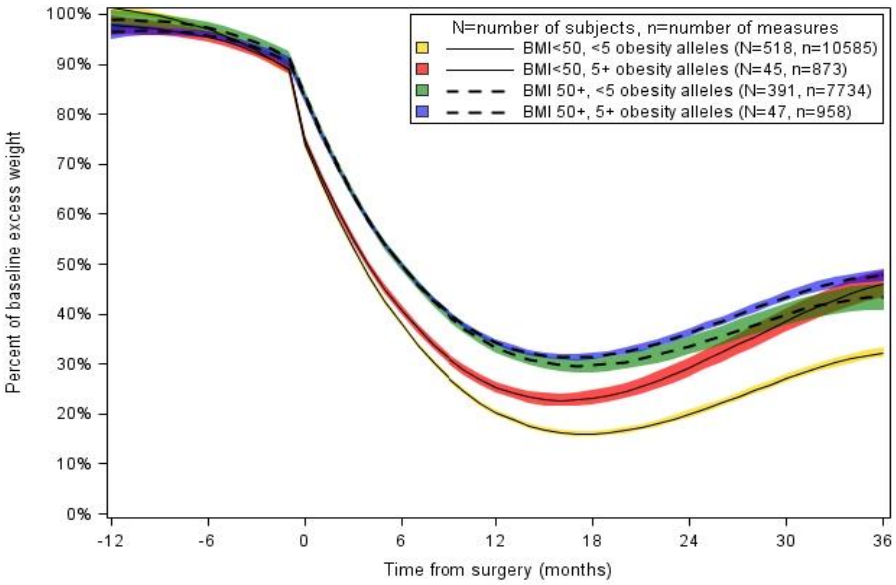
i. Examine plasma unacylated ghrelin in bariatric surgery patients 1. on the day of surgery and 2. in a separate group 6-12 months post surgery

ii. Measure liver tissue levels of ghrelin, leptin, insulin, and proinflammatory cytokines in patients with fatty liver disease and insulin resistance or without either clinical diagnosis

iii. Genotype 1400 patients for two GHSR SNPs and correlate these genotypes with clinical measures of weight loss success during dietary intervention and after surgery, initial BMIs, and obesity-related disease presence.

There are two caloric restriction situations occurring in the human bariatric surgery protocol. Patients at Geisinger Medical Center (Danville, PA), where this research will be conducted, are required to lose 10% of their body weights prior to surgery in a three-month dietary weight loss period. Some patients reach this goal quickly; some require administration of a severe energy-restricted liquid diet to reach the weight loss goal during this time and some never reach this goal and do not undergo surgery (Still, personal communication). The second weight loss period is after the surgery. Weight loss is dramatic in the first year and most patients near their target weight loss (see Figure below). The first aim investigates the actions of serum ghrelin during these key times. Serum unacylated ghrelin will be measured by standard ELISA.

Figure 6-1. Weight trajectories based on genotype in post-bypass surgical patients.



Another point of interest in the graph above is the weight trajectories which emerge from six months post surgery and continue in the following years. Some patients only regain a small amount of weight and some regain more moderate amounts. The third aim begins to examine how genetic contributions may in fact impact not only absolute pre- and post-surgical BMI, but the ability to lose weight during the pre-surgical dietary intervention, and weight regain trajectories in the months to years post surgery. Two single nucleotide polymorphisms (SNPs) in the promoter region of the ghrelin receptor gene, rs490683 and rs9819506, have been identified as contributing significant variation to dietary weight loss effectiveness and measures of insulin resistance (Mager et al., 2008).

Additionally, the second aim addresses ghrelin's role in the liver. A significant portion of the patients undergoing gastric bypass at GMC have clinical diagnoses of Type II Diabetes and Non-alcoholic steatohepatitis (NASH). NASH is a condition characterized by inflammation and increased lipid deposition in the liver potentially leading to impaired liver functioning and Fatty Liver Disease. The decrease in proinflammatory cytokines may be important in the reversal of this condition post-surgery. Since ghrelin is known for its anti-inflammatory effects, high plasma levels of ghrelin may provide a mechanism. Tissue levels of total ghrelin, cytokines known to be involved in fatty liver disease progression (IL-6 and TNF-α), as well as leptin, known to induce these cytokines, will be measured by Luminex. The Luminex technique combines fluorescent labeling of polystyrene microspheres with flow cytometry, where the fluorescent labels act as the identifiers for the simultaneous measurement of multiple analytes and flow cytometry quantifies concentration (System. 2010).

Therefore, the goal of studying ghrelin's actions in the roux-en-y technique of gastric bypass surgery is to classify its actions peri-surgically. As previously described, many physiological changes occur during the first year post surgery including fast and marked weight loss, improvement in clinical diagnoses of weight-related disease, and an overall change in metabolic physiology. Additionally, pre-surgical characteristics may predispose a patient to success in these various areas. Measurement of genetic and physiological properties of patients may help identify characteristics of success.

CHAPTER VII. INTRODUCTION SUMMARY

There is convincing overlap between the actions of caloric restriction and the actions of ghrelin. Levels of plasma ghrelin change with various CR paradigms which suggest ghrelin could be an important player in CR's numerous effects. The aim of this work is to investigate changes in ghrelin levels in various models of CR and then further characterize some specific disease- and CR-related actions of ghrelin. These studies will span rodents and humans, males and females, young, middle and old ages, multiple models of CR, genetics and physiology. Results from these diverse subjects, studies, and techniques are advantageous for providing evidence that ghrelin may indeed play an important role in CR physiology.

CHAPTER VIII. THE ARMY STUDY

METABOLIC CHANGES DURING SHORT-TERM, SEVERE CALORIC RESTRICTION IN MICE.

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Introduction

The long-term effects of restricted feeding, or calorie restriction (CR) have been exhaustively studied and have been shown to have many beneficial effects (Masoro 2005). Short-term restriction of nutrient input has been less well-studied and is certainly of importance for men and women under duress in different situations. For example, under battlefield conditions, soldiers may have limited access to food or supplies and may experience significant caloric deficiency for a short period of time while needing to maintain high levels of physical performance. Under-consumption of meals, combined with increased energy expenditure in strenuous physical activity will lead to severe energy deficit. Physiological changes and associated ability to function may result from severe, acute restriction of food intake. Prior research has established that animals having reduced food intake for long periods, such as in CR, exhibit altered functional and metabolic states (McCarter, Masoro et al. 1985; Ingram, Weindruch et al. 1986;

McCarter, Shimokawa et al. 1997). The purpose of this study was to gain insight into physiological changes associated with moderate and severe restriction of nutrient input by investigating select metabolic, body composition, and hormonal changes associated with short-term, moderate and severe caloric restriction in mice.

Background

Human studies of CR are primarily limited to (1.) post-famine cross-sectional data (Roseboom, van der Meulen et al. 2001; Painter, Roseboom et al. 2005), (2.) obese or overweight individuals placed on low energy diets (Wadden 1993), and (3.) normal-weight individuals who self-select to adopt a low calorie diet for improved health and longevity (Fontana, Meyer et al. 2004). While these models provide information about aspects of CR, each has limitations to its usefulness and none sufficiently describes the metabolic properties of normal weight adults exposed to a short-term severe calorie deficit. Therefore, this study was designed to provide information about the unique metabolic properties of healthy adult animals spontaneously exposed to moderate and severe CR.

Some metabolic changes have been characterized in short-term (weeks-months) moderate to severe caloric restriction. In humans, metabolic rate (MR) has been shown to decrease in obese individuals on very low calorie diets (Geleibter, Maher et al. 1997; Bryner, Ullrich et al. 1999). However, in long-term human CR studies, decreases in metabolic rate are transient and ultimately do not exceed body weight decreases (Wadden, Foster et al. 1990; Jebb, Goldberg et al. 1991). Similar evidence in long-term

restricted rodents has demonstrated that MR per kilogram body weight decreases in the short-term but is the same if not higher in the long term (McCarter, Masoro et al. 1985). Respiratory quotient (RQ), the rate of carbon dioxide production to the rate of oxygen consumption, is a measure of fuel usage at a given time. Rodent studies of CR have shown RQ to be both unchanged between CR and ad libitum feeding over a 24-hour period but to favor carbohydrate fuel use at different times of the day, suggesting improved insulin sensitivity in long-term restricted animals (Duffy, Feuers et al. 1989; Masoro, McCarter et al. 1992).

Despite no changes in metabolic rate, long-term CR induces increased physical activity (McCarter, Shimokawa et al. 1997). On the contrary, short-term CR in a variety of species suggest activity may be decreased (Goodrick, Ingram et al. 1983; Duffy, Feuers et al. 1990; Kemnitz, Weindruch et al. 1993). Twenty-four-hour energy expenditure (EE) has been shown to be decreased in obese and non-obese humans undergoing four and six month CRs, respectively, and restricted non-obese also demonstrated decreased physical activity (Ravussin, Burnand et al. 1985; Martin, Heilbronn et al. 2007). Both of these schemes are considered short-term CR for humans relative to the duration required to match long-term rodent CR. This short duration of CR does not allow for the expected physiological defenses (i.e. rebound in MR, increased physical activity) to protect against body weight loss and ensure survival, as has been demonstrated in the rodent studies.

Severe CR schemes in humans result in expected reductions of both body fat and lean mass. The addition of resistance training mitigates major loss of fat-free mass (Martin, Heilbronn et al. 2007). Previous work in non-obese humans demonstrated a

low-calorie diet of 890 kcal/day, aimed to reduce body weight by 15%, reduced both body weight and fat mass more than both a moderate 30% CR and a 15% CR + 15% exercise diet over six months (Redman, Veldhuis et al. 2010). Additionally, bone growth has been demonstrated to be impaired in young C57BL/6 mice undergoing ~25% CR and bone mineral density decreased in adult CR animals (Ferguson, Greenberg et al. 1999).

In addition to fat mass, vital organs exhibit reduced mass in CR. Rodents on long-term CR demonstrate shrinkage of vitally essential tissues such as the liver, heart, lungs, and kidneys (Yu, Masoro et al. 1985), whereas the brain tissue apparently is protected. On the contrary, one recent study reported that C57/B6 mice on a six to eight-month 40% restriction had increased overall stomach mass (Yang, Youm et al. 2007), especially that of the upper stomach. This observation correlated with previous findings that plasma ghrelin is increased in CR. Ghrelin protein, primarily derived from stomach tissue (Kojima, Hosoda et al. 1999), circulates in plasma and is responsive to changes in both short- and long-term changes in energy balance. Plasma ghrelin is negatively correlated with body weight, save for congenital obese conditions such as Prader-Willi Syndrome (Cummings, Clement et al. 2002). Ghrelin exists in two forms: unacylated and acylated. The acylated form is responsible for most of the identified major actions of ghrelin: appetite stimulation, growth hormone release, and suppression of proinflammatory cytokines, but is rapidly converted to the unacylated form, which has less potent actions (Kojima, Hosoda et al. 1999; Akamizu, Takaya et al. 2004; Li, Gavrila et al. 2004; Asakawa, Inui et al. 2005). Plasma ghrelin is inversely correlated with body weight; ie. high in lean and low in obese (Tschoep, Weyer et al. 2001; Shiya, Nakazato et al. 2002). Similarly, dieting increases plasma ghrelin concomitant with weight loss,

suggesting that ghrelin is responsive to both short- and long-term energy status(Otto, Cuntz et al. 2001; Cummings, Weigle et al. 2002; Hansen, Dall et al. 2002a). Here we investigated plasma unacylated ghrelin and stomach ghrelin mRNA expression during short-term 60% and 40% CR. Because the amount of acylated to unacylated ghrelin is typically maintained at approximately 10-20%, the relative ease of unacylated ghrelin measurement supported its use (Akamizu, Shinomiya et al. 2005). Top (fundus) and bottom (antrum) stomach sections were separately evaluated, as it has been shown that the regions differ in ghrelin production (Yang, Youm et al. 2007).

Another prominent hormone involved in body weight control is insulin-like growth factor-1 (IGF-1), which has been implicated in growth, development, and the aging process (Butler and Roith 2001). Knockout models of various aspects of the growth hormone-IGF-1 axis, including its receptor (*Igf-1r*), demonstrate decreased signaling along this pathway impairs growth and extends longevity in several species: *Drosophila melanogaster, C. elegans,* and various rodents (Kimura, Tissenbaum et al. 1997; Bartke 2001; Tatar, Kopelman et al. 2001; Holzenberger, Dupont et al. 2003). Dieting and other restriction paradigms decrease plasma IGF-1 (Hursting, Switzer et al. 1993). Together these changes implicate IGF-1 as a factor in the link between energy restriction and altered function. Although the involvement of IGF-1 in the mechanism of this process has not been confirmed, interest remains in further investigating IGF-1's role. Therefore, we measured plasma IGF-1to characterize its actions in short-term, severe CR.

The aim of this investigation is to characterize metabolic properties that are known to be altered with CR. A drastic 60% reduction of calories may accelerate

changes induced by more moderate, longer-duration CR. Therefore, measures of energy balance, physical performance, body composition, and hormones related to metabolism will be described between restriction levels in HS mice.

Methods

Animals & Food Consumption

Male and female adult (ages 12-18 months, 13.5 ± 8.5 months mean ± SEM) heterogeneous stock (HS) mice were used in this study. This genetically-diverse mouse strain was used to more accurately reflect the variable genetic heritage in the human population than is possible with the use of inbred mouse strains (McClearn, Wilson et al. 1970). All animals were housed in a specific pathogen-free barrier facility on a 12-hour light/dark cycle. Animals in the control group were given standard rodent chow (Purina 5001) and water *ad libitum*. Animals in one of two experimental groups were subjected to either a 40% or 60% food restriction from *ad libitum* feeding (control group) and were provided with water *ad libitum*. Individual mice destined for restriction underwent three days of baseline measurements, averaging the grams of food freely consumed daily, and multiplying by either .6 or .4, respectively, to achieve 40% and 60% restriction levels.

Testing Protocol

Animals were put on one of three diets for ten days. A ten-day scheme was employed after a 14-day protocol resulted in ~30% fatalities in the 60% restriction group. Prior to

the onset of diet, each animal underwent measurements of body weight, body composition, 24-hour metabolic rate, and 24-hour physical activity. At the end of the ten-day period, each animal underwent repeat measures for body weight and composition, and 24-hour metabolic rate and physical activity just prior to sacrifice. Animals were sacrificed by decapitation and trunk blood was collected for metabolite analysis. Stomachs, livers, right and left kidneys and heart were systematically excised for weighing.

Body Weight & Composition

Body weight was measured by standard balance to the nearest tenth of a gram (Mettler PM 4600, Mettler-Toledo INC, Columbus, OH). Body composition was measured by small animal dual emission x-ray absorptiometry (DEXA) (GE Lunar PIXImus). Body composition included measurements of body fat mass (g), fat-free or lean mass (g), region of interest (ROI) body fat percentage (%), and bone mineral density (BMD) (mg/cm^2). Body fat percentage was determined in the ROI to measure changes in abdominal visceral fat. This region was defined on the X-ray scan as between the rostral tips of the scapulas to the caudal end of the second caudal vertebrae.

Metabolic Rate and Physical Activity

To determine daily energy expenditure, measurements of physical activity and metabolic rate were made continuously over 24- hours. Physical activity was measured by beam

breaks in a physical activity monitor (Digiscan; Omnitech Electronics, Columbus, OH). A transparent cage was placed in a field with a grid of interlocking infrared beams tracking movement in both lateral directions and vertically. Movement was recorded every minute as distance traveled (cm) which consisted of computer analysis of beam breaks in the horizontal plane. Hourly averages were calculated and a 24-hour cumulative value was obtained. Metabolic rate was measured by indirect calorimetry using carbon dioxide and oxygen analyzers and airflow controls from AEI technologies and the modular respirometry system software (MARS, AEI technologies, Naperville, IL). Air flowed continuously through the cage (500ml/min) and changes in percent oxygen and carbon dioxide entering and leaving the cage were measured per second. Minute and hourly averages were calculated and described as kcal/hour/gram body weight. Substrate analysis was determined using the respiratory quotient (RQ), calculated by the equation ($RQ = (rate\ of\ CO_2\ produced)/(rate\ of\ O_2\ consumed)$).

HORMONAL ANALYSIS

Plasma levels of IGF-1, glucose, and unacylated ghrelin were measured from trunk blood provided at time of sacrifice. Unacylated ghrelin was measured by a standard, commercially-available ELISA kit (BioVendor LLC, Chandler, NC). Glucose was measured by standard glucose assay and spectroscopy (SIGMA, St. Louis, MO) and IGF-1 was analyzed using Luminex technology (Millipore Corporation, St. Charles, MO). Unacylated ghrelin, IGF-1 and glucose values are reported as means ± standard error of the mean (SEM).

PCR

Total RNA was isolated from ~15mg of tissue from each animal's upper fundus and lower antrum stomach regions. Samples were homogenized and purified using TRI Reagent® (Sigma, St. Louis, MO). RNA quantity and 260/280 ratios were measured on a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). Equal amounts of total RNA (0.5ug) was synthesized into cDNA (Fermentas, Hanover, MD). ABgene Absolute qPCR SYBR Green Master Mix (ABgene, Surrey, UK) with ROX dye was used for all qRT-PCR. Forward and Reverse qRT-PCR Primers for transcripts of interest were designed using NCBI gene sequences and the Primer Design platform provided by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA). NCBI BLAST searches were performed on primer sequences to ensure gene and species specificity. β-Actin, a constitutively expressed gene, was used as a reference standard. Amplification reactions were performed on a Stratagene MX3000P (Agilent Technologies, La Jolla, CA) with the following designed primers: Ghrelin: Forward- 5'-AGG AAT CCA AGA AGC CAC CAG CTA-3', Reverse- 5'- ATG CCA ACATCG AAG GGA GCATTG-3'; Ghrelin Receptor: Forward- 5'- ATG GGT GTC GAG CGT CTT CTT CTT -3', Reverse- 5'- CAA ACA CCA CCA CAG CAA GCA TCT-3'; β-Actin: Forward-5'- AGC CAT GTA CGT AGC CAT CC-3', Reverse- 5'- CTC TCA GCT GTG GTG GTG AA-3'. A melting curve was performed at the end of each reaction to screen for multiple PCR products or primer dimers. The differences in mRNA expression between upper and lower stomach regions, and diet group (40% CR, 60% CR, ad libitum) were determined by the relative quantification method utilizing the threshold

cycle (CT) method and real-time PCR efficiencies of the target gene normalized to the housekeeping gene β-Actin for each sample (top vs. bottom, 40% CR or 60% CR vs. ad libitum). Expression of β-Actin was not different between fundus and antrum regions of the stomach, or restriction groups.

STOMACH WEIGHTS

Stomachs were collected immediately after sacrifice. Each organ was meticulously cut directly at the junction of any connecting tissues (ie esophagus and duodenum) and excess tissue was trimmed. Stomach contents were also removed. Stomach mass was weighed by a precision balance (Mettler PM 4600) to the nearest 0.0001 gram. Additionally, stomachs were cut in half to separate the upper fundus region from the lower antrum. A change in coloration clearly delineated these two regions and the incision was made along this line. Stomach halves were separately analyzed for ghrelin and ghrelin receptor mRNA expression via quantitative PCR.

STATISTICS

All data were analyzed by sex and diet group using analysis of variance in SPSS Statistics 17.0 (IBM, Chicago, Illinois). When sexes did not differ, males and females were pooled for greater power. Pre- and post-restriction comparisons were measured by paired T-test. Student-Newman-Keuls (SNK) posthoc tests were used for multiple comparisons unless otherwise noted.

Results

Food Consumption & Body Weight

Twenty-four hour food consumption was measured for each animal on three consecutive days and average daily food consumption was calculated. No differences in food consumption were measured between diet groups before the start of the study (data not shown). Body weight also did not differ between diet groups before the start of the study, although males were significantly heavier than females (34.7g ± 0.59 vs 30.2g ± 0.50, respectively, p=0.000) *(Figure 1).* Body weight significantly decreased for animals in all diet groups at the end of the study (p=0.001). Female 40% and 60% CR mice weighed less than control females post-treatment ([60% CR] 23.17g ± 1.02, [40% CR] 25.0g ± 0.68, [control] 28.2g ± 0.66). Post-diet, control females were heavier than 40% CR females, p=0.021 and 60% CR females, p=0.000. Body weights of 40% and 60% CR groups did not differ significantly from each other, p=.264. Control males (32.49 ± 0.87g) weighed more than both the 40% CR males (28.91 ± 0.96g) and 60% CR males (27.45 ± 0.86) post-diet (p=0.018 and p=0.001, respectively), with no observable difference between the CR groups (p=0.49).

Body Composition

No pre-restriction differences by diet group on any parameter existed in males or females, respectively. Females had significantly less total lean mass pre-restriction than males

(p=0.000), but no other sex differences were present for pre- or post-restriction total fat mass, ROI body fat percentage, or bone mineral density. Within diet group, total lean mass and total fat mass decreased significantly from pre- to post-restriction in both 40% females (p=0.001 and p=0.026, respectively) and males (p=0.000 and p=0.009) and 60% CR females (p=0.004 and p=0.002) and males (p=0.000 and p=0.001), n= 8-9 in each sex x diet group. Post-restriction, 40% and 60% restriction groups had significantly less lean mass than control-fed animals (p=0.000) *(data not shown).*

Abdominal fat, as detected by ROI, did not decrease in control males or females but did in 40% CR males (p=0.017), 60% CR males (p=0.001), 40% CR females (p=0.026) and 60% CR females (p=0.017) as determined by one tail t-test *(data not shown).*

BMD did not decrease in control males or females, 40% CR females or males, or 60% CR females, but did in 60% males (p=0.031), via one-tail t-test (*data not shown).*

METABOLIC RATE, RESPIRATORY QUOTIENT, & PHYSICAL ACTIVITY

Twenty-four hour metabolic rate, respiratory quotient, and physical activity were measured continuously just before initiating the ten-day diet condition and again in the twenty-four hours before sacrifice. Pre-diet metabolic rates did not differ between sex nor diet group (*data not shown).* Sexes within the 40% and 60% CR diets did not differ in post-restriction analysis, however females on the control diet had a higher metabolic rate than male controls in post-diet analysis (0.0317 kcal/hr/g vs. 0.0260 kcal/hr/g, p=0.034) (*Figure 2).* Males in the 60% CR group also had a higher body-weight adjusted

MR than ad libitum males post-restriction (0.0340 vs 0.0260 kcal/hr/g, p=0.045). Metabolic rates in males and females on the *ad libitum* diet were the same pre and post restriction (*Figure 3)*. Metabolic rates of male and female animals in the 40% and 60% restriction groups were similar post-restriction, so sexes were pooled for analysis (n=4-5 per sex x diet group). Both diets showed a significant increase in post-restriction metabolic rate from baseline [40% CR pre-diet 0.0295 vs. 40% CR post-diet 0.0331 kcal/hr/g, p=0.000 & 60% pre-diet 0.0281 vs. 60% CR post-diet 0.0331 kcal/hr/g, p=0.000]. RQ did not differ between any sex or diet group prior to restriction (n=5 per sex-diet group). There was also no significant change in RQ from pre-restriction to post-restriction in any sex-diet group (*data not shown*). Post restriction analysis showed increased RQ during hours 17, 20, and 22-24 in ad libitum-fed females versus males but not between sexes in any other diet group (*Figure 4)*. Additionally, post-diet, the 60% CR group significantly differed from the ad libitum group during hours 11-16. No differences were seen in the 24-hour average RQ between any groups before or after restriction and within any group from pre- to post-restriction (*data not shown).*

There were no differences in physical activity between sexes or diet groups (n=4-6) at baseline or after restriction. Analysis within diet groups from baseline to post-restriction also indicated no change in activity level occurred as a result of energy status (*data not shown).*

PLASMA GLUCOSE AND IGF-1

Plasma glucose did not differ between sexes within diet groups. With sexes pooled (n=12 per diet group), AL-fed animals had significantly higher plasma glucose levels than 60% CR animals (10.7 ± 1.13 vs 6.25 ± 1.28 mg/ml, p=0.036). This difference was attributed to the difference in plasma glucose values in females ([AL] 10.6 ± 1.85 vs [60%CR] 4.57 ± 1.21 mg/ml, p=0.05 SNK posthoc test) but not males ([AL] 10.85 ± 1.35 vs [60% CR] 7.68 ± 2.08 mg/ml, p=NS). There was a trend toward decreasing plasma glucose in both males and females with increasing levels of restriction *(Figure 5)*. Males and females did not differ in any diet group on levels of plasma IGF-1 *(Figure 6)*. Sexes were pooled resulting in n= 11-13 for each diet group. SNK post-hoc tests revealed each diet level significantly differed from the others on plasma IGF-1 ([AL] 566.35 ± 43.19 vs [40% CR] 336.43 ± 44.43 vs 182.54 ± [60% CR] 50.68 pg/ml, $p<0.05$ for each comparison).

PLASMA UNACYLATED GHRELIN AND STOMACH GHRELIN & GHRELIN RECEPTOR EXPRESSION

Plasma unacylated ghrelin was measured by standard ELISA in male and female mice of each diet group (n=5-6 per sex-diet group). No diet effects were observed (*Figure 7*). Males and females differed overall (617.4 ± 145.8 vs. 1750.1 ± 314.9 pg/ml, p=0.003, respectively), a result attributed to the sexual dimorphism in the control (females 1660.0 ± 362.5 vs. males 579.8 ± 160.7 pg/ml, p=0.034) and 60% restriction groups (females 2322.4 ± 616.3 vs males 532.7 ± 303.3 pg/ml, p=0.012). Stomach tissue was analyzed

for ghrelin and ghrelin receptor mRNA expression *(Figures 8 and 9)*. Fundus and antrum sections of stomachs were separately analyzed (n=6 per sex-diet-stomach half group). Ghrelin mRNA expression was upregulated approximately twelve-fold in the bottom portion of stomachs of 60% CR animals vs. *ad libitum*-fed animals, $p<0.05$. Additionally, ghrelin was increased 4.5-fold in the bottom portion of 40% CR animal stomachs versus the AL group, $p<0.05$. For ghrelin receptor gene expression in stomach tissue, there were no statistically significant differences.

STOMACH WEIGHTS

Post-sacrifice stomachs were analyzed for differences in sex and diet groups (n= 9-11 per sex x diet group). There were no significant differences between males and females within any diet group. Sexes were pooled yielding sample sizes of ~20 in each diet group. Tukey HSD post-hoc tests revealed a significant increase in stomach size per body size in the 40% and 60% CR groups vs. controls ([40% CR] 0.0091 ± 0.0004, [60% CR] 0.0088 ± 0.0004, [AL] 0.0074 ± 0.0004). The 40% CR group differed from controls at p= .008 and 60% CR versus controls at p= .04 *(Figure 10)*. Animals in the 40% and 60% CR groups did not differ.

Discussion

Several metabolic changes occurred with a 10-day moderate and severe CR. The most significant observation was that hormone levels were more affected by diet than

measures of energy expenditure or body composition. In general, the 60% restriction produced greater changes from AL-fed animals than a 40% restriction, suggesting animals that could survive this level of restriction may experience even greater CR benefits across the lifespan.

Nearly one-third of animals on the 60% restriction diet did not survive 14 days of this level of restriction. Any stress of baseline measurements likely did not contribute to early deaths, as most animals survived to 10 days of the restriction. Because of these early deaths, the diet duration was scaled back to a 10-day restriction.

The short-term severe diet method allowed the investigation of an altered metabolic phenotype in HS mice and yielded some unexpected findings. As expected, body weight significantly decreased with both the 40% and 60% CR diets. However, body weight also significantly decreased in the control group. Weight loss in all groups can be partially attributed to stress of measurement, as animals underwent two 24-hour measurement periods within eleven days and data collection was conducted in a different cage from their home cage. Body composition measurements indicated loss of fat and lean mass in males and females in both restriction groups, most likely attributed to the severity of the restriction imposed, since physical activity was unchanged.

METABOLIC RATE, RESPIRATORY QUOTIENT, AND PHYSICAL ACTIVITY

Although metabolic rate in the long term is not decreased relative to body weight (McCarter, Masoro et al. 1985), it has been repeatedly reported as lower in short-term restriction models in various animal experiments (Wadden, Foster et al. 1990; Roark and

Bjorndal 2009). Our results indicated metabolic rate adjusted for body weight was significantly higher in both sexes on short-term 40% and 60% CR, but absolute MR presented a non-significant decreasing trend with increasing CR. A recent study of short-duration (1-5 weeks) 30% CR in C57Bl/6 and DBA/2 mice also demonstrated no short-term decrease in MR relative to body weight, although DBA/2s were significantly higher overall. Failure to detect a decrease in adjusted MR could be due to either a greater decrease in body weight compared to absolute MR or variation in measurement in HS animals. The mixed genetic background may partially explain the unexpected results; CR effects have been shown to be strongly influenced by genetic background (Liao, Rikke et al. 2010).

Prior evidence has shown a positive correlation with plasma ghrelin levels and RQ. Daily infusion of ghrelin in mice and rats demonstrated a temporary increase in RQ that was additionally associated with weight gain due to reduced fat utilization (Tschoep, Smiley et al. 2000). In normal-weight men, a positive relationship between total ghrelin levels and RQ was reported (Doucet, Pomerleau et al. 2004). However, after a four-day 800 kcal reduction, RQ was decreased but plasma total ghrelin levels were not. Our results indicate RQ significantly decreased with implementation of the 60% CR diet from ad-libitum feeding. The shift in fuel use towards increased fat utilization could be due to preferential metabolic utilization of available energy stores. As plasma unacylated ghrelin was not also decreased in the 60% animals, a direct positive relationship between RQ and ghrelin is not be supported by our data.

Physical activity in rodents is typically increased in long-term CR, proposed to be due to a foraging effect (Haigis and Guarente 2006). The foraging phenomenon has been

proposed by anthropologists as an instinctual survival response to decreased proximal food availability and the energy preservation physiological state as a type of hibernation or torpor in times of low food availability (Overton and Williams 2004). Data in humans suggest that short-term calorie restriction leads to reduced 24-hour energy expenditure (Leibel, Rosenbaum et al. 1995; Heilbronn, de Jonge et al. 2006). Our data demonstrate no change in physical activity, a result in disagreement with our hypothesis that performance will be impaired during short-term restriction. It is possible that the CR protocol implemented here was not sustained long enough to either induce a 'foraging effect' or long enough to induce an energy conservation mechanism in the animals.

HORMONAL CHANGES

Although the duration of the restriction period employed here was only ten days, plasma IGF-1 levels decreased significantly and step-wise in the 40% and 60% CR groups, indicating the response of IGF-1 to changes in energy status occurs rapidly. Other rodent studies of long-term CR have demonstrated a marked reduction in plasma IGF-1 with CR (Breese, Ingram et al. 1991; Sonntag, Lenham et al. 1992; D'Costa, Lenham et al. 1993). However, CR in humans has been shown to have no effect on blood levels of IGF-1, although moderate restriction may increase plasma IGF-1 (Fontana, Weiss et al. 2008; Redman, Veldhuis et al. 2010).

The sexual dimorphism in plasma glucose levels of 60% CR mice was unexpected. Plasma glucose levels have been shown to be decreased in CR, but no evidence for a sexual dimorphism has been reported in the literature (Masoro, McCarter

et al. 1992). A complex relationship between plasma ghrelin levels and plasma glucose may partially explain this effect. Plasma glucose is decreased in response to CR and plasma ghrelin is increased (Masoro, McCarter et al. 1992; Kelley, Wing et al. 1993; Cummings, Clement et al. 2002; Yang, Youm et al. 2007). The decrease in plasma glucose is suggested to be due to low energy intake and not from a direct inhibitory effect of ghrelin or insulin on glucose levels. One report found administration of unacylated ghrelin and acylated ghrelin to obese, non-diabetic individuals, decreased plasma insulin but not plasma glucose, suggesting increased insulin sensitivity (Kiewiet, van Aken et al. 2009). Whether there is a direct relationship between glucose and ghrelin and not a secondary one in response to change in energy intake is unknown. However, the low plasma glucose in females is not entirely surprising given the high plasma ghrelin levels in this study, if such a relationship exists.

The robust sexual dimorphism in plasma ghrelin levels described here was also an important finding. Prior reports have shown this effect in humans, but no such data were found for rodents (Akamizu, Shinomiya et al. 2005). One explanation may be an interaction of ghrelin and sex hormones. Plasma ghrelin levels have been documented to be increased as much as three-fold higher in women in the late follicular phase of the menstrual cycle than men (Barkman, Dimaraki et al. 2003). The late follicular phase occurs just before ovulation and is characterized by high estrogen and low progesterone, follicle-stimulating hormone, and luteinizing hormone. Murine estrous cycles are of much shorter duration (4-5 days) versus women (~28 days). Therefore, high ghrelin levels may be partially attributed to capturing female mice at all cycle points. The increased variation in measurements in our HS females versus males, evidenced by

greater standard errors of the mean, support this possibility. Additionally, our laboratory has measured plasma ghrelin levels in female mice undergoing other CR paradigms and have not seen such a robust sex effect (unpublished data). One explanation for this sexual dimorphism in the effect of CR on plasma ghrelin may be associated with the single versus multiple housing of mice. Singly-housed female mice continue to cycle whereas multiply-housed female mice become anestrous until exposure to male pheromones, a phenomenon known as the Whitten effect (Bruce 1963). Because this diet paradigm was based on an individual mouse's average daily food consumption, animals were singly-housed. However, the possibility that changes in estradiol levels led to increased ghrelin in females in this study is speculative. Some studies suggest estradiol is decreased with long-term CR in rodents while other studies did not find evidence for this effect (Rocha, Bonkowski et al. 2007; Guevara, Valle et al. 2008). We did not measure estradiol levels and do not know why the sexual dimorphism in ghrelin exists.

The lack of response of unacylated ghrelin to diet was in contrast to the expected step-wise increase in the 40% and 60% CR groups from baseline. No effects in both diet groups may be attributed to the measurement of unacylated ghrelin and not its active form, acylated ghrelin. Most studies of ghrelin and metabolism refer to the measurement of total ghrelin, which includes both acylated and unacylated forms. Because the acylated form only accounts for about 10% of total ghrelin, the increase in total ghrelin with fasting or dieting is attributed to both forms (Tschoep, Smiley et al. 2000; Toshinai, Mondal et al. 2001; Cummings, Clement et al. 2002). Contrary to initial findings of ghrelin's actions, unacylated ghrelin may be involved with upregulated anorexigenic agents in the hypothalamus, decreased body weight, fat pad mass, food intake, and gastric

emptying in mice overexpressing unacylated ghrelin (Asakawa, Inui et al. 2005). Therefore, an expected increase in unacylated ghrelin during CR may be unfounded, despite increases in total and acylated ghrelin with CR.

Plasma levels of IGF-1, glucose, and uancylated ghrelin were measured to determine whole organism changes in hormonal activity. Ghrelin and ghrelin receptor mRNA was measured in stomach to identify any potential change in ghrelin production or autocrine action. Stomach mRNA content of ghrelin and its receptor were quantified separately in fundus and antrum stomach sections. One prior report demonstrated disparate actions between fundus and antrum sections in response to long-term CR in mice (Yang, Youm et al. 2007). Fundi showed greater hypertrophy and CR animals had a higher plasma level of ghrelin as well as increased production of ghrelin from stomach tissue. These findings served as a basis for our examination of mRNA expression of ghrelin and ghrelin receptor in fundus and antrum stomach tissue sections. Ghrelin mRNA expression increased in the antrum region of 40% and 60% CR stomachs.

Ghrelin production is suggested to be greater in the fundus (Inui, Asakawa et al. 2004), although the antrum as well as the small intestines have also been identified as sites of production (Korbonits, Bustin et al. 2001). Because this was an acute CR protocol, it is possible the antrum was recruited to provide additional ghrelin protein during times of energy stress while the fundus is primarily responsible for constitutive circulating ghrelin levels. The increase in ghrelin receptor mRNA expression may be a function of ghrelin's additional actions of promoting gastric motility (Masuda, Tanaka et al. 2000) as gastric motility has been shown to increase in some models of CR, but not others (Laferrere, Teixeira et al. 2008). However, since we only measured mRNA levels

of ghrelin and its receptor, it is not known if transcriptional changes will result in changes at the protein level. It is possible that increases in mRNA were compensating for losses in ghrelin protein as a result of the severe CR.

STOMACH WEIGHT

Prior reports have indicated long-term CR decreases mass of several organs: heart, liver, kidneys, etc, but the duration of CR required for this effect is unknown (Yu, Masoro et al. 1985). On the contrary, brain tissue has been shown to be protected from CR-induced tissue shrinkage and stomach mass may be actually be increased (Yu, Masoro et al. 1985; Yang, Youm et al. 2007). Our data indicate stomach mass was increased in the 40% and 60% diet groups, suggesting ten days is sufficient to induce changes in stomach mass in response to alterations in energy status. Increased stomach mass may support additional ghrelin production.

In summary, several metabolic alterations were observed with this short-term, severe CR study. The results from body weight and composition, physical activity, and metabolic rate measurements were surprising. The lack of effect from CR could be due to the duration of time it takes to achieve expected changes in these whole animal physiological measures. The most robust effects observed were from the hormonal measurements, potentially linked to sex of the animal. This study provides a unique description of a sexual dimorphism between male and female plasma glucose and plasma unacylated ghrelin during diet restriction. It is unclear whether these effects have additional physiological, morphological or behavioral consequences. Other metabolic

interventions have been identified as disparately affecting males and females (Albu, Heilbronn et al. 2010) and these results contribute to the justification for further investigation. Alternatively, the results may be a consequence of the genetic heterogeneity of mice used in this study in agreement with the recent results of Liao et al (Liao, Rikke et al. 2010). Finally in conclusion, our results suggest short-term moderate to severe restriction of food intake would not be expected to adversely affect physiological performance.

FIGURES

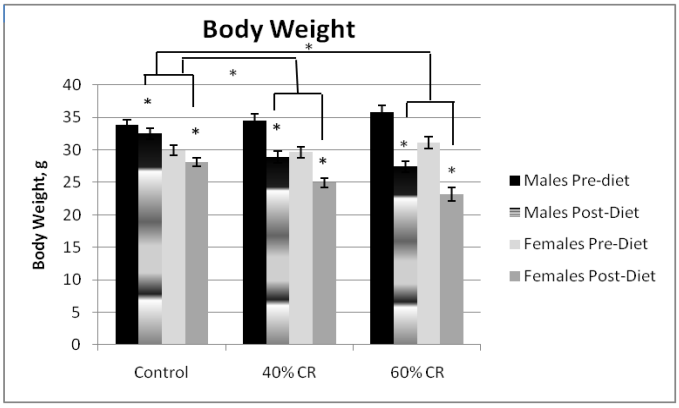
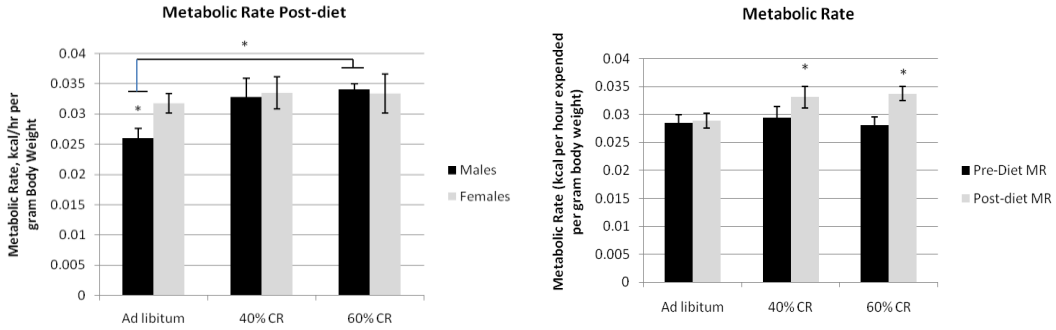


Figure 8-1. Body Weight

Body weights prior to initiation of diet did not differ between diet groups. Males were significantly heavier than females (p=0.000). Post-diet analysis indicated all groups weighed less than pre-diet measurements (p=0.000). Additionally, males and females in both the 40% and 60% CR groups were lighter than control group animals post-restriction (p-values = .000 to .021), n=19-21/ sex-diet group.



Figures 8-2 and 8-3. Post-Diet Metabolic Rate and Change in Metabolic Rate

Males in the ad libitum diet group had a significantly lower MR than female ad libitum-fed animals or male 60% CR animals in post-diet analysis. Evaluation of change in metabolic rate before and after the diet period revealed an increase in post-diet MR in the 40% and 60% CR groups which was not seen in the AL-fed group, n= 4-5/ sex-diet group; * p<0.05.

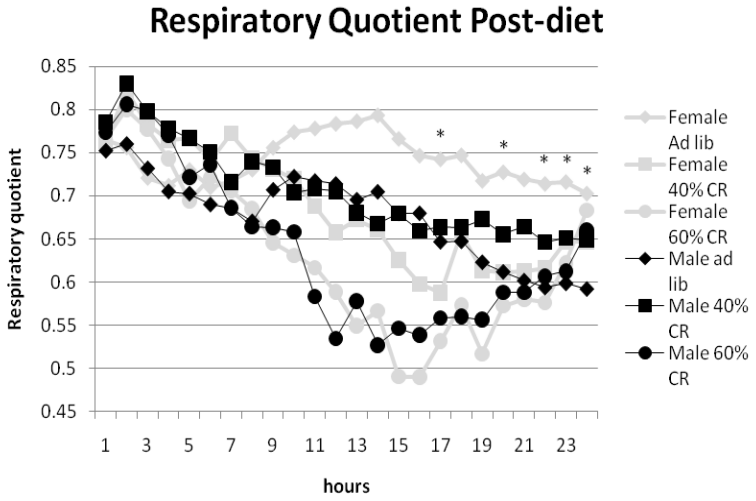


Figure 8-4. Respiratory Quotient

Pre-diet RQ did not differ between any sex-diet group (n=5) prior to the onset of diet (*data not shown)*. RQ also did not change within any sex-diet group from pre-post restriction (*not shown)*. Post-restriction analysis demonstrated a significant difference in ad libitum-fed males and females during hours 17, 20, 22-24, denoted by *. Animals in the 60% CR group significantly differed from AL-fed animals during hours 11-16, p<0.05 (*averages not shown*). No differences were seen in the 24-hour average between any groups.

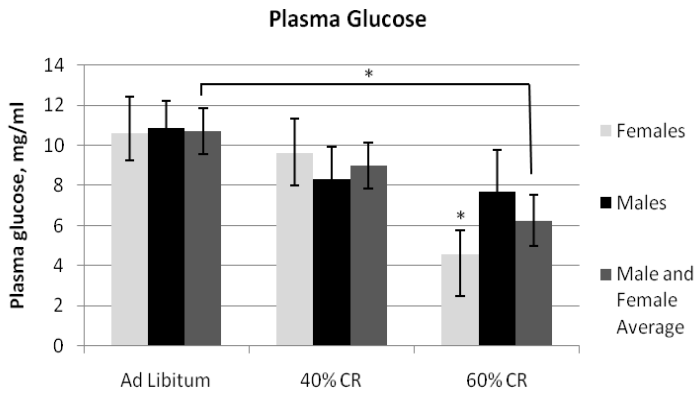


Figure 8-5. Plasma glucose

Plasma glucose did not differ between sex for any diet group. With sexes pooled (n=12 per diet group), AL-fed animals had significantly higher plasma glucose levels than 60% CR animals, an effect attributed to the low value in 60% CR females. Animals at the 40% CR level did not differ significantly from either group, although a non-significant step-wise trend can be observed, *p<0.05.

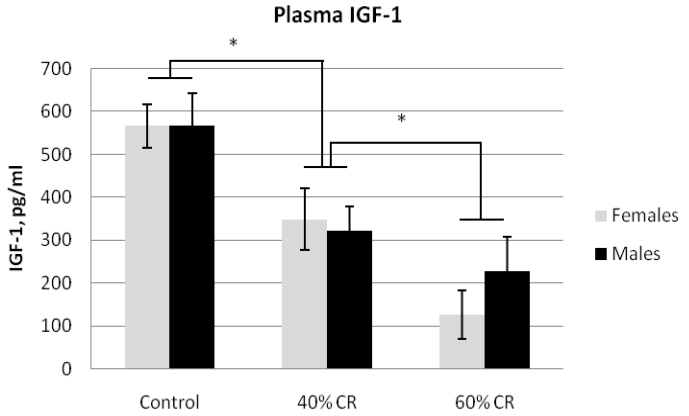


Figure 8-6. Plasma IGF-1

IGF-1 did not differ between sexes for any diet group. With sexes pooled (n=11-13 per sex-diet group), posthoc tests indicated a significant trend for decreasing levels of IGF-1 with increasing restriction. Each group significantly differed from the two remaining diet groups, *p<0.05.

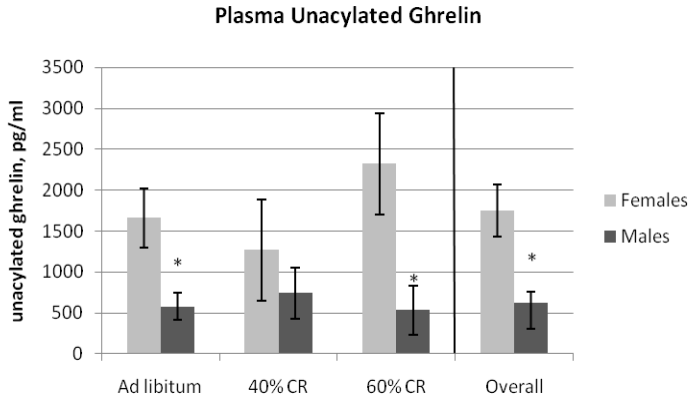


Figure 8-7. Plasma unacylated ghrelin

There was a sex effect for plasma unacylated ghrelin. Plasma unacylated ghrelin did not differ between diet groups for males or females (n=5-6/sex/diet). Females had significantly higher levels of plasma unacylated ghrelin than males, which is attributed to differences in the AL and 60% CR diet groups, *p<0.05.

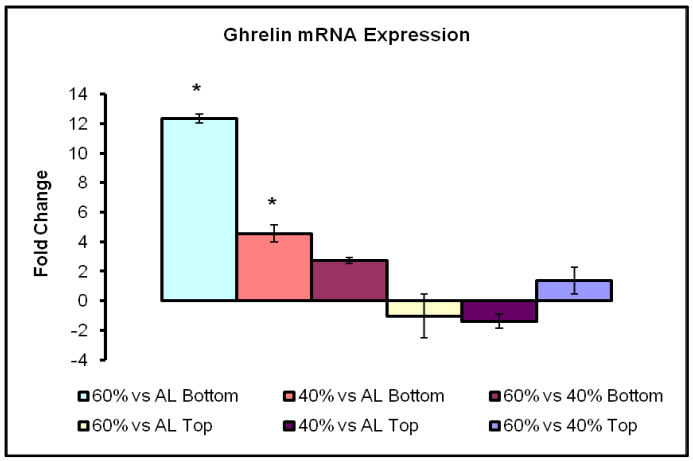


Figure 8-8. Ghrelin mRNA expression

Stomachs were divided into fundus and antrum sections. Ghrelin mRNA expression is enhanced in the 60% and 40% CR antrums (bottoms) compared to the AL group. There were no differences in ghrelin mRNA expression between the 60% and 40% CR groups' antrums or any fundi sections. Sample size was n=12/diet/stomach half, *p<0.05.

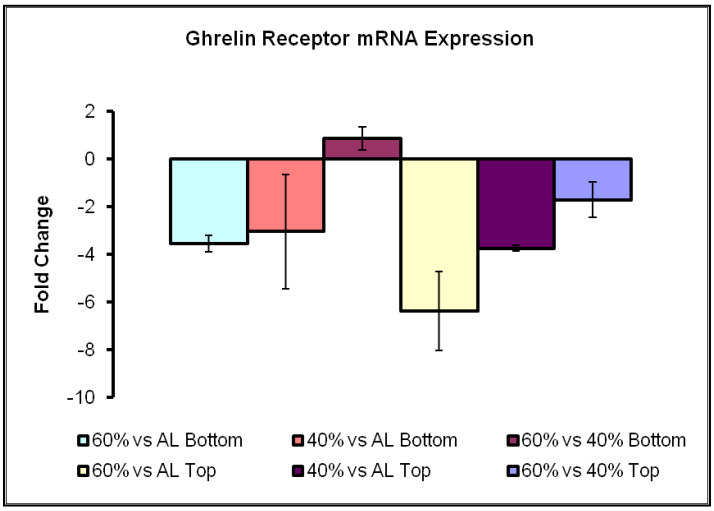


Figure 8-9. Ghrelin Receptor mRNA Expression

For ghrelin receptor gene expression in stomach tissue, there were no significant decreases in any stomach portion, n=12/sex/stomach half.

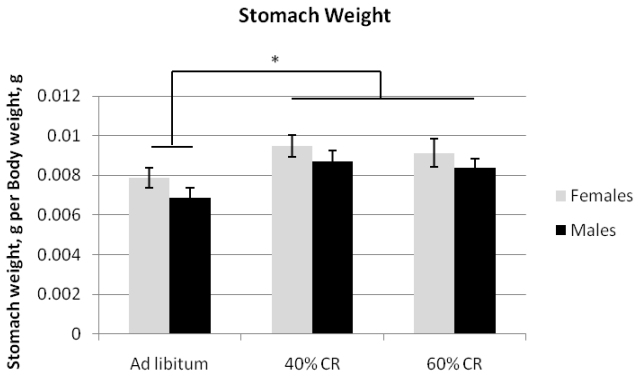


Figure 8-10. Stomach Weight

Post-CR stomach weight, as a fraction of body weight, did not differ between sexes within any diet group. Animals in both the 40% and 60% CR groups had increased relative stomach size compared to AL-fed animals, but were not different from each other, n=9-11/sex/diet group, *p<0.05.

"The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Army or the Department of Defense."

CHAPTER IX. THE GHRELIN COMPARISON STUDY

COMPARISON OF PLASMA GHRELIN IN MOUSE MODELS AND MIMETICS OF CALORIC RESTRCTION

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Introduction

Caloric restriction (CR) has identified physiological benefits for diverse animal species including invertebrates, rodents, and humans (Weindruch and Walford 1988; Hursting, Lavigne et al. 2003; Masoro 2005). Beneficial effects include a healthier metabolic phenotype, decreased incidence of age-related disease, and extension of both healthspan and lifespan. However, the mechanism by which CR confers its effects is unknown. Several adaptive changes may be coordinated via a redundant pathway, and the metabolic hormone ghrelin may be a factor mediating CR and its beneficial effects during aging. Therefore, we propose ghrelin is a hormone regulating major actions of CR and we investigate its actions during several models of CR.

Background

Ghrelin is a stomach-derived hormone which initiates effects in multiple physiological systems. Originally identified for its stimulation of growth hormone

release from the pituitary, its appetite-stimulating actions are now considered its primary physiological role (Kojima, Hosoda et al. 1999; Tschoep, Smiley et al. 2000). Ghrelin exists in two forms: acylated (AG) and unacylated (UAG). An octanoylation at the position three serine produces AG, which is the form primarily responsible for signaling through its major receptor, growth hormone secretagogue receptor (GHSR)-1α. The conversion of unacylated ghrelin to acylated depends on the presence of Ghrelin-*O*-acyl transferase (G*O*AT), which is regulated by a lipid-rich stomach environment and hence may be increased in CR (Gutierrez, Solenberg et al. 2008; Kirchner, Gutierrez et al. 2009). Identified actions of AG include potent appetite stimulation via hypothalamic signaling, growth hormone release, adipocyte differentiation and deposition, inhibition of proinflammatory cytokines, and regulation of the gonadotropic axis during hypoenergetic states (Kojima, Hosoda et al. 1999; Tschoep, Statnick et al. 2002; Dixit, Shaffer et al. 2004; Fernandez-Fernandez, Tena-Sempere et al. 2004; Thompson, Gill et al. 2004). Such actions serve to favor positive energy balance and growth and each is similar to changes seen during CR. UAG has some effects overlapping those of the acylated form (such as CV effects, stimulation of cell proliferation, promotion of adipogenesis) and circulates at a level ~3-5 times higher than AG (Broglio 2004; Akamizu, Shinomiya et al. 2005). Additionally, the presence of UAG can decrease food intake, delay gastric emptying, and attenuate increases in plasma glucose and decreases in plasma insulin caused by AG via a yet unknown mechanism (Broglio 2004; Asakawa, Inui et al. 2005).

The actions of ghrelin and CR are linked in several ways. Plasma total ghrelin levels are increased in both short-term energy deficit and long-term CR (Ariyasu, Takaya et al. 2001; Cummings, Weigle et al. 2002; Hansen, Dall et al. 2002a). CR, in addition to

its alterations of short-term metabolism, renders a unique long-term physiological status with broad physiological manifestations: reduced reproductive fitness, decreased chronic low-grade inflammation but also impaired adaptive immunity, increased glucose effectiveness and insulin sensitivity and reduced growth hormone (GH) and insulin-like growth factor-1 (IGF-1) signaling (Holliday 1989; Wing, Blair et al. 1994; Holzenberger, Dupont et al. 2003; Jolly 2004). Increased ghrelin signaling affects some of these actions in the same way: suppression of proinflammatory and reproductive functioning and improved glucose and insulin homeostasis; and others in the opposite direction: GH/IGF-1 stimulation and sustainment of adaptive immunity (Kojima, Hosoda et al. 1999; Broglio, Arvat et al. 2001; Dixit and Taub 2005; Tena-Sempere 2008). Despite some discrepancy in the direction of response, each of these actions is nonetheless affected by both CR and circulating ghrelin levels.

Plasma ghrelin levels have been evaluated in various models of human CR. Ghrelin levels increase in most identified models of CR except those which mechanically separate the ghrelin-secreting portion of the stomach (i.e. restrictive gastric bypass surgery) (Cummings, Weigle et al. 2002; Hansen, Dall et al. 2002a). Contrary to dietary weight loss which increases plasma ghrelin, maintenance of low ghrelin levels for up to six months after Roux-en-Y gastric bypass surgery likely contributes to appetite suppression and subsequent weight loss success (Cummings, Weigle et al. 2002). Few rodent studies of CR and ghrelin exist and none has proposed ghrelin as a coordinating hormone of CR's effects. The goal of this study was to evaluate the possible role of ghrelin in this important aspect, i.e. as a coordinating hormone of the metabolic effects of

CR. Thus, ghrelin's actions in three separate rodent studies investigating metabolic changes associated with various models/mimetics of CR will be characterized.

The aim of the first study was to determine if mice genetically altered for low plasma levels of insulin-like growth factor-1 (IGF-1), i.e. 'midi' mice, exhibit CR-like alterations in several metabolic parameters. Low plasma IGF-1 is characteristic of animals undergoing sustained CR and deletions along the growth hormone (GH)/IGF-1 axis have demonstrated life extension in several species (Breese, Ingram et al. 1991; Holzenberger, Dupont et al. 2003; Fontana, Weiss et al. 2008). Midi mice have previously exhibited modest increases in lifespan, but other physiological parameters have not yet been described (Sell 2003). The aim of this study was to measure plasma ghrelin levels of mice with approximately 50% circulating levels of IGF-1 at various ages to determine if the known metabolic effects that accompany CR, including elevated ghrelin levels, are also present in these animals.

Study 2 compares the physiological changes associated with a low-methionine (LM) diet to those of long-term 40% CR and *ad libitum* (AL) diets. Diets deficient in essential amino acids like methionine have been reported to exhibit similar effects on lifespan as CR but without reduced food intake (Orentreich, Matias et al. 1993). However, many of the physiological changes that accompany an LM diet have not yet been characterized. Study 2 compares food intake and ghrelin levels associated with LM, 40% CR, and AL diet initiated in middle age in mice of a mixed genetic stock.

Study 3 addresses metabolic changes associated with a short-term, severe CR in young adult male and female mice. While the metabolic outcomes resulting from

extended CR are relatively well-known, ten-day 40% and 60% CR diets were employed to elucidate early changes in response to different levels of CR. Changes in ghrelin levels associated with short-term fasting were measured, as prior reports demonstrated no clear increase in the ghrelin response to short-term decreases in food intake (Doucet, Pomerleau et al. 2004; Blom, Mars et al. 2006).

Each of the above studies was designed to investigate metabolic changes that stem from a range of CR and CR-like manipulations. Ghrelin measurements were taken throughout three studies and were compared to describe any uniform changes in ghrelin levels during disparate models of CR. The increase of ghrelin levels in response to CR in these studies may suggest (1) that ghrelin is, in fact, a signal involved with CR's actions and (2) the studies designed as mechanisms or mimetics of classic CR indeed share similar pathways of CR.

Methods

Animals, Diet, and Food Consumption

IGF-1 Hypomorph study: Seventeen male and female IGF-1 hypomorphic or 'midi' mice and eighteen male and female I29/C57/CD-1 wildtype control mice were acquired at three months of age. IGF-1 hypomorphic mice were generated as described earlier by Sell (Sell 2003). Plasma IGF-1 values are approximately ~50% reduced in addition to a reduced body size, but mice appeared otherwise normal. Same sex and genotype animals were housed two-to-four per cage and were kept on a 12-hour light/dark cycle. Water and normal rodent chow (LabDiet 5001, PMI Nutrition, Branson, MO) were provided *ad*

libitum to both groups for the duration of the study. Average daily food consumption was measured at 6-12 months of age and was averaged over a five-day duration.

Low methionine study: Thirty-five male and female heterogeneous stock (HS) mice were born and remained in a specific-pathogen free animal facility. At approximately 14 months of age, animals began respective diets of

- low methionine food provided *ad libitum* (LabDiet 5010 specially formulated for 0.15% methionine, PMI Nutrition, Branson, MO)
- normal, autoclavable rodent chow provided *ad libitum* (Labdiet 5010, PMI Nutrition), or
- normal, autoclavable rodent chow (Labdiet 5010) provided daily at the 40% CR level.

In comparison, normal rodent chow for ad libitum (AL)-fed animals and 40% CR animals contained .4% methionine. Water was provided *ad libitum* for all animals. Daily food consumption was measured for a period of five days before the start of the study and for the LM and 40% groups at two month intervals thereafter.

Acute CR study: Thirty-five male and female HS, young adult mice were randomized to one of three diets: AL-fed, 40% CR (receiving 40% less food than the *ad libitum* level), or 60% CR (receiving 60% less food) for ten days. Normal rodent chow (LabDiet 5001, PMI Nutrition, Branson, MO) was provided to all three groups. Each mouse was individually housed in a conventional animal facility on a 12-hour light/dark cycle. Food consumption was measured and averaged over three consecutive days prior to the start of

the study. Animals were sacrificed by decapitation at day ten of the intervention for post-diet analysis.

Blood Collection Measures

IGF-1 Hypomorph study: Blood was collected at Baseline (7-11 months) and at Time 2 (15-19 months). At Baseline, four timepoints (0, 600, 1200, and 1800 hours) were collected to yield a diurnal picture of unacylated ghrelin. At Time 2, sampling only occurred at 1200 hours for both acylated and unacylated ghrelin. At each sampling, blood was collected via tail vein puncture in a microcentrifuge tube with EDTA and centrifuged at 3500rpm for 10 minutes at 4°C. Whole blood for acylated ghrelin measurement was collected in tubes with EDTA and p-hydroxymercuribenzoic acid [1mM in final sample volume] and 100µL of 1M HCl to prevent protein degradation. Plasma was recovered and samples were assayed with Rat Acylated Ghrelin ELISA (BioVendor, Candler, NC) according to manufacturer's recommendations. Whole blood for unacylated ghrelin measurement was collected with EDTA only and centrifuged at 3500rpm for 10 minutes at 4°C. Plasma was extracted and analyzed with Rat Unacylated Ghrelin ELISA kit (BioVendor, Candler, NC).

Low methionine study: Blood was sampled every four months starting at 17 months of age, approximately six months after initiation of the diets. Samples for unacylated ghrelin were collected at 17 and 21 months of age. Blood for acylated ghrelin measurement was collected at 25 months of age. Blood was collected and processed as was described for the IGF-1 hypomorphic mice and all blood was collected between the

hours of 9am and 12pm. Acylated and unacylated plasma ghrelin kit specifications are identical to those listed above (Rat Acylated/Unacylated Ghrelin ELISA-BioVendor).

Acute CR: Trunk blood was collected upon animal decapitation on the 10^{th} day of the restriction for analysis of unacylated ghrelin. Blood was preserved with EDTA, spun, and stored as plasma via identical protocol as described above. All sample collections took place between 9am and 12pm. Unacylated ghrelin assay was performed using Rat Unacylated Ghrelin ELISA (BioVendor) with identical kit specifications as above.

STATISTICS

Ghrelin levels were compared between sexes and diet or genotype groups. Where no sex differences were apparent, sexes were pooled. Normality was assessed and either Kruskal-Wallis and Mann-Whitney tests or ANOVAs were applied to compare mean differences. Tukey and SNK post-hoc tests were used for multiple comparisons after ANOVA. Significance was determined at the $p<0.05$ level. Values are reported as means ± SEM.

Figure 9-1. Table of Study Characteristics.

Study	Sex	Genotype	Diet	Testing Scheme	Housing	Plasma Ghrelin
Low IGF-1	Female n=18 Male n=17	Midi (IGF -/0) n=17 or WT (C57/I9/CD-1) n=18	Ad libitum rodent chow	Longitudinal; 7-11 months (baseline) & 15-19 months (time 2)	2-3 per cage, conventional animal facility	Unacylated diurnal curve at baseline; unacylated & acylated at 12pm at time 2
Short-term, severe CR	Female n=18 Male n=17	Heterogeneous Stock	Ad libitum n=11 40% CR n=12 60% CR n=12	Cross-sectional; 8-12 months	Singly-housed, conventional animal facility	Plasma unacylated at 9am at 8-12 mos
Low Methionine	Female n=17 Male n=18	Heterogeneous Stock	AL (.4% methionine) n=11 40% CR (.4% methionine) n=12 AL-fed LM (.15% methionine) n=12	Longitudinal at 17, 21, and 25 months	2-3 per cage, specific-pathogen-free barrier facility	Unacylated at 9am at 17 & 21 mos Acylated at 9am at 25 mos

IGF-1 insulin-like growth factor-1; WT, wild type; CR, calorie restriction; AL, ad libitum; LM, low methionine; mos, month

RESULTS

Body weight and food consumption in low IGF-1'midi' mice

Midi mice had significantly lower body weights than controls [midi: 20.6 ± 0.53g vs. controls: 27.4 ± 1.02g, p=0.000], but this was not due to a CR effect: absolute food consumption in male and midi female mice was not different from that of wildtype controls [midi: 4.76 ± 0.486 grams/day versus controls: 5.96 ± 0.351g/d, p=0.117]. Additionally, food consumption relative to body weight did not differ between midi and control animals [midi: 0.223 ± 0.024 grams/grams body weight vs. control: 0.219 ± 0.016, p=NS] *(data not shown).*

Ghrelin levels in low IGF-1 mice

Unacylated ghrelin levels were measured in 37 mice at 7-11 months of age at four timepoints: 0, 600, 1200, and 1800 hours. Plasma levels did not differ between any sex*genotype subgroup within any timepoint. Additionally, there was no major effect by sex at 0, 600, 1200, or 1800 hours. With sexes pooled, there was a significant difference between midi and control mice at the 1200 hours measurement (midis 684.4 ± 91.7 pg/ml versus controls 1027.9 ± 106.1 pg/ml, p=0.023), but not at any other time *(Figure 1)*.

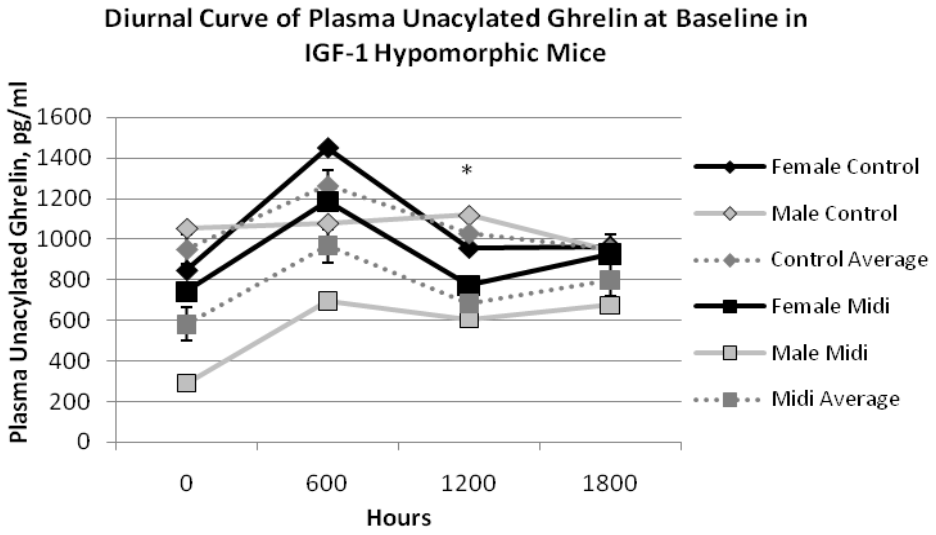

Figure 9-1. Diurnal curve of ghrelin at baseline. Plasma unacylated ghrelin did not differ between sexes within genotype at any timepoint. Sexes were pooled to provide 17-18 mice per genotypic group. Controls differed from midis at 1200 hours only. *p<0.05

Based on the significance at 1200 hours during baseline measurements, blood was collected at 1200 hours at time 2 (mouse age 15-19 months). At time 2, there were no major differences by sex or genotype group, or between any sex*genotype subgroups. Plasma unacylated ghrelin at time 2 was significantly higher than baseline unacylated ghrelin levels for female and male midis. Differences from baseline to time 2 were:

[female midi] 771.8 ± 81.6 vs. 1434.0 ± 167.6, p=0.011; [male midi] 606.7 ± 158.1 vs. 1273.8 ± 238.8, p=0.047. Baseline to time 2 values did not change in control animals *(Figure 2)*.

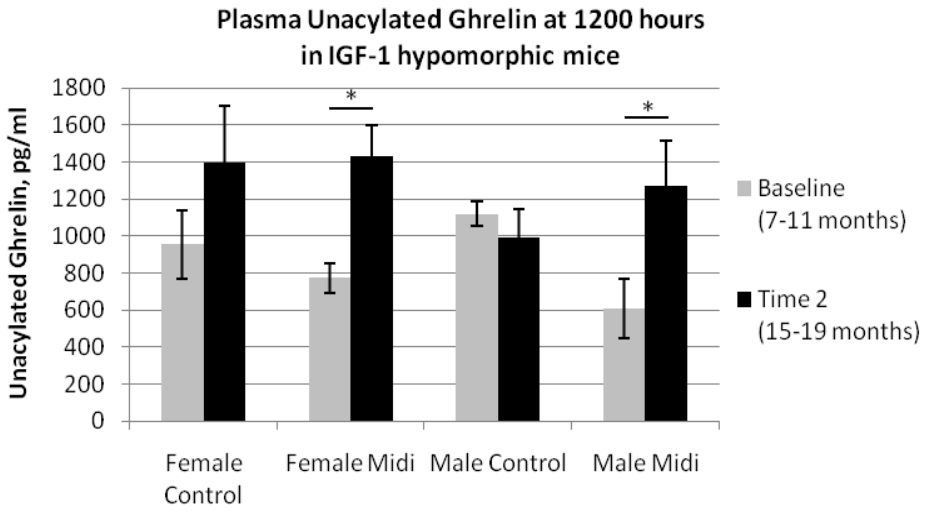

Figure 9-2. Comparison of unacylated ghrelin at baseline and time 2. There were no significant differences between any subgroup within baseline or within time 2 measurement at 1200 hours. Paired t-test comparison from baseline to time 2 yielded significant differences within female midi mice and male midi mice. n=7-10 per sex/genotype/timepoint subgroup, *p<0.05.

Acylated plasma ghrelin levels were compared to unacylated plasma ghrelin levels at time 2. Unacylated ghrelin levels did not differ between any sex*genotype subgroup at time 2 and there were no major effects by sex or genotype. Acylated ghrelin levels did not differ by sex or genotype, or between any sex* genotype subgroups at time 2. However, unacylated plasma ghrelin levels were significantly higher than acylated ghrelin levels for each subgroup *(Figure 3)*. The Mann-Whitney U test was employed to test mean differences between acylated and unacylated ghrelin levels at time 2. With

sexes and genotypes pooled, unacylated ghrelin was ~3.6 fold higher than acylated ghrelin in plasma (1263.7 ± 103.8 vs. 352.6 ± 41.4, p=0.000).

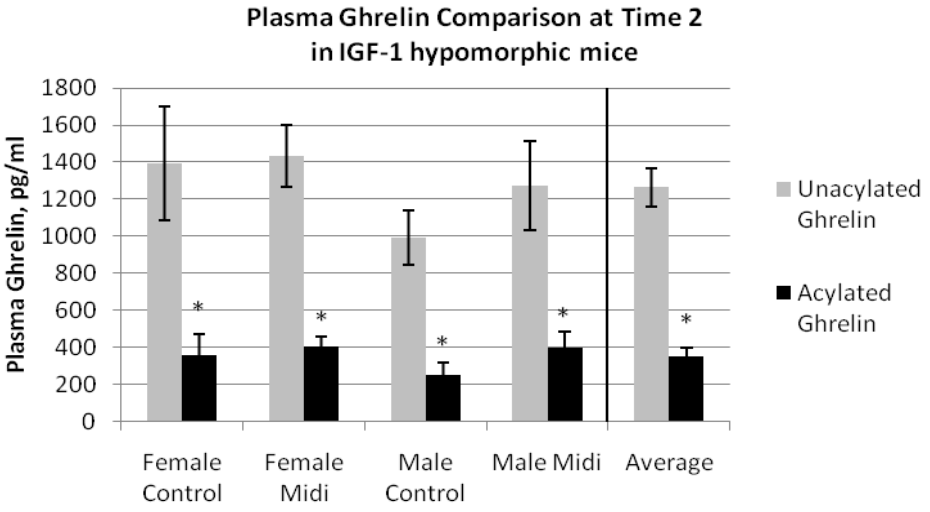

Figure 9-3. Comparison of acylated and unacylated ghrelin at time 2. Unacylated plasma ghrelin did not differ between any sex or genotype groups, n=7-10/sex/genotype group. Likewise, acylated ghrelin did not differ between any sex of genotype groups, n=8-9/sex/genotype. Unacylated ghrelin was significantly higher than acylated ghrelin in every group and overall, *p<0.05.

Body weight and food consumption in low methionine mice and 40% CR mice

Body weight was significantly lower in 40% CR and LM-fed animals versus AL-fed controls [40% CR (28.2 ± 0.63g) vs AL (33.8 ± 0.94g), p=0.000; LM (30.1 ± 0.91g) vs AL, p=0.001]. Body weight did not significantly differ between animals in the 40% CR and AL-fed groups (p=.114), although there was a trend for lowest body weight in 40% CR animals. Food consumption was significantly higher (g/day) in LM-fed animals versus AL controls [LM, 7.61 ± 1.37 g/day vs AL 2.50 ± 0.64 g/day, p=0.000]. After adjustment for body weight, differences were even more pronounced [LM 0.26 ± 0.05g food/g body weight vs. AL 0.08 ± 0.02g food/g body weight, p=0.001, *data not shown*].

Ghrelin levels in low methionine and 40% CR mice

Plasma unacylated ghrelin was measured in male and female LM, 40% CR and AL-fed mice. Mice were measured at 17 months of age and had been on their respective diets for approximately six months. There was no major effect by sex or by diet. Subgroup analysis via Mann-Whitney U test for two samples with multiple comparison correction demonstrated LM males (1500.9 ± 372.0pg/mL) had significantly higher plasma ghrelin levels at 17 months than AL females (503.5 ± 124.0pg/mL, p=0.017) and 40% CR females (487.0 ± 140.1pg/mL, p=0.009). AL males, LM females, and 40% CR males did not differ from any other subgroup.

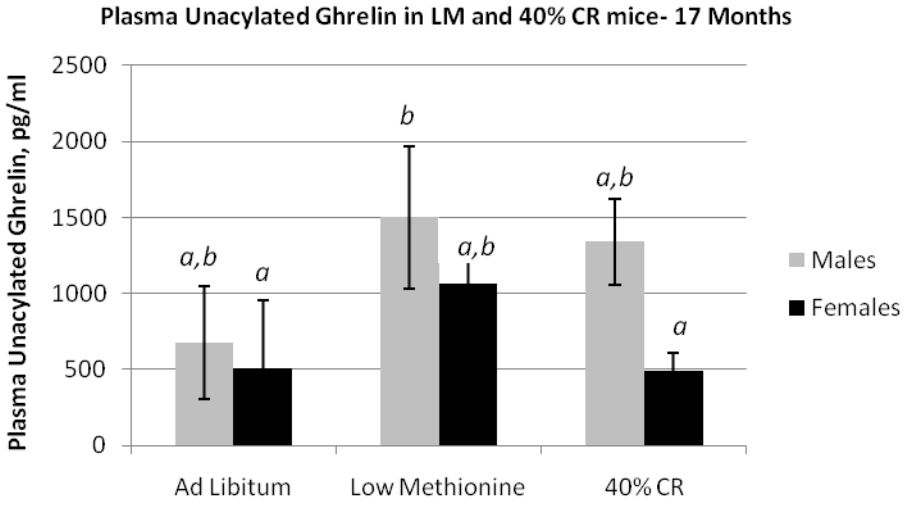

Figure 9-4. Plasma unacylated ghrelin at 17 months. Plasma unacylated ghrelin did not differ overall by sex or by diet. There was a significant difference between male LM mice and AL and 40% CR female mice. n=5-6/sex/diet group, *p<0.05

Plasma unacylated ghrelin was additionally measured at 21 months. Non-parametric tests revealed no differences by sex or diet in plasma ghrelin levels. Additionally, there were no significant differences between any sex*diet subgroups.

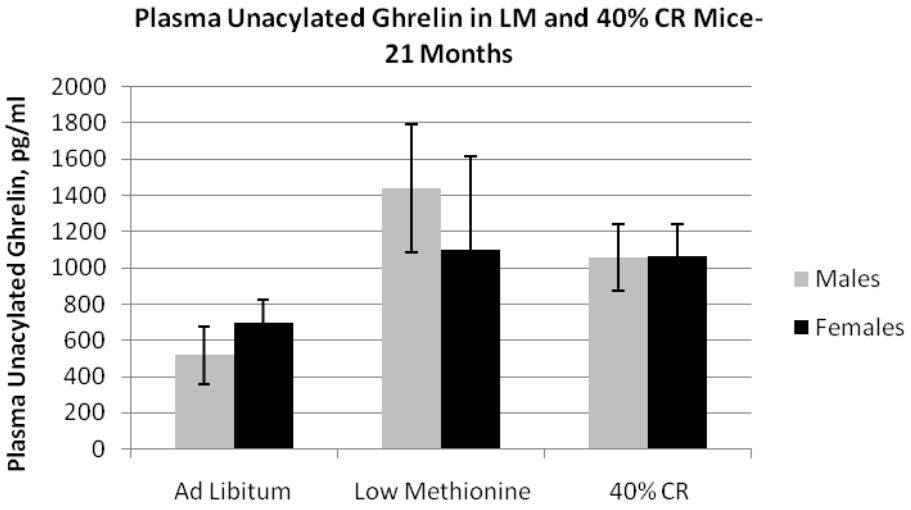

Figure 9-5. Plasma unacylated ghrelin at 21 months. No effect by sex was determined with the Mann-Whitney U test. Effects by diet and sex*diet subgroup were analyzed by Kruskal-Wallis and no differences by diet nor subgroup were detected. n=4-6/sex/diet group, *p<0.05

Plasma unacylated ghrelin did not change between 17 and 21 months for any diet group. Therefore, timepoints were pooled to yield 22-24 per diet group. Plasma unacylated ghrelin was significantly higher in the LM group (1331.7 ± 205.1pg/mL) and 40% CR group (987.2 ± 144.2) versus controls (605.4 ± 92.1pg/mL, p=0.009 & p=0.029, respectively). Experimental groups did not differ from each other.

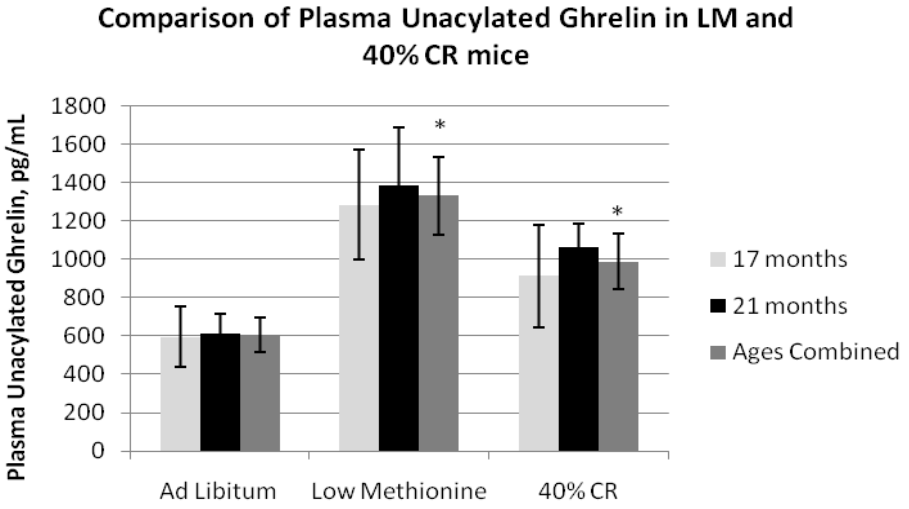

Figure 9-6. Comparison of unacylated plasma ghrelin at 17 and 21 months. Plasma ghrelin did not differ from 17 to 21 months for any diet group. Timepoints were pooled resulting in significant differences between LM-fed and 40% CR-fed animals versus controls. LM and 40% CR animals did not differ from each other. n=22-24 per sex/diet/timepoint group, *p<0.05

Plasma acylated ghrelin was measured at 25 months of age to determine if any subgroup differences in acylated ghrelin were responsible for observed differences in feeding behavior. There were no significant differences in plasma acylated ghrelin between sex or diet groups or sex*diet subgroups at 25 months of age.

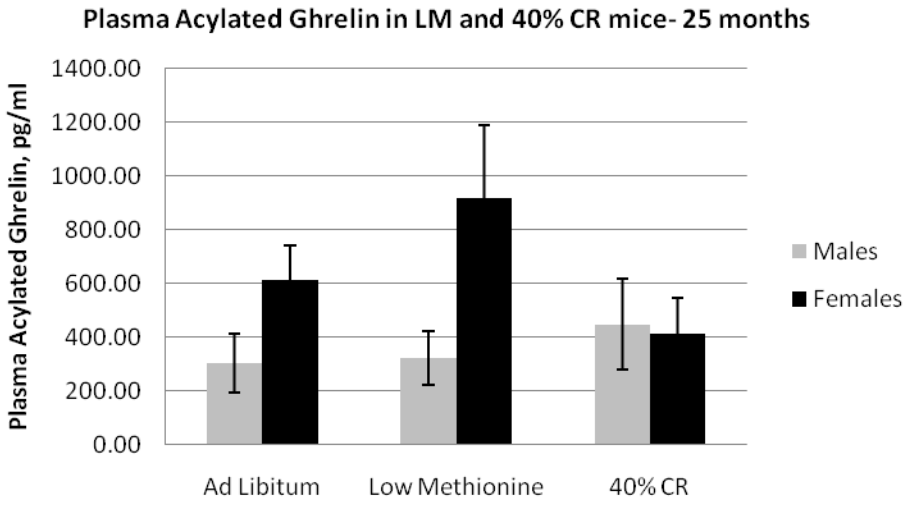

Figure 9-7. Plasma acylated ghrelin at 25 months. Plasma acylated ghrelin at 25 months did not significantly differ overall by sex or diet using non-parametric tests. Sub-group comparisons showed no significant differences between any sex*diet groups, n= 3-5/sex/diet group, *p<0.05.

Body weight and food consumption in short-term, severe CR animals

Absolute food consumption (grams) differed between males (5.84 ± 0.14 grams) and females (4.94 ± 0.12 grams, p= 0.000) but after adjusting for consumption by body weight, differences were no longer observed (p= 0.382). Neither absolute food consumption nor food consumed per body weight differed between diet groups before initiation of the diet *(data not shown).* Body weight differed between males and females before the start of the study [males] 35.7 ± 0.6 grams vs. [females] 30.4 ± 0.5 grams (p=0.000). Body weight did not differ before the start of the study between diet groups *(data not shown).*

Ghrelin levels in short-term, severe CR mice

Plasma unacylated ghrelin was measured from trunk blood collection at the end of the 10-day diet period. Males and females significantly differed overall (males, 617.4 ± 145.8 pg/mL vs. females, 1750.1 ± 314.9, p=0.002) and hence, were analyzed separately. By diet, males and females in the 60% CR group differed (males, 532.7 ± 303.3pg/mL vs. females, 2322.4 ± 616.3, p=0.017) but not in the 40% CR group (p=0.792) or AL-fed group (p=0.052). There was no effect by diet within females or within males.

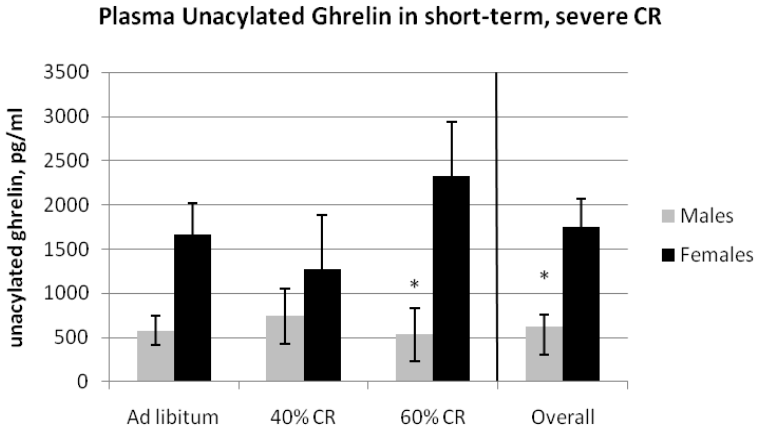

Figure 9-8. Plasma Unacylated ghrelin after 10-day CR. Females had significantly higher plasma unacylated ghrelin levels versus males (p=0.02 via Mann Whitney U test). Specifically, 60% CR females had significantly higher ghrelin levels than 60% CR males (p=0.017) and there was a trend for a sex difference in AL-fed animals (p=0.052). There was no effect by diet within males or within females. n= 5-6 per sex/genotype group, *p<0.05.

DISCUSSION

The data from multiple CR or CR-like studies present no overall trend in ghrelin responses to diet in mice. Several prior studies have found that ghrelin levels increase in response to long-term negative energy balance as well as fasting in both humans and rodents (Tschoep, Smiley et al. 2000; Tschoep, Weyer et al. 2001; Cummings, Weigle et al. 2002; Purnell 2007; Yang, Youm et al. 2007; Kirchner, Gutierrez et al. 2009).

Comparing direct models of CR: long term 40% CR in the low methionine study and short-term 40% and 60% CR in the severe CR study, our results suggest no uniform increase in ghrelin levels in these mice. Long-term 40% CR (Low Methionine study) initiated an increase in plasma unacylated ghrelin levels versus AL-fed controls, an effect only demonstrated after pooling data from two timepoints and both males and females. Although this finding supports existing evidence that ghrelin increases with long-term CR, an expected increase in acylated ghrelin could not be detected. Likewise, data from our short-term, severe CR study demonstrated no significant increase in unacylated ghrelin for either the 40% CR or 60% CR groups.

Results were additionally mixed for the indirect models of CR: low IGF-1 as a CR mechanism and low methionine as a CR mimetic. In low IGF-1 mice, plasma unacylated ghrelin levels only differed at the 1200 hours timepoint during the 7-11 month baseline measurement. Noontime measurement disrupted the animals' sleep cycle and lower ghrelin levels in midi mice at that time may reflect a difference in metabolism that contributes to their stunted growth. Additionally, the overall diurnal rhythm reflects known changes in ghrelin in response to feeding: ghrelin levels were lower during feeding periods (1800-0 hours) and non-significantly increased during fasting periods (600 hours). The increase in unacylated ghrelin from baseline to time 2 (15-19 months) in midi mice is a surprising result. Ghrelin levels are typically decreased in older animals, perhaps contributing to the anorexia of aging, and this decline is typically not initiated until late life (Hays and Roberts 2006). While food consumption relative to body weight was not different between midis and wildtypes at the baseline measurement, no data on food consumption were subsequently collected. The interaction between food

consumption behavior, ghrelin levels, and age may be an important direction for future research in these growth-impaired, long-lived mice.

Acylated ghrelin is the 'bioactive' form of ghrelin, binding to the GHSR-1a receptor with high affinity and initiating appetite stimulation, growth hormone secretion, proinflammatory cytokine suppression and fat deposition (van der Lely, Tschoep M et al. 2004). Its actions have been well-characterized in studies of human CR, which define an increase in plasma acylated ghrelin with negative energy balance states (Marzullo, Verti et al. 2004). In rodents, mixed effects have been reported (Lutter, Sakata et al. 2008; Kirchner, Gutierrez et al. 2009). Here we demonstrate that acylated ghrelin levels did not respond to a low methionine or 40% CR diet in late-middle aged HS mice nor were altered in midi mice. However, the expected ratio of plasma uacylated ghrelin: plasma acylated ghrelin (~3-5 fold) was confirmed by our 3.6 fold difference.

The only marked diet effect on plasma unacylated ghrelin was demonstrated after pooling multiple sex and timepoint values, suggesting a large sample size (>20 animals per group) may be required to detect mean differences using ELISA techniques in mouse plasma. Other studies in our laboratory have yielded significant results in metabolic hormone alteration with a similar sample size, but ghrelin's unique release mechanism (in relation to changing energy status) and metabolism may contribute to added variance in mean values. Ghrelin is known to be a hormone with sizable variation due to its release in advance of meals and underlying diurnal rhythm (Murakami, Hayashida et al. 2002; Sanchez, Oliver et al. 2004). Accurate measurement of acylated ghrelin is even less reliable, because fluctuations in its status are dependent on unacylated ghrelin production as well as the degradation of the n-octanylation on its serine-3 (Yang, Brown et al. 2008).

Additionally, the half-life of acylated ghrelin is ~ten minutes, so high variability in its plasma levels is not unexpected (Nagaya, Kojima et al. 2001).

Despite high variability with ghrelin measurements within studies, several aspects of study design allowed for direct comparison of ghrelin levels between studies. Heterogeneous stock mice derived from the same colony were used in both the low methionine and the short-term, severe CR studies. Although housing conditions were different (multiply-housed in barrier facility vs. singly-housed in normal animal facility), mice were fed similar diets and had identical light/dark cycles. The increased plasma unacylated ghrelin levels in females that were revealed in the short-term, severe CR study were not apparent in the 40% CR or AL-fed animals of the low methionine study. In fact, there was a trend toward males having higher ghrelin levels than females. This difference may be attributed to the mixed genetic background of the HS mice, the age at which the animals were evaluated (young adult versus late middle age), group vs. single housing accommodations, or a combination thereof. Sex differences in ghrelin levels have been previously reported in humans, with females have higher ghrelin levels than males, but similar data for rodents are not reported (Akamizu, Shinomiya et al. 2005). This finding is possibly attributed to estrogen/estradiol's stimulatory effect on ghrelin (Barkman, Dimaraki et al. 2003). Additionally, housing conditions have been shown to affect release of sex steroids in rodents, so the interaction of circulating sex steroids on ghrelin levels due to housing conditions (female mice cease cycling when multiply housed) may contribute to the variability in ghrelin measurement (Bruce 1963). Finally, the unpredictable contribution of genetics to complex outcomes is clearly demonstrated

in a recent paper which showed a wide range of lifespan responses to CR (unfavorable to favorable) depending on mouse strain (Liao, Rikke et al. 2010).

Each of these models was initially designed to address a specific aspect of CR in relation to aging. Because CR is the only known modulator of lifespan and ghrelin and CR are closely correlated, measuring ghrelin in these studies may help elucidate which models are truly CR-like (Weindruch and Walford 1988). Mice genetically altered for low plasma IGF-1 demonstrate extended lifespan, an effect noted thus far in females (Sell 2003). However, other metabolic parameters of these animals (metabolic rate, physical activity, body temperature) do not resemble CR animals (Motch, Sell et al. 2010). Because ghrelin levels also did not respond in a CR-like way in the low IGF-1 mice, low plasma IGF-1 is unlikely to be a major component of the mechanism linking CR and extended lifespan.

Low methionine-fed animals have been reported to have increased daily food intake, therefore any extension in lifespan on this diet is due to an effect independent of CR (Orentreich, Matias et al. 1993). In our study, LM animals displayed increased food intake and an increase in plasma unacylated ghrelin greater than that of animals on long-term 40% CR. Since experimental data have shown effects of LM diets on lifespan, it is possible that a CR-like mechanism is engaged. In this case, the increase in ghrelin levels beyond that of 40% CR combined with non-food restricted life extension strongly supports a role for ghrelin as an integrating metabolic hormone during CR.

Short-term, severe CR did not elicit an expected increase in ghrelin levels in either the 40% CR or the 60% CR groups. This is a surprising result as others have

shown a short-duration restriction can increase ghrelin (Hosoda, Kojima et al. 2000; Kirchner, Gutierrez et al. 2009). It is possible that the metabolic disturbance in the 60% CR group is so severe that normal metabolic compensatory mechanisms are unable to counteract rapid and dramatic changes in energy balance.

In conclusion, ghrelin may integrate processes of CR. Several changes occur with long-term moderate CR including increased appetite, decreased adaptive immune function, and changes in GH/IGF-1 signaling. Ghrelin is a unique metabolic hormone with multi-system physiological consequences including similar alterations in appetite, immunity, and growth. Our data support some role for ghrelin in models of CR and its function may be even more pronounced during a CR-like methionine diet. It is unlikely that the mice genetically altered for low IGF-1 have metabolic pathways engaged that are identical to that of CR, as several CR features, including increased ghrelin, were not demonstrated. However, these data point toward a coordinating role for ghrelin in the interaction between CR, metabolism, and lifespan and ghrelin may emerge as a key regulatory hormone in future studies.

CHAPTER X. THE GASTRIC BYPASS STUDY

"Genetic and Hormonal Aspects of Ghrelin in Roux-en-Y Gastric Bypass Patients"

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Introduction

Recent reports indicate the prevalence of adult overweight and obesity in America is currently 68% and 34%, respectively (Flegal 2010). The prevalence of several conditions such as non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), insulin resistance (IR), and type II diabetes (T2D) is also increased with obesity, emphasizing the hazard to public health of this condition. In the morbidly obese, the risk of NASH and T2D is 2-3 fold and 5-10 fold higher, respectively, and complications from these conditions contribute to increased morbidity and mortality characteristic of these patients (Diabetes 1975; Statement 1985; Brunt, Janney et al. 1999). Gastric bypass surgery is an effective treatment for the reversal of these co-morbid conditions and several others: hypertension, heart disease, sleep apnea, hyperlipidemia, depression, etc (Benotti 1992; Charuzi 1992; Gleysteen 1992; Kral,

Sjostrom et al. 1992). The multiple actions of bypass surgeries have shown the greatest improvements for NAFLD and T2D, with 80% and 84% of Roux-en-Y patients having resolution of T2D and steatosis, respectively, within two years (Furuya 2007; Buchwald 2009). The metabolic hormone ghrelin has important, possibly mechanistic, connections between the Roux-en-Y procedure, obesity, and relief from T2D and NASH: (1) plasma levels are differentially affected during dietary versus surgically-induced weight loss (Cummings, Weigle et al. 2002; Cummings, Overduin et al. 2004; Purnell 2007); (2) plasma levels are decreased in obesity, as well as in T2D and NASH independent of BMI (Uribe 2008; Vestergaard, Djurhuus et al. 2008; Vestergaard, Gormsen et al. 2008) and (3) its actions include antagonizing the release of the proinflammatory cytokines Interleukin (IL)-6 and tumor necrosis factor (TNF)-α which are increased in obesity and have been shown to induce insulin resistance and advance NAFLD (Kern 2001; Yalniz 2006; Leclercq, Da Silva et al. 2007).

Ghrelin has additional actions that may contribute to the feed-forward cycle of obesity and metabolic disease. Such actions include: hypothalamic stimulation of appetite, promotion of fatty deposition (stimulation of adipogenesis *in vivo*, inhibition of lipolysis *in vitro*, facilitation of triglyceride storage in fat and lean tissue), and possible inhibition of insulin (Tschoep, Smiley et al. 2000; Muccioli, M et al. 2002; Choi, Roh et al. 2003; Reimer, Pacini et al. 2003; Thompson, Gill et al. 2004; Barazzoni, Bosutti et al. 2005). Ghrelin further promotes insulin resistance by encouraging lipid deposition in lean tissue (e.g. liver), activating the insulin-signaling cascade, and inducing expression of glucogenic genes in hepatocytes (Murata, Okimura et al. 2002; Barazzoni, Bosutti et al. 2005). Exogenous administration in humans has been shown to decrease plasma

insulin levels and increase plasma glucose although causality in this complex relationship has not been established (Broglio, Arvat et al. 2001; Kiewiet, van Aken et al. 2009). However, as a compensatory response to excess energy stores, ghrelin circulates in low levels with increasing BMI (Muccioli, M et al. 2002). Dietary weight loss substantially elevates ghrelin levels, increasing these anabolic effects and impairing the physiological recovery from obesity (Angulo 2002). Evidence suggests ghrelin levels decrease immediately after Roux-en-Y and Biliopancreatic Diversion-type gastric bypass surgeries and may remain low for several months (Pournaras and le Roux 2010). Low ghrelin levels following surgery have been suggested to aid weight loss by suppressing appetite and prevent the weight loss-related rebound of lipid deposition in lean tissue (Angulo 2002; Cummings, Weigle et al. 2002).

Allelic variation in ghrelin-related genes may independently contribute to obesity and metabolic disease. Previously considered important only for monogenic conditions, recent evidence indicates single nucleotide polymorphisms (SNPs) are important factors in complex clinical traits. SNPs in genes related to obesity and/or diabetes have been shown to have a significant impact on the etiology of these conditions and may affect post-surgical weight loss and disease mitigation success (Chu 2008; Franks, Jablonski et al. 2008; Freathy, Timpson et al. 2008; Renstrom, Payne et al. 2009; Zhao, Bradfield et al. 2009). Several SNPs lead to non-synonymous amino acid substitutions affecting transcription rate or other regulatory actions. It is unknown how SNPs in ghrelin and ghrelin receptor genes exert effects but variation at these sites have been linked to obesity, eating behavior and appetite, blood triglycerides, fasting insulin, and insulin resistance (Baessler, Hasinoff et al. 2005; Vartiainen, Kesaniemi et al. 2006; Baessler,

Fisher et al. 2007; den Hoed, Smeets et al. 2008; Zavarella, Petrone et al. 2008). Specifically, two ghrelin receptor promoter SNPs, rs490683 and rs9819506, have been significantly correlated with BMI, weight loss efficacy during dietary intervention, and measures of insulin resistance (Mager, Degenhardt et al. 2008).

The presence of specific allelic variants in ghrelin-related genes and secondly, plasma-level changes in ghrelin protein may impact weight, weight loss during surgical intervention, and progression of metabolic disease. The liver microenvironment, as a site of T2D and NASH disease action, is indirectly affected by ghrelin's actions on insulin, insulin resistance, and suppression of inflammation. Plasma changes in ghrelin have been well-studied following gastric bypass surgery, but liver tissue changes have not. The complex interaction of ghrelin, liver-tissue proteins and cytokines, and weight during gastric bypass is important to characterize with the goal of understanding mechanisms of action and thereby improving surgical outcomes. In this study, we have (i) characterized ghrelin levels in plasma and liver tissue in surgical patients in the presence or absence of NASH and T2D, (ii) compared ghrelin, insulin, leptin, TNF-α and IL-6 levels in liver tissue, and (iii) correlated two ghrelin receptor promoter SNPs with weight and weight loss, prevalence of comorbid conditions (hyperinsulinemia, diabetes, and Prader-Willi syndrome), and clinical measures of disease severity (glycosylated hemoglobin (HbA1c) and fasting insulin and glucose). The data demonstrate the importance of both physiological and genetic actions of ghrelin relevant to gastric bypass surgical outcomes.

Materials & Methods

Patients

Study patients included a total of 694 individuals who underwent gastric bypass surgery at Geisinger Medical Center, Danville, PA from 2004-2010. Thirty-seven of the 694 patients have recently (2009-2010) undergone Roux-en-Y surgery and provided liver biopsy and blood samples for hormonal analysis. The remaining 657 patients provided blood samples for genetic analysis. Approval was provided by the Institutional Review Board of Geisinger Medical Center and patients provided informed consent. Demographic data (mean ± SEM or frequency) are provided in Table 1 for both patient populations.

Table 1. Baseline patient demographics

	Patients for hormonal analysis (n=37)			Patients for genetic analysis (n=657)		
	Females n= 34 (91.8%)	Males n= 3 (8.2%)	*p-value*	Females n= 535 (81.4%)	Males n= 122 (18.6%)	*p-value*
Age	42.9 ± 2.1	61.3 ± 1.2	= .013	45.5 ± 0.49	48.0 ± 1.11	=.036[a]
Race *White* *Black* *Hispanic, mixed* *Other or unknown*	 32 1 1 0	 3 0 0 0	--	 502 13 5 15	 111 3 4 4	--
Baseline BMI	49.2 ± 1.5	46.3 ± 1.7	= .979[a]	48.2 ± 0.35	50.1 ± 0.76	= .018
BMI at ~4 months prior to surgery	47.8 ± 1.4	44.5 ± 1.4	= .498[a]	46.3 ± 0.37	46.4 ± 0.74	= .545[a]
Prevalence of Type II Diabetes	16 (47%)	2 (67%)	= .604[b]	169 (31%)	59 (48%)	= .002[b]
Presurgical avg. HbA1c	6.24 ± 0.2	7.43 ± 0.7	= .041	6.2 ± 0.06	6.7 ± 0.16	= .078
Presurgical avg. HOMA	2.42 ± 0.25	3.5 ± 1.3	= .371	--	--	--

[a]Analyzed by MannWhitney U test.
[b]Analyzed by χ^2 test
HbA1c, hemoglobin A1c; HOMA, homeostasis model assessment

Hormonal Analyses

Patients

Thirty-seven patients were stratified by presence of T2D and NAFLD activity into four groups: presence of T2D and NAFLD (+D/+N), presence of T2D but not NAFLD (+D/-N), presence of NAFLD but not T2D (-D/+N) and absence of both conditions (-D/-N). A positive T2D diagnosis was obtained from electronic medical record data (EpicCare electronic medical records, EpicSystems, Verona, WI). Patients identified with metabolic syndrome or hyperinsulinemia but not explicit T2D were classified as positive for T2D, but gestational diabetics were not. NAFLD/NASH activity was quantified by a clinical pathologist using the criteria outlined by Kleiner et al. (Brunt EM 1999; Kleiner 2005). Briefly, the NAFLD activity score (NAS) is the sum of a steatosis

score (0-3), a lobular inflammation score (0-3), and a hepatocyte ballooning score (0-2). The overall NAS score (0-8) is defined as (0-2) largely not considered diagnostic for steatohepatitis, (3-4) includes not diagnostic, borderline, or positive for steatohepatitis, or (5-8) largely considered positive for steatohepatitis. This project was not specifically designed to study patients based on clinical diagnosis for steatohepatitis but with some level of liver impairment (i.e. fatty infiltration), therefore a NAS score of 2 or above was considered positive for NAFLD. The classification of the 37 patients was as follows: (+D/+N), n=9; (+D/-N), n=9; (-D/+N), n=8; and (-D/-N), n=11.

Blood Collection and Analysis

Blood samples from thirty patients were collected at a clinic visit prior to surgery (mean time before surgery 4.1 ± 0.5 months, range 0.5-1 months). A second sample was collected at the 5 or 12-month post-operative clinic visit (mean time post-surgery 6.0 ± 0.3 months, range 4.5-12 months). Whole blood was collected in serum separator tubes containing EDTA preservative. Serum was extracted and stored at -80°C. Because of restrictive hospital laboratory procedures, pre- and post-operative serum samples were collected in standard serum separator tubes with no protease inhibitor or HCl, which are recommended for total ghrelin measurement. Samples were analyzed in duplicate for total ghrelin via ELISA with the Human Ghrelin (Total) ELISA (Millipore Corp., Billerica, MA; Luminex Corp., Austin, TX). Sensitivity was rated at 100 pg/ml for des-

octanoyl ghrelin in serum. Expected yield of ghrelin without protease inhibitor or HCl is ~30% less via manufacturer's specifications.

Liver Collection, Classification, and Protein Extraction

Liver wedge biopsies were collected from the operating room during gastric bypass surgery, snap frozen in liquid nitrogen, and stored at -80°C. A separate portion was used for NAFLD diagnosis (vide supra). Frozen liver tissues (~0.5g sections) were mechanically homogenized with 500µL 1x phosphate buffered saline (PBS) assay buffer containing Complete Mini Protease Inhibitor Cocktail (1 tablet per 3mL PBS-final concentration, 3.5x protease inhibitor, Roche Diagnostics, Indianapolis, IN.). Buffer solution was free of organic solvents and detergents. Homogenized samples were centrifuged at 14000 rpm for 10 minutes and protein-containing supernatant was recovered for simultaneous measurement of leptin, IL-6 and TNF-α. Samples were prepared in triplicate according to manufacturer's recommendations for the Human Metabolic Hormone panel with Luminex xMAP technology (Millipore Corp.). Intra-assay precision was <9% and accuracy 82%, 86%, and 74% for IL-6, TNF-α, and leptin, respectively. Sensitivity was as follows: IL-6, 2.4 pg/ml; TNF-α, 0.6 pg/ml; and leptin, 160 pg/ml. Total ghrelin from liver tissue homogenate (with no additional HCl or protease inhibitor to maintain comparability with serum samples) was analyzed in duplicate by ELISA (Human Ghrelin (Total) ELISA, Millipore Corp.) using PBS cocktail as assay buffer. Kit specifications are identical to those listed above.

Clinical Laboratory Values, Disease Diagnoses, and HOMA

Pre-surgical values for fasting insulin, glucose, and HbA1c were measured by standard clinical laboratory techniques and accessed from each patient's electronic health record. Weight and associated BMI values were carefully measured at several clinical visits: the initial clinic encounter (BMI_baseline, e.g.), the bariatric surgery evaluation visit (BMI_BSE), a visit during the post-evaluation dietary weight loss period (BMI_pBSE), at the time of surgery (BMI_surgery), and at the time of post-operative blood collection (BMI_postop). Clinical diagnoses for T2D, hyperinsulinemia, and Prader-Willi were obtained from medical record data. Ideal body weight was calculated for each patient based on height and a BMI of 25. Excess body weight (actual body weight-ideal body weight) and percentage of excess body weight lost [EBWL—(pounds lost/excess body weight)*100] were markers for success at various points, e.g. EBWL at 12 months post surgery.

The homeostasis model assessment (HOMA) was used to assess pancreatic beta-cell function, insulin sensitivity and insulin resistance. The quantification of IR was calculated by the HOMA Calculator, version 2.2 (Diabetes Trial Unit, University of Oxford, UK) using clinical values of fasting insulin (µU/ml) and fasting glucose (mg/dl). This software calculates HOMA for glucose values between 54.1 and 450.5mg/dl and insulin values between 2.9 and 57.6µU/ml.

Genetic Analysis

Patients

Patients undergoing Roux-en-Y or laparoscopic adjustable band surgery from 2004 to present consented to provide blood samples during the preoperative dietary weight loss period. Clinical measures of BMI at several timepoints: baseline, evaluation for surgery, pre-operative weight loss visit, surgery, 12- and 24- month follow-up visits, along with fasting insulin, glucose, and HbA1c were compared by genotype. Measures of weight loss success including percent of excess body weight lost at various post-surgical timepoints were also compared between genotypes.

DNA Isolation and Genotyping

DNA was isolated from 350µL of EDTA-preserved whole blood or fixed liver tissues according to manufacturer's specifications using Qiagen MagAttract DNA Blood MidiM48 Kit and Qiagen BioRobot M48 Workstation (Qiagen, Valencia, California). For liver tissues, samples were first digested with 1µg/µL of proteinase K in 350µL of Qiagen Tissue Lysis Buffer (Qiagen) and incubated at 55ºC overnight. Quantification of DNA for each sample was performed on a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Following DNA isolation, samples were genotyped for two ghrelin receptor promoter SNPs, rs490683 and rs9819506. Reagents were provided by Applied Biosystems (rs490683-C__2169194_10 and rs9819605-C__2863288_10, Carlsbad, CA) and assayed according to protocol with DNA (10ng), TaqMan Genotyping MasterMix (5µL, Applied Biosystems), 40x assay mix (0.25µL),

and water to a total volume of 10µL. Thermocycler settings were: 50ºC for two minutes, 95ºC for ten minutes, and 40 cycles of 95ºC for 15 seconds followed by 60ºC for 1 minute. Results were determined using Applied Biosystems Sequence Detection Software.

Statistics

Data were analyzed using SPSS Statistics 17.0 (IBM, Chicago, IL). Normality was assessed and non-parametic tests were applied as appropriate. Pre- and post- surgical values were evaluated using paired t-tests and the post-hoc Student-Newman-Keuls (SNK) was employed for multiple comparisons after ANOVA, unless otherwise noted. Values are reported as mean ± standard error of the mean (SEM).

RESULTS

Hormonal Analysis

Baseline Sex Differences

There was a significant sex difference for age and pre-surgical HbA1c in patients in the hormonal analysis group. There were no initial differences between men and women on BMI at baseline visit or four months prior to surgery, prevalence of diabetes, or average pre-surgical HOMA (*Table 1).*

Post-surgical Differences by Disease Classification

Individuals were significantly older in the diabetic and NAFLD positive group (+D/+N) versus the non-diabetic, NAFLD group (-D/+N) (50.7 ± 3.1 vs. 36.4 ± 4.4 years, $p<0.05$, respectively). Age differences between other groups were not significant. BMI did not differ between diabetes*NAFLD groups at the time of surgery or at the 5-12 month post-operative blood draw. Plasma glucose was significantly higher in the double-positive [(+D/+N), 154.5 ± 10.9] group compared to both non-diabetic groups [(-D/+N), 115.6 ± 7.8, p=0.014 and (-D/-N), 119.2 ± 4.5, p=0.008]. HbA1c significantly differed between the diabetic/NAFLD positive group [(+D/+N), 7.29 ± 0.27] and both non-diabetic groups [(-D/+N), 5.76 ± 0.30, p=0.003 and (-D/-N), 5.92 ± 0.19, p=0.027]. Both single positive groups differed from each other [(-D/+N), 5.76 ± 0.30 versus (+D/-N), 6.47 ± 0.034, p=0.020]. Insulin and HOMA differed between the double positive and double negative groups [Insulin: (+D/+N), 29.9 ± 6.7 versus (-D/-N), 14.0 ± 1.8, p=0.004 and HOMA: (+D/+N), 3.35 ± 0.75 versus (-D/-N), 1.81 ± 0.22, p=0.046]. A comparison of surgical characteristics and post-surgical results stratified by T2D and NASH diagnosis is provided in table 2.

Table 2. Summary data of post-surgical characteristics

Characteristic	**(+D/+N)**	**(+D/-N)**	**(-D/+N)**	**(-D/-N)**
Age	50.7 ± 3.1[b]	48 ± 4.6	36.4 ± 4.4[d]	42.1 ± 3.7
Sex *n*	7 females, 2 males	9 females	7 females, 1 male	11 females
BMI, kg/m^2 *at surgery* *at post-op blood draw*	 48.1 ± 2.5 36.1 ± 2.3	 47.5 ± 2.5 36.2 ± 2.3	 46.8 ± 2.1 33.7 2.2	 46.4 ± 3.2 36.7 ± 2.6
Pre-surgical HbA1c	7.29 ± 0.27[a,b]	6.47 ± 0.34[b]	5.76 ± 0.30[c,d]	5.92 ± 0.19[d]
Pre-surgical Glucose, mg/dL	154.5 ± 10.9[a,b]	135.4 ± 11.0	115.6 ± 7.8[d]	119.2 ± 4.5[d]
Pre-surgical Insulin, µU/mL	29.9 ± 6.7[a]	19.0 ± 4.5	23.6 ± 4.3	14.0 ± 1.8[d]
Pre-surgical HOMA	3.35 ± 0.75[a]	2.08 ± .26	3.0 ± 0.54	1.81 ± 0.22[d]
[Serum Ghrelin, ng/mL] *before surgery* *after surgery*	 1.7 ± 0.49 3.0 ± 0.35	 1.9 ± 0.40 2.7 ± 0.42	 1.6 ± 0.33 2.2 ± 0.26	 1.9 ± 0.41 2.9 ± 0.39
[Liver Protein, pg/mL] *Leptin* *Insulin* *TNF-α* *IL-6* *Total Ghrelin*	 2505.0 ± 417.1 -- 4.7 ± 1.2 -- 1407.1 ± 51.9	 2451.6 ± 241.1 -- 7.7 ± 1.6 58.1 ± 29.6 1496.8 ± 65.0	 2473.6 ± 557.6 -- 5.3 ± 1.1 35.2 ± 19.1 1465.2 ± 10.2	 1959.4 ± 346.2 -- 4.3 ± 0.2 54.9 ± 15.5 1555.2 ± 37.2

[a] Significantly differs from –D/-N group
[b] Significantly differs from –D/+N group
[c] Significantly differs from +D/-N group
[d] Significantly differs from +D/+N group

Serum Ghrelin

Pre-surgical total serum ghrelin did not differ between diabetic and NAFLD groups. Additionally, total ghrelin did not differ by diabetic status or NAFLD status within presurgical measurements. Post-surgical total serum ghrelin did not differ between diabetic and NAFLD groups nor by diabetic or NAFLD status independently. Pre- to post-surgical ghrelin levels were compared for each subgroup. There was no significant increase from pre-post for each individual group, but with all groups pooled,

post-surgical levels were significantly higher than pre-surgical serum ghrelin levels [post-surgery, 2.7 ± 0.19 ng/mL vs. pre-surgery, 1.8 ± 0.19 ng/mL, p=0.003, *Figure 1*.]

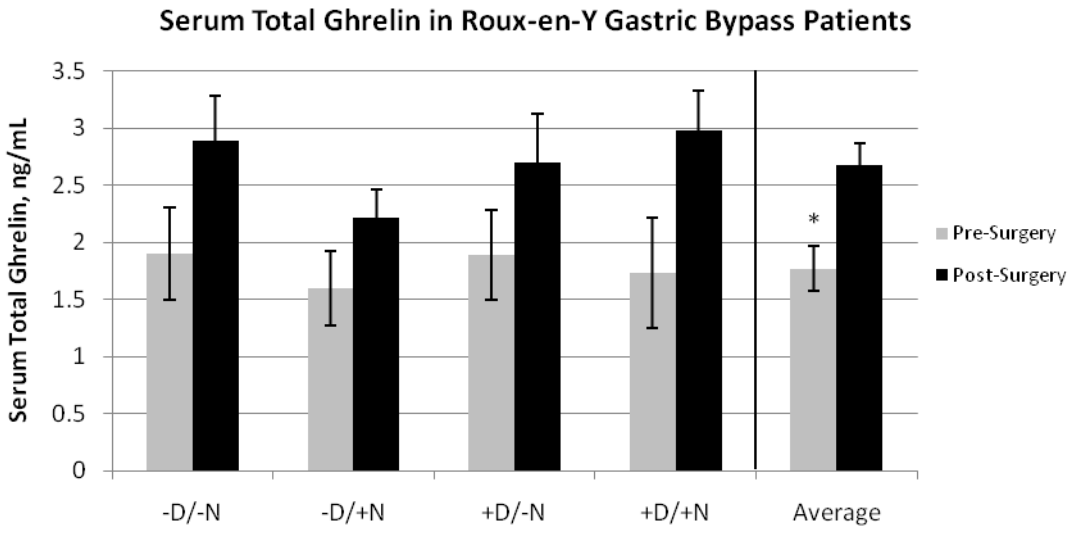

Figure 10-1. Serum total ghrelin levels pre- and post-surgery. There were no differences between groups within either pre- or post-surgical measurements. Post-surgical serum ghrelin levels were significantly higher than pre-surgical levels; n=4-8/group/timepoint; *p<0.05.

Liver Ghrelin

Total ghrelin from liver tissue homogenate was measured. Liver ghrelin levels did not significantly differ from pre-surgical serum ghrelin levels *(data not shown)*. Liver tissue ghrelin levels did not significantly differ by group, although there was a trend toward lower liver ghrelin levels in NAFLD patients (p=0.1, *Figure 2*).

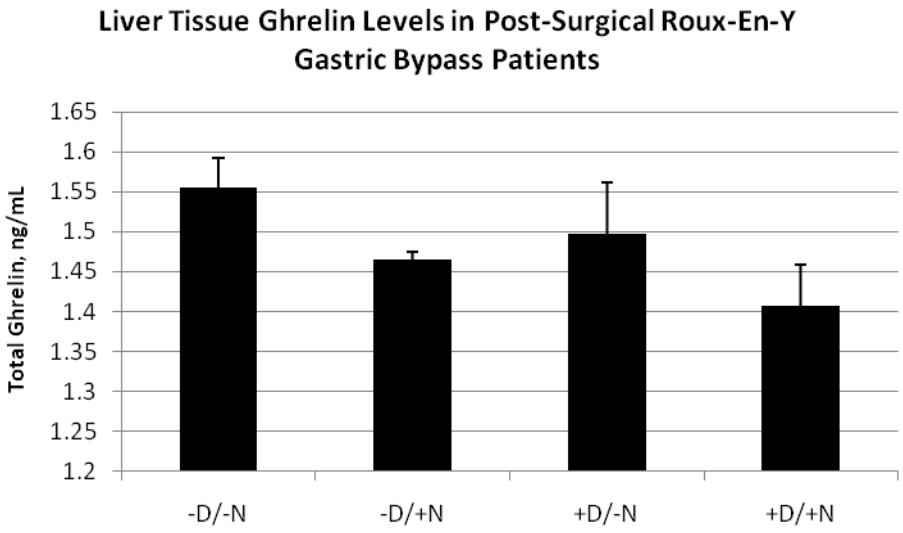

Figure 10-2. Liver tissue total ghrelin levels. Ghrelin levels did not differ based on diabetic status, NAFLD status, or diabetes*NAFLD interaction; n=5-8/group; *p<0.05.

Liver Leptin, TNF-α, and IL-6

Liver values for leptin were detected in all samples and did not differ by diabetes or NAFLD status or between diabetes*NAFLD groups. TNF-α levels were detected in 80% of samples (range, 2.74 -14.0 pg/mL) and did not differ by diabetic or NAFLD status or between diabetes*NALFD groups. IL-6 was detected in 87% of samples from the +D/-N, -D/+N, and -D/-N groups and groups did not differ. IL-6 was not detected in any +D/+N sample *(Figure 3).*

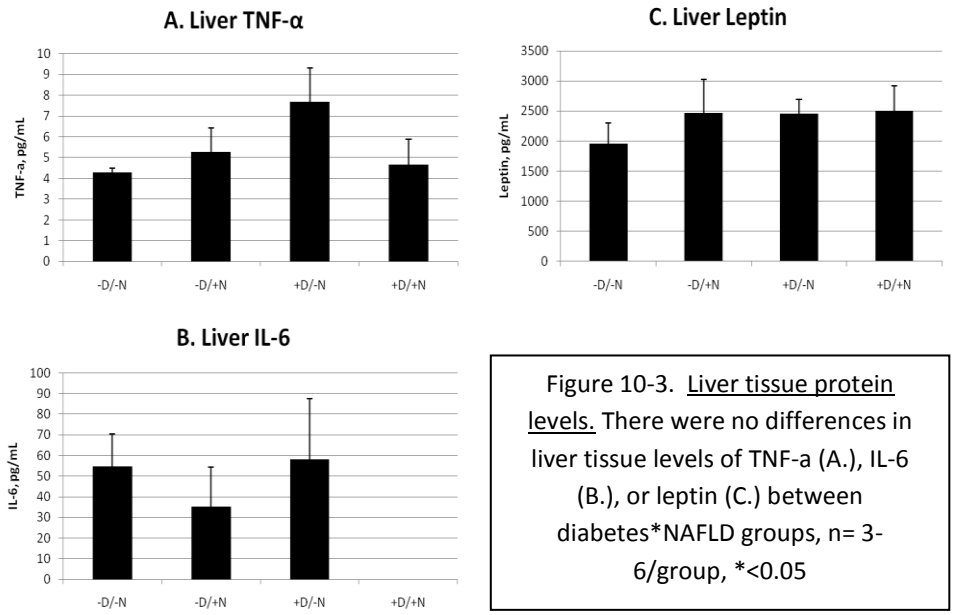

Figure 10-3. Liver tissue protein levels. There were no differences in liver tissue levels of TNF-a (A.), IL-6 (B.), or leptin (C.) between diabetes*NAFLD groups, n= 3-6/group, *<0.05

Genetic Analysis Group

Baseline Differences

Men were significantly older (48.0 ± 1.1 vs 45.5 ± 0.5 years, p=0.036), had a higher surgical BMI (50.1 ± 0.76 vs 48.2 ± 0.35kg/m^2, p=0.018), and had more diabetes (48% vs 31%, p=0.002) than women in patients genotyped for two ghrelin receptor promoter SNPs. BMI before surgery, presurgical HOMA and HbA1c did not differ *(Table 1).*

SNP Results

Allelic variant analysis of two GHSR promoter SNPs (rs490683 and rs9819506) was performed in 657 subjects. Genotype identification was possible in 94% of patients

(617 out of 657) for rs490683 and in 96% (633 of 657) of patients at location rs9818506. Frequency data are provided in Table 3.

Table 3. Frequency data of GHSR promoter SNPs rs490683 and rs9819506.

Allele	rs480683	rs9819506
Major/Major	G/G = .51 *n=335*	C/C = .47 *n=312*
Major/Minor	G/C = .36 *n=235*	C/T = .40 *n=266*
Minor/Minor	C/C = .07 *n=47*	T/T = .08 *n=55*
Undetermined	.06	.04

Non-Caucasians, undetermined genotypes, and patients without clinical values for the examined outcome were removed from analyses. There were no cases of Prader-Willi Syndrome diagnosed in this cohort. No significant associations were found with rs9819506 and BMI or EBWL at any time point, hyperinsulinemia, or diabetic status. For rs490683, individuals homozygous for the major allele G/G significantly differed from heterozygotes on BMI and EBWL at the 12 month post-surgical time point. The G/G genotype was significantly lighter (G/G BMI 32.2 ± 0.40 vs. G/C 33.6 ± 0.50, p=0.017) and had a greater percentage of excess body weight loss at 12 months post surgery (G/G 73.4 ± 1.5% versus G/C 65.5 ± 2.0% EBWL, p=0.010), than the G/C genotype group *(Figures 4 and 5)*. No other significant differences emerged between genotype groups for rs490683.

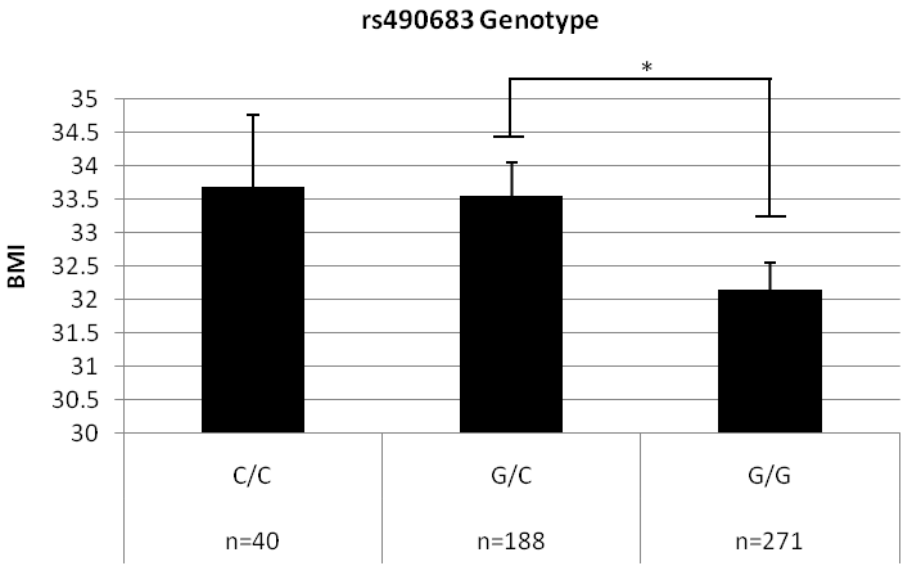

Figure 10-4. BMI by rs490683 genotype. BMI differed at 12 months post-surgery between G/C and G/G genotypes, $p<0.05$ via MannWhitney U test.

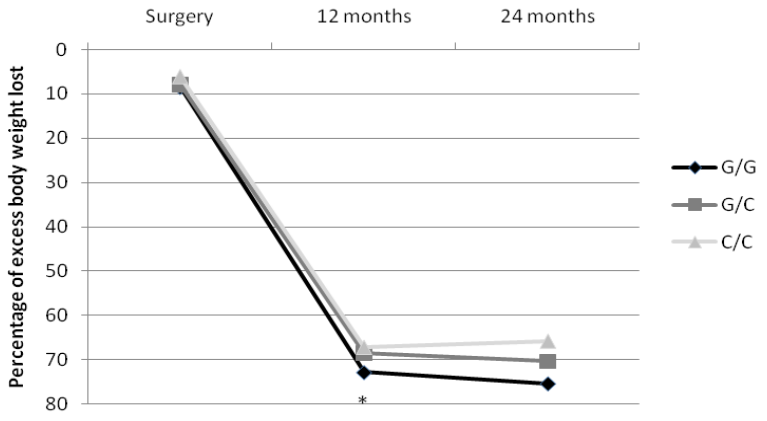

Figure 10-5. Percent excess body weight loss by genotype. Individuals homozygotic for the major allele G/G at rs490683 lost a significantly greater percentage of excess body weight at 12 months post-surgery than heterozygotes.

For the six GHSR194*GHSR288 allelic combinations, only one significant difference emerged. Individuals heterozygous for GHSR194 (C/G) and homozygous for the major allele at GHSR288 (C/C) had a significantly higher BMI and less EBWL at 12 months post surgery than the GHSR194 G/G and GHSR288 C/T haplotype. BMI at 12 months post-surgery for the C/G:C/C group was 34.5 ± 0.71 versus 31.9 ± 0.58 for the G/G:C/T haplotype, p=0.036. EBWL at 12 months for the C/G:C/C group was 65.6 ± 2.2% versus 74.1 ± 2.2% for the G/G:C/T combination, p=0.015 *(data not shown).* No other haploytypes demonstrated statistical differences in BMI or EBWL at 12 months.

The difference in BMI between males and females existing prior to surgery was maintained at 12 months post surgery. Males had a significantly higher average BMI at 12 months [males 34.0 ± 0.63 vs females 32.7 ± 0.34, p=0.032]. Males additionally had a reduced percentage of excess weight loss at 12 months versus females [males 64.9 ± 2.2% vs females 73.1 ± 3.1%, p=0.006] although absolute body weight loss was greater in males than females (107.1 ± 4.3 vs. 92.5 ± 1.7 lbs, p=0.001, *data not shown).*

Discussion:

Changes in ghrelin levels have been previously linked to greater weight loss after bariatric surgery and other studies describe ghrelin's effects on IR and NASH progression. The above results suggest variation in the ghrelin receptor gene and a post-operative change in serum ghrelin may impact weight loss success in the first year after surgery. However, there is no evidence for a relationship between serum ghrelin levels and NAFLD and/or diabetes in this patient population. Moreover, in the present work,

liver ghrelin, leptin, and the proinflammatory cytokines IL-6 and TNF-a did not correlate to diabetes and NALFD disease status.

Blood ghrelin levels are low in obesity, markedly lower in BMI-matched diabetics or individuals with NASH, and increase with weight loss (Cummings, Weigle et al. 2002; Marchesini, Pagotto et al. 2003; Yalniz 2006). In our population, serum ghrelin levels increased significantly after Roux-en-Y gastric bypass surgery and were comparable to literature values for similar populations (Vendrell, Broch et al. 2004). Initial work by Cummings and colleagues reported a decrease in plasma ghrelin levels six months after Roux-en-Y surgery (Cummings, Weigle et al. 2002). However, subsequent studies reviewed by Pournaras and le Roux presented mixed results (Pournaras and le Roux 2010). Some studies have shown decreases in fasting ghrelin levels post surgery (Leonetti, Silecchia et al. 2003; Fruehbeck, Rotellar et al. 2004; Lin, Gletsu et al. 2004; Morinigo, Casamitjana et al. 2004), others no change (Faraj, Havel et al. 2003; Sotoeckli, Clianda R et al. 2004; Korner, Bessler et al. 2005; Le Roux, Aylwin et al. 2006; Le Roux, Welbourn et al. 2007; Korner, Inabnet et al. 2009), and yet others significant increases in post-surgical ghrelin levels (Holdstock, Engstrom et al. 2003; Vendrell, Broch et al. 2004; Sundbom, Holdstock et al. 2007; Ybarra, Bobbioni-Harsch et al. 2009). Differences in response may be related to conformation and size of the remaining pouch, variation in ghrelin measurement, changes in plasma insulin and its regulation of ghrelin, and intactness/function of the vagal nerve peri-surgically (Pournaras and le Roux 2010). The appetite-stimulating actions of ghrelin and its increase during fasting require signaling through vagal afferents and efferents, respectively (Williams, Grill et al. 2003). One study demonstrated ghrelin levels decreased immediately after surgery, returned to

pre-operative levels by one month post-surgery and were further elevated after 12 months (Sundbom, Holdstock et al. 2007). Disruptions in vagus nerve signaling during surgery may have been responsible for the post-surgical ghrelin changes described above and/or discrepancies in ghrelin's response between studies.

Ghrelin levels were measured directly in liver tissue via ELISA. Although liver has not been identified as a major location of ghrelin action, both receptor subtypes 1a and 1b are expressed in liver tissue and ghrelin has direct actions on the liver-acting proteins insulin, TNF-α and IL-6 (Gnanapavan, Kola et al. 2002; Reimer, Pacini et al. 2003; Dixit, Shaffer et al. 2004; Dezaki, Kakei et al. 2007; Kiewiet, van Aken et al. 2009). We report total ghrelin levels in liver did not significantly differ from pre-operative serum levels. Whether ghrelin is active at the level of the liver or liver ghrelin levels merely reflect hepatic blood ghrelin levels, is unknown. Identifying disparate actions of liver versus serum ghrelin following gastric bypass surgery may clarify ghrelin's role in the NASH, IR, and gastric bypass process. Ghrelin may contribute to the NASH progression feed-forward cycle by increasing triglyceride content in the liver via upregulation of fat deposition promoting acetyl coA carboxylase (ACC) and fatty acid synthase (FAS) and reducing the actions of carnitine palmiyoyl transferase I (CPTI), a mitochondrial transporter leading to degradation of fatty acids (Barazzoni, Bosutti et al. 2005). The etiology of NASH is considered a two-hit process and ghrelin actions have implications for both (Day and James 1998). Initially, excess blood lipids combined with a local physiology promoting lipid deposition (insulin resistance, increased TNF-α) favors fat deposition in liver tissue (Ahima 2007; Carter-Kent, Zein et al. 2008). The addition of a proinflammatory state progresses NAFLD to NASH and resulting oxidative

damage to liver tissue can lead to cirrhosis or hepatocarcinoma (Day and James 1998; Mendez-Sanchez, Arrese et al. 2007). Increased ghrelin levels after weight loss may instigate both increased fat deposition via ghrelin's inhibition of lipid metabolism (contributing to rebound fatty liver) and a decrease in proinflammatory cytokines, preventing the advancement of benign steatosis to steatohepatitis (Dixit and Taub 2005; Theander-Carillo, Wiedmer et al. 2006). Blood and hepatic ghrelin levels relate to the degree of IR and NASH, respectively, and hence were investigated here as a central factor in the local transformation of healthy liver to NASH liver (Marchesini, Pagotto et al. 2003; Mendez-Sanchez, Chavez-Tapia et al. 2006).

NASH has been described as the hepatic manifestation of insulin resistance and IR occurs in 98% of NASH patients (Chitturi, Abeygunasekera et al. 2002). With the exception of biliopancreatic diversion/duodenal switch, Roux-en-Y gastric bypass surgery has the greatest impact on restoring insulin sensitivity versus other surgical procedures (gastric banding, gastroplasty, etc) and the improvement in IR and T2D is correlated with the amount of weight lost (Buchwald 2009). For all surgical types, the post-surgical improvement in diabetes has been estimated at 78% for full resolution and 87% for marked improvement and full resolution, results maintained for two or more years. Our findings demonstrating that serum ghrelin levels are elevated after surgery may be a significant factor for the resolution of diabetes both directly via its actions on insulin as well as indirectly via suppression of proinflammatory cytokines. The latter may be more important as proinflammatory cytokines have been shown to exacerbate impaired glucose tolerance through several (autocrine, paracrine, endocrine) pathways (Cummings, Overduin et al. 2004; Pittas, Joseph et al. 2004).

The adipocytokines leptin, TNF-α and IL-6 have been suggested to encourage the development of IR and NASH (Pittas, Joseph et al. 2004). Levels of TNF-α in adipose tissue and IL-6 in plasma increase with increased severity of IR/T2D (Streetz, Luedde et al. 2000; Leclercq, Da Silva et al. 2007). Both IL-6 and TNF-α may impair insulin sensitivity by releasing excess free fatty acids (FFAs), inhibiting the insulin sensitizing hormone adiponectin, and interfering with insulin signaling (Greenberg and McDaniel 2002; Bruun, Lihn et al. 2003). Administration of IL-6 has been shown to reverse fat deposition in rats, but mobilize FFAs and induce peripheral IR in humans (Tsigos, Papanicolaou et al. 1997; Boden and Shulman 2002; Hong, Radaeva et al. 2004). Hepatic IL-6 expression correlates with blood IL-6 levels and increasing stages of inflammation and fibrosis in NASH (Wieckowska, Papouchado et al. 2008). TNF-α has also been demonstrated to interfere with insulin signaling, thereby promoting steatosis, increasing the proinflammatory response, and advancing NAFLD (Crespo, Cayon et al. 2001). Additionally, leptin likely contributes to peripheral insulin resistance. Leptin, like TNF-α and IL-6, is secreted by adipocytes and its secretion is affected by weight status (Considine, Sinha et al. 1996). Leptin levels are increased in NASH and increased levels impair lipid turnover, leading to hepatic leptin resistance (Chitturi, Abeygunasekera et al. 2002). Following gastric bypass surgery, blood levels of leptin ,TNF-α, and IL-6 decrease with weight loss, likely contributing to post-surgical improvements in insulin resistance (Cottam, Mattar et al. 2004). Our study detected no significant relationship between liver tissue levels of leptin, TNF-α, and IL-6 and NAFLD and diabetes disease status. Prior studies have demonstrated no changes with leptin levels in NAFLD, but increased leptin in NASH (Chitturi, Farrell et al. 2002; Angulo, Alba et al. 2004). One

explanation of this difference may be that our liberal definition of NAFLD activity did not achieve the severity of disease activity required to instigate the metabolic changes characteristic of advanced NASH.

The genotypic results reported here indicate significant SNP associations between rs490683 and BMI and EBWL at 12-months post-surgery. From prior observation, patients at our center reach their weight nadir one year after surgery and by two years have experienced little, moderate, or significant weight regain. The association between variants of the rs490683 SNP and (1) BMI and (2) amount of excess body weight loss at 12 months after surgery are in agreement with results in the existing literature. Mager et al. (Mager, Degenhardt et al. 2008) found the C/C genotype of rs490683 was significantly associated with amount of weight lost during a three-year dietary intervention in both the diet and control groups although the experimental group lost significantly more. Our data suggest the G/G genotype is the protective form. Although the direction of response is inconsistent for this SNP, these results indicate the ghrelin receptor promoter gene SNP rs490683 may be involved with regulation of weight. Functional studies to determine the physiological consequences of variance in the rs490683 SNP may help indentify physiological mechanisms associated with favorable weight status. No significant associations were found with rs9819506. Prior reports indicate individuals homozygous for the minor allele of 9819506 have lower BMIs and may have a more favorable response to weight loss than other genotypes (Mager, Degenhardt et al. 2008).

Other groups have found significant associations between ghrelin and ghrelin receptor SNP haplotypes and diabetes and risk for myocardial infarction and/or coronary

artery disease, respectively (Baessler, Hasinoff et al. 2005; Garcia, King et al. 2009). The number or functions of ghrelin-related SNPs with the greatest impact on BMI, weight loss success, or insulin resistance measures is yet unclear. One study demonstrated GHSR promoter SNP genotypes may alter expression of the GHSR gene. GHSR expression in rat hypothalami was 745% lower in animals with the C allele, attributed to a disruption in the TF nuclear factor-1 binding site (Mager, Degenhardt et al. 2008). The physiological outcomes of less GHSR expression is unclear, but less ghrelin signaling may have favorable effects on body weight regulation but negative consequences for immune regulation.

Conclusion:

Our results from serum and liver measurement indicate that ghrelin action is not a major component of the post-operative improvements in weight and metabolic disease following Roux-en-Y gastric bypass surgery. In this population, we report an increase in post-operative serum ghrelin levels, similar to increases observed during dietary weight loss and no differences in liver proteins related to NASH: leptin, ghrelin, IL-6, and TNF-α between diabetes*NAFLD groups. A major finding was that the genetic contribution of the rs490683 SNP in the promoter region of the ghrelin receptor gene may play a role in weight and weight loss in men and women who are obese. Future functional studies should be directed at identifying mechanisms by which GHSR SNPs affect changes in complex metabolic phenotypes.

CHAPTER XI. DISCUSSION & CONCLUSION

This body of work characterizes ghrelin's involvement in several models of CR. Data from the rodent studies provides information regarding plasma changes and stomach mRNA expression of ghrelin in three different models of CR. Data from humans undergoing gastric bypass surgery examines serum and liver ghrelin changes peri-surgically and evaluates the contribution of two ghrelin receptor promoter SNPs to obesity and metabolic disease. Together the studies allow for the comparison of different ghrelin actions in a broad spectrum of conditions: different human and mouse genotypes, different measurement techniques, and different models of CR.

Ghrelin has well-established roles in appetite stimulation, growth hormone release, and to some extent inhibition of immune function. Additional roles continue to be revealed, e.g. anxiolytic properties, pancreatic regulation of insulin, cardiovascular effects, fetal growth characteristics. Changes plasma ghrelin levels in response to hunger and fed states are also well-characterized. The diverse and disparate actions of ghrelin make it unique among metabolic hormones and open the possibility that ghrelin could coordinate changes in multiple physiological systems. The physiological changes accompanying caloric restriction precede ghrelin's discovery and thus far, the postulation that ghrelin may be a coordinating signal of CR's major physiological effects has not been evaluated.

Long-term CR induces several physical and metabolic changes that lead to an altered physiological state. Data across animal models suggest that, to some degree, this altered state is similar between species. Extension of lifespan, low GH/IGF-1 axis

activity, increased hunger/appetite, increased physical activity, short-term decreases in metabolic rate, increased tolerance to stress, improved adaptive immunity and impaired innate immunity have all been established in multiple species (Weindruch and Walford 1988; Masoro 2005). However, metabolic effects of CR are not consistently found and the principle action of CR: extension of lifespan, is not necessarily the outcome for all animals on moderate, long-term CR. Animal strains within the same species reflect high heterogeneity in response to CR and a recent report demonstrated mouse strains had a variety of lifespan responses to CR. Some strains exhibited moderately shortened lifespan while others significantly extended (Liao, Rikke et al. 2010). The interaction of genes and environment for individual animals may contribute to very unique outcomes following treatment and is one consideration for unexpected results in these studies. Below several outcomes of CR treatment are reviewed together with their possible relationship to ghrelin action as identified in our multiple CR studies.

Evaluation of CR Phenotypes & Relationship to Ghrelin

Metabolic Rate

We first identified ghrelin as a possible regulatory factor for hunger during CR based on changes in metabolic rate that accompany CR. Correlational data in underweight athletes suggests resting metabolic rate is significantly lower, and ghrelin levels higher, compared with healthy controls (Lebenstedt, Platte et al. 1999; De Souza, Leidy et al. 2004). However, ghrelin has been shown to directly affect MR. Administration of a ghrelin antibody which degrades the acyl form to des-acyl ghrelin

induces an increase in metabolic rate in fasting mice (Mayorov, Amara et al. 2008). Data from the short-term, acute CR study showed absolute body weight-adjusted MR significantly increased in both the 40% and 60% CR groups after 10 days. This is a unique result in demonstrating MR is not decreased in the short-term by moderate CR. Lack of increase in plasma ghrelin levels may have affected the lack of decrease in MR, if such a direct relationship between ghrelin and MR exists. The mechanism by which MR changes in response to CR is unknown, and ghrelin may be an important factor in this response.

Respiratory Quotient

Respiratory quotient is a measure of the type of fuel utilized at a given time. Differences in RQ between individuals have been noted based on body composition, macronutrient distribution of the diet, and endocrine (insulin) functioning, but no overall sex difference is reported in the literature (Flatt 1995). Assuming a constant rate of protein turnover, high RQ values represent carbohydrate, and low numbers lipids, as the primary source of fuel burned and harnessed for cellular processes. RQ has a unique diurnal pattern in mice undergoing CR. In AL-fed animals, RQ remains steady at a middle value indicating a constant mix of both carbohydrate and fat utilization (Duffy, Feuers et al. 1990; Masoro, McCarter et al. 1992; McCarter, Mejia et al. 2007). CR animals demonstrate increased RQ during feeding, suggesting ingested carbohydrates are being immediately metabolized. In the absence of food, CR mice demonstrate a marked decline in RQ, indicating fuel partitioning has shifted toward fat as the primary substrate.

Exogenous administration of ghrelin markedly increases RQ in mice and rats but does not affect RQ in humans (Tschoep, Smiley et al. 2000; Wren, Seal et al. 2001a). RQ was measured pre- and post-ten day diet in AL, 40% CR and 60% CR HS mice. There were no differences between sex-diet groups prior to diet onset nor changes within a group from pre-diet to post-diet measurement. RQ was significantly lower in the 60% CR group than AL-fed mice during nighttime feeding hours. Low RQ during feeding hours in restricted mice is an unexpected result. Prior literature establishes carbohydrates as the primary fuel source during feeding hours in CR animals (Masoro, McCarter et al. 1992). However, because the 60% restriction was unusually severe and ghrelin levels did not increase, typical metabolic shifts, including corresponding increases in RQ, may not be characteristic of this dietary scheme. It is additionally possible that animals on a 60% CR diet for a duration of 10 days will maintain fat oxidation as their primary method of fuel use.

Physical Activity

Physical activity has been demonstrated to increase in rodents undergoing CR (McCarter, Shimokawa et al. 1997). The mechanisms inducing this response are currently unknown, but engagement of an evolutionary foraging effect has been proposed. Physical activity was measured in the young adult HS mice undergoing 40% and 60% CR for ten days. No change in activity was observed compared to AL-fed mice. The duration of restriction required to detect significant changes in activity is not clear. Rodent studies which report increases in activity do so after several months of restriction.

Data from human studies suggest an opposite response. Obese individuals undergoing severe restriction demonstrated decreased activity levels (Ravussin, Burnand et al. 1985). Similarly, exogenous ghrelin administration in rats decreases spontaneous locomotor activity, suggesting an energy conservation status may be engaged during fasting. The difference between human and rodent behavior regarding activity levels during CR may be explained by psychological factors, energy levels, duration and severity of restriction, etc, but the mechanisms behind these differences are not clear.

Feeding Behavior

Ghrelin and CR both increase appetite. Ghrelin's actions through vagal afferents and hypothalamic signaling have been suggested to produce increased appetite and feeding behavior activities in both rodents and humans (Tschoep, Smiley et al. 2000; Wren, Seal et al. 2001a; Cummings, Frayo et al. 2004). CR also manifests hunger: in rodents via excess food intake upon refeeding and in humans via self-reporting of hunger and increased appetite (Weindruch and Walford 1988; Pasman, Saris et al. 1999). In this study, food consumption was measured in low IGF-1 animals and their wildtype siblings and in AL-fed HS mice and their long-term LM-fed littermates. No differences in food consumption were detected for low IGF-1 animals and controls. Food consumption adjusted for body weight was slightly, but non-significantly higher for midi mice compared to wildtypes, which negated the explanation of a possible CR effect for their reduced body size. Not unexpectedly, plasma unacylated ghrelin was not different between groups. Low methionine-fed mice had significantly increased food consumption

at ~six months after diet initiation compared to controls, but accurate measurement was confounded by a unique feeding behavior exhibited by LM animals. LM animals moistened their food pellets with water, created a food-water paste mixture, and smeared the paste over the cage walls. Pellet and paste were recovered, dried, and measured, but accuracy of measurement after this unusual behavior may have been compromised. Regardless, accurate measurements were made where possible and revealed a step-wise increase in food consumption per body weight in the AL, 40% CR and LM groups, respectively. Plasma unacylated ghrelin pooled over two timepoints (mouse ages) revealed an identical stepwise increase in these groups.

Food consumption was not directly measured in men and women undergoing gastric bypass surgery, but dietary restriction is a component of two phases of the complete surgical process. Surgical patients are required to lose 10% of their excess body weight prior to surgery to (1) confirm compliance with prescribed nutritional recommendations, vital to the post-surgical period, and (2) reduce risk of adverse events during surgery. The second phase of CR is the mechanical restriction imposed by decreased stomach size after surgery. Patients adhere to a strict post-surgical diet which is restricted in both volume and calories but contains adequate amounts of essential nutrients. Weight loss in both CR periods contributes to the sum of excess body weight lost to reach ideal body weight. Compliance with guidelines for reduced consumption is critical for successful loss of excess body weight and maintenance of the new weight after surgery. Reduced post-surgical ghrelin levels demonstrated in some studies may suppress appetite, aiding adherence to the low calorie and low volume diet post-surgery

(Cummings, Weigle et al. 2002), although post-surgical actions of ghrelin are not consistent between studies, including the increased ghrelin levels we report here.

Body Weight

Weight loss during CR was one of the initial changes identified as a potential mechanism responsible for the physiological benefits of CR, as overweight conditions are linked to many metabolic conditions that improve with CR. However, several lines of evidence emerged that separate weight or BMI from the improved physiological status attributed to CR. First, diabetes-prone, obese rodents undergoing CR have dramatically decreased disease incidence than their AL counterparts (Okauchi, Mizuno et al. 1995). Additionally, obese ob/ob mice on CR live as long as lean CR mice, but have significantly more body adiposity. AL-fed ob/ob mice live significantly shorter than AL-fed lean mice, suggesting CR, but not reduced fat mass, is responsible for overcoming the ob/ob genotype's life-shortening effect (Harrison, Archer et al. 1984). In humans, obese individuals with diabetes have marked improvement in insulin sensitivity within days following gastric bypass surgery, preceding any substantial weight loss (Schauer, Burgeura et al. 2003). While weight remains a significant predictor of development of several life-shortening conditions, as well as overall morbidity and mortality, consequences of weight loss itself have been uncoupled from those specifically of CR. Our data demonstrate low IGF-1 animals weigh significantly less than their IGF-1 normal siblings. GH and IGF-1 are the primary signals responsible for growth during development. Animals partially deficient in IGF-1 from birth exhibit a dwarf phenotype,

similar to the size of animals beginning CR at an early age (Brown-Borg, Borg et al. 1996; Coschigano, Clemmons et al. 2000; Flurkey, Papaconstantinou et al. 2001; Holzenberger, Dupont et al. 2003). A CR effect does not explain the smaller stature in organisms with impaired IGF-1 signaling; animals genetically deficient for GH, IGF-1 or their receptors exhibit normal feeding compared to genetically-intact counterparts (Brown-Borg, Borg et al. 1996). Additionally, LM-fed and 40% CR animals in our study had significantly lower body weight than AL-fed control animals, with 40% CR animals being the lightest. Although CR initiated in middle age (as was applied here) alters only body weight and not growth, significant reductions in body size due to fat and lean mass loss can still be observed. Restriction of methionine does not produce the dramatic decrease in body weight that CR does and any reduction in body weight is not due to a CR effect. LM-fed animals eat significantly more food than AL controls, both reported here and previously, likely due to feeling unsatisfied with the incomplete amino acids provided by the LM diet (Orentreich, Matias et al. 1993). Body weight significantly decreased in both severely restricted (40% and 60% CR) groups above and beyond a slight but significant decrease in the control group over the 10-day diet. Weight loss in the control group is likely attributed to stress of measurement, as all animals spend two 24-hour periods (at the start of the study and in the final day before sacrifice) in a metabolic chamber separate from their home cage. The two CR groups lost significantly more weight than the control group, but did not differ from each other, suggesting this ten-day diet protocol is of a long enough duration to alter body weight, but not long enough to expose differences between the 40% and 60% restriction levels.

Body weight in the gastric bypass population was evaluated based on sex, incidence of disease (T2D and NASH) prior to surgery, GHSR SNP genotype groups before surgery, and weight trajectories based both on disease and genotype classifications following surgery. Body weight significantly differed by sex: men had higher BMIs than women prior to surgery in patients in the genotyping study. No BMI difference could be determined with the small sample size of men (n=3) and overall (n=37) in the hormonal analysis study. Although normal-weight men on average weigh more than normal-weight women, in this extreme population (~top 10% heaviest individuals), the weight difference between sexes is less pronounced. Additionally, men comprise only 20% of the patients who undergo gastric bypass surgery, a number that reflects the national average, although this percentage continues to rise (Tiwari, Goede et al. 2010). Because the national rates for obesity between males and females do not differ, men undergoing surgery may have significantly more health problems (including higher weight) than the average female gastric bypass surgical patient. The difference in BMI was maintained at 12 months post surgery. Percentage weight loss was higher in females but actual weight loss was higher in males, reflecting men having a greater amount of excess body weight to lose. Because of these differences, the BMI disparity between men and women remained. Ghrelin levels are inversely associated with body weight, except in Prader-Willi syndrome, anorexia nervosa, and bulimia nervosa, where plasma ghrelin levels are higher than BMI-matched controls (Cummings, Clement et al. 2002; Tanaka, Naruo et al. 2002; Misra, Miller et al. 2005). Plasma ghrelin levels increase with weight loss in humans and decrease with body weight gain associated with recovery from anorexia nervosa or other underweight conditions (Hansen, Dall et al. 2002a; Hansen,

Dall et al. 2002b). Ghrelin has been shown to be a factor indirectly affecting body weight through stimulation of feeding behavior in both humans and rodents (Wren, Seal et al. 2001a; Wren, Small et al. 2001b). Our results do not uniformly confirm an increase in ghrelin levels with CR. The differences between the types of restrictions that were implemented and the duration and severity of the diets may explain the disparity.

Body Composition

Body fat and lean mass both decrease with CR, although most total body weight loss is attributed to loss of fat mass (Redman, Heilbronn et al. 2007). Bone mineral density also decreases with long-term CR, a result both demonstrated in humans and rodents (Jensen, Quaade et al. 1994; Ferguson, Greenberg et al. 1999; Villareal, Fontana et al. 2006). Total fat and lean mass and bone mineral density were measured in HS mice before and after undergoing 10 days of 40% CR, 60% CR or AL feeding. Animals in both the 40% CR and 60% CR groups demonstrated significant loss of total fat and lean mass, a result attributed to the severity of the restriction and not changes in physical activity. Bone mineral density was unchanged. The duration required for significant changes in bone mineral density is unknown, but it is considered a negative attribute of long-term, but not short-term, CR (Lane, Black et al. 2001; Mattison, Lane et al. 2003). In humans, BMI was used to capture weight as a function of height, but this metric does not reflect body composition: e.g. fat and lean mass, bone mineral density, etc, and these parameters were not directly measured. Ghrelin has been shown to induce adiposity in rodents via increased food consumption and weight gain (Tschoep, Smiley et al. 2000).

In vitro studies provide evidence that ghrelin additionally acts at the cellular level to increase fatty deposition in lean tissue (liver and skeletal muscle) (Barazzoni, Bosutti et al. 2005). Our rodent studies indicate CR induced loss of lean and fat mass and the lack of increase in ghrelin may have protected lean tissue from fatty infiltration.

Genotypes

Two mouse strains have been evaluated here. Inbred I29/C57/CD-1 mice and their IGF-1 hypomorphic littermates were used to examine lifelong physiological changes associated with reduced IGF-1 signaling. Heterogeneous stock mice were compared on several diets: long-term low methionine and 40% CR diets initiated in middle age as well as 40% CR and 60% CR 10-day diets begun in young adulthood. The HS mice are derived from the 78th generation of an eight-strain cross and the genetic diversity is used to reflect the diversity of the human population (McClearn, Wilson et al. 1970). The use of HS mice from the same colony in two studies allowed for the comparison of measures between them. For example, plasma unacylated ghrelin was measured in *ad libitum* fed animals in both the short-term, severe CR study and the low methionine study. Plasma levels were similar for males in the acute CR study and at both 17 and 21 months in the low methionine study (~500pg/mL). However, the sexual dimorphism that was evident in the acute CR study was not apparent in the low methionine study (for discussion, see 'Sexes'). The use of identical strains allows the exclusion of genotype as a possible explanation for the disparity and permits focus on a potentially true source of the variability: here, possibly age. However, because these are animals of mixed genetic

stock and because of well-known strain differences in most physiological outcomes, there still may be significant variation in outcomes measured in HS mice (Liao, Rikke et al. 2010).

The results from the genotyping portion of the gastric bypass surgery study suggest SNP genotypes play an important role in the outcome of complex phenotypes. In recent years, multiple studies have provided evidence that individual SNPs and SNP haplotypes can account for a significant proportion of variance in complex conditions like obesity and diabetes (Chu 2008; Franks, Jablonski et al. 2008; Freathy, Timpson et al. 2008). Variation in one SNP located in the promoter region of the GHSR gene accounted for significant differences in BMI at 12 months post surgery and the amount of excess body weight lost from the initial clinic visit to the 12-month post-operative visit. The second GHSR promoter SNP evaluated was not significantly linked to any outcome measured in our study. In general the ghrelin and GHSR SNP literature is sparse, but both of these specific locations had reported strong correlations to metabolic outcomes. The identification of additional relevant SNPs in the ghrelin or ghrelin receptor genes could provide the basis for haplotype analysis, which is likely to reveal more significant associations with complex traits than single SNPs alone.

Sexes

Historically males were used in both rodent and human physiological studies to eliminate any contribution of cycling hormones to a phenotype. The trend in recent decades has been to evaluate females separately, as males and females have very different

physiologies relating to health, disease, lifespan, etc. Our rodent studies were designed to include both males and females with the ability to detect potential sex effects in various CR-related outcomes. There is some evidence for sex differences in outcomes that were evaluated in these studies: metabolic rate, respiratory quotient, body weight, plasma ghrelin levels, and plasma glucose levels all demonstrated a significant sexual dimorphism. Metabolic rate measurements indicated female AL mice had a significantly increased MR compared to male AL-fed HS mice. However, in the 40% and 60% CR groups, no such difference existed. There is evidence in the literature for a slight sexual dimorphism with regard to MR (Cunningham 1980), but evidence of that effect in our study was abolished with the alteration in metabolism induced by CR. A sex difference in metabolic rate has been linked to differences in lean mass, and not sex, per se, so it is possible the CR manipulation led to similar outcomes in lean body mass post-restriction (Das, Roberts et al. 2003). Likewise, RQ differed by sex in the AL-fed mice of the acute CR study. Female AL mice had significantly increased RQ during early morning hours versus male AL mice. There has been no prior evidence for a sexual dimorphism in RQ in CR animals. Our results may be influenced by sex disparities in ghrelin, as females demonstrated increased ghrelin levels and ghrelin has been shown to increase RQ (Tschoep, Smiley et al. 2000). Data from all studies revealed body weight differences in male and female mice. Baseline measurements prior to initiation of the short-term severe CR diets demonstrated an approximate five gram difference between male and female body weights, which was maintained after the 10-day diet period. This baseline difference was similar to the body weight difference exhibited by male and female mice

prior to the LM and 40% diets. Body weight in LM and 40% CR female mice remained lower than male LM and 40% CR mice after several months of the diet.

Additionally, both men and women were included in the hormonal and genetic analysis groups of the gastric bypass surgery study. Because women make up ~80% of gastric bypass surgical patients at our site, the number of men in the hormonal analysis group did not allow for adequate comparison between sexes on parameters measured in that study. However, BMI at the time of surgery, percent of excess body weight loss by 12 months post-surgery, and absolute amount of body weight loss significantly differed between men and women analyzed by genotypic classification. Men were heavier at the time of surgery, lost significantly more weight in pounds, but less in percentage excess body weight. This indicates men may respond better to the surgery than women, but the effect may be attributed to heavier weight and not specifically sex. Matching sexes by BMI will identify if men have more success in weight loss in the first year after surgery or if the effect is attributed to higher starting weight. A sex difference in success during dietary weight loss favoring men has been established in the literature and was seen in our study. Differences may be partially explained by starting BMI, metabolic rate, or lean body mass (Serdula, Mokdad et al. 1999). Metabolic rate has been demonstrated to be reduced in men and women post-surgically, due to changes in fat and fat-free mass and not baseline rates (Das, Roberts et al. 2003). Therefore, sex differences in, and restoration of, metabolic rate post-surgery can be overcome by maintenance of lean body mass.

Plasma glucose decreased significantly in female mice undergoing severe 60% CR in comparison with controls, but the same effect was not observed for male 60% CR

mice in the same 10-day period. 40% CR mice had a modest but non-significant decrease from controls. There have been no differences in plasma glucose levels by sex reported in the CR literature, and it has also been established that low plasma glucose is not the primary mechanism by which CR confers physiological benefits (McCarter, Mejia et al. 2007). Exogenous ghrelin administration has been demonstrated to increase plasma glucose levels, possibly mediated by decreased circulating insulin levels, but the relationship between these circulating metabolic hormones is not yet fully understood (Broglio, Arvat et al. 2001; Kiewiet, van Aken et al. 2009).

Duration of Diet

Current expected lifespan in humans in the US is approximately 78 years, with women living slightly longer than men (Xu, Kochanek et al. 2010). The lifespan of an average mouse is 2-3 years, with no apparent sex difference under laboratory conditions. Therefore, there is an approximate 30-fold difference in time as a percent of lifespan between humans and mice. The 10-day diet duration in mice is considered a short-term intervention and is comparable to dietary interventions of less than a year in humans. Restrictions of short duration identify changes that rapidly occur with CR and others which require a longer exposure to the restriction. To that end, several outcomes typically associated with CR, e.g. increased plasma ghrelin levels, were unable to be detected after just 10 days. Hence, translation of the rodent findings may require an experimental period of more than one year to elicit change in humans, a duration which far exceeds the length of most published dietary studies.

Diets

A minimum of one group in each of the three rodent studies was provided a nutrient-complete chow *ad libitum.* Others were provided complete chow at the 40% and 60% CR levels and others LM-deficient chow *ad libitum*. Similarly, humans entering the gastric bypass study were eating *ad libitum* and were subjected to dietary CR prior to surgery and surgically-induced CR after the Roux-en-Y procedure. Despite both rodents and humans eating *ad libitum,* rodents in our AL groups in each of the three studies were not obese while all humans entering surgery were morbidly obese with obesity-related complications. Mechanisms of dysregulation leading to the severity and prevalence of obesity observed in men and women consuming a Western diet have been evaluated in humans, but only mice with genetic predispositions for obesity overconsume resulting in morbid obesity. Therefore, there are important differences between the body weights, diets, and food consumption of mice and humans across these CR studies. These differences lead to diversity in evaluation of ghrelin during CR models but also allow for many potential sources of variation in ghrelin across studies.

Stomach Size

CR has been shown to impact organ size. Most organs, excluding brain and heart, decrease in size proportional to the severity of CR imposed (ie. 30% CR, organs shrink ~30%) (Yu, Masoro et al. 1985). The brain is protected from reductions in size and hence is proportionately larger in CR animals than AL-fed animals. One report found the stomach is increased with long-term, moderate CR, and the increase is attributed to

hypertrophy of the fundus, the upper-stomach region where ghrelin-producing cells primarily reside (Yang, Youm et al. 2007). In that study, stomach ghrelin mRNA and plasma ghrelin levels were markedly increased. Data from our short-term 40% and 60% CR animals show significant enlargements in relative stomach size (per body weight) are seen in restricted groups after the 10-day restriction. One possible explanation for the increase in stomach size is the production of additional ghrelin to stimulate appetite. In the acute CR study, significant increases in ghrelin mRNA production in the bottom half of 60% CR versus AL-fed animals' stomachs were revealed, but no significant increases in the top halves for either diet group were demonstrated. This result may be attributed to baseline expression. It may be that the bottom half of the stomach is recruited for rapid and excess production of ghrelin protein during fasting or CR states, beyond the constitutively high production of the stomach fundi. However, increased plasma unacylated ghrelin in the 60% CR group that would substantiate this theory was not found.

Stomach size after Roux-en-Y bypass is significantly smaller, both in the effort to control volume of food consumed and delay gastric emptying and extend intestinal transit time (Suzuki, Ramos et al. 2005). This procedure, depending on the conformation of stomach pouch remaining, has been shown to excise significant portions of ghrelin-secreting cells (Korner, Inabnet et al. 2009). It is unclear if such alternate surgical techniques, and their subsequent effects on circulating ghrelin levels, result in striking differences in weight loss success in long-term post-surgical follow-up. How ghrelin levels are differentially affected between and within surgical procedures is also unclear but identifying differences may help describe variation in reported appetite post-surgery.

Obesity-Related Disease

Both serum and liver total ghrelin levels and GHSR SNPs were compared with incidence of disease peri-surgically. While there has been much evidence with regard to ghrelin's actions during obesity and metabolic disease (specifically, low circulating levels), the evidence in this study did not find a significant association between serum or liver tissue ghrelin levels and presence or absence of disease in the morbidly obese. Moreover, SNP allele variants that were associated with significant losses in body weight and BMI were not additionally associated with presence of hyperinsulinemia, T2D, or Prader-Willi syndrome, a monogenic cause of obesity characterized by very high ghrelin levels and appetite. While CR in rodents and gastric bypass surgery in humans have shown dramatic reductions in age- and weight-related co-morbid conditions, our data do not support the case that ghrelin is involved with these outcomes.

Other Related Hormones

Plasma glucose and plasma IGF-1 have both been linked to actions of CR. While both are low in CR animals, there is some evidence that low IGF-1 signaling may be a part of the mechanism of action of CR. Animals with genetic alterations for low plasma GH or IGF-1 or dysfunctional IGF-1 receptors exhibit extended lifespan, manifest in a dwarf phenotype, and have plasma levels of IGF-1 similar to genetically-intact CR animals (Brown-Borg, Borg et al. 1996; Flurkey, Papaconstantinou et al. 2001; Holzenberger, Dupont et al. 2003). However, animals genetically altered for low plasma

glucose do not closely resemble CR animals, such that low plasma glucose has been negated as a possible mechanism of CR (McCarter, Mejia et al. 2007). We report both plasma glucose and plasma IGF-1 levels were lower in 60% CR animals versus controls after 10 days, with a significant stepwise trend for 40% CR animals with IGF-1 and a non-significant stepwise trend in plasma glucose. These decreases are akin to expected values for animals undergoing CR, and are not unexpectedly decreased in a 10-day CR, as both are relatively responsive to short-term changes.

Liver values of leptin, IL-6 and TNF-α were compared between diabetes*NAFLD groups in ~30 gastric bypass patients. Each of these proteins has been demonstrated to be increased in obesity and obesity-related conditions and may have direct actions on hepatic insulin resistance and progression of NAFLD to NASH in liver (Pittas, Joseph et al. 2004). Our study detected no differences in any of these hormones based on disease status. However, differences between groups did exist for clinical values of markers for disease. Hemoglobin A1c (HbA1c), fasting glucose, fasting insulin, and a calculated HOMA value are predictors of metabolic disease and are routinely measured to monitor disease progression (Lorenzo, Williams et al. 2007). Overall, there were significant differences between the double positive group (+D/+N) and the double negative group (-D/-N) for glucose, insulin, HbA1c, and HOMA. The +D/+N group also differed from the NAFLD only group (-D/+N) on glucose and HbA1c. The NAFLD only group (-D/+N) differed from the diabetes only group (+D/-N) on HbA1c. Because these clinical features are predictors of disease severity, it is not surprising each significantly differed between the disease-burdened group and the disease-free group. Additionally, glucose and HbA1c are specific to diabetes, therefore differences between the +D/+N group and the non-

diabetic groups are expected. HbA1c levels were evaluated from clinical data in regard to the GHSR SNPS and were not associated with a specific genotype. Although ghrelin has been demonstrated to be low in obesity and obesity-related conditions independent of BMI, little evidence for direct relationships between ghrelin and these related proteins exist outside of the framework of disease. Hence, changes in levels of ghrelin may or may not directly affect levels of each of these proteins without a concomitant change in disease or metabolic status.

Evaluation of Ghrelin Across Studies

The following table (Table 11-1) relays information about the change in ghrelin levels of specific diet groups relative to *ad libitum* controls:

Table 11-1. Comparison of ghrelin across all studies.

Diet	Ghrelin Form	Females	Males	Overall
10-day 40% CR	Plasma unacylated	N.C.	N.C.	N.C.
10-day 60% CR	Plasma unacylated	N.C.	N.C.	f>m
10-day 40% CR	Stomach top mRNA	*NA*	*NA*	N.C.
10-day 60% CR	Stomach top mRNA	*NA*	*NA*	N.C.
10-day 40% CR	Stomach bottom mRNA	*NA*	*NA*	+
10-day 60% CR	Stomach bottom mRNA	*NA*	*NA*	+
10-day 40% CR	Stomach (top & bottom) GHSR mRNA	*NA*	*NA*	N.C.
10-day 60% CR	Stomach (top & bottom) GHSR mRNA	*NA*	*NA*	N.C.

Low IGF-1 mice at ~9 mos	Plasma unacylated	N.C.	N.C.	--
Low IGF-1 mice at ~17mos	Plasma unacylated	N.C.	N.C.	N.C.
Low IGF-1 mice from ~9 mos to ~17 mos	Plasma unacylated	N.C.	N.C.	For midis: Time1>Time 2
Low IGF-1 mice at ~17mos	Plasma acylated	N.C.	N.C.	N.C; unacyl > acyl
LM at 17 mos	Plasma unacylated	N.C.	N.C.	LM males> AL females
40% CR at 17 mos	Plasma unacylated	N.C.	N.C.	N.C.
LM at 17 mos	Plasma unacylated	N.C.	N.C.	N.C. (trend)
40% CR at 17 mos	Plasma unacylated	N.C.	N.C.	N.C. (trend)
LM at 17 & 21 mos combined	Plasma unacylated	N.C.	N.C.	+ ; + vs 40% CR
40% CR at 17 & 21 mos combined	Plasma unacylated	N.C.	N.C.	+
LM at 25 mos	Plasma acylated	N.C.	N.C.	N.C.
40% CR at 25 mos	Plasma acylated	N.C.	N.C.	N.C.
Diabetes*NAFLD Groups 1-10 mos before surgery	Serum total	N.C.	N.C.	N.C.
Diabetes*NAFLD Groups 5-12 mos after surgery	Serum total	N.C.	N.C.	Post- > pre-surgery
Diabetes*NAFLD Groups during surgery	Liver total	N.C.	N.C.	N.C.

N.C., no change; *NA*, not applicable; mos, months; f, females; m, males; +, increased; --, decreased.

Based on this table, there are no clear consistent trends of ghrelin action during CR. Females and males were compared separately since separate evaluation of sexes was a common feature of all studies. While there were modest and nuanced effects of ghrelin relating to sex differences in HS mice, increases in 40% CR and LM long-term feeding, and a post-surgical increase in Roux-en-Y gastric bypass patients, there were no obvious effects of ghrelin by disease state or fully-fed vs. restricted state.

The Army or short-term, severe CR study found that hormonal outcomes (plasma IGF-1, plasma glucose) responded more rapidly to alteration in diet vs. whole-body measures of energy expenditure or body composition (metabolic rate, physical activity, RQ, body fat and lean mass, BMD). The severity of restriction altered the degree of response, such that 60% CR animals experienced more diet-induced physiological changes than the 40% CR group. Plasma unacylated ghrelin was unaffected by diet but females exhibited constitutively higher ghrelin levels than males, the first result reporting a sex disparity. Ghrelin mRNA expression was greater in the bottom half of stomachs for the restricted groups than AL-fed animals; GHSR mRNA expression was unchanged. Based on modest and inconsistent changes in ghrelin levels during short-term, moderate and severe CR, ghrelin was not demonstrated to be a major factor coordinating alterations in this acute CR model.

Plasma changes in unacylated and acylated ghrelin were measured at various ages and times throughout the day in low IGF-1 mice, long-term LM and 40% CR diets begun in middle aged, and in HS mice restricted 40% and 60% for 10 days. The data from these multiple CR or CR-like studies present no overall trend in ghrelin responses to diet in mice. Long-term LM and 40% CR (Low Methionine study) induced an increase in plasma unacylated ghrelin levels versus AL-fed controls, with LM-fed animals displaying the highest plasma unacylated ghrelin levels. Plasma unacylated ghrelin levels were not altered by a 10-day 40% or 60% CR or in genetically-altered low plasma IGF-1 mice. Therefore, there were no consistent trends in ghrelin action across these multiple CR-like manipulations.

The genetic contribution of ghrelin's action, but not blood or liver levels of ghrelin, protein was determined to be an important factor conferring success in the post-surgical period in gastric bypass patients. Serum levels of ghrelin and liver levels of ghrelin, TNF-a, IL-6, and leptin did not differ by the presence or absence of obesity-related disease. One of two ghrelin receptor promoter SNPs was positively linked to lower BMI and greater percent excess body weight loss at12 months post surgery. Further investigation of SNP haplotypes, including the mechanisms by which they confer physiological change, would provide further insight into ghrelin's roles in weight loss after gastric bypass surgery.

The results of these studies demonstrate, ghrelin, in large part, does not have a unifying or consistent action across CR studies. While data regarding ghrelin's response to CR and its actions across several physiological systems that are similarly affected in CR point to ghrelin as a coordinating hunger signal during CR, the data do not support this hypothesis. Future models may further investigate this theory. The future use of novel models, e.g. ghrelin knockout animals for evaluation of various CR diets would provide further explanation of the actions of ghrelin during CR.

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Select Publications & Presentations

"Ghrelin as a Signal during Human CR: Gastric Bypass Surgery" — 22 November 2010
Gerontological Society of America Annual Meeting — New Orleans, Louisiana

Lerner C, Lorenzini A, Torres C, Bitto A, Malaguti M, Roel M, Hrelia S, Ikeno Y, **Matzko ME,** McCarter RJ, Sell C. [Submitted]. Increased Life Span and Evidence of Enhanced Autophagy in Mice with Reduced Levels of Insulin-Like Growth Factor-1.

"Hormonal Control of Aging & Disease" — 21 November 2009
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