Anti-ghrelin Therapeutic Vaccine: A Novel Approach for Obesity Treatment

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Contents

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Abstract

Obesity is currently a major public health problem, due to the worldwide increasing rates of the disease and burden of the associated comorbilities, such as, type 2 diabetes, cardiovascular disease and cancer. Despite its increasing clinical relevance, there are still very few tools to treat obesity. The cornerstones for obesity treatment are still diet and exercise; anti-obesity drugs, which cause anorexia or malabsorption of nutrients, can be used as adjuvant therapy, however achieve only a modest weight loss and often short-term due to weight regain. For severe obesity the only proven effective therapy is bariatric surgery, an invasive procedure that carries inherent risks and is only recommended for selected patients.

Ghrelin is the only known hormone that stimulates food intake. In physiological conditions, ghrelin levels rise with fasting and decrease after meals. Most obese individuals have low fasting ghrelin levels that rise after food restriction and weigh loss, an explanation for the difficulty of weight loss maintenance. In contrast, in spite of major weight loss, the increase in ghrelin levels is prevented by some bariatric surgery techniques, which could contribute to sustain weight loss.

As ghrelin is the only known orexigenic hormone, it has been hypothesized that blocking reactive ghrelin increase could induce a sustained weight control.

Previous attempts to neutralize ghrelin orexigenic effects included passive immunizations

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by the inoculation of monoclonal anti-ghrelin antibodies and mixtures of monoclonal antibodies targeting different ghrelin haptens, which were able to decrease ghrelin-mediated and deprivation-induced food intake, while promoted an increase in energy expenditure, but had the limitation of having only acute effects; use of ghrelin receptor antagonists that demonstrated to improve glucose tolerance, suppress appetite and promote weight loss; and active immunization against ghrelin using keyhole limpet hemocyanin and bovine serum albumin as carrier proteins, which required the use of adjuvants that may be responsible for inflammatory responses and have limited use in humans.

A novel molecular approach is the use of an anti-ghrelin vaccine using Virus-like Particles as immunogenic carrier, which appears to be well-tolerated, decrease food intake and increase energy expenditure in both normal weight and diet-induced obese (DIO) mice. Vaccinated DIO mice also display a significant decrease of NPY gene expression in the basal hypothalamus reflecting a decrease in central orexigenic drive. All together, data suggests that this novel therapeutic anti-ghrelin vaccine is safe, has a positive impact on energy homeostasis and may be a useful tool for obesity treatment.

28.1 Introduction

The prevalence of overweight, obesity, and extreme obesity has been increasing worldwide in the last decades, affecting not only adults but starting as early as in childhood and adolescence [1, 2]. More recent estimates suggest that body weight gain will continue to increase, particularly in the younger people [3].

Obesity is a well-known risk factor for many chronic conditions including type 2 diabetes mellitus, hypertension, metabolic syndrome, cardiovascular diseases, and cancer [4].

The adverse health consequences occur not only in overweight individuals but start to increase at the upper limit of the normal body mass index (BMI 22–24.9 kg/m^2), and weight loss improves or resolves several comorbid conditions associated with the disorder [4].

The estimated economic impact of obesity in health-care systems due to both direct (personal health care, hospital care, physician services, allied health services and medications) and indirect costs (lost output as a result of a reduction or cessation of productivity due to morbidity or mortality) is enormous [5, 6]. These can be attributed not only to obesity per se, as excess physician visits, lost of workdays, restricted activity, and in-bed days, but particularly to the associated comorbidities such as type 2 diabetes mellitus, coronary heart disease, breast, endometrial and colon cancer, and osteoarthritis [7]. Furthermore, obesity is also associated with denial of employment, restriction of career advancement and higher insurance premiums [7].

Obesity and overweight are associated with large decreases in life expectancy, independently of comorbidities such as hypertension and diabetes that are major, potentially preventable, causes of premature morbidity and death [8]. The decrease in life expectancy and increase in early mortality associated with obesity is similar to those seen of smokers [8].

In view of the fact that obesity is a leading cause of preventable death worldwide, authorities have now considered the disease as one of the most serious public health problems of the century [9].

28.2 Obesity: Diagnosis and Classical Therapy

Obesity is defined as a medical condition characterized by accumulation of excess body fat to the extent that it may have adverse effects on health [2].

The body mass index (BMI), calculated by the ratio of weight (kg) for the square of height (m^2), is a measurement tool routinely used in the clinic to diagnose overweight and obesity, which in spite of providing no information concerning body fat distribution, with few exceptions,

correlates well with the percentage of body fat. Body mass index defines people as normal weight if their BMI is between 18.5 and 24.9 kg/m^2, overweight if their BMI is between 25 and 29.9 kg/m^2, and obese when it is greater than 30 kg/m^2 [10, 11].

Obesity is most often the result of a combination of excessive food energy intake and a lack of physical activity in genetically predisposed individuals. Only a limited number of cases are due primarily to monogenetic causes; endocrine disorders, such as Cushing syndrome and hypothyroidism; or previous use of drugs that cause weight gain [4].

Obesity is a chronic disease, as evidenced by the high likelihood of weight regain after weight loss attained by medical therapies, and therefore, there is a need for a long-term approach to the disease [12]. However, clinicians have few tools to fight obesity. Diet and exercise are still the cornerstones for obesity treatment, and current antiobesity drugs achieve only relative short-term weight loss and are often followed by weight regain [10, 12].

The available weight loss treatments include different combinations of diet, exercise, behavioral modification and pharmacotherapy. Many diets with different macronutrient compositions have demonstrated efficacy in weight loss; even though there is currently no evidence that clearly supports a superiority of a single dietary approach above the other diets used for weight loss. The degree of adherence to the prescribed calorie reduction appears to be the most important determinant of success [12]. Physical activity is also a valuable aid for weight loss; however, it is even more important for weight maintenance once weight loss is achieved [12]. Pharmacotherapy for obesity includes drugs that can either suppress appetite or alter nutrient absorption, with the purpose of inducing weight loss.

These drugs generally are capable to induce 5–10 % weight loss, the minimum requirement for a drug to be approved for weight loss by the regulatory authorities such as the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA). In addition to weight reduction, these drugs should provide a good safety profile and beneficial actions on several cardiovascular risk factors [13]. Even when there is only a modest weight loss and the patient does not reach a normal body weight, these are able to confer health benefits to the patient and improvement of obesity comorbidities [13]. Weight loss in people suffering from obesity is associated with a reduction in low-density lipoprotein cholesterol, total cholesterol, and blood pressure, with decreased risk of development of type 2 diabetes and may be beneficial for cardiovascular disease in the long term [14].

At the present, there are two antiobesity drug classes available in the market approved by the FDA, appetite suppressors and the lipase inhibitor orlistat, of which the former is the only drug authorized by EMEA and marketed in Europe. Appetite suppressant drugs include the central nervous system stimulants, such as phentermine, phendimetrazine, and diethylpropion. Phentermine is the most widely used drug for weight loss, owing to the fact of being in the US market for decades and to its low cost. Phentermine hydrochloride is a noradrenergic sympathetic amine approved for the short-term treatment of obesity. Phentermine belongs to a class of drugs that stimulate the central nervous system and conveys a response of "fight or flight" which blocks the feeding drive and induces anorexia. The most common side effects associated with phentermine include insomnia, irritability, and increase in blood pressure [12]. Sibutramine and rimonabant are another two appetite suppressant drugs that were recently withdrawn from the market due to safety concerns, after suspension being recommended due to cardiovascular and psychiatric side effects, respectively.

Orlistat is a lipase inhibitor that prevents hydrolysis of dietary triglycerides and consequently prevents the absorption of dietary fat that is excreted unaltered by the gastrointestinal tract. The drug is widely available and approved for long-term use; nevertheless, it produces only a modest weight loss and is associated with high rates of gastrointestinal side effects, such as steatorrhea, fecal incontinence and flatulence [15].

The FDA has recently approved two new drugs that will be available in the market in the near future, which are lorcaserin, a selective 5-hydroxytryptamine receptor 2c agonist [16], and a combination of phentermine and topiramate [17]. Topiramate is a sulfamate-substituted monosaccharide marketed since 1996 and formerly approved by the FDA for seizure disorders and prevention of migraine headaches. Topiramate, among other drugs for which previous clinical trials data suggested that could promote weight loss as a side effect of their therapeutic usage, was used off-label as adjuvant therapy in obesity treatment, along with the antidepressants fluoxetine, sertraline, and bupropion and the antidiabetic drug metformin [18].

For morbid obesity, the surgical approach, termed bariatric surgery, is the only therapy that provides sustainable weight reduction [19]. In 1992, the National Institutes of Health Consensus Development Conference, in a Position Statement, affirmed the superiority of surgical over nonsurgical approaches in this condition [20, 21]. Bariatric surgery is reserved for patients in whom medical weight loss treatments are known to fail and have not shown long-term effectiveness, namely, in patients with BMI > 40 kg/m^2 or >35 kg/m^2 associated with high-risk comorbid conditions [22].

In severe obesity, surgical treatments, by decreasing the risk for development of new obesity associated comorbidities and improving the existing ones, are also more cost-effective at producing and maintaining weight loss [23]. Obesity surgery provides a significant risk reduction for the development of new health-related conditions, namely, cardiovascular, cancer, endocrine (including diabetes mellitus and hypertension), respiratory, musculoskeletal, infectious, psychiatric, and mental disorders [24]. Weight loss, after bariatric surgery, usually results in improvement or resolution of multiple medical conditions that eliminates the use of medications and absenteeism from work in patients who were previously morbidly obese. When compared to matched controls, the patients submitted to surgery display a significant reduction in health-care use rates and total direct health-care costs. Bariatric surgery also significantly decreases overall 5-year mortality rate, with a reduction in the relative risk of death of 89 % compared to controls [24].

Understandably, in response to the relative ineffectiveness of medical therapy in severe obesity, the demand for bariatric surgery has greatly increased in recent years [25].

28.3 Anti-ghrelin Therapeutic Vaccine

28.3.1 Regulation of Food Intake and Energy Homeostasis

The physiological systems that regulate energy homeostasis include brain centers, such as the hypothalamus, brainstem, and reward centers in the limbic system, which regulate food intake and energy expenditure through the secretion of neuropeptides. These centers are modulated by neural and hormonal signals coming from the periphery. Hormones synthesized by the adipose tissue, like leptin, reflect the long-term nutritional status of the body and are able to influence long-term body weight regulation, while gastrointestinal hormones, like ghrelin, peptide tyrosine-tyrosine (PYY) and glucagon-like peptide 1 (GLP-1), among several other hormones, modulate these pathways acutely and are able to regulate food intake and energy expenditure [26] (Fig. 28.1). In the hypothalamus are located the most important food intake-regulating nuclei, the arcuate (ARC) and the paraventricular nuclei (PVN). The ARC of the basal hypothalamus receives signals from the periphery and plays an integrative role in appetite regulation. The ARC projects second-order neurons to the PVN, which is involved in the regulation of visceral efferent activity. In the ARC, there are two well-characterized neuronal populations involved, one appetite stimulating that co-expresses neuropeptide Y and Agouti-related protein (NPY/AgRP) and another appetite inhibiting that co-expresses proopiomelanocortin and cocaine- and amphetamine-regulated transcript (POMC/CART) [27] (Fig. 28.2).

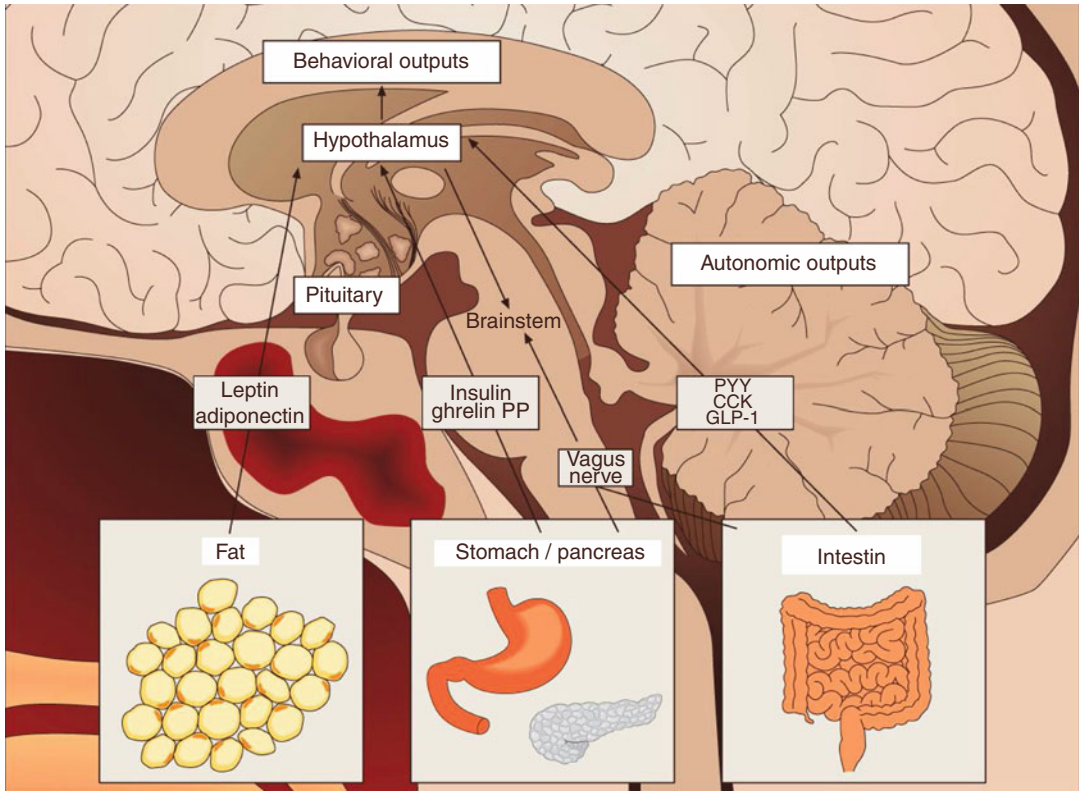

Fig. 28.1 The physiological systems that regulate energy homeostasis. Brain centers, such as the hypothalamus, brainstem and reward centers in the limbic system, which regulate food intake and energy expenditure through the secretion of neuropeptides. These centers are modulated by neural and hormonal signals coming from the periphery. Hormones synthesized by the adipose tissue, like leptin, and gastrointestinal hormones, like ghrelin, peptide tyrosine-tyrosine (*PYY*) and glucagon-like peptide 1 (*GLP-1*), modulate these pathways and are able to regulate food intake and energy expenditure

28.3.2 Ghrelin

Ghrelin is a gastrointestinal hormone that promotes food intake and decreases energy expenditure [28]. Ghrelin is produced predominantly in the gastric fundus [29] and conveys orexigenic signals to the hypothalamus [30].

Ghrelin acts in the ARC of the basal hypothalamus, stimulating the production and release of NPY and suppressing POMC [31]. NPY is the most potent signal in the central nervous system that stimulates food intake and decreases energy expenditure, while POMC is a precursor protein that through proteolytic cleavage originates various peptides, among which α-MSH that decreases appetite and increases energy expenditure [32, 33].

Ghrelin plasma levels rise before meals and are suppressed after food intake [34] in lean but not in obese patients [35]. There is a negative correlation between fasting ghrelin levels and body mass index. Fasting serum ghrelin levels are usually lower in obese subjects compared with controls [36], and fasting plasma levels rise after diet-induced weight loss [34, 37] and in patients with nervous anorexia [38]. Therefore, with the exception of patients with Prader-Willi syndrome [39], ghrelin does not seem to play a causative role in obesity in general, and its decreased concentrations are believed to represent a physiological adaptation to the positive energy balance.

Recent studies suggest that weight loss attained after bariatric surgery is also due to

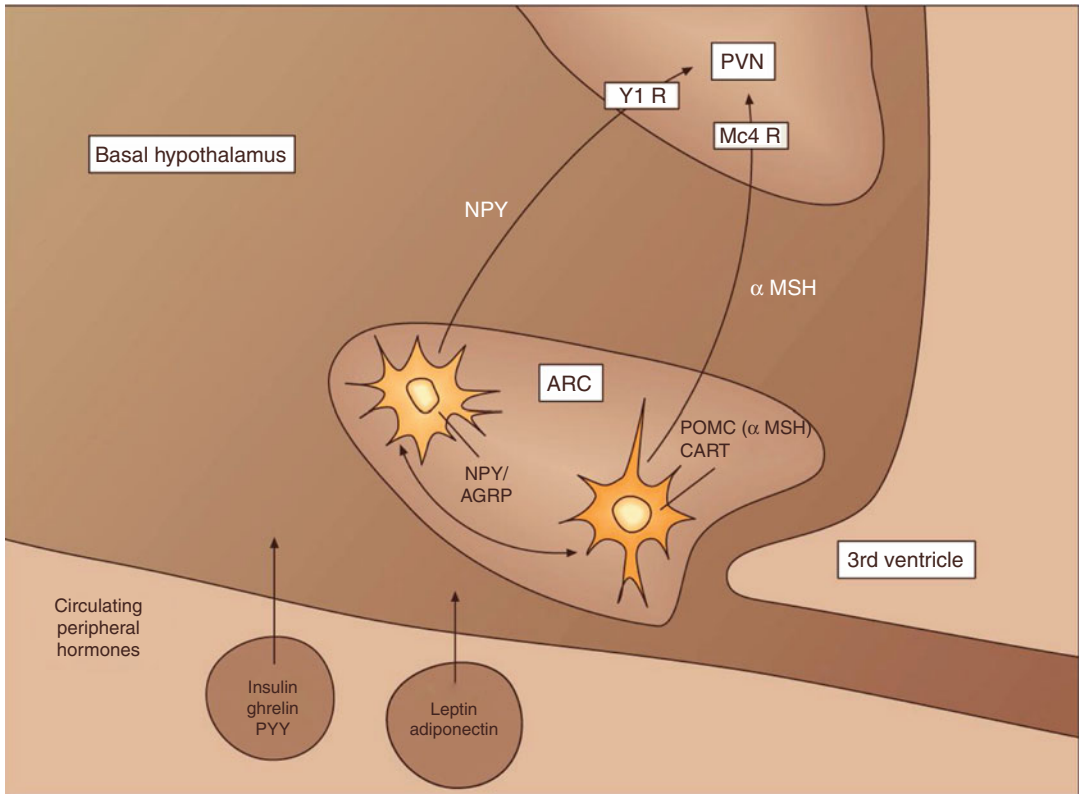

Fig. 28.2 The arcuate (ARC) and the paraventricular nuclei (PVN) are located in the basal hypothalamus. The ARC receives signals from the periphery and plays an integrative role in appetite regulation. The ARC projects second-order neurons to the PVN, which is involved in the regulation of visceral efferent activity. In the ARC, there are two well-characterized neuronal populations involved, one appetite stimulating that co-expresses neuropeptide Y and Agouti-related protein (NPY/AgRP) and another appetite inhibiting that co-expresses proopiomelanocortin and cocaine- and amphetamine-regulated transcript (POMC/CART)

endocrine effects of the surgery, which are able to interfere with appetite pathways by suppressing the rise in ghrelin levels that is usually observed after caloric deprivation [34, 40].

28.3.3 Rational for the Use of an Anti-ghrelin Vaccine

In view of the fact that ghrelin is the only orexigenic hormone identified so far, it has been considered a promising target in the development of new treatments for obesity.

As a proof of this concept, it was demonstrated that inoculation of monoclonal anti-ghrelin antibodies in mice inhibited acute ghrelin-mediated orexigenic effects, but it was unable to change long-term food intake [41]. More recently, another study suggested that the use of a mixture of monoclonal antibodies targeting different haptens, but not the antibodies individually, promotes not only an increase in energy expenditure but also reduced deprivation-induced food intake [42]. Ghrelin receptor antagonists, GSH-R1, demonstrated improved glucose tolerance, suppressed appetite and promoted weight loss [43], thus confirming the potential of ghrelin blocking as a potential treatment target for obesity. The suppression of endogenous ghrelin bioactivity with anti-ghrelin vaccines using keyhole limpet hemocyanin (KLH) as carrier protein [44] or bovine serum albumin (BSA) [45] was also tested in mice and pigs, respectively. These vaccines were able to induce the development

of antibodies against the active form of ghrelin [44] and also to decrease body weight gain and fat mass [45]. However, these vaccines required the use of adjuvants, such as alum and Freund incomplete adjuvant, which may be associated of inflammatory responses or have limited use in humans.

28.3.4 Anti-ghrelin Vaccine Using Virus-Like Particles

Virus-Like particles (VLPs) have been used as immunogenic molecules in several recombinant vaccines in the last few years in order to induce the production of specific antibodies against endogenous molecules with a preponderant role in chronic diseases [46], such as the anti-angiotensin vaccine developed for arterial hypertension treatment [47].

The main goal of our research work was to develop an effective anti-ghrelin vaccine using a chemical conjugate of active ghrelin with protein tubules of NS1 of the bluetongue virus (BTV) [48]. Although this protein is not part of the viral capsid, NS1 tubules possess the same immunogenic characteristics as classical VLPs [49].

28.3.5 Animal Models and Feasibility Study

Male adult C57BL6/J mice (Charles River, Barcelona, Spain), normal weight mice, and diet-induced obesity mice (DIO) ($n=18$/group) were randomized into three weight-matched groups ($n=6$/group). Normal weight mice had unrestricted access to tap water and regular rat chow and DIO mice to a hypercaloric diet with 60 % of fat (Charles River, Barcelona, Spain), after weaning and until a week before the first immunization study when food was switched to regular rat chow.

Mice received three intraperitoneal (i.p.) injections with 2-week intervals, containing 500 µl of 75 µg of immunoconjugate, 75 µg of NS1 protein alone, or PBS. A dose was chosen after performing a dose-finding study in which the 75 µg dose of the immunoconjugate has demonstrated to be adequate in inducing the development of anti-ghrelin antibodies and reducing food intake.

After the immunizations, energy expenditure was accessed by indirect calorimetry. For that mice were individually placed in a small grid cage to limit locomotor activity, which was placed into a sealed chamber containing a sodium hydroxide recipient to adsorb carbon dioxide. The lid of the chamber was sealed and pierced by a volumetric pipette to measure the volume of oxygen consumed. The time elapsed until 1 ml was consumed was registered and repeated until five concordant values were obtained. The energy expenditure was then calculated considering that 4.82 kcal is the average energy released per liter of O_2 consumed.

Anti-ghrelin antibodies titer was determined 2 weeks after each immunization. Plasma levels of active ghrelin (EZRGRA-90K, Linco Research, St. Charles, Mo, USA, range 25–2,000 pg/ml), leptin (EZML-82K, Linco Research, St. Charles, Mo, USA, range 0.2–30 ng/ml), insulin (EZRMI-13K, Linco Research, St. Charles, Mo, USA, range 0.2–10 ng/ml), growth hormone (EZRMGH-45K, Linco Research, St. Charles, Mo, USA, range 0.07–50 ng/ml), IGF-1 (E25, Mediagnost, Reutlingen, Germany, range 0.5–18 ng/ml), and TNF-α (Quantikine, R&D Systems, Abingdon, United Kingdom, range 15.6–1,000 pg/ml) were determined by ELISA using specific commercial kits according to the manufacturer instructions.

Two weeks after the third immunization, mice were sacrificed and the stomach fundus and the hypothalamus were recovered and immediately frozen by immersion in liquid nitrogen to evaluate ghrelin, neuropeptide Y (NPY) and proopiomelanocortin (POMC) expression.

28.3.6 Safety and Efficacy of the Vaccine

Normal weight mice treated with the immunoconjugate displayed a significant decrease in daily food intake (0.44 g NS1-Ghr vs. 0.14 g PBS vs. 0.16 g NS1, $p<0.001$) (Fig. 28.3). In addition, after the first two inoculations, there was also an acute decrease in food intake in the group of mice that received the Immunoconjugate when compared to

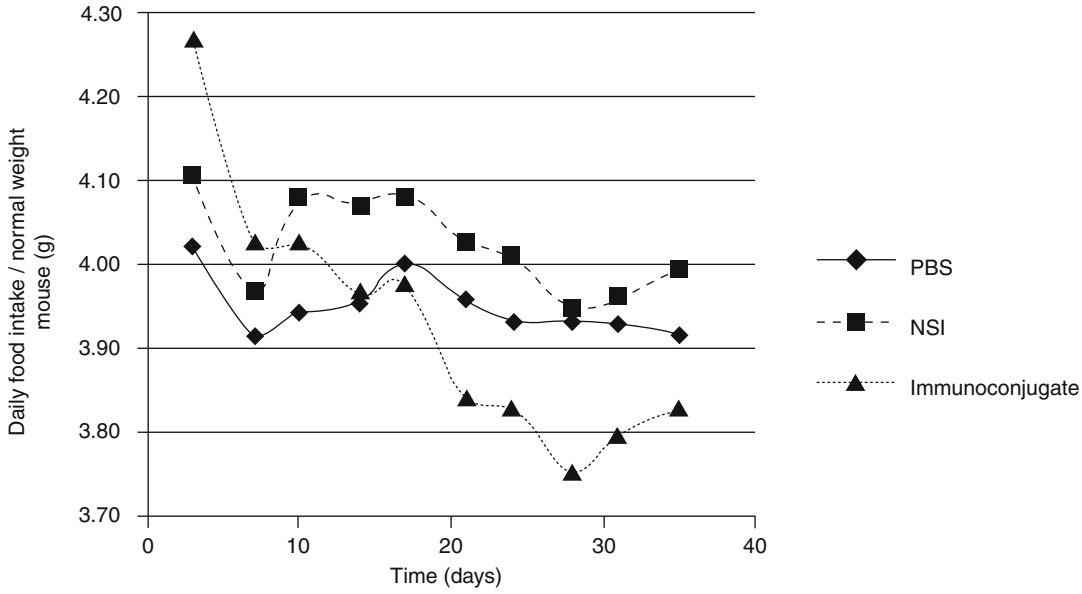

Fig. 28.3 Normal weight mice treated with the immunoconjugate displayed a significant decrease in daily food intake when compared to control mice (0.44 g NS1-Ghr vs. 0.14 g PBS vs. 0.16 g NS1, $p<0.001$)

the PBS control, corresponding to 95.3 and 94.8 % of the PBS control, respectively, although without reaching statistical significance. There were no significant differences in body weight gain between the different groups of mice during the study span (3.83 g ± 0.40 g NS1-Ghr vs. 5.00 g ± 0.26 g PBS vs. 5.33 g ± 0.49 g NS1, p=NS) [50].

In DIO mice, after changing from the hypercaloric to the standard diet, there was an increase in daily food intake followed by rapid stabilization. DIO mice inoculated with the immunoconjugate did not display a significant decrease in cumulative food intake when compared to controls (147.64 ± 2.46 g NS1-Ghr, 147.80 ± 5.89 g NS1, 150.37 ± 3.65 g PBS, p=NS), although there was a significant decrease of food intake in the 24 h immediately after each inoculation of the immunoconjugate, corresponding to 66.16 % ($p=0.036$), 82.22 % ($p=0.008$) and 50.09 % ($p=0.039$) of the food intake of the PBS group, after the three inoculations, respectively. DIO mice body weight decreased in response to the change from the hypercaloric to the standard diet (13.84 % compared to baseline), although after the inoculations, there were no significant differences in body weight among the different experimental groups (32.17 ± 0.872 g NS1-Ghr vs. 31.33 ± 1.282 g NS1 vs. 31.83 ± 0.833 g PBS, p=NS) [50].

Normal weight mice inoculated with the immunoconjugate developed specific anti-ghrelin antibodies, with increasing titers after each inoculation, reaching a maximum of 1,265 ± 492 2 weeks after the last inoculation. The control groups that received either NS1 protein alone or PBS presented basal titers of 332 ± 114 and 324 ± 143, $p=0.035$, respectively, which were maintained throughout the study and were not altered by the immunizations, which suggests nonspecific bindings related to the detection method. DIO mice inoculated with the immunoconjugate also developed specific anti-ghrelin antibodies in increasing titers until reaching a maximum after the third inoculation in contrast with control groups that maintained their basal titers (2,680 ± 1,197 NS1-Ghr, 458 ± 31 NS1 and 257 ± 78 PBS group, respectively, $p=0.03$) [50].

Energy expenditure was significantly higher in normal weight mice inoculated with immunoconjugate when compared with controls (0.0146 ± 0.001 kcal/h/kg NS1-Ghr,

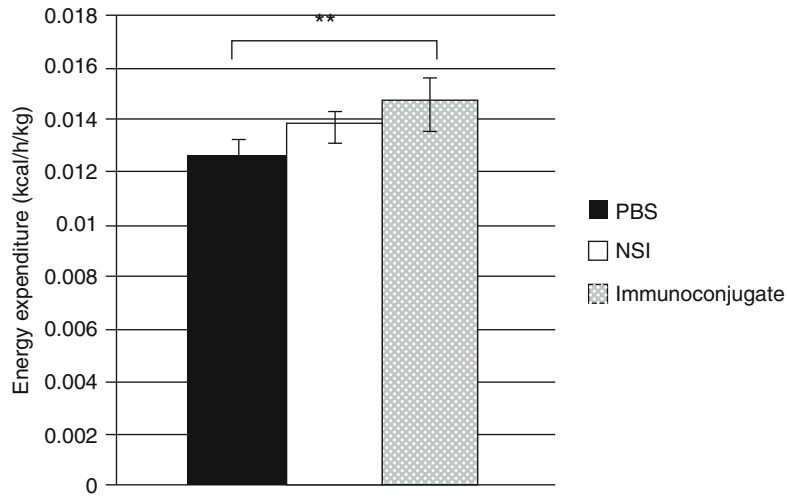

Fig. 28.4 Energy expenditure was significantly higher in normal weight mice inoculated with immunoconjugate when compared with controls (0.0146 ± 0.001 kcal/h/kg NS1-Ghr, 0.0138 ± 0.001 kcal/h/kg NS1, 0.0129 ± 0.001 kcal/h/kg PBS, $p = 0.038$)

0.0138 ± 0.001 kcal/h/kg NS1, 0.0129 ± 0.001 kcal/h/kg PBS, $p = 0.038$) (Fig. 28.4). DIO mice inoculated with the immunoconjugate also showed higher energy expenditure when compared to the control groups (0.0207 ± 0.01 kcal/h/kg NS1-Ghr, 0.0140 ± 0.002 kcal/h/kg NS1, 0.0159 ± 0.002 kcal/h/kg PBS; $p = 0{,}044$, NS1-Ghr vs. PBS and $p = 0{,}008$, NS1-Ghr vs. NS1) [50].

Fasting plasma levels of active ghrelin were significantly higher in normal weight mice that received the immunoconjugate (361.3 ± 79.9 pg/ml) when compared to control groups (186.9 ± 14.8 pg/ml NS1 and 114.1 ± 27.9 pg/ml PBS, $p = 0.009$). DIO mice inoculated with the immunoconjugate also presented higher levels of fasting plasma ghrelin than the controls (429.63 ± 179.27 pg/ml NS1-Ghr, 147.29 ± 53.17 pg/ml NS1, 105.88 ± 27.76 pg/ml PBS, $p =$ NS) although not statistically significant. There were no significant differences in plasma levels of leptin, insulin, glucose, growth hormone, IGF-1 or TNF-α between the groups. ELISA confirmed the presence of circulating immune complexes of ghrelin-anti-ghrelin antibodies in the plasma of normal weight mice inoculated with the immune conjugate. There was also a positive correlation between ghrelin plasma levels and the titer of circulating immune complexes ($r = 0.846$) (Fig. 28.5). Search for immunoglobulins deposits in the kidney by immunohistochemistry failed to reveal any evidence of deposited immune complexes on the glomerular basement membranes [50].

In normal weight mice, there was no significant difference in ghrelin expression in the gastric fundus between the three experimental groups of mice (0.94 ± 0.17 NS1-Ghr, 1.79 ± 0.35 NS1, 1.00 ± 0.30 PBS, $p =$ NS). There was also no significant difference in NPY expression in the basal hypothalamus between the study groups (1.32 ± 0.17 NS1-Ghr, 0.94 ± 0.10 NS1, 1.00 ± 0.20 PBS, $p =$ NS). In contrast, POMC mRNA expression was significantly lower in mice inoculated with the immunoconjugate when compared to controls (0.20 ± 0.17 NS1-Ghr, 0.93 ± 0.17 NS1, 1.00 ± 0.10 PBS, $p < 0.05$). In DIO mice the expression of ghrelin after normalization for GAPDH expression in stomach cells was also not significantly different among the different study groups (1.27 ± 0.30 NS1-Ghr, 0.38 ± 0.13 NS1, 1.00 ± 0.12 PBS, $p =$ NS). However, DIO mice inoculated with the Immunoconjugate had a lower expression of NPY in the basal hypothalamus when compared to control groups (0.59 ± 0.09 NS1-Ghr, 1.03 ± 0.12 NS1, 1.00 ± 0.13 for PBS, $p < 0.05$). The expression of POMC in the basal hypothalamus was not significantly different between the different groups in study (1.04 ± 0.14 NS1-Ghr, 1.32 ± 0.25 NS1, 1.00 ± 0.12 PBS, $p =$ NS) [50].

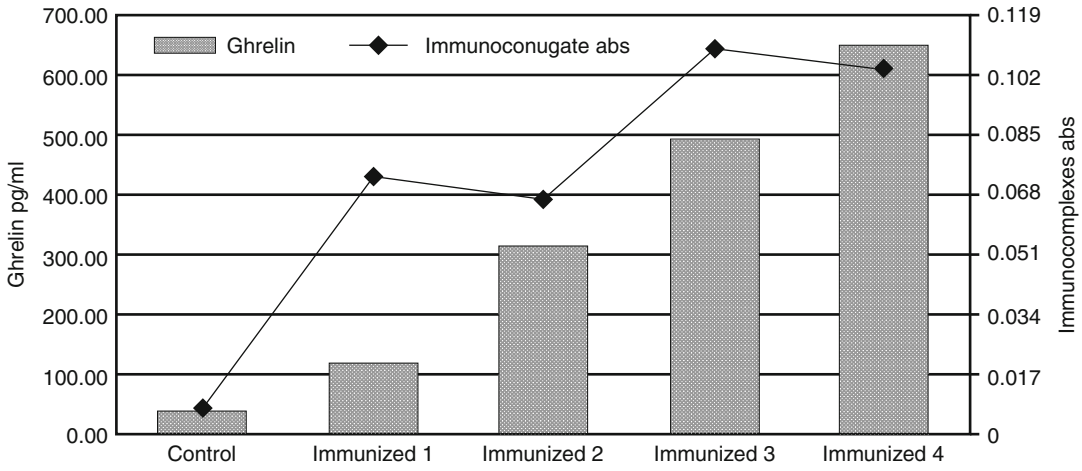

Fig. 28.5 Circulating ghrelin-anti-ghrelin antibodies immune complexes titers and plasma ghrelin levels. The presence of circulating immune complexes of ghrelin-anti-ghrelin antibodies in the plasma of normal weight mice inoculated with the immune conjugate was confirmed by ELISA. There was also a positive correlation between ghrelin plasma levels and the titer of circulating immune complexes ($r=0.846$)

28.4 Strengths and Weaknesses

Obesity is nowadays a major public health problem [11, 51] for which there is a lack of medical therapeutic resources [12, 18]. Since ghrelin is the only orexigenic hormone identified so far, it has been pointed as a promising treatment target for obesity [52]. Several research groups have previously attempted ghrelin neutralization. Passive transfer of monoclonal anti-ghrelin antibodies was unable to change long-term food intake in mice [41]. Antibodies targeted to hydrolyze the octanoyl moiety of ghrelin to form desacyl ghrelin, which has no biological activity, resulted in increased metabolic rate and suppressed 6 h refeeding after 24 h of food deprivation in mice, but this approach would imply the need of periodic antibodies administration [53].

More recently, another study concluded that an oligoclonal response is required to maintain increased energy expenditure during fasting and deprivation-induced food intake as well as to reduce overall food intake upon refeeding [42]. Ghrelin receptor antagonists have also been tested, and GSH-R1a decreased food intake and body weight and improved glucose tolerance due to increased glucose-dependent insulin secretion [43]. Anti-ghrelin vaccines using KLH or BSA as immunogenic substances decreased body weight gain by decreasing feed efficiency in rats [44] and food intake and body weight in pigs [45].

However, these anti-ghrelin vaccination and neutralization strategies present several limitations when applied to humans because of the need to use adjuvants, the risk of exacerbated immune response against an endogenous substance, and, in the case of passive immunization, acquired tolerance and lack of long-term effectiveness. When compared with classic immunization techniques, VLPs are safe due to the lack of genetic material, since VLPs consist only of viral proteins and induce an efficient B cell activation. The highly repetitive nature of these structures has the advantage of allowing B cell receptor cross-linking due to the ordered presentation of epitopes in molecule surface and a high immunogenicity regardless of the route of the immunization, which allows the use of a low number of immunizations and a lower quantity of vaccine, making this type of vaccination protocol more efficient and cost-effective [54].

The main goal of the current vaccine approach was to develop a safer and more effective anti-ghrelin vaccine that could be used for human treatment. For that we developed an immunoconjugate composed of ghrelin and NS1 protein of

BTV. The choice of NS1 tubules as VLP-like carrier protein was driven by its previous use as a distribution system for molecules of prophylactic vaccines against common human infectious diseases, such as proteins of the foot-and-mouth disease and influenza A virus [49, 55].

The ability of the vaccine to trigger an immune response was tested in normal weight and DIO male mice that developed increasing titers of specific anti-ghrelin antibodies, confirming the hypothesis that a vaccine consisting of immunoconjugate only is able to trigger an immune response without the need adjuvants. Furthermore, antibody titers attained after the immunization protocol were not very high, when compared to antibodies titers after common infectious diseases, which is also reassuring in safety concerns, since complete neutralization of ghrelin was not the purpose of an anti-ghrelin vaccination strategy for obesity treatment as ghrelin also intervenes in several key biological processes besides appetite regulation, such as growth hormone secretion and gastrointestinal and cardiovascular functions [52].

Vaccinated mice showed significantly higher energy expenditure than the animals of the groups that received either NS1 protein alone or PBS. Higher energy expenditure usually translates into greater ease of weight loss and maintenance. Ghrelin is known to suppress energy metabolism, and ghrelin replacement partially reverses the reduction in body weight and body fat in gastrectomized mice [56]. Ghrelin has been shown to have a long-term effect on energy homeostasis by increasing the respiratory quotient, through decreasing utilization of fat as energy [30]. In addition, ghrelin knockout mice compared to wild-type mice present no change in food intake but have a decreased respiratory quotient when fed with high-fat diet, suggesting that endogenous ghrelin plays a more prominent role in determining the type of metabolic substrate that is used for maintenance of energy balance than in the regulation of food intake [57]. Although vaccinated animals gained less weight when compared with control animals, this difference failed to reach statistical significance, which may be explained by the short follow-up time or the activation of compensatory mechanisms of energy homeostasis pathways.

Paradoxically, vaccinated mice had higher ghrelin levels compared to controls. Given that these increased levels of ghrelin did not appear to have a biological effect, we hypothesize that circulating ghrelin could be in the form of immune complexes of ghrelin-anti-ghrelin antibodies, which was confirmed. Previous reports on anti-ghrelin vaccines have also documented an increase of ghrelin in immunized animals, although the presence of circulating immunocomplexes has not been documented [58]. The presence of circulating immunocomplexes, which could be due to a lower rate of elimination, raised the concern of renal toxicity due to the deposition in the glomerular basement membrane that has been excluded. Since there was no difference in ghrelin expression in the stomach, ghrelin appears to be synthetized in immunized animals as in controls, and after neutralization of ghrelin biological activity, there is no upregulation of ghrelin expression in order to maintain the homeostasis.

In vaccinated normal weight mice, there were no significant differences in the genetic expression of NPY gene in the basal hypothalamus in comparison to control mice. However, in vaccinated DIO mice, there was a significant decrease of NPY gene expression in the basal hypothalamus compared with controls reflecting a decrease in central orexigenic signals [59]. The expression of POMC in the basal hypothalamus was significantly lower in vaccinated normal weight animals compared to controls that could represent a compensatory mechanism to the decreased peripheral orexigenic signals in order to prevent the reduction in feeding threshold of normal weight mice, which could also explain why these findings only occurred in the normal weight mice but not in DIO mice.

Ghrelin is a growth hormone secretagogue [52] and ghrelin neutralization could induce alterations in GH/IGF-1 axis. Since this vaccine appears to have no effect in GH and IGF-1 levels, this suggests that our vaccine is unlikely to cause endocrine adverse effects on the growth hormone axis.

The regulatory mechanisms of energy homeostasis and appetite control are very complex processes that include highly redundant signalling pathways [31]. Therefore, it is possible that the lack of significant differences in some biological parameters, such as food intake and body weight, may be due to activation of compensatory mechanisms for the decrease in available active ghrelin similar to that which occurs in ghrelin knockout mice [57, 60].

28.5 Concluding Remarks

This anti-ghrelin vaccine appears to be well tolerated by the animals, and there were no signs of inflammatory reaction or toxicity. The production of anti-ghrelin antibodies was effective in decreasing acute food intake and increasing energy expenditure in the vaccinated animals compared to control animals, which are important contributions to establish a negative energy balance and thus promote weight loss.

Most obese patients have low ghrelin levels; therefore, it is not expected for the vaccine to be effective in the absence of diet-induced ghrelin rise, so an anti-ghrelin vaccine would be beneficial for patients enrolling a diet and exercise program as adjuvant therapy for weight loss and prevention of weight regain [34]. Additionally, obese patients with high ghrelin levels could benefit from ghrelin blockade through this anti-ghrelin vaccine, such as individuals with Prader-Willi syndrome [39].

In conclusion, these results suggest that this anti-ghrelin vaccine has a positive impact on energy homeostasis and may be a useful tool for obesity treatment.

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