Improvement in insulin resistance and reduction in plasma inflammatory adipokines after weight loss in obese dogs

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Received 2 May 2009; received in revised form 3 July 2009; accepted 3 July 2009

Abstract

Obesity is now a major disease of dogs, predisposing to numerous disorders including diabetes mellitus. Adipocytes are active endocrine cells, and human obesity is characterized by derangements in inflammatory adipokine production. However, it is unclear as to whether similar changes occur in dogs. The purpose of the current study was to assess insulin sensitivity and inflammatory adipokine profiles in dogs with naturally occurring obesity and to investigate the effect of subsequent weight loss. Twenty-six overweight dogs were studied, representing a range of breeds and both sexes. All dogs underwent a weight loss program involving diet and exercise. Body fat mass was measured by dual-energy x-ray absorptiometry; plasma concentrations of insulin, glucose, and a panel of inflammatory adipokines (including acute-phase proteins, cytokines, and chemokines) were also analyzed. Body fat mass before weight loss was positively correlated with both plasma insulin concentrations (Kendall $\tau = 0.30$, $P = 0.044$) and insulin:glucose ratio (Kendall $\tau = 0.36$, $P = 0.022$), and both decreased after weight loss ($P = 0.0037$ and 0.0063, respectively). Weight loss also led to notable decreases in plasma tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), haptoglobin, and C-reactive protein concentrations ($P < 0.05$ for all), suggesting improvement of a subclinical inflammatory state associated with obesity. This study has demonstrated that in obese dogs, insulin resistance correlates with degree of adiposity, and weight loss improves insulin sensitivity. Concurrent decreases in TNF-\(\alpha\) and adipose tissue mass suggest that in dogs, as in humans, this adipokine may be implicated in the insulin resistance of obesity.

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Keywords: Adipose tissue; Acute phase protein; Metabolic syndrome; Obesity; Diabetes mellitus

1. Introduction

Obesity is defined as a disease in which excess body fat has accumulated such that health may be adversely affected [1]. Obese humans are known to suffer from several associated clinical conditions including hypertension, coronary heart disease, certain cancers (eg, breast, ovarian, prostate), osteoarthritis, respiratory disease, and reproductive disorders [1]. Similarly, obesity
has detrimental effects on the health of dogs, with disease associations including orthopedic diseases, respiratory disease, urinary tract disorders, and decreased longevity [2–4]. However, a possible association between obesity and cancer is controversial in dogs, with one study suggesting an association [2], whilst another suggested no relationship [5].

In humans, the most important disease association is that of the metabolic syndrome, a group of metabolic and vascular disorders, which increase the risk of an individual developing type 2 diabetes and cardiovascular disease [6]. The risk of developing diabetes increases with increasing body mass index (BMI); individuals with a BMI > 30 kg/m² are 10 times more likely to develop type 2 diabetes than those with a BMI < 25 kg/m² [7]. In contrast, dogs more commonly suffer from a form of diabetes resembling type 1 diabetes in man [8–10], and although common, its pathogenesis is poorly understood [11–13]. Although dogs do not commonly suffer from type 2 diabetes [11–13], a previous study has reported an association between canine diabetes and excess body weight [2]. The reason for such an association is not clear but may be owing to insulin resistance, which can be induced experimentally in dogs through dietary manipulation [14–17]. Further, lifelong dietary energy restriction has been shown to improve insulin sensitivity and glucose tolerance [18], and a relationship between obesity, glucose tolerance, and insulin response was reported in one study of dogs with naturally occurring diabetes [19]. However, in that study, the degree of excess weight was estimated subjectively using breed standards, which is a notoriously unreliable method for quantifying adiposity. Therefore, further studies are clearly required to clarify the link between insulin resistance in naturally occurring canine obesity.

White adipose tissue (WAT) has recently been recognized as an active endocrine organ that is capable of secreting a wide range of hormones and protein factors, collectively termed adipokines [20,21]. Of particular note is the range of cytokines, chemokines, and other inflammation-related proteins secreted by WAT, such that a state of chronic low-grade systemic inflammation is now known to exist in obesity [22,23]. Systemic concentrations of acute-phase proteins (eg, C-reactive protein [CRP], haptoglobin) and pro-inflammatory cytokines (eg, IL-6 and tumor necrosis factor-α [TNFα]) are elevated in obese individuals [24]. Since adipose tissue is an important source of these factors [23], this organ system may contribute significantly to the elevated circulating concentrations [23], providing a link between obesity, insulin resistance, and the metabolic syndrome in humans [25,26]. Further, weight loss in these subjects has been shown to reverse this low-grade inflammatory state and to improve insulin sensitivity [27].

Our recent work has demonstrated that genes encoding key inflammatory adipokines are expressed in canine WAT samples and in canine adipocytes differentiated in culture [28,29]. However, studies examining adipokine profiles in canine obesity and associated disease, including insulin resistance, are limited. Therefore, the purpose of the current study was to assess insulin sensitivity and inflammatory adipokine profiles in dogs with naturally occurring obesity and to determine the effect of subsequent weight loss.

2. Materials and Methods

2.1. Study animals

Twenty-six dogs were included in this study; all were referred to the Royal Canin Weight Management Clinic (RCMWC), University of Liverpool, United Kingdom (UK), for the investigation and management of obesity or obesity-related disorders. Dogs were enrolled if they were systemically well, euthyroid, and had no significant abnormalities on complete blood count, serum biochemical analysis, and urinalysis. The study was performed in adherence to the University of Liverpool Animal Ethics Guidelines and was approved by the WALTHAM ethical review committee, in accordance with European Community Directive 86/609/EEC for Animal Experiments. All clinical procedures performed were for the direct benefit of the dogs in the study, and the owners of all participating animals gave written informed consent.

2.2. Weight loss regimen

Full details of the regimen used for weight loss have been previously described [30]. Initially, all patients were weighed by electronic weigh scales (Soehnle Professional), which were calibrated on a weekly basis using test weights (2 kg, 5 kg, 10 kg, and 50 kg; guaranteed accuracy ≤ 0.5%; Blake and Boughton Ltd., Thetford, UK). A body condition score (BCS) was assigned to each patient using a 9-integer system as previously described [31], where 4/9 and 5/9 represent ideal condition; scores above 5/9 represent differing degrees of excess weight (6/9 = overweight to 9/9 = grossly obese); and scores below 4/9 represent differing degrees of suboptimal condition (3/9 = underweight to 1/9 = emaciated). Body composition was analyzed by fan-beam dual-energy x-ray absorptiometry (DEXA; Lunar Prodigy Advance; GE Lunar) and associated computer software (enCORE...
Target weight was estimated with reference to DEXA results, as previously described [32]. Thereafter, an individually tailored weight management protocol was instigated, using caloric energy restriction, also as previously described [30].

All dogs received either of 2 commercially available, purpose-formulated weight loss diets: 21 dogs received a high-protein, medium-fiber weight loss diet, whereas the remaining five dogs received a high-protein, high-fiber weight loss diet (Table 1). The initial energy allocation given was tailored to the individual patient, where sex and neuter status were factored in [30]. Further adjustments were made, based upon other factors, for example, ability to exercise (greater restriction if concurrent orthopedic disease) and sanctioned use of additional treats. The owner was also educated about the significance of obesity (as a disease), associated diseases, and about altering lifestyle to assist in weight loss. The type of exercise varied and could include play sessions, lead walking, and swimming.

All animals were reassessed every 7 to 21 d, when they were weighed and changes were made to the dietary plan if necessary. Throughout the weight loss period, owners maintained a diary covering diet ration fed, activity, and any additional food that had been consumed (either given as treats or stolen). A detailed evaluation was then conducted after the period of weight loss. Dogs were confirmed to have remained healthy based upon physical examination, routine hematological analysis, routine serum biochemical analysis, and urinalysis. Body weight and body condition were recorded, and body composition was assessed by DEXA.

2.3. Sample collection, storage, and preparation for analysis

Blood samples were collected by jugular venipuncture prior to and after the weight loss period. All blood samples were taken after a fast of at least 16 h. Immediately after collection, heparinized samples were centrifuged, and the plasma was harvested and then divided into 4 aliquots. Most samples (> 90%) were frozen and stored at -20°C within 30 min of sample collection, although for a minority, there was a slightly longer delay before freezing (between 30 min and 3 h of sample collection). Samples remained frozen until the time of analyte measurement, when 1 aliquot from each dog was transported on dry ice to MD Biosciences, Inc. (St. Paul, MN, USA), and the remaining plasma was defrosted and used for the assays conducted in-house. When the analyses were performed, pre- and post-weight loss samples were all run at the same time to minimize assay variability.

2.4. Plasma analysis of insulin and glucose, and calculation of insulin:glucose ratio

Fasting plasma insulin concentrations were measured by MD Biosciences, Inc., using a Luminex-based assay, according to the manufacturer’s instructions (canine endocrine hormone Linco-plex assay, Millipore, USA). Intra- and interassay coefficients of variation were 4.7% and 17.6%, respectively. The limit of detection was 1.8 μU/mL (12 pmol/L), and the assay reference range was 5.0–25.0 μU/mL (35–175 pmol/L). Fasting plasma glucose concentrations were analyzed by the Depart-

Table 1

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Abbreviations: DM, dry matter; FOS, fructo-oligosaccharides; HPHF, high-protein, high-fiber (Satiety Support, Royal Canin); HPMF, high protein, medium fiber (Obesity Management, Royal Canin); ME, metabolizable energy content, as measured by animal trials according to the American Association of American Feed Control Officials protocol (AAFCO, 2003).

Note: Diets were analyzed for ash, crude protein (calculated from Dumas nitrogen values), and total lipid content using Association Française de Normalisation (AFNOR) methods. Total dietary fiber (TDF) was assayed using methods that adhered to Association of Analytical Chemists (AOAC) guidelines.
ment of Veterinary Pathology, University of Liverpool, using a Kone Specific Supra biochemistry analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The assay reference range was 55-100 mg/dL (3.0-5.5 mmol/L). As an indirect assessment of insulin resistance, insulin:glucose ratios (I:G) were calculated, as previously described [14], using the following formula:

\[ I : G = \frac{\text{Insulin} \, [\mu U/mL] \times 100}{\text{Fasting glucose} \, [mg/dL]} \]

2.5. Plasma analysis of acute-phase proteins, cytokines, and chemokines

C-reactive proteins and serum amyloid A (SAA) were measured by solid-phase sandwich immunoassays (Tridelta Phase Range canine CRP kit and Tridelta Phase Range serum amyloid A assay, Tridelta Development, Ltd.), again as previously described [33,34]. For CRP, intra- and interassay variability (low to high concentration samples) was 6.5%-7.7% and 7.8%-8.2%, respectively. The limit of detection was 0.05 mg/L, and the assay reference range was 0.0-3.0 mg/L. For SAA, intra- and interassay variability (low to high concentration samples) was 2.7%-7.7% and 3.8%-10.8%, respectively. The limit of detection was 0.15 µg/mL, and the assay reference range was 5.0-80.0 µg/mL. A colorimetric assay (Tridelta Phase Haptoglobin Assay; Tridelta Development Limited, Ireland) was used to determine haptoglobin concentrations, as previously described [33,34]. The assay was based on the finding that formation of a hemoglobin–haptoglobin complex preserves the peroxidase activity of hemoglobin against inactivation at low pH. Intra- and interassay (for low to high concentrations) variability was 0.9%-1.4% and 7%-11%, respectively. The limit of detection was 0.05 mg/mL, and the assay reference range was 0.3-3.5 mg/mL.

Plasma TNF-α concentration was measured by sandwich ELISA specific for the canine protein (R&D Systems, Abingdon, UK). Intra- and interassay variability (low to high concentration samples) was 5.3%-8.1% and 9.3%-10.0%, respectively. The limit of detection was 0.9 pg/mL, and the assay reference range was 0.0-4.5 mg/L. Plasma concentrations of IL-6 and macrophage chemotactic protein-1 (MCP-1) were measured by MD Biosciences, Inc., using a Luminex-based assay (canine cytokine/chemokine Linco-plex assay, Millipore, USA). Data were collected using a Luminex 100 (Luminex Corporation, Austin, TX, USA). Intra-assay variability was 3.9% and 13.8%, interassay variability was 15.8% and 19.1%, and the limits of detection were 12.1 pg/mL and 8.6 pg/mL for IL-6 and MCP-1, respectively.

2.6. Plasma analysis of adiponectin and leptin

Fasting plasma adiponectin and leptin were measured by MD Biosciences Inc., using Luminex-based assays according the manufacturer’s instructions (canine endocrine hormone and canine adipokine Linco-plex assays, Millipore, USA). For adiponectin, intra- and interassay coefficients of variation were 7.9% and 9.3%, respectively. For leptin, intra- and interassay coefficients of variation were 4.6% and 17.6%, respectively. The limits of detection were 22.1 pg/mL and 148 pg/mL for adiponectin and leptin, respectively.

2.7. Statistical analysis

Statistical analysis was performed with the Stats Direct computer software package, version 2.6.2 (Stats Direct Ltd., Altrincham, UK). Complete blood count and clinical biochemistry data were normally distributed, either before or after logarithmic transformation, and results prior to and after weight loss were analyzed with the paired t-test. Results for all remaining variables were not normally distributed and, instead, nonparametric methods were used; the Wilcoxon signed rank test was used to compare plasma adipokine concentrations prior to and after weight loss, whereas the Kendall rank correlation was used to compare the association between body fat mass and both pre-weight loss insulin concentrations and I:G ratio. The Fisher exact test was used to compare the proportions of samples positive for TNF-α prior to and after weight loss. The level of statistical significance was set at \( P < 0.05 \), and all significance values quoted are 2-sided.

3. Results

3.1. Baseline characteristics of the diet groups

Of the 26 dogs in the study, breeds represented included crossbred (7), Labrador retriever (6), Cavalier King Charles spaniel (4), Yorkshire terrier (4), golden retriever (2), border collie (1), cocker spaniel (1), and German shepherd dog (1). The median (range) age was 83 mo (19-166 mo). Fifteen dogs were male (11 neutered), whereas all of the 11 female dogs were neutered. Full details of the outcome of the weight loss program are given in Table 2. The mean (± SD) percentage of weight lost was 22% ± 9%, whereas the mean
decrease in body fat mass was 43% ± 13%. Twenty-three of 26 dogs reached their target weight and were BCS 5/9 at the end of weight loss. In the other three dogs, the owners chose to discontinue the program early, when they were close to target weight. All of these dogs had lost a significant amount of weight by the time of their follow-up (ie, 14%, 22%, and 28%), and these three dogs were BCS 6/9 at the time of their final assessment. Signalment factors (ie, age, sex, neuter status), type of diet received, and whether or not a dog had reached target weight did not have any significant effect on the plasma concentrations of any of the measured plasma variables (data not shown).

### 3.2. CBC and clinical biochemistry

Table 3 shows the results of CBC and clinical biochemistry before and after weight loss. Although occasional abnormal results were found with most variables, most abnormalities were only mild and unlikely to be of clinical significance. Although mean white blood cell numbers were within reference limits both before and after weight management, weight loss did lead to a significant decrease in cell count \( (P = 0.0041) \). Differential cell counts revealed that this change was a result of significant declines in neutrophils and macrophages (data not shown). Albumin \( (P = 0.016) \), cholesterol \( (P = 0.0062) \), and alkaline phosphatase (ALP, \( P = 0.042 \)) also decreased significantly after weight loss.

### 3.3. Insulin, glucose, and I:G

Prior to weight loss, median fasting plasma insulin concentration was 27 μU/mL (187 pmol/L), with a range of 9-71 μU/mL (63-493 pmol/L), and results from 13 dogs (50 %) were above the assay reference range. Median fasting plasma glucose concentration was 88 mg/dL (4.9 mmol/L), with a range of 74-113 mg/dL (4.6-6.2 mmol/L), and results from four dogs (15 %) were above the assay reference range. However, in all cases glucose concentrations were only marginally elevated and of doubtful clinical significance. Median (range) I:G was 28 (11-66). Percentage body fat prior to weight loss was positively correlated with both fasting plasma insulin concentrations (Kendall \( \tau \) correlation = 0.30, \( P = 0.044 \); Fig. 1) and I:G (Kendall \( \tau \) correlation = 0.36, \( P = 0.022 \); Fig. 2); however, there was no association with fasting plasma glucose concentrations (Kendall \( \tau \) correlation = 0.00, \( P = 0.99 \)).

After weight loss, median fasting plasma insulin concentration was 18 μU/mL (123 pmol/L), with a range of 4-75 μU/mL (27-517 pmol/L), and results from four...
dogs (15%) were above the assay reference range. Median fasting plasma glucose concentration was 91 mg/dL (5.0 mmol/L), with a range of 61-120 mg/dL (3.6-6.6 mmol/L), and again results from four dogs (15%) were above the assay reference range, none of which was at a level likely to be clinically significant. Median (range) I:G was 19 (4-73). Both fasting plasma insulin concentration (Fig. 3; \( P = 0.0037 \)) and I:G ratio (Fig. 4; \( P = 0.0063 \)) decreased significantly with weight loss, by a median of 32% and 40%, respectively. However, there was no significant change in fasting glucose concentrations (Fig. 5; \( P = 0.99 \)).

### 3.4. Plasma acute-phase protein concentrations

Prior to weight loss, median (range) plasma CRP concentration was 2.8 mg/L (0.2-18.0 mg/L), and the results from nine dogs (35%) were above the assay reference range. After weight loss, median (range) plasma CRP concentration was 1.4 mg/mL (0.1-2.0 mg/mL), and the results of four dogs remained above the reference range (15%). Statistical analysis demonstrated that median CRP concentration decreased significantly after weight loss, with a median decrease of 29% (\( P = 0.046 \), Fig. 6).

Median (range) pre- and post-weight loss plasma haptoglobin concentrations were 1.6 g/L (0.1-3.2 g/L) and
1.4 g/L (0.1-2.0 g/L), respectively. Although statistical
analysis demonstrated that haptoglobin concentrations
were significantly lower post-weight loss, with a
median decrease of 22% \( (P=0.014, \text{ Fig. 7}) \), the results
of all dogs were within the assay reference range
throughout the study. Median (range) pre- and post-
weight loss plasma SAA concentrations were 0.2 mg/mL
(0.05-90.0 mg/mL) and 0.2 mg/mL (0.06-1.8 mg/mL),
respectively. The result of one dog (4%) was above
the assay reference range prior to weight loss, whereas
all post-weight loss results were within reference lim-
its. There was no significant difference between pre-
and post-weight loss SAA concentrations \( (P=0.84, \text{ Fig. 8}) \).

3.5. Plasma cytokine and chemokine concentrations

Plasma TNF-\( \alpha \) was above the detectable limit of the
assay in 11 dogs (42%) prior to weight loss, and in only
three dogs (12%) after weight loss (Fisher exact test,
\( P=0.016 \)). The pre-weight loss TNF-\( \alpha \) concentrations
ranged from \( \leq 0.9 \) to 6.6 pg/mL, and two dogs (8%)
had plasma TNF-\( \alpha \) above the assay reference limit. Post-
weight loss plasma TNF-\( \alpha \) concentrations (range \( \leq 0.9-
3.8 \) pg/mL, 0 dogs with plasma TNF-\( \alpha \) above the assay
reference limit) were significantly lower than prior to
weight loss (Wilcoxon signed rank test, \( P=0.002 \)).

Plasma MCP-1 concentrations varied greatly among
dogs in the current study, both before (median
128 pg/mL, range 39-14,600 pg/mL) and after weight
loss (median 103 pg/mL, range 27-22,200 pg/mL). In
contrast to TNF-\( \alpha \), no significant changes in plasma
concentrations of MCP-1 occurred with weight loss
(Wilcoxon signed rank test, \( P=0.32 \)). An attempt was
made to quantify plasma IL-6 concentrations using the
multiplex protocol described above. Most of the sam-
pies were below the detectable limit of the assay (data
not shown) and, as a consequence, differences before and
after weight loss were not assessed statistically.
3.6. Plasma adiponectin and leptin concentrations

Plasma adiponectin concentrations did not change significantly in obese dogs undergoing weight loss (median [range] adiponectin pre-weight loss 10 pg/mL [1-46 pg/mL] vs post-weight loss 11 pg/mL [1-36 pg/mL]; \( P = 0.46 \)). A multiplex assay was used to quantify plasma leptin concentrations but, as with IL-6, a substantial number of the results were below the detectable limit of the assay (data not shown). As a consequence, differences before and after weight loss were not assessed statistically.

4. Discussion

The current study has demonstrated the presence of insulin resistance, as judged by elevated fasting plasma insulin concentration and I:G ratio, in obese dogs prior to weight loss. Nonetheless, although glucose concentra-

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Fig. 8. Box-and-whisker plots demonstrating pre- and post-weight loss plasma serum amyloid A (SAA) concentration in a cohort of obese dogs. The boxes depict median (horizontal line) and interquartile range (top and bottom of box), the vertical lines show range, and outliers are shown as separate points. There was no change in plasma SAA concentration after weight loss ($P = 0.84$).

study in cats has demonstrated that both correlate well with both glucose clamping and minimal model analysis and can thus provide a useful indirect marker of insulin resistance in clinical studies [37].

A second potential advantage of experimental studies is the fact that a homogenous group of animals can be studied in a in a controlled environment. In such studies, a young, sexually intact, healthy, inbred colony population of dogs is overfed to produce a modest degree (typically 15% overweight) of weight gain. The short-term weight gain is then rapidly corrected, in a controlled setting, without the influence of owner factors. Although the use of such a cohort undoubtedly keeps between-subject variability to a minimum, such work may not be reflective of the situation in the domestic canine population. In contrast, the current study group was diverse and representative of the at-risk population typically seen by veterinarians. Obesity was naturally occurring, usually long-standing (in some cases lifelong), patients were more markedly overweight than for a typical experimental study (eg, up to 45% overweight), and a standard weight loss regimen was used for management. Overall, this marked diversity is likely to have increased between-subject variability, with the likely effect of blunting the pre- versus post-weight loss differences. Nonetheless, the results are arguably more representative of the true clinical picture. A final advantage of using experimental animals is that a control group can readily be included for comparison. However, given that the current study used client-owned dogs, a healthy, lean control population (ie, a population that could undergo the same procedures such as blood sampling and body composition analysis) was not available for study. It was also not possible to treat some of our clinical cases with a placebo. Despite this limitation, the fact that we were able to sample dogs both before and after weight loss meant that each individual could act as its own control. Therefore, although our findings should rightly be interpreted with caution, we do still believe them to be valid.

The current study is only the second study of its kind to examine insulin sensitivity in dogs with naturally occurring obesity. A major strength of this work, over the previous study [20], is the fact that degree of adiposity was assessed objectively, namely, using DEXA. In the earlier work, breed standards were used to determine target weight [19], and this method correlates poorly with the degree of adiposity [38]. In contrast, DEXA has reasonably good precision and reliability as a method of body fat mass estimation in dogs [32], and its use in the current study enabled us to show that increasing adiposity correlated with the degree of insulin resistance. Nonetheless, the association was relatively weak (Kendall $\tau = 0.30$-0.36), suggesting that body fat mass may be only one of many factors which may affect insulin sensitivity in dogs. In many respects, these findings are not surprising. Most notably, as detailed above, a diverse outbred population of dogs was included in the study, and we used only indirect “surrogate” markers of insulin sensitivity in dogs. Further, all tests have an inherent degree of imprecision, which may induce additional variability. Finally, the degree of insulin resistance was minor and not likely to be of the same magnitude as that likely to be observed with clinical type 2 diabetes. Thus, the most we could achieve would be subtle improvements in insulin sensitivity. Despite these issues, our findings agree with some [39], but not all [40], previous studies that have demonstrated that insulin concentrations decrease after weight loss in dogs made obese in an experimental setting.

In addition to the weak association between insulin sensitivity and adiposity, improvements in insulin sensitivity, as judged by fasting plasma insulin concentration and I:G ratio, were documented after weight loss in pet
dogs with naturally occurring obesity. It is of particular note that the degree of reduction in plasma insulin concentrations (32% decrease from pre-weight loss concentrations) is of a magnitude similar to that seen in a previous experimental study (27% decrease) in which dogs lost a similar amount of weight [39]. Further, these findings parallel those of human studies, where a 38% decrease in plasma insulin concentration was seen when patients lost a similar (25%) proportion of body weight in the 12 months after bariatric surgery [41].

The significant decrease in plasma cholesterol concentrations may also provide further evidence of an improved metabolic status of these dogs on weight loss, although since the majority of results were within the reference range, its clinical significance is not known. Nonetheless, similar findings have been seen in other canine studies, with increases in cholesterol and triglycerides noted, but again not exceeding the upper limit of the reference range [40,42,43]. It is also possible that the difference seen was simply because of the change to a low-fat diet. Further work, for example by examining lipoprotein profiles, would be required to determine the significance of this result.

A number of putative adipokines were also analyzed in the current study, including cytokines (TNF-\(\alpha\), IL-6), chemokines (MCP-1), acute-phase proteins (CRP, haptoglobin, SAA), leptin, and adiponectin. We were able to document alterations in some analytes, but results were variable for other adipokines. The reason for these variable results is not known but might be the result of study methodology and poor specificity of the assays. In this respect, for some of the assays we used, the product guidelines recommended that samples be centrifuged and frozen within 30 minutes of collection; although this recommendation was achieved for the majority of our samples, the additional delay for other samples may have lead to unreliability in some results. Further, we used heparinized plasma in the current study, but the recommendation for the Linco-plex system is to use EDTA plasma. Again, this difference may have contributed to variability in results. Finally, there may have been issues with cross-reactivity of some assays, particularly with regard to the analytes measured in the Linco-plex system (see below).

Despite these concerns, this study was able to demonstrate that weight loss, in pet dogs with naturally occurring obesity, is associated with decreases in the circulating level of the inflammatory markers TNF-\(\alpha\), haptoglobin, and CRP. As reported in previous studies, circulating TNF-\(\alpha\) concentrations in healthy dogs were typically low and often at or below the detection limit of the assay [15,44]. In the current study, we found that detectable concentrations of TNF-\(\alpha\) were more often found in obese dogs prior to weight loss than after weight loss. Further, a proportion of the obese dogs had TNF-\(\alpha\) concentrations above the assay reference range, but all such values had normalized after weight loss. These results are similar to those of a previous laboratory study, which demonstrated that increases in plasma TNF-\(\alpha\) concentrations occur in dogs made experimentally obese through overfeeding [15]. The role of TNF-\(\alpha\) in insulin resistance is well established in other species: for instance, studies in obese rodents have shown that neutralizing TNF-\(\alpha\) production from WAT leads to improved insulin sensitivity [45]. Further, in obese human beings, diet therapy and drug treatment have both been shown to improve insulin sensitivity and correlate with a decreased adipose tissue production of TNF-\(\alpha\) [46]. The concurrence of decreased insulin resistance and reductions in plasma TNF-\(\alpha\) concentrations with weight loss in the dogs of this study may suggest that, as in humans, TNF-\(\alpha\) is causally linked to the insulin resistance seen in canine obesity.

In humans, adipose tissue production accounts for 10% to 30% of systemic levels of IL-6 [47]. Plasma IL-6 concentrations are elevated in human obesity [48], and this cytokine is thought to play a direct role in insulin resistance by altering insulin signaling in hepatocytes [49]. Our previous studies have demonstrated IL-6 expression by canine adipocytes differentiated in primary culture [28], but no changes in plasma IL-6 concentrations were seen in the present study after weight loss. This finding may result from concerns over assay reliability and sample handling as detailed above. An alternative explanation is that plasma IL-6 concentrations were undetectable in many of the samples, may suggest that normal circulating levels for IL-6 are low in this species. Indeed, in a recent canine study examining pro-inflammatory cytokine responses in dogs with sepsis, plasma IL-6 concentrations were similarly undetectable in all 12 healthy control dogs, and in a significant number of dogs that survived during the study [50]. Further canine-specific work is, therefore, required to study the true significance of this cytokine in canine obesity.

Work in human beings has demonstrated that there are derangements in circulatory acute-phase protein concentrations in obesity. C-reactive protein concentration is elevated in obese patients [51,52] and in those with features of the metabolic syndrome [53,54]. Gene expression for haptoglobin has been documented in both human and rodent white adipocytes, with up-regulation in obesity [55,56]. Previous work in dogs has demonstrated that gene expression for both of these acute-phase proteins is seen in canine adipose tissue and, more
specifically can be synthesized by canine adipocytes themselves [28]. In the current study, weight loss in obese dogs was associated with modest reductions in both CRP and haptoglobin concentrations of a magnitude similar to the reductions seen during weight loss (30%) in obese, insulin-resistant women [57]. However, given that most of the acute-phase protein concentrations measured were within the reference limits of the assay, the clinical relevance of these results is unclear. Nonetheless, it is tempting to speculate that, as in humans, obesity predisposes to a state of subclinical inflammation [23], which can be resolved by weight loss [27]. Such a theory of subclinical inflammation would be supported by the fact that the median leukocyte count was in the upper-end reference range prior to weight loss, and it declined significantly after weight loss.

Human adipocytes have been shown to secrete the chemokine MCP-1 [58], and circulating concentrations are elevated in human obesity [59], although the effect of subsequent weight loss has not yet been examined. The current study also attempted to examine the effect of weight loss in obese dogs, on circulating concentrations of a number of other adipokines including MCP-1, adiponectin, and leptin. Our previous work has demonstrated gene expression for all of these adipokines in all 5 of the main WAT depots in the dog, whereas canine adipocytes also express mRNA in primary culture [28,29]. Weight loss in obese dogs had no significant effect on circulating levels of this chemokine. However, high variability was noted in MCP-1 concentrations, which may in part have been related to sample handling and issues with cross-reactivity of the assay used. Given the degree of variability encountered among study dogs, we cannot exclude the possibility of a type II statistical error. In this respect, based on the inherent variability we have identified in circulating MCP-1 concentrations, we would estimate that a future study would need to include paired results from approximately 400 dogs to achieve an 80% chance of finding a difference, if one existed (data not shown). Thus, more work is required to examine the role of this adipokine in obesity-associated inflammation in this species.

Unlike most adipokines, circulating adiponectin concentrations in humans are inversely related to body weight, with reduced expression occurring in obesity and type 2 diabetes mellitus [60]. A previous study has shown that, in a manner similar to humans, plasma adiponectin concentrations in dogs are inversely correlated with increasing body weight, and weight loss results in a rise in serum adiponectin concentrations [61]. The plasma concentrations observed in the current study (1.3-46.0 μg/mL) were of an magnitude equivalent to those found in this study [61], although other authors have reported much lower serum concentrations (0.85 to 1.5 μg/mL) [62]. The reason for such discrepancies is not clear but could suggest differences in assay sensitivity for the different circulating forms. In humans, adiponectin is known to circulate as 3 discrete protein complexes in humans [63], and both low-molecular-weight and high-molecular-weight forms are suspected in dogs [62]. Differences in assay sensitivity could also explain why, in the current study, no change in adiponectin concentration was seen during weight loss. Further studies are recommended, therefore, to clarify the role of this adipokine in canine obesity and its disease associations.

Using assays that are different from that employed here, previous studies have documented measurable plasma concentrations of leptin in dogs [39,64]. However, in many cases, we were unable to detect leptin, which likely reflects the sensitivity of the assay; there is also a suspicion that the multiplex assay that we used only detects free (unbound) circulating leptin (E. Bensen, personal communication). Previous work on leptin in dogs has used a species-specific antibody that is not commercially available [39,64], and unfortunately, this assay was not available for the current study.

In conclusion, we have confirmed the presence of insulin resistance in obese dogs, shown that its severity correlates with the degree of adiposity, and demonstrated that improvements occur after weight loss. In the same population, the reduction in fat mass also leads to decreases in TNF-α, suggesting that, in dogs as in humans, this adipokine might be implicated in the insulin resistance of obesity. Other changes observed during weight loss include reductions in both haptoglobin and CRP concentrations, suggesting that canine obesity might be associated with a subclinical inflammatory state, which improves when normal adipose mass is restored.

Conflict of interest statement

The following conflicts of interest apply: A.J.G.’s senior lectureship is funded by Royal Canin; the diet used in this study is manufactured by Royal Canin; P.J.M. is an employee of WALTHAM; and V.B. is employed by Royal Canin.

Acknowledgments

The authors wish to acknowledge the referring veterinarians for referring cases, the owners of all dogs for allowing them to participate, and the clinical staff at the University of Liverpool for assistance with case.
management. Renaud Sergheraert and John Rawlings are acknowledged for their assistance. The contribution made by each author is as follows: A.J.G: designed study, collected clinical data, analyzed results, drafted paper; S.L.H: collected clinical data, reviewed manuscript; M.H.: performed plasma adipokine assays, analyzed results, reviewed manuscript; L.H.: performed plasma adipokine assays, reviewed manuscript; P.J.M.: designed study, reviewed results, reviewed manuscript; V.B.: designed study, reviewed results, reviewed manuscript; P.T.: reviewed results, reviewed manuscript. The study was funded by a grant from WALTHAM (VCR10030). A co-author employed by the funders was directly involved in the study (see above).

References


