

UNIVERSIDAD DE MURCIA



FACULTAD DE VETERINARIA

**Metabolic and inflammatory biomarkers
in canine and feline obesity
and associated diseases**

**Biomarcadores metabólicos y de inflamación
en la obesidad canina y felina
y enfermedades asociadas**

Memoria presentada por
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1. Introduction

Obesity is the most common nutritional disorder in dogs and cats (Roudebush et al., 2008; Lusby et al., 2009) being a growing concern nowadays. Its increasing incidence appears to be mirroring the trend observed in humans (Rennie and Jebb, 2005). In humans almost two-thirds of adults in the United States are overweight or obese (Flegal et al., 2002). Similarly, studies in companion animals from various parts of the world have estimated the incidence of obesity in the dog and cat population to be between 22 and 40% (Burkholder et al., 2000; McGreevy et al., 2005).

Main *risk factors* associated with weight gain in dogs and cats are: 1) uncontrolled food intake producing an excess calories intake, 2) lack of activity and exercise, 3) spaying/neutering leading to reduced metabolic rate, and 4) breed pre-disposition (Gossellin et al., 2007). Excess of body weight in dogs and cats is associated with endocrine, cardio-respiratory, orthopaedic or other diseases (Kopelman, 2000; German, 2006). There is also evidence of obesity-associated shortened lifespan in dogs (Kealy et al., 2002; Lawler et al., 2008). Obesity can *predispose to disease* by two main mechanisms (German et al., 2010): firstly, the deposition of excess fat can have mechanical/physical effects that exacerbate orthopaedic disease, constrict upper airways, restrict grooming and reduce heat dissipation. Secondly, and most importantly, the perturbation of the normal endocrine function of white adipose tissue can trigger a number of associated conditions, such as insulin resistance, and increased blood pressure among others.

Adipose tissue is not only a storage organ for excess energy, but an active endocrine organ known to produce and release a number of bioactive proteins. Proteins, hormones, and cytokines derived from adipose tissue are called *adipokines* (Halberg et al., 2008). Adipokines may contribute to the regulation of diverse biological processes, including energy balance, metabolism, inflammation and immune function, hematopoiesis, and cell proliferation and angiogenesis (Lago et al., 2007). Although many adipokines have been discovered, extensive research has been conducted in only a few of them, being the three most studied *leptin*, *adiponectin*, and *ghrelin* (Lusby et al., 2009). In companion animals, similarly as in humans, increasing adiposity is related to increased plasma leptin concentration (Sagawa et al., 2002) and decreased ghrelin concentration (Hoenig et al., 2007). Furthermore, weight loss results in decreased leptin concentrations (Jeusette et al., 2006; Hoenig et al., 2007) whilst the ghrelin concentration are increased (Jeusette et al., 2005).

There are also some other analytes that, although their primary production site is not fat, are intimately related with obesity. One example is *insulin-like growth factor 1* (IGF-1), a peptide hormone which is synthesized in the liver. It is involved in the regulation of growth and metabolism, and mediates many of the anabolic effects of growth hormone in different tissues (Klapper et al., 1983). Low concentrations of IGF-1 have been associated with insulin resistance in both humans (Janssen et al., 2002) and cats (Reusch et al., 2006). Nutrition also plays an important role in the regulation of IGF-I, with both caloric and protein restriction known to decrease IGF-1 concentration in humans and a variety of other species (Prewitt et al., 1982).

When data about obesity in companion animals were revised before starting this PhD work, 4 areas in which a gain of knowledge could benefit the progress and advances in the understanding of this disorder were detected:

- A. Validation studies of commercially available assays for determination of the main adipokines (leptin, adiponectin, and ghrelin) and other analytes related with obesity such as IGF-1.** For determination of these analytes in dog or cat sera, only radioimmunoassay (RIA) methods (Jeusette et al., 2005) or locally developed ELISAs existed (Ishioka et al., 2006). Situation that limited the use of these biomarkers for research studies in dogs and cats.
- B. Possible variations of adipokines and analytes related to obesity due to other but obesity factors.** For example diurnal variation, effect of food intake, castration, renal failure, and sepsis.
- C. How obesity and obesity-associated disorders can influence adipokines, acute phase proteins (APPs) and other analytes.** When reviewing literature two controversies and three aspects not studied were detected in this point:
 - Contradictions were found about: (1) the existence of relationship between the adipokine adiponectin and canine obesity. Some authors described a negative association between adiponectin and canine obesity (Ishioka et al., 2006), similarly as occur in humans (Chandran et al., 2003), while others deny it (Verkest et al., 2011). (2) Also the link between obesity and inflammation remains controversial in human and veterinary medicine. For example, in humans an increase C-reactive protein (CRP) in obesity has been described. However, this increase in many cases is mild and inside the range of non-inflammatory values (Esposito et al., 2003). In the dog a similar situation occurs, since very mild increases and even decreases in CRP has been described in obese individuals (Veiga et al., 2008; German et al., 2009).

- The three not studied aspects were: (1) existence of metabolic syndrome (MS) in obese dogs. (2) association between excess adiposity and renal functional alterations in dogs. (3) the behaviour of adipokines and inflammatory markers in obesity-related diseases, such as hypothyroidism.

D. Study and identification of new potential serum biomarkers involved/linked to obesity. In human medicine, two enzymes: paraoxonase type 1 (PON1) and butyrylcholinesterase (BChE), were found to be affected in obesity. BChE activity is increased (Randell et al., 2005), while PON1 activity is decreased in this situation (Kotani et al., 2009). Both enzymes seem to be functionally related since BChE is inherently protected from oxidative stress by PON1 (Ofek et al., 2007). However, to our knowledge, no studies have examined BChE or PON1 in canine or feline obesity.

In addition, in order to identify new potential serum biomarkers involved/linked to obesity, proteome analysis had been performed in studies of obesity in humans and experimental animal models such as mice and rats (Dayarathna et al., 2008), but not in dogs or cats.

2. Objectives

This PhD Thesis was designed to try to produce advances in the areas mentioned in introduction section and therefore its main objectives were:

1. To validate commercially available assays for adipokines and IGF-1 for use in dogs and cats (Articles 2-6).
2. To analyze possible causes of the variations due to other but obesity factors, (such as diurnal variation, effect of food intake, castration, renal failure, and sepsis). For this purpose the analytes that gave adequate validation results in point 1 and were more economic, easy and fast to perform would be selected (Articles 7-10).
3. To clarify the possible effect of obesity on adiponectin and acute phase proteins in dogs and cats. Also to explore if obese dogs suffer from metabolic syndrome, evaluate if obesity could be related with changes in renal biomarkers, and to study how adipokines and acute phase proteins are affected by an obesity-related disease, such as hypothyroidism (Articles 11-17).
4. To study and identify new potential serum biomarkers involved/linked to obesity (Articles 18-23).

3. Articles

3.1 Review of methods for measuring the degree of obesity in dogs

3.1.1. Article 1

MÉTODOS PARA MEDIR EL GRADO DE LA OBESIDAD EN PERROS: ENTRE LA FÍSICA Y LA BIOQUÍMICA

Methods for obesity grade measurement in dogs: between physics and biochemistry

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RESUMEN

La obesidad se define como un acumulo excesivo de tejido adiposo en el cuerpo. La causa de obesidad normalmente es una excesiva ingesta o un metabolismo inadecuado, que ocasiona un balance positivo de energía. Además de las múltiples enfermedades que se asocian con la obesidad en el perro, se ha visto que los animales obesos viven hasta dos años menos en comparación con animales sanos. Esto hace que, hoy en día, sea de gran importancia la correcta determinación, valoración y tratamiento de los perros obesos.

Para medir grado de obesidad se han descrito muchos métodos que pueden ser divididos en dos grupos: físicos y bioquímicos. En este trabajo se van a analizar los métodos mas utilizados en veterinaria para medir grado de obesidad en el perro; así dentro de los métodos físicos se estudiarán el peso corporal, las medidas antropométricas, escalas morfológicas, absorciometría de rayos X de doble energía (DXA) y dilución de isótopos de oxido de deuterio (D_2O). Por otra parte dentro de los métodos bioquímicos, nos centraremos en dos proteínas que en el futuro podrán ser utilizados como biomarcadores de la obesidad: la leptina y adiponectina.

Palabras clave: obesidad, perro, métodos, leptina, adiponectina.

ABSTRACT

Obesity is defined as an exesive accumulation of adipose tissue in the body. Frequently the main cause of obesity in dogs is an execive ingest or an inadequate metabolism, that causes positive energy balance. Besides of the high number of associated diseases that obesity can cause, longevity of obese animals is two years shorter than normal weight animals. For these reasons the correct determination, valoration and treatment of obesity in dogs is of great importance nowadays in routine practice.

In this review we will deal with several methods for obesity grade measurement that can be divided into two groups: physical and biochemical. In physical we will include body weight, morphometric methods, tape measurements, dual X-ray absorptiometry (DXA), deuterium isotope dilution (D_2O). Regarding the biochemical methods, we will focus on two proteins: leptin and adiponectin that in the future could be used as biomarkers of obesity in canine species.

Keywords: obesity, dog, methods, leptin, adiponectin.

INTRODUCCIÓN

La obesidad es la enfermedad nutricional mas frecuente en los perros y se define como una acumulación excesiva de tejido adiposo en el cuerpo (Burkholder y Toll, 2000). De forma similar a lo que ocurre en humana, la obesidad en el perro es un problema en continuo aumento, afectando a entre el 22 y 40 por ciento de la población según diversos estudios (McGreavy et al., 2005). Es importante detectar y controlar la obesidad, ya que los perros obesos tienen más riesgo de padecer problemas de salud tales como enfermedades ortopédicas traumáticas y degenerativas, cardiovasculopatías, enfermedades dermatológicas, distocias, descenso de la tolerancia al calor y ejercicio; predisposición a diabetes mellitus, hipertensión, hiperlipidemia, descenso de respuesta inmune y aumento del riesgo en anestesia (Edney y Smith, 1986; Fettman et al., 1997; Burkholder 2001; Kuruvilla y Frankel, 2003; Ishioka et al., 2002).

En humana se han establecido criterios para definir el “sobrepeso” y la “obesidad”. Estos criterios normalmente están basados en medidas de tejido adiposo tales como el índice de masa corporal (BMI, body mass index) que se calcula en base a la relación entre el peso y la altura ($BMI = \text{peso (kg)} / \text{altura}^2 \text{ (m)}$). Por ejemplo, un individuo adulto se define con sobrepeso cuando su $BMI > 25 \text{ kg/m}^2$, y obeso cuando sobrepasa 30 kg/m^2 (Eknoyan, 2008).

Sin embargo, en el perro todavía no se ha definido un “gold standard” para determinar el grado de obesidad. Así, existen numerosos métodos descritos, algunos muy simples, que pueden ser utilizados en la práctica clínica ha-

bitual, y otros más complejos que necesitan un equipamiento específico y que se realizan en centros de investigación. El objetivo de este trabajo será describir los métodos actualmente más utilizados hoy en día en veterinaria para detectar y medir el grado de la obesidad canina y los que pueden tener más potencial en el futuro. Dividiremos los métodos en físicos y químicos, y realizaremos un énfasis especial en estos últimos, basados en la medición de metabolitos relacionados con la obesidad, que han sido descubiertos en los últimos años y que pueden tener un futuro prometedor en la clínica de pequeños animales.

MÉTODOS FÍSICOS

Existen numerosos métodos para medir el grado de obesidad en animales de compañía (Tabla 1). El objetivo de todos estos métodos es comparar la cantidad de grasa con la masa de los tejidos corporales. Desde un punto de vista práctico, se pueden dividir en:

Métodos que no necesitan equipos complejos:

1 Peso corporal. El peso corporal del perro se compara con el peso óptimo de la raza y se calcula el porcentaje de aumento o descenso del peso del animal. Es muy importante que las condiciones de pesado del animal estén estandarizadas: al mismo tiempo del día, con el mismo sistema de peso y por la misma persona. En perros se indica que hay sobrepeso cuando pesan un 15% (Laflamme 2001; Simpson et al., 1993) más de su “peso óptimo”, y obesos cuando sobrepasa el 30% (Burkholder y Toll, 2000).

Tabla 1. Métodos de análisis de composición corporal en perros y gatos (adaptada de German et al., 2006 b

Métodos de análisis de composición corporal en perros y gatos más utilizados en veterinaria
<i>Métodos físicos</i> Peso corporal Medidas antropométricas Escala morfológicas: -Escala de 9 puntos -Escala de 5 puntos -Sistema 7 letras o S.H.A.P.E (size, health and phisical evaluation)¹⁹ Absorciometría de rayos X de doble energía (DXA o DEXA) Dilución de isótopos de oxido de deuterio (D₂O) <i>Métodos químicos</i> Leptina Adiponectina

Sin embargo, estos criterios no han sido confirmados con un estudio epidemiológico riguroso, y tampoco hay datos suficientes y fiables sobre el peso ideal de los perros de raza, además los perros mestizos carecen de estándar y por lo tanto no se puede saber su peso ideal (German 2006 a).

2 Medidas antropométricas. En perros las medidas antropométricas se toman utilizando una cinta de medir graduada en centímetros (Burkholder y Toll, 2000; Pendergrass et al., 1982). Las medidas que se suelen utilizar son: la altura del perro a nivel del hombro; la longitud desde el centro de la parte craneal de la escápula hasta base de cola; la longitud desde la protuberancia occipital hasta la base de cola; perímetro a nivel del flanco; y la longitud desde tuberosidad del calcáneo hasta el ligamento patelar (Burkholder y Toll, 2000). Estos datos permiten calcular:

- Índice de masa corporal adaptado para perros (BMI) = peso corporal (PC)_{kg} / (altura a nivel del hombro_{cm} × longitud desde la protuberancia occipital hasta la base de cola_{cm}).

- Masa de grasa corporal (GC), que se calcula dependiendo del sexo del animal:
Machos (%GC) = -1.4 (longitud desde tuberosidad del calcáneo hasta ligamento patelar_{cm}) + 0.77 (alrededor del flanco_{cm}) + 4
Hembras (%GC) = -1.7 (longitud desde tuberosidad d calcáneo hasta ligamento patelar_{cm}) + 0.93 (alrededor del flanco_{cm}) + 5

Se ha apreciado que existe una correlación entre estas medidas morfométricas y la cantidad de grasa corporal (Burkholder y Toll, 2000; Pendergrass et al., 1982). Incluso se ha visto correlación baja, aunque significativa, entre las medidas antropométricas y los datos obtenidos con absorciometría de rayos X de doble energía (DXA) ($r^2=0.54$, $P<0.001$) (Mawby et al., 2004).

Las medidas antropométricas parecen ser más objetivas para ver la composición corporal que la utilización de otros sistemas como los de puntos, pero sus principales problemas radican en la gran variedad de tamaños y formas de perros, y en el hecho de ser un método laborio-

so que hace que en numerosas ocasiones no se pueda utilizar de forma rutinaria en la clínica veterinaria.

3 Escalas morfológicas. Estos sistemas se basan en evaluar la obesidad en base a unas características morfológicas externas (Tabla 2). Hay métodos que otorgan a los perros un valor numérico en base a escalas de 5 o 9 puntos, llamados sistemas de condición corporal (BCS, body condition score) y también se puede utili-

zar un sistema basado en 7 letras para valorar el estado corporal del animal (German et al., 2006 a) (Tabla 3). Todos estos sistemas incluyen características visuales y palpables como la grasa subcutánea, grasa abdominal y musculatura superficial, y por esta razón son muy subjetivos; pero se ha demostrado que los resultados obtenidos por DXA y por los sistemas de 9 puntos y 7 letras tienen correlación significativa (German 2006 a; Laflamme 1997).

Tabla 2. **Sistemas de evaluación corporal de cinco y nueve puntos en perros (Tams 2003).**

Rasgo	Descripción	5 puntos	9 puntos
Caquéctico	Las costillas se palpan con facilidad sin cobertura grasa; las estructuras óseas son prominentes y de fácil identificación; tono y masa musculares a menudo deprimidos; poco o nada de grasa subcutánea; manto piloso de mala calidad; abdomen muy recogido.	1	1
Subpeso	Las costillas se palpan con facilidad con escasa cobertura grasa; abdomen recogido; estructuras óseas palpables pero no prominentes; manto piloso de mala calidad; tono y masa musculares normales o algo deprimidos.	2	3
Ideal	Las costillas se palpan con facilidad, pero hay cobertura grasa; forma de reloj de arena y abdomen recogido, pero no pronunciado; las prominencias óseas son palpables pero no visibles; hay grasa subcutánea pero no grandes acumulaciones; tono y masa musculares normales; manto piloso de buena calidad.	3	5
Sobrepeso	Las costillas se palpan con dificultad debido a la acumulación de grasa superpuesta; la forma de reloj de arena no es prominente, abdomen no recogido; grasa subcutánea evidente en algunas áreas de acumulación; tono y masa musculares normales; la calidad de manto piloso puede estar reducida; no se pueden identificar prominencias óseas.	4	7
Obeso	Las costillas son imposibles de palpar debido a la grasa superpuesta; falta la forma de reloj de arena y el animal puede tener apariencia redondeada; la grasa subcutánea es evidente y hay acumulaciones en el cuello, base del rabo y región abdominal; tono y masa musculares pueden estar reducidos; la calidad del manto piloso puede estar deprimida.	5	9

Tabla 3. Sistema de 7 letras para evaluar condición corporal en perros (adaptada de German et al., 2006a).

Pasando los dedos en la dirección contraria del crecimiento de los pelos sin utilizar la presión se notan la caja torácica, columna y huesos de la cadera prominentes.	A	EXTREMA DELGADEZ
Pasando los dedos en la dirección contraria del crecimiento de los pelos sin utilizar la presión se notan la caja torácica y columna, pero los huesos de la cadera no son evidentes.	B	MUY DELGADO
Pasando los dedos en la dirección contraria del crecimiento de los pelos sin utilizar la presión se notan solamente las costillas, pero ni la columna, ni los huesos de la cadera no se notan.	C	DELGADO
Pasando los dedos en la dirección contraria del crecimiento de los pelos utilizando leve presión se puede notar las costillas. No existe capa de grasa encima de las costillas.	D	IDEAL
Pasando los dedos en la dirección contraria del crecimiento de los pelos utilizando leve presión se puede notar las costillas. Existe pequeña capa de grasa encima de las costillas. Acariciando animal por debajo de cavidad torácica y los lados se nota concavidad delante de las extremidades posteriores y concavidad en la cintura	E	LIGERAMENTE OBESO
Pasando los dedos en la dirección contraria del crecimiento de los pelos utilizando leve presión se puede notar las costillas. Existe importante capa de grasa encima de las costillas. Acariciando animal por debajo de cavidad torácica no se nota concavidad delante de las extremidades posteriores, pero se nota la concavidad en la cintura.	F	MODERADAMENTE OBESO
Pasando los dedos en la dirección contraria del crecimiento de los pelos utilizando leve presión no se puede notar las costillas. Existe gran cantidad de grasa encima de las costillas. Acariciando animal por debajo de cavidad torácica no se nota concavidad delante de las extremidades posteriores, ni la concavidad en la cintura.	G	SEVERAMENTE OBESO

Métodos que necesitan equipos complejos

1 Absorciometría de rayos X de doble energía (DXA). Se basa en el uso de dos niveles diferentes de energía de rayos X (70 y

140 kVp) para diferenciar el tipo y cantidad de cada tejido en la parte de cuerpo escaneado. Así permite diferenciar tejidos corporales: masa de grasa corporal, masa de tejidos no grasos sin hueso, contenido mineral de hueso, y densidad

ósea. El uso de DXA en perros ha sido validado por Lauten et al. (2001), apreciando una buena correlación entre los datos obtenidos por esta técnica y los niveles de grasa corporal obtenidos durante necropsia. En la Tabla 4 aparece breve descripción de la técnica de DXA aparece en la Tabla 4.

2 Técnica de dilución de isótopos de oxido de deuterio (D₂O) esta basada en el hecho de que el agua corporal está asociada predominantemente con los tejidos no-grasos del cuerpo; por esto la medición de agua total corporal mediante D₂O proporciona una estimación indirecta

de la masa de los tejidos no-grasos. Esta técnica ha sido aplicada en el perro por Burkholder y Thatcher (1998) y se describe en Tabla 5.

MÉTODOS QUÍMICOS

1 Leptina

Metabolismo y función. La leptina es una proteína, sintetizada y secretada por el tejido adiposo como respuesta al balance energético positivo. Estudios en humana y animales de laboratorio confirman que la leptina, aparte de en

Tabla 4. Breve descripción de realización de técnica DXA

Para efectuar el procedimiento, los perros tienen que ser anestesiados o sedados profundamente para evitar movimientos ya que el proceso dura unos 20 minutos. Se utiliza el programa para personas adultas de todo el cuerpo, ya que se ha visto que es adecuado para los perros de más de 10 kg (Speakman et al., 2001). El porcentaje de masa grasa se calcula según la formula siguiente (g son gramos):

$$[\text{Masa de la grasa(g)} / (\text{masa de la grasa(g)} + \text{masa de los tejidos no grasos sin hueso(g)} + \text{contenido mineral de hueso(g)}) \times 100]$$

Tabla 5. Breve descripción de realización de técnica D₂O

El óxido de deuterio se administra intravenoso (IV) a dosis de 0.275 g/PC_{kg}, (PC es el peso corporal). Este compuesto es estable, no tóxico y se intercambia con agua fácilmente.

Después de dos horas post-inyección se saca sangre para análisis con espectroscopía de resonancia magnética nuclear (RMN) (Son 1998). El agua total corporal se calcula según la siguiente formula:

$$\text{Agua total corporal (g)} = (\text{g D}_2\text{O inyectado}) - (m_0 - m_1) \times ((D_1 - D_0)/100) \times 0.985 \times 18/20 \div (D_1 - D_0)/100$$

Donde m_0 = peso del perro justo antes de inyectar D₂O; m_1 = peso del perro justo antes de sacar muestra de sangre; D_1 = % de los átomos de D₂O en el plasma obtenido después de administración de D₂O y equilibrio de de D₂O; D_0 = % de los átomos de D₂O en el plasma antes de administrar la dosis de D₂O; 0.985 = corrección de la incorporación de deuterio a los constituyentes orgánicos no cambiables; 18/20 = factor de corrección de la diferencia de peso molecular de H₂O y D₂O. Asumiendo que tejidos no-grasos tienen 73.2% de humedad, el porcentaje de grasa corporal (%GC) se calcula con la siguiente formula:

$$\%GC = 100 - \% \text{ agua corporal total}/0.732$$

Donde 0.732 es factor de corrección.

tejido adiposo, también se sintetiza en otros órganos como placenta, ovarios, músculo esquelético, estómago, hígado, y glándula pituitaria (Muio y Lynis, 2002; Ishioka et al. 2006). La leptina actúa como estímulo aferente inhibitorio del centro de la saciedad y afecta a los circuitos centrales del hipotálamo, bajando la ingesta de alimento y aumentando el gasto de energía. Así la leptina controla los depósitos de grasa corporal y el balance energético (Friedman y Halaas, 1998).

Aparte de los efectos neuroendocrinos mencionados anteriormente, en humana se ha visto que la leptina se une a receptores en pulmones, intestino, riñones, hígado, piel, estómago, corazón, bazo y otros órganos (Dal Farra et al., 2000; Masuzaki et al., 1997; Smith-Kirwin et al., 1998; Bado et al., 1998; Bado et al., 2005), actuando en la regulación de la pubertad y reproducción, aumentando la resistencia a la insulina en las células musculares y hepáticas, previniendo la deposición ectópica de los lípidos, y vinculando el sistema endocrino e inmune para la reparación de la piel (Bjorbaek y Kahn, 2004; Margetic et al., 2002).

Se ha observado un aumento de la expresión de la leptina en casos de incremento de grasa corporal en distintas especies como humanos, roedores y perros (Frederich et al., 1995b, Considine et al., 1996, Ishioka et al., 2002). Los niveles de leptina en sangre en perros con sobrepeso son aproximadamente el doble de los valores de animales con peso normal y en los obesos puede llegar a aumentar hasta 3.5 veces (Ishioka et al., 2006, Juesette et al., 2006). Los niveles de leptina sérica también pueden aumentar secundariamente a la acción de hormonas (como insulina, estrógenos, glucocorticoides) o mediadores inflamatorios (como α TNF, IL-1) (Margetic et al., 2002). En contraste, se ha apreciado una disminución de los niveles de leptina como respuesta a los agonistas de beta-adrenoreceptores, andrógenos, frío, thiazolidinediones, y al humo del tabaco (Margetic et al., 2002).

Niveles normales. Los niveles normales de leptina obtenidos por diferentes autores aparecen en la Tabla 6. En el estudio realizado por Sagawa et al. (2002) con 20 beagle hembras con BCS 3/5, los niveles de leptina en sangre fueron de 0.59 ± 0.9 ng/ml. Sin embargo en el estudio de Ishioka et al. en 2007 con 166 animales la concentración de leptina en perros con BCS 3/5, fue mas alta, de 3.0 ± 0.4 ng/ml; aunque en algunos de los animales incluidos la concentración de leptina era más baja que el límite de detección. Las diferencias entre los niveles de leptina en sangre obtenidos por diferentes autores se pueden atribuir al diferente tiempo transcurrido después de la última comida antes de la extracción de la sangre para el análisis. Ya que durante la inanición, las concentraciones de leptina en suero bajan, pero suben en unas horas después de la ingesta de comida (Ishioka et al., 2005; Ishioka et al., 2006). Nishii et al. (2006) han descrito que los niveles de leptina empiezan subir a las dos horas y alcanzan el máximo (528% en comparación con los niveles en ayunas) seis horas después de la última comida.

Influencias fisiológicas:

Sexo. Está descrito que en humanos (Ostlund et al., 1996) y ratones (Frederich et al., 1995a) las concentraciones de leptina son más altas en las hembras. La causa no está muy clara, pero se sospecha que la diferencia puede ser causada por la síntesis de leptina en tejidos no-adiposos. Así, existen publicaciones que indican que la leptina se sintetiza en la placenta (Masuzaki et al., 1997) y glándula mamaria de las mujeres (Smith-Kirwin et al., 1998) y en el estómago de las ratas (Bado et al., 1998; Bado et al., 2005). En perros no se han detectado otras fuentes de síntesis de leptina distintas al tejido adiposo, pero los órganos analizados no incluyeron ni placenta, ni el estómago (Iwase et al., 2000). De todas formas en perros tampoco se observó diferencia significativa de los nive-

Tabla 6. Niveles de leptina y adiponectina en animales sanos y obesos.

	Estado (BCS)	Numero de animales	Peso, kg	Leptina, ng/mL	Adiponectina, μ g/mL	Referencia
Perros con obesidad inducida experimentalmente	Sanos	5	12.5 $\pm$ 0.3	2.4 $\pm$ 1.2		Ishioka et al. 2002
	Obesos	5	14.3 $\pm$ 0.2	4.9 $\pm$ 0.9		
	Sanos	22	10.1 $\pm$ 4	2.0 $\pm$ 0.4	37.7 $\pm$ 2	Ishioka et al. 2006
	Obesos	22	13.8 $\pm$ 0.6	5.8 $\pm$ 0.7	28.1 $\pm$ 2.3	
	Sanos	4	13.6 $\pm$ 0.8	4.8 $\pm$ 2.1		Jeusette et al. 2006
	Obesos	4	17.8 $\pm$ 0.6	10.9 $\pm$ 3.7		
	Sanos	10	9.3 – 29.5		0.85 – 1.5 (media 1.22)	Brunson et al. 2007
Perros que se presentaron en clínica	Sanos (3/5)	34			33.4 $\pm$ 2.9	Ishioka et al. 2006
	Con sobrepeso (4/5)	20			24.0 $\pm$ 2.8	
	Obesos (5/5)	17			16.8 $\pm$ 3.5	
	Sanos (3/5)	45		3.0 $\pm$ 0.4		Ishioka et al. 2007
	Con sobrepeso (4/5)	46		8.6 $\pm$ 0.7		
	Obesos (5/5)	75		12.8 $\pm$ 0.8		
	Sanos (3/5)	36		2.7 $\pm$ 0.3		Ishioka et al. 2002
	Con sobrepeso (4/5)	8		9.7 $\pm$ 0.7		
	Obesos (5/5)	11		12.3 $\pm$ 1.5		

les de leptina entre hembras y machos (Ishioka et al., 2002; Ishioka et al., 2007).

Edad. Ishioka et al (2007) en un estudio retrospectivo utilizando 166 perros, no encontraron una correlación significativa entre la edad y los niveles de leptina en sangre en perros con diferentes condiciones corporales.

Raza. Ishioka et al (2007) han analizado si existen diferencias de niveles de leptina entre razas. En este estudio se seleccionaron seis razas (teckel miniatura, shih tzu, pastor de shetland, golden retriever, labrador retriever, y mestizos) no viéndose diferencias significativas entre animales con BSC 3/5.

Relación con parámetros de obesidad. Está descrito que en perros que se presentan a consulta veterinaria o en condiciones experimentales, la concentración de leptina se correlaciona con el BCS (body condition score) y que la concentración de leptina sérica refleja la grasa corporal mejor que el peso del animal, ya que el tamaño y peso corporal varía mucho según la raza del perro (Ishioka et al., 2007, Ishioka et al., 2002, Sagawa et al., 2002).

2 Adiponectina

Metabolismo y función. La adiponectina es una adipocitoquina que está sintetizada exclusivamente en el tejido adiposo blanco, sus cantidades en sangre superan tres veces las cantidades de los demás adipocitoquinas y representa el 0.01% de las proteínas totales circulantes en los mamíferos (Phillips et al., 2003). La secuencia de genes de la adiponectina es altamente homóloga entre las distintas especies: ratón:perro 84-85%; rata:perro 83%, humano:perro 87%, rata:ratón 92% (Ishioka et al., 2006, Brunson et al., 2007).

En estudios realizados en humana y en animales de laboratorio se ha observado que los niveles sanguíneos de adiponectina disminuyen en la obesidad (Arita et al., 1999; Chandran et al., 2003; Ishioka et al., 2006). En perros con sobrepeso la disminución de adiponectina es de un 25 por ciento y de un 50 por ciento

en obesos (Ishioka et al., 2006). Los niveles sanguíneos de adiponectina también bajan en animales que presentan resistencia a la insulina (Weyer et al., 2001; Kondo et al., 2002), diabetes tipo II (Hotta et al., 2000; Hotta et al., 2001) y dislipidemia (Matsubara et al., 2002), y están especialmente bajos en pacientes con patologías en arterias coronarias (Ouchi et al., 2001; Zoccali et al., 2002). En cambio, la pérdida de peso (Yang et al., 2001) y el tratamiento con tiazolidinediones para aumentar la sensibilidad a la insulina están asociados con aumentos de los niveles de adiponectina en humana (Maeda et al., 2001; Combs et al., 2002; Yang et al., 2002).

Se ha indicado que la adiponectina puede tener una función estimuladora de la acción de la insulina, ya que se ha observado que el fragmento C-terminal de la adiponectina es capaz de disminuir la concentración plasmática de la glucosa en todo el cuerpo, aumentar la oxidación de ácidos grasos en el músculo y favorecer la inhibición de la secreción de la glucosa por los hepatocitos inducida por la insulina (Lu et al., 2006; Fasshauer y Paschke, 2003).

Niveles normales. Los niveles normales de adiponectina obtenidos por diferentes autores aparecen en la Tabla 6. Se ha descrito que los niveles normales de adiponectina en humanos son de 5 a 30 µg/ml (Gil-Campos et al., 2004) y en ratas de 9 a 17.4 µg/ml (Qi et al., 2004). Los niveles medios de adiponectina en perros con un peso ideal según Ishioka et al. (2006) son de 33.4 ± 2.9 µg/ml. Sin embargo Brunson et al. (2007) encontraron valores más bajos, de 0.85 a 1.5 µg/ml.

Influencias fisiológicas:

Sexo. Se ha visto que la testosterona inhibe la secreción de adiponectina de adipocitos in vitro (Nishizawa et al., 2002), y también se ha apreciado un efecto similar “in vivo” en humanos (Böttner et al., 2004; Cnop et al., 2003) y ratones (Combs et al., 2003). Así, en adultos, los

niveles de adiponectina son significativamente más bajos en machos que en hembras. Sin embargo, en perros no se han observado diferencias significativas de los niveles de adiponectina entre hembras ($32.9 \pm 3.5 \mu\text{g/ml}$) y machos ($33.8 \pm 4.6 \mu\text{g/ml}$) (Ishioka et al., 2006).

Edad. En estudios realizados en humanos (Böttner et al., 2004; Cnop et al., 2003) y ratones (Combs et al., 2003) se observó que los niveles de adiponectina bajan en los machos después de la madurez sexual, debido a que la testosterona inhibe la secreción de adiponectina en los adipocitos (Nishizawa et al., 2002). No existen estudios que valoren diferencias de niveles de adiponectina en perros según su edad.

Raza. No existen estudios que valoren diferencias de niveles de adiponectina en perros según su raza.

Relación con otros parámetros de obesidad. Existe una correlación negativa entre los niveles sanguíneos de adiponectina y leptina (Ishioka et al., 2006), siempre cuando la muestra de sangre para medir leptina se obtenga 14 horas después de la última comida. Ya que, como se ha mencionado anteriormente, las concentraciones de leptina en suero bajan en ayunas, pero suben en unas horas después de ingesta de la comida (Ishioka et al., 2005; Ishioka et al., 2006).

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3.2. Validation studies

3.2.1. Article 2

Validation of 2 commercially available enzyme-linked immunosorbent assays for adiponectin determination in canine serum samples

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Abstract

The aim of this study was to validate 2 commercially available enzyme-linked immunosorbent assays (ELISAs) for adiponectin in dogs, 1 canine-specific and 1 originally designed for measurements in humans. Intra-assay and interassay precision was evaluated by multiple measurements in canine serum samples, and assay accuracy was indirectly determined by linearity under dilution. Interference caused by hemolysis and lipemia was also studied. Both assays were subsequently used for measuring adiponectin concentrations in clinically healthy dogs and those with different grades of obesity. The intra-assay and inter-assay precision was less than 7.5% and 13.5% in serum samples with low and high adiponectin concentrations, respectively. Lipemia and hemolysis did not affect the results of any of the assays. Both assays were able to differentiate lean dogs from those that were overweight or obese on the basis of the measured adiponectin concentrations. From these results it can be concluded that canine adiponectin concentrations can be measured reliably by means of the 2 ELISAs evaluated in this study.

Résumé

L'objectif de la présente étude était de valider deux troussees commerciales d'épreuve immuno-enzymatique (ELISA) pour mesurer l'adiponectine chez les chiens, une spécifique à l'espèce canine et une développée au départ pour utilisation chez l'humain. La précision intra- et inter-essais a été évaluée par des mesures multiples d'échantillons de sérum canin, et la précision de l'épreuve a été mesurée indirectement par la linéarité après dilution. L'interférence causée par l'hémolyse et la lipémie a également été examinée. Les deux épreuves ont par la suite été utilisées pour mesurer les concentrations d'adiponectine chez des chiens cliniquement en santé et d'autres avec différents grades d'obésité. La précision intra-essai et inter-essai était respectivement de moins de 7,5 % et 13,5 % dans des échantillons de sérum avec des concentrations faibles et élevées d'adiponectine. La lipémie et l'hémolyse n'ont pas affecté les résultats des deux troussees. Les deux épreuves étaient en mesure de différencier les chiens maigres de ceux avec un surplus de poids ou obèses à partir des concentrations d'adiponectine mesurées. À partir de ces résultats il peut être conclu que les concentrations d'adiponectine canines peuvent être mesurées de manière fiable à l'aide des deux troussees ELISA évaluées dans cette étude.

(Traduit par Docteur Serge Messier)

Introduction

Adiponectin is a cytokine produced in adipocytes that reduces plasma glucose concentrations and increases fatty acid oxidation (1–3). It also inhibits the inflammatory process of arteriosclerosis by suppressing the expression of adhesion molecules on endothelial cells (4). The serum concentration of adiponectin decreases with body fat accumulation and is considered a biomarker for obesity in humans (5,6). In addition, levels of adiponectin decrease with resistance to insulin (7,8), type 2 diabetes (9,10), dyslipidemia (11), and abnormalities of the coronary arteries (4,12). In dogs, adiponectin has gained attention recently after reports that its concentrations are lower in obese dogs than in dogs with a normal body condition score (BCS) (13,14).

Most of the published studies in animal species, including dogs, have used multispecies radioimmunoassay (RIA) for adiponectin quantification (14–17). Use of enzyme-linked immunosorbent assay

(ELISA) instead of RIA has several advantages, such as nonexposure to radioisotopes, easy integration into laboratory functions, and rapid turnaround (18).

Although different ELISA methods are commercially available for adiponectin measurement, to our knowledge no validation studies of their use in dogs have been published. However, adiponectin is highly homologous among different species, with 87% homology in the case of humans and dogs (13,14). The objective of this study was to validate 2 commercially available ELISAs for adiponectin in dogs, 1 canine-specific and 1 originally designed for measurement in humans, the postulation being that the latter could be used to quantify canine adiponectin. For the validation, the analytical performance of the assays was determined. In addition, the effects of hemolysis and lipemia on the assays, as well as the ability of the assays to differentiate low and high adiponectin concentrations, corresponding to obesity and optimal BCS, respectively, were studied.

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Materials and methods

Dogs and samples

Dogs with potentially high and low serum adiponectin concentrations were selected for the study. The client-owned dogs, of different sexes, breeds, and ages, had presented at private clinics in southern Spain for routine checkups between January and July 2008. None had evidence of acute or chronic disease (except for obesity) from physical and clinical examination or from routine hematologic and biochemical analysis. Assessment of their nutritional status was based on a 5-scale BCS: 1 — thin; 2 — underweight; 3 — optimal (lean); 4 — overweight; 5 — obese (19). Serum specimens with a potentially high adiponectin concentration were obtained from dogs with an optimal BCS; specimens with a potentially low concentration were obtained from dogs that were overweight or obese. Of the 30 dogs selected, 22 were overweight or obese [12 females (6 neutered) and 10 males (1 neutered); BCS > 3; body weight, 3.2 to 50 kg; age, 1 to 16 y; mixed breeds]; the other 8 were of optimal weight for health (4 females and 4 males; BCS = 3; body weight, 5 to 18.9 kg; age, 1.2 to 7 y; mixed breeds).

Blood samples were collected the morning after an overnight fast of at least 12 h by puncture of the jugular or saphenous vein and placed in tubes containing a clotting accelerator (TapVal; Aquisel, Barcelona, Spain). Samples were centrifuged at $2000 \times g$ for 10 min at room temperature to obtain serum, which was stored in aliquots in plastic vials at -20°C . On the day of analysis, the aliquots were brought to room temperature and thoroughly vortexed before the adiponectin measurement.

Adiponectin analysis

Canine-specific assay — The commercially available canine sandwich ELISA (Canine Adiponectin ELISA Kit; Millipore, St. Charles, Missouri, USA) used in our study has the following sequence of steps: 1) capture of canine adiponectin by polyclonal antibodies against adiponectin; 2) washing away of unbound materials; 3) binding of antibodies with horseradish peroxidase (HRP); 4) quantification of immobilized antibody-enzyme conjugates by monitoring of HRP activity in the presence of the substrate 3,3',5,5'-tetramethylbenzidine (TMB); and 5) addition of an acidic solution (0.3 M HCl) to stop the reaction. The enzyme activity is measured spectrophotometrically by increased absorbance at 450 nm, corrected for the absorbance at 590 nm, in a microtiter plate reader (PowerWave XS; Bio-Tek Instruments, Winooski, Vermont, USA). Since the increase in absorbance is directly proportional to the amount of captured canine adiponectin, the concentration in the studied serum sample can be calculated from the reference curve generated in the same assay with reference standards of known concentrations of canine adiponectin. The calibration curve was established by duplicate determination of calibrators.

High-sensitivity assay of human adiponectin — This assay (Human Adiponectin ELISA, High Sensitivity Kit; BioVendor-Laboratorni medicina, Modrice, Czech Republic) has the following sequence of steps: 1) capture of adiponectin by polyclonal antibodies against adiponectin; 2) washing away of unbound materials; 3) binding of antibodies with HRP; 4) washing away of unbound materials;

5) reaction of HRP-conjugated antibodies with a hydrogen peroxide/TMB substrate; and 6) addition of an acidic solution (0.2 M H_2SO_4) to stop the reaction. The enzyme activity is measured spectrophotometrically at 450 nm in a PowerWave XS microtiter plate reader. A standard curve is constructed from duplicate determination of calibrators by plotting absorbance values against the adiponectin concentrations of the calibrators, and the concentrations in studied samples are determined by means of this standard curve.

Species-specific standards are highly recommended to achieve similar affinity of antiserum against standards and samples (20), and the use of serum samples as a standard is preferred in many cases instead of purified protein (it is more economical, yields a more suitable physiological matrix, and can ensure a consistent, long-lasting supply of standard material) (20–22). A canine serum sample with the adiponectin concentration determined by the canine-specific adiponectin ELISA was evaluated as a standard for curve calibration of the human adiponectin ELISA.

For this purpose, 12 canine samples were analyzed with the assay for human adiponectin and the use of 2 different standard curves: the original curve from the kit (human standard), which has an upper value of 150 ng/mL, and a curve prepared from a canine serum sample with an adiponectin concentration of 30 $\mu\text{g/mL}$ as measured by the commercially available canine-specific ELISA. This canine serum sample was serially diluted with dilution buffer, and a standard curve was constructed with a range of 0.137 to 30 $\mu\text{g/mL}$. This canine standard was used for the remainder of the analytical and clinical validation of the human assay.

Analytical validation

For analytical validation of both methods the following parameters were calculated.

Precision — The intra-assay coefficient of variation (CV) was calculated after analysis of 2 samples of low adiponectin concentration and 2 of high concentration 5 times in a single assay run. The inter-assay CV was determined by analyzing the same samples in 5 separate runs, carried out on different days.

Accuracy — Owing to the lack of a reference method or commercially available certified reference material for canine adiponectin, which would be optimal for testing accuracy (23), accuracy of the assays was evaluated indirectly by linearity under dilution (24). Two canine serum samples with high adiponectin concentrations were serially diluted with the corresponding sample diluent provided with each assay.

Limit of detection — This was calculated on the basis of data from 11 replicate determinations of the zero standard (dilution buffer) as the mean value plus 2 standard deviations.

All samples used for repetitive analysis were frozen in aliquots, and only the vials needed for each run were used, to avoid possible changes due to repetitive thawing and freezing.

Effects of hemolysis and lipemia

To investigate the effects of hemolysis and lipemia on the assays, 2 canine serum samples were mixed with different concentrations of hemoglobin or lipid solution, and each preparation was run in duplicate.

A fresh hemolysate was prepared by adding distilled water to packed saline-washed canine erythrocytes from a healthy dog. The concentration of hemoglobin in the hemolysate was determined with the Vet ABC Animal Blood Counter (ABX Diagnostics, Montpellier, France) and adjusted to 200 g/L by adding assay buffer to produce a stock solution. The stock solution was serially diluted with sample diluent buffer, and 10 µL of each dilution was added to 2 samples of 90 µL of canine serum with high and low adiponectin concentrations. The final hemoglobin concentrations were 8, 4, 2, 1, 0.5, and 0.0 g/L (10 µL of sample diluent buffer was added to the samples to give a 0.0 g/L concentration), which would correspond to slight hemolysis (0.5 g/L), moderate hemolysis (1 and 2 g/L), and marked hemolysis (4 and 8 g/L).

A commercial fat emulsion (Lipofundina 20%; Braun Medical, Barcelona, Spain) with a triglyceride concentration of 200 g/L was serially diluted with sample diluent buffer, and 10 µL of each dilution was added to 2 samples of 90 µL of canine serum with high and low adiponectin concentrations. Homogeneity was achieved by vortexing. The final triglyceride concentrations were 5, 2.5, 1.25, 0.625, and 0.3125 g/L, which would correspond to slight lipemia (0.3125 and 0.625 g/L), moderate lipemia (1.25 and 2.5 g/L), and marked lipemia (5 g/L).

Statistical methods

Arithmetic means, medians, and intra-assay and interassay CVs were calculated by means of routine descriptive statistical procedures and software [Excel (Microsoft, Redmond, Washington, USA) and GraphPad Prism (GraphPad Software, San Diego, California, USA)]. Differences between results obtained with human and canine standards and comparison of the results for lean dogs with those for overweight or obese dogs obtained with the assay for human adiponectin were studied by use of Student's *t*-test with a significance level of $P < 0.05$. In addition, Bland-Altman plots were used to evaluate the clinical agreement between results obtained in the ELISA for human adiponectin with 2 different standards and to reveal any bias. Bias was computed as the mean difference between scores for the 2 methods. Values for 95% limits of agreement (LOA) were computed as the mean difference $\pm$ 1.96 standard deviations of the difference. Linearity under dilution was accomplished by ordinary linear regression analysis comparing the measured levels of adiponectin with the expected levels. Interferograms were prepared to show the differences in adiponectin concentration when hemoglobin or lipid was added. On the graphs the X-axes show increasing concentrations of hemoglobin or lipid; the Y-axes show the percentage change in adiponectin [$V_f(V_o) \times 100$]. Additionally, regression analysis was applied in the comparison of results of the canine and human ELISAs to derive regression data. The significance level used in each case was $P < 0.05$.

Results

Assay characteristics

Canine-specific ELISA — The time needed to perform the assay was 4 h. The intra-assay and inter-assay CVs were 4.40% and 13.31% for samples with low adiponectin concentrations and 6.26% and 8.52%

for samples with high concentrations (Table 1). The linearity under dilution of 2 canine samples is shown in Figure 1: dilution of the samples with different adiponectin concentrations resulted in linear regression equations with correlation coefficients of 0.999 and 0.997. The detection limit was 0.098 (mean, 0.019; standard deviation, 0.039) µg/mL.

High-sensitivity ELISA of human adiponectin — In the evaluation of canine serum as a standard for curve calibration in this assay, the use of canine standards resulted in significantly higher adiponectin values ($P < 0.001$) than the use of human standards for the same 12 canine serum samples. In addition, Bland-Altman analysis indicated a proportional bias (Figure 2). The mean difference between adiponectin values obtained with the canine and human standards was 4.95 (95% LOA, -2.04 to 11.95) µg/mL.

The time needed to perform the assay was 2.5 h. The intra-assay and inter-assay CVs were 2.48% and 10.60% for samples with low adiponectin concentrations and 7.45% and 9.52% for samples with high concentrations (Table 1). The linearity under dilution of 2 canine samples is shown in Figure 3: dilution of the samples with different adiponectin concentrations resulted in linear regression equations with correlation coefficients of 0.996 and 0.999. The detection limit was 0.74 (mean, 0.49; standard deviation, 0.13) µg/mL.

Effects of hemolysis and lipemia on adiponectin analysis

The different degrees of hemolysis and lipemia tested in this study did not affect the measured levels of adiponectin in either the canine-specific assay or the high-sensitivity assay of human adiponectin, as shown by the interferograms presented in Figures 4 and 5, respectively.

Ability of the assays to differentiate lean and obese dogs

The adiponectin concentration in the 30 serum samples from the client-owned dogs ranged from 0.12 to 28.52 µg/mL as determined by the canine-specific ELISA and 2.37 to 26.69 µg/mL as determined by the ELISA for human adiponectin. Regression analysis (Figure 6) demonstrated high correlation ($r = 0.9173$) between the results with the 2 methods. With both methods, the samples from the overweight or obese dogs showed significantly lower adiponectin concentrations ($P < 0.005$ and 0.0005, respectively) than the samples collected from the lean dogs (Figure 7).

Discussion

There are numerous methods to determine grade of obesity in dogs, but most of them are complicated, expensive, or time-intensive (25). So in recent years it has become of great interest to find new, rapid, and reliable methods to diagnose and monitor obesity in this species. Adiponectin is decreased in obese dogs and can be used as an obesity marker (13,14). But the measurement of adiponectin concentrations is not very common in companion animal practices, which often use RIA kits. These kits have the inherent disadvantages of the need to have special installations and to dispose of radioactive wastes, as well as being labor-intensive and having a long processing time. The development of adiponectin assays based on the ELISA

Table 1. Intra-assay and inter-assay coefficients of variation (CVs) in the adiponectin concentration of serum samples with high and low values [mean and standard deviation (s) for 2 samples] as determined by a canine-specific enzyme-linked immunosorbent assay (ELISA) and an ELISA originally designed to measure the adiponectin concentration in human serum

ELISA	Comparison	Number of samples	Obtained in present study			Reported by manufacturer	
			Adiponectin concentration (µg/mL)			Mean adiponectin concentration (µg/mL)	
Canine-specific	Intra-assay	2	Mean	s	CV (%)	13.39	2.86
		2	20.20	1.26	6.26		
	Inter-assay	2	7.11	0.31	4.40	1.58	4.21
		2	25.81	2.20	8.52		
Human	Intra-assay	2	5.59	0.74	13.31	13.47	0.46
		2	18.58	1.39	7.45		
	Inter-assay	2	5.45	0.135	2.48	6.59	6.7
		2	18.54	1.76	9.52		
		2	5.52	0.59	10.60	19.08	9.5
		2					
		2				6.41	7.6
		2					

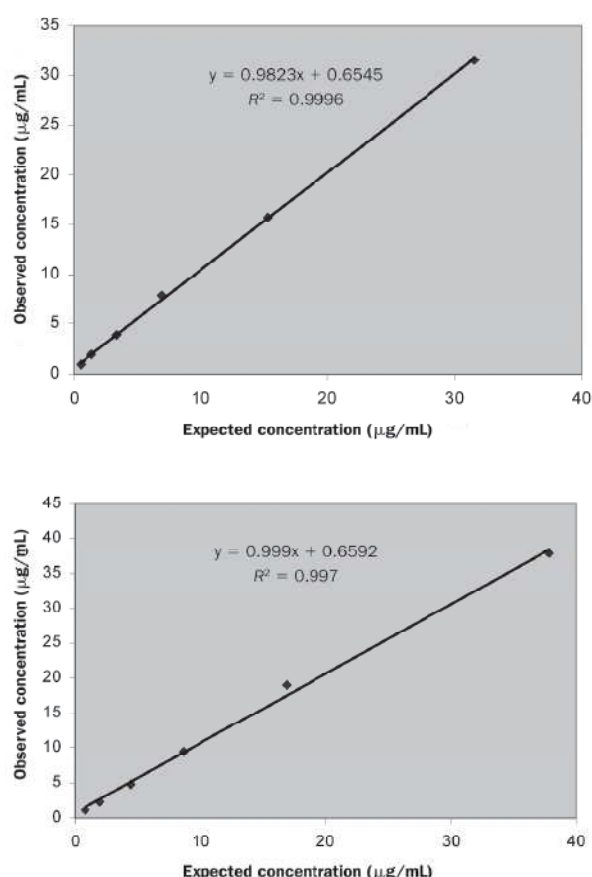


Figure 1. Linearity under dilution of 2 canine serum samples with different adiponectin concentrations measured with the canine-specific adiponectin enzyme-linked immunosorbent assay (ELISA).

technique represents substantial progress from the technologic, ecologic, and economic viewpoints (18), but the commercially available ELISA methods have not been validated for use in dogs. Validation studies must ensure that analytical methods are able to detect the

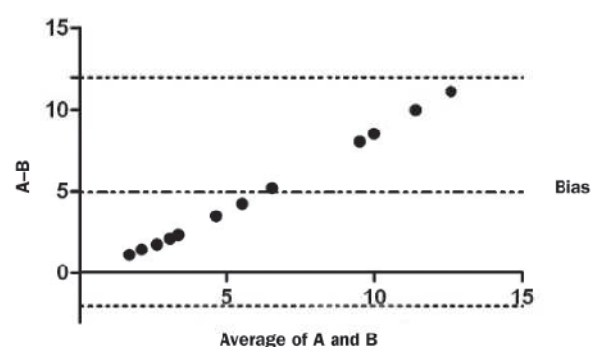


Figure 2. Bland-Altman plot illustrating the 95% limits of agreement (LOA) for adiponectin values (µg/mL) determined by the ELISA for human adiponectin with canine (A) and human (B) standards in 12 canine blood samples. The top and bottom horizontal dotted lines represent the 95% values (calculated as the mean difference $\pm$ 1.96 $\times$ the standard deviation of the difference); the middle line represents the mean difference (bias).

corresponding analyte, provide repeatedly accurate results, and discriminate between healthy and diseased animals (26).

This study validated 2 different ELISA kits for adiponectin measurement in dogs: 1 canine-specific and 1 designed for measuring adiponectin in humans. The use of a canine serum sample with a known adiponectin concentration measured by the canine-specific assay to calibrate the human adiponectin ELISA was evaluated. A proportional bias was detected between the human and canine standards. Although the reasons for this bias are not clear and should be further investigated, from a practical point of view this bias could indicate that the use of a canine serum sample as a standard might contribute to better differentiation between low and high adiponectin concentrations in overweight or obese and lean dogs, respectively.

In this study similar CVs were obtained in the 2 assays. However, except for the intra-assay CV for low adiponectin values in the human assay, the CVs obtained with both ELISA kits for intra-assay and interassay precision were higher than those reported by the manufacturers. A murine RIA kit previously used for quantification of adiponectin in dogs gave a higher intra-assay CV (9.5%) but a slightly lower interassay CV (9.7%) than the kits in our study (14).

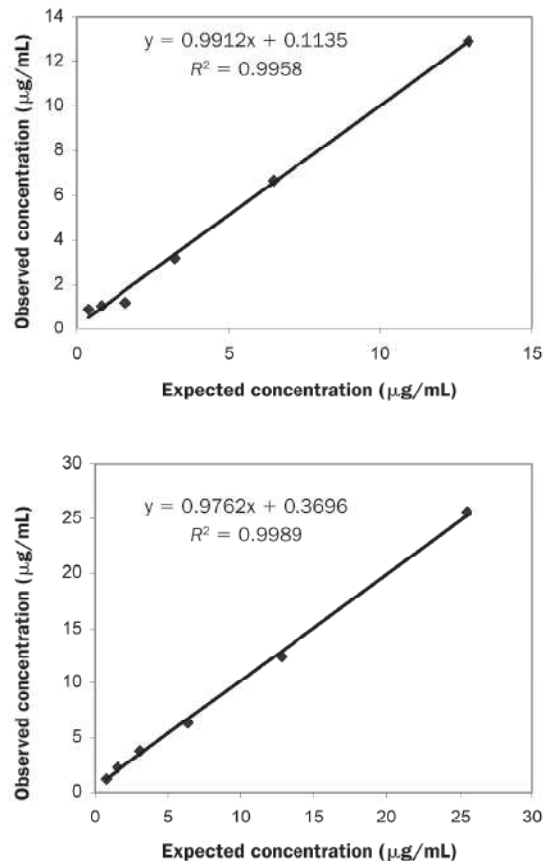


Figure 3. Linearity under dilution of 2 canine serum samples with different adiponectin concentrations measured with the ELISA originally designed to measure the adiponectin concentration in human serum.

Nevertheless, both ELISA kits appear to be suitable for measuring canine adiponectin concentrations, as the CVs did not exceed 15%, the limit of the analytical objective performance standard for precision (27).

The accuracy of the adiponectin ELISAs was indirectly evaluated by linearity under dilution. This procedure is an alternative used with canine adiponectin measurements when no reference method or commercially available certified reference material is available (24,28). Regression analysis revealed high accuracy of both assays: $r = 0.99989$ and 0.9958 for the canine-specific ELISA and $r = 0.997$ and 0.9996 for the ELISA for measurement in humans. The canine-specific assay had a lower limit of detection ($0.098 \mu\text{g/mL}$) than the assay for human adiponectin ($0.74 \mu\text{g/mL}$), which could suggest that the canine assay detects low adiponectin concentrations more efficiently.

Veterinary clinical pathology laboratories usually have to deal with hemolytic or lipemic serum. In dogs, hemolysis can be attributed to various diseases or to complications at the time of blood sampling. Lipemia is usually related to recent ingestion or endocrine diseases related to obesity. For these reasons it is important to know the influence of hemolysis and lipemia on adiponectin values in dogs. We found that concentrations up to 5 g/L of triglycerides and

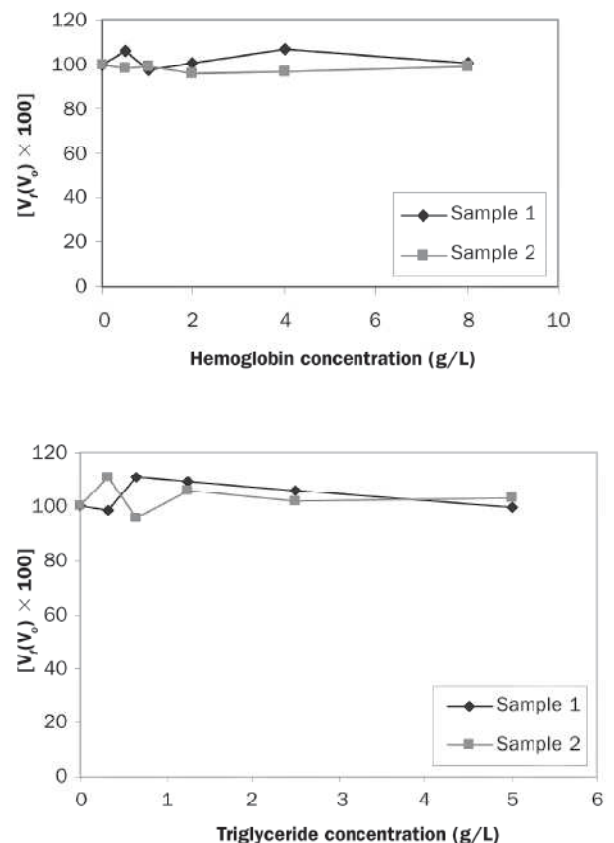


Figure 4. Interferograms showing the effect of hemoglobin and triglyceride on adiponectin determinations by the canine-specific ELISA. The Y-axes show the percentage change in adiponectin concentration for a given concentration of hemoglobin or triglyceride. V_f — final value; V_o — original value.

up to 8 g/L of hemoglobin did not cause differences in the adiponectin values in the 2 assays, which suggests that lipemia and hemolysis would not influence the adiponectin values measured in canine samples with either of the assays used in our study. These results agree with those of previous reports on the same high-sensitivity ELISA for human adiponectin and a latex particle-enhanced turbidimetric immunosorbent assay for human adiponectin: neither method showed significant interference of hemoglobin concentrations up to 500 mg/dL (18,29). In addition, it was reported that triglyceride levels up to 2000 formazin turbidity units did not alter adiponectin values obtained with the human assay used in our study (29). This could be of practical importance because obese dogs frequently have lipemic serum, and lipemia predisposes to hemolysis, so both interferences could often be present in obese dogs.

The results of the 2 assays evaluated in the present study showed high correlation ($r = 0.9173$) in regression analysis. However, this finding should be interpreted with caution because there may be a dependence between the methods since calibration material used for the human assay was established by the canine-specific ELISA (21).

With both assays, the overweight or obese dogs in this study had significantly lower serum adiponectin concentrations than the lean dogs, as has been reported previously (13,14). The mean

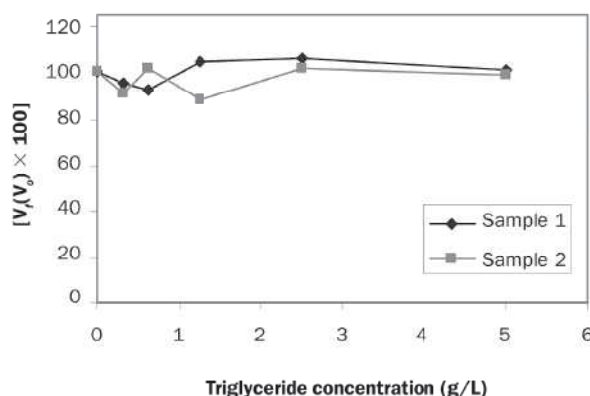
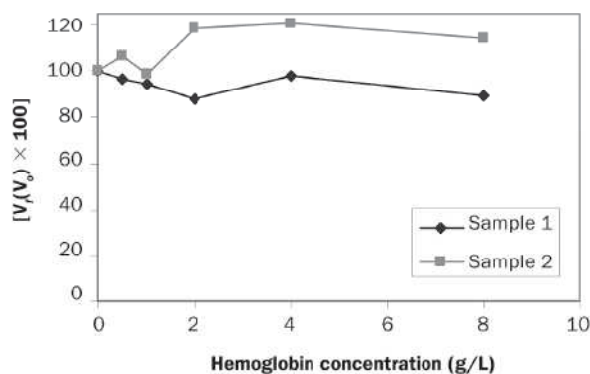


Figure 5. Interferograms showing the effect of hemoglobin and triglyceride on adiponectin determinations by the ELISA for human adiponectin.

concentrations in the 2 groups of dogs were higher than those reported by another group of investigators (14), who found an adiponectin concentration in normal dogs of $1.22 \mu\text{g/mL}$, but lower than those reported by others (13), who found means of $16.8 \pm 3.5 \mu\text{g/mL}$ and $33.4 \pm 2.9 \mu\text{g/mL}$ in obese and lean dogs, respectively. The differences can be attributed to different methods used in the studies.

The main limitations of the validation performed in this study are that neither method was compared with a reference method for adiponectin measurement in dogs (but, to the authors' knowledge, such a method does not exist) and that spiking recovery tests with reference materials of known adiponectin concentrations were not performed (because canine adiponectin is not commercially available). However, the linearity under dilution, which in cases of lack of a reference method or reference materials could be an estimation of accuracy, provided very high correlations, indicating that both methods could measure adiponectin in a linear and proportional way.

In summary, the 2 commercially available ELISAs for adiponectin determination in dogs that were validated in this study exhibit analytical characteristics that allow their use in the laboratory, with adequate precision, linearity under dilution, and ability to discriminate between high and low adiponectin values, corresponding to

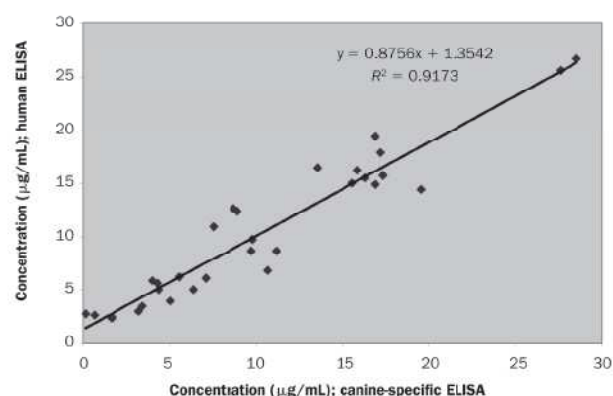


Figure 6. Regression analysis of the adiponectin concentrations determined by the 2 ELISAs.

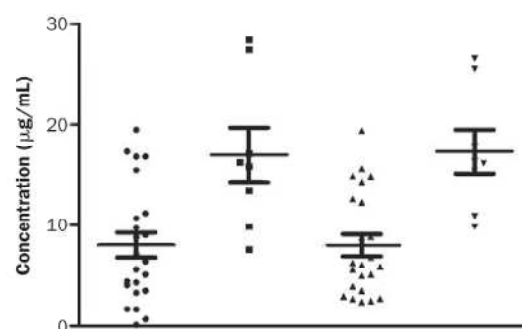


Figure 7. Serum adiponectin concentrations in lean dogs (those of optimal body condition score) determined by the canine-specific (■) and human (▼) ELISAs and in overweight or obese dogs determined by the canine-specific (●) and human (▲) ELISAs.

lean and overweight or obese dogs, respectively. It is expected that validation of these 2 assays will increase the analytical options of veterinary laboratories for canine adiponectin measurement and will contribute to a wider use of adiponectin as a marker of obesity and related diseases in canine clinical practice.

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3.2.2. Article 3

Assessment of five ELISAs for measurement of leptin concentrations in dogs

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Objective—To evaluate 5 commercially available ELISAs for determination of leptin concentrations in serum samples from dogs.

Sample Population—Serum samples from overweight-obese and thin-ideal weight client-owned dogs.

Procedures—Serum samples with high and low leptin concentrations ($n = 7$ samples each) were used for validation of the assays. Intra- and interassay precision, linearity under dilution, spiking recovery, and limit of quantification were determined. In addition, leptin concentrations in thin-ideal weight ($n = 8$) and overweight-obese (37) dogs were quantified.

Results—Use of 2 of the 5 ELISAs (A and B) revealed reactivity with canine leptin. Intra- and interassay coefficients of variation were $< 6.1\%$ and 7.6% , respectively, for assay A and 14.0% and 13.7% , respectively, for assay B. In assays A and B, dilutions of canine serum pools were used to determine linear regression equations. Recoveries were 77% to 101% for assay A and 67% to 125% for assay B. Significant differences in leptin concentrations between thin-ideal weight and overweight-obese dogs were detected only when analyzed with assay A.

Conclusions and Clinical Relevance—Among 5 leptin ELISAs evaluated, a canine-specific leptin ELISA had adequate precision, linearity, and ability to discriminate between high and low leptin concentrations corresponding to overweight-obese and thin-ideal weight dogs, respectively. (*Am J Vet Res* 2011;72:169–173)

Obesity is the most common nutritional disorder in dogs and a major risk factor for a number of diseases.¹ Data published in 2006 for 21,754 dogs examined in US veterinary practices revealed that 29.0% of adult dogs were overweight and 5.1% were obese.² To diagnose and monitor obesity, veterinarians have used BCS systems that correlate with more complex measures of adiposity, such as dual-energy x-ray absorptiometry.^{3,4} However, BCS systems are subjective because visual and tactile cues are used to assign a numeric value to a dog's degree of adiposity. Recently, the possible use in dogs of serum markers for obesity such as leptin or adiponectin, which correlate with the degree of adiposity, has gained attention.^{5–10}

Leptin is a protein that has similarities to cytokines and is mainly synthesized and secreted by adipose tissue.¹¹ Leptin concentrations are markedly increased in obesity and, because they are positively correlated with body fat content, are considered a good adiposity marker.^{5,6,12} Therefore, the measurement of leptin concentrations may be used in obesity control programs to ensure adequate fat loss or to study obesity-associated diseases.

Previous studies^{5–8} that investigated leptin in lean and obese dogs used ELISAs that unfortunately are not

ABBREVIATIONS	
BCS	Body condition score
CV	Coefficient of variation

commercially available. However, several ELISAs for leptin quantification in dogs as well as other species are presently available commercially. The purpose of the study reported here was to evaluate 5 leptin ELISAs (2 canine specific, 2 human specific, and 1 designed for mice or rats) for determination of leptin concentrations in canine serum samples.

Materials and Methods

Dogs and samples—Dogs were of different sexes, breeds, and ages and were evaluated at private clinics in southern Spain for routine checkups between February and July of 2008. The dogs had no remarkable findings via physical, clinical (with the exception of obesity), hematologic, or serum biochemical evaluations. Assessment of the nutritional condition of dogs was performed by use of a 5-point BCS following a weight guide chart based on the characteristics of ribs, tail base, side view, and overhead view. Dogs were classified as follows: 1/5, very thin; 2/5, thin; 3/5, ideal weight; 4/5, overweight; and 5/5, obese.¹³ Serum samples with expected high and low leptin concentrations were used for analytic validation of the assays. Low leptin concentration specimens were obtained from 7

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thin or ideal-weight dogs (BCS, 2/5 or 3/5), and high leptin concentration specimens were obtained from 7 overweight or obese dogs (BCS, 4/5 or 5/5). None of the samples were of sufficient volume for all analyses required for complete analytic validation of all 5 ELISAs, so samples with expected low leptin concentrations were pooled and samples with expected high leptin concentrations were pooled.

After food was withheld overnight for at least 12 hours, blood samples were collected in the morning via saphenous venipuncture with a 21-gauge needle and placed into 5-mL tubes containing a clotting accelerator.^a No later than 15 minutes after collection, samples were centrifuged at 2,000 × *g* for 10 minutes at 20°C to obtain serum, and serum was stored in aliquots in plastic vials at -20°C until analysis. Duration of storage was < 6 months. On the day of analysis, samples were thawed (1 hour at 20°C) and thoroughly vortexed prior to leptin measurement.

Leptin analysis—Five commercially available ELISAs developed for serum leptin determinations in dogs (A^b and B^c), humans (C^d and D^e), and mice or rats (E^f) were evaluated for leptin determination in the canine serum samples (Appendix). All spectrophotometric measurements were performed in a microtiter plate reader.^g

Analytic validation—To evaluate the existence of reactivity between assay antibodies and leptin in canine sera, 3 pools prepared from overweight-obese dogs' serum samples were analyzed in duplicate by use of all 5 ELISAs. Because purified canine leptin was not commercially available, it could not be used for test reactivity, so it was supposed that a positive analytic signal corresponded to leptin present in the canine sera. The ELISAs that yielded a positive signal were further tested for precision, accuracy, linearity under dilution, spiking recovery, and limit of quantification.

For evaluation of precision, intra-assay CV was calculated after analysis of 2 serum pools with high and low leptin concentrations, 5 times in a single assay run. Interassay CV was determined by analysis of the same serum pools in 5 runs performed on different days.

Accuracy was indirectly evaluated by investigation of linearity under dilution and spiking recovery because neither a certified species-specific reference material nor a reference method was available.¹⁴ To assess linearity under dilution, 2 canine serum pools with high leptin concentrations were serially diluted. In assay A, pools were diluted with the corresponding sample diluent provided with the assay, and in assay B, saline solution (NaCl, 9 g/L) was used because no diluent was provided by the manufacturer.

To evaluate the ability of the assays to recover the amount of analyte added to baseline serum samples, a spiking recovery was performed. Because purified canine leptin was not commercially available, this test was based on the addition of a calibrator.¹⁵ For this purpose, 2 samples were divided into 4 aliquots and each aliquot was mixed with an equal amount of calibrator (calibrator included in each assay kit [A or B] was used for the corresponding assay) at 3 concentrations or dilution buffer. Test recovery (percentage) was

calculated for each dilution for comparison of expected versus measured leptin concentrations.

Limit of quantification was calculated as the amount of analyte that could be measured with an intra-assay variation < 15%.^{16,17} Six dilutions (100%, 75%, 50%, 37.5%, 18.75%, and 9.38%) of 2 serum pools (1 for each assay tested) were prepared and analyzed 5 times. The CV of each dilution was calculated and plotted as a function of leptin concentration.

All pools used for repetitive analysis were frozen in aliquots, and only vials needed for each run were used to avoid possible changes caused by repetitive thawing and freezing.

Comparison of assays with positive reactivity and ability of assays to differentiate between thin-ideal weight and overweight-obese dogs—Samples from 45 client-owned dogs were included for method comparison and to determine whether leptin ELISAs were able to differentiate between thin-ideal weight and overweight-obese dogs. Thirty-seven mixed-breed dogs were overweight or obese (20 females [3 neutered] and 17 males [4 neutered]; BCS, 4/5 or 5/5; body weight range, 3.0 to 48.5 kg; age range, 1.5 to 11 years), and 8 were thin-ideal weight, healthy mixed-breed dogs (4 females [1 neutered] and 4 males; BCS, 2/5 or 3/5; body weight range, 5 to 14.1 kg; age range, 1.0 to 6.0 years).

Statistical analysis—Arithmetic means, medians, and intra- and interassay CVs were calculated by use of routine descriptive statistical procedures and software.^h Comparison of leptin concentrations between thin-ideal weight and overweight-obese dogs was determined by use of the Mann-Whitney test because data did not pass the Kolmogorov-Smirnov normality test. Visual inspection of results of linear regression analysis comparing measured and expected concentrations of leptin was used to evaluate the linearity under dilution. Bland-Altman plots were used to compare the results obtained with the assays that had reactivity with canine leptin. The 95% limits of agreement were calculated as mean difference ± 1.96 × SD of the difference. For all tests, a value of *P* < 0.05 was considered significant.

Results

Assay characteristics—Mean results obtained from 3 canine serum pools from overweight-obese dogs that were analyzed to evaluate the existence of reactivity between assay antibodies and leptin, along with mean values of blank samples from all kits evaluated, were determined (Table 1). Because a positive result that could be differentiated from that obtained with the blank was not detected by use of any of the human or mouse or rat leptin ELISAs, the rest of the study was focused on evaluation and validation of assays A and B.

Assays A and B had intra-assay CVs < 6.1% and 14.0% and interassay CVs < 7.6% and 13.7%, respectively (Table 2). Linearity under dilution of 2 canine serum pools for assays A and B was determined (Figures 1 and 2). In assay A, dilution of pools with different leptin concentrations resulted in linear regression equations with correlation coefficients of

0.99 and 0.99; in assay B, these correlation coefficients were 0.97 and 0.95. Recovery between observed and expected leptin concentrations ranged from 77% to 101% for assay A and from 67% to 125% for assay B (Table 3). Limits of quantification were 3.6 µg/L for assay A and 11.6 µg/L for assay B (Figure 3).

Comparison of canine leptin ELISAs and ability of the assays to differentiate between thin-ideal weight and overweight-obese dogs—Seven of 8 serum samples collected from thin-ideal weight dogs did not yield a result when analyzed with assay A (leptin concentrations of these samples and all samples with results less than the limit of quantification were set to 0 µg/L for further statistical analysis; however, values are reported as less than or equal to the limit of quantification). For the 45 clinical samples, ranges of leptin concentrations obtained with assays A and B were ≤ 3.6 to 45.9 µg/L and ≤ 11.6 to 103.8 µg/L, respectively. A Bland-Altman plot of comparison between the methods revealed a

proportional error indicated by differences in leptin concentrations increasing relative to the increase in mean value (Figure 4).

When assay A was used, samples collected from overweight-obese dogs (median, 7.2 µg/L; 10th to 90th percentile range, 4.2 to 25.0 µg/L) had significantly ($P < 0.001$) higher leptin concentrations than those collected from thin-ideal weight dogs (median, ≤ 3.6 µg/L; 10th to 90th percentile range, ≤ 3.6 to 5.4 µg/L). In contrast, no significant ($P = 0.89$) difference was observed in leptin concentration when serum samples

Table 1—Mean leptin concentrations (µg/L) obtained by use of 5 ELISAs from 3 pools prepared from canine serum samples and blank samples.

Sample	Assay A	Assay B	Assay C	Assay D	Assay E
Pool 1	6.4	21.9	ND	0.1	1.2
Pool 2	5.1	26.2	ND	0.6	1.2
Pool 3	4.2	28.1	ND	0.2	1.2
Blank	ND	6.5	7.5	0.1	1.1

ND = Not detected.

Table 2—Intra- and interassay variability of leptin concentrations (µg/L) determined by use of 2 ELISAs in canine serum pools with different concentrations of analyte (high and low).

Method	Comparison	Pool	Mean	CV (%)
Assay A	Intra-assay	Low	4.0	4.8
		High	10.9	6.1
	Interassay	Low	3.7	6.9
		High	10.9	7.6
Assay B	Intra-assay	Low	13.0	14.0
		High	31.8	5.9
	Interassay	Low	12.4	9.7
		High	29.6	13.7

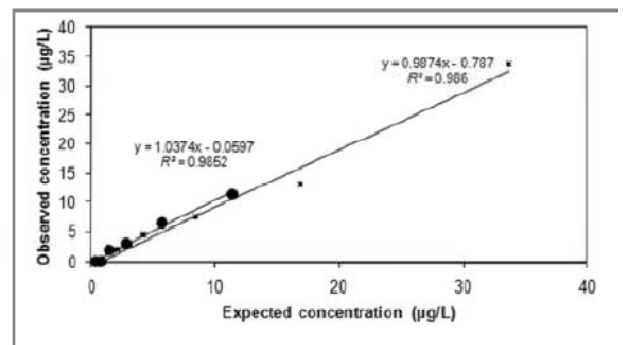


Figure 1—Linearity under dilution of 2 canine serum pools (circles and squares) with different leptin concentrations measured by use of an ELISA (assay A).

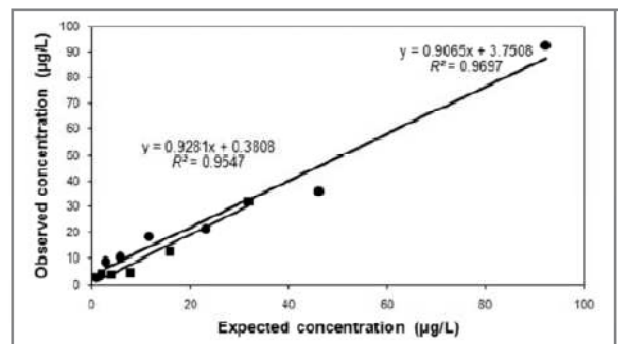


Figure 2—Linearity under dilution of 2 canine serum pools (circles and squares) with different leptin concentrations measured by use of an ELISA (assay B).

Table 3—Percentage recovery of leptin by use of 2 ELISAs. Calibrators with different amounts of leptin were mixed with serum samples with known leptin concentrations.

Method	Leptin concentration (µg/L)		Expected value (µg/L)	Observed value (µg/L)	Recovery (%)
	Sample	Calibrator			
Assay A	5.79	25	15.4	11.8	77.0
		12.5	9.1	8.9	97.5
		3.1	4.5	4.3	95.6
		0	2.9	2.4	81.6
	11.75	25	18.4	15.2	82.6
		12.5	12.1	10.3	85.0
		3.1	7.4	7.2	96.2
	33.95	0	5.9	5.9	101.1
		50	41.9	31.6	75.4
		25	29.5	25.1	85.2
		5	19.5	22.2	113.9
	88.28	0	17.0	20.1	118.2
		50	69.1	86.1	124.5
Assay B	33.95	25	56.6	37.7	66.6
		5	46.6	35.1	75.3
		0	44.1	31.4	71.2
	88.28	50	69.1	86.1	124.5
		25	29.5	25.1	85.2
		5	19.5	22.2	113.9

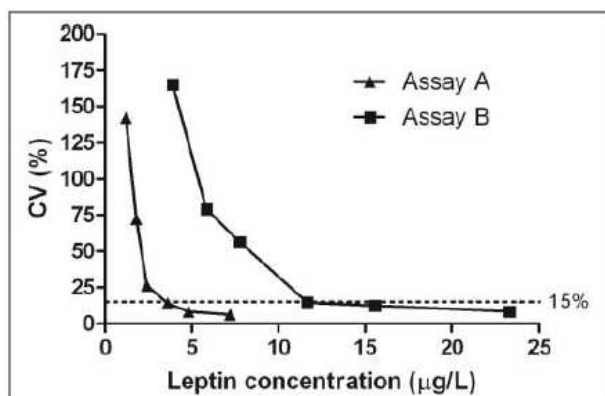


Figure 3—Limits of quantification for 2 ELISAs used to measure canine leptin concentrations. Different dilutions of 2 serum pools were assayed with assay A and B, and the CVs were plotted as a function of leptin concentration. Dotted line indicates 15% CV, which is considered the acceptable limit of quantification.

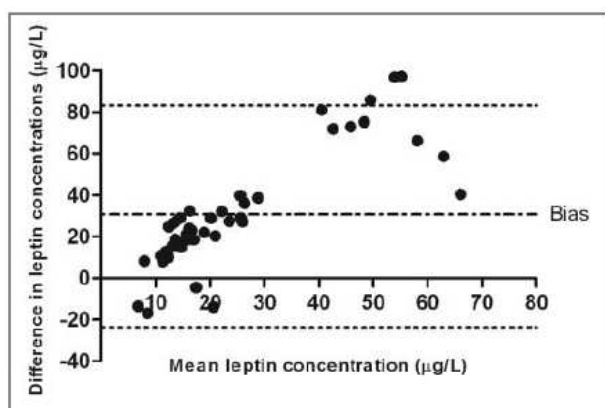


Figure 4—Bland-Altman plot illustrating the 95% limits of agreement for leptin concentrations determined by use of 2 ELISAs (A and B). Upper and lower horizontal dashed lines represent 95% limits of agreement, and the middle horizontal dotted-dashed line represents mean difference (bias).

from overweight-obese (median, 28.1 µg/L; 10th to 90th percentile range, 13.7 to 92.3 µg/L) and thin-ideal weight dogs (median, 27.6 µg/L; 10th to 90th percentile range, 20.8 to 81.0 µg/L) were analyzed with assay B.

Discussion

Leptin concentration is high in obese dogs and can be used in the study of obesity and obesity-associated diseases in this species.^{5,6} However, leptin concentration is still not widely used in canine practice, perhaps because, to the authors' knowledge, no validation studies for use in dogs have been published for any leptin ELISA methods that are presently commercially available. Validation studies are needed to ensure that analytic methods can successfully measure the analyte and provide reproducible and accurate results that are useful to discriminate between healthy and diseased animals.¹⁸

In the study in dogs reported here, we tested 5 ELISA kits for leptin measurement: 2 for use in dogs, 2 for use in humans, and 1 for use in mice or rats. Despite an 82% and 79% homology in amino acid sequence between canine and human or mouse leptin,

respectively, only canine-specific ELISAs (A and B) yielded a positive reaction with canine serum. These results could suggest that antibodies of the different kits recognize leptin epitopes located in regions with a high heterogeneity in the protein sequence between species.¹⁹ It must be pointed out that canine serum with potentially high leptin concentrations was used instead of purified canine leptin to evaluate the presence of reactivity between assay antibodies and leptin; therefore, it was supposed that the analytic signal obtained did indicate the presence of leptin because antibodies of the positive reacting kits were canine leptin specific.

The CVs reported by assay A manufacturers (5.9% and 6.7% at mean concentrations of 3.1 and 20.8 µg/L, respectively) were slightly higher than those observed in the present study. Manufacturers of assay B do not provide such data with the ELISA kit. However, obtained results indicated that assay A was more precise in measuring leptin in canine serum samples than assay B, and assay B imprecision was near 15%, which is considered the maximum acceptable value for analytic methods.²⁰

The accuracy of A and B ELISAs was indirectly evaluated by use of linearity under dilution and spiking recovery.^{14,15} Regression analysis revealed that both assays yielded linear results when measuring leptin in different dilutions of canine serum samples. The recovery study revealed that assay A slightly underestimated serum leptin concentrations because most recovery values were < 100%. The low recovery found at the expected value of 2.9 µg/L could be attributable to the fact that it was less than the limit of quantification of the assay (3.6 µg/L). Leptin values obtained with assay B were highly under- or overestimated (reaching up to 33% of the expected value), compared with expected values.

When assay A was used, overweight-obese dogs had higher plasma leptin concentrations, compared with thin-ideal weight dogs, which was similar to previous reports.⁵⁻⁷ In the present study, the median leptin concentration in overweight-obese dogs (7.2 µg/L) obtained with assay A was lower than reported by Ishioka et al^{6,7} in dogs with a BCS of 5/5 (12.3 ± 1.5 µg/L and 12.8 ± 0.8 µg/L, respectively). However, no comparison could be made in thin-ideal weight dogs between assay A and reports from Ishioka et al^{6,7} because median values obtained in our assay were less than the limit of quantification of the method.

No significant difference in leptin concentrations was detected between overweight-obese and thin-ideal weight dogs when analyzed with assay B. Factors that might explain these results were the high imprecision and poor performance in recovery studies. In addition, this assay's high limit of quantification reflected a limited ability to measure low concentrations of leptin.

Of the 5 leptin ELISAs evaluated in the present study, 1 canine-specific ELISA had adequate analytic characteristics of precision, linearity, recovery, and ability to discriminate between high and low leptin concentrations corresponding to overweight-obese and thin-ideal weight dogs, respectively. Although these results should be interpreted with caution because no purified canine leptin was available for cross-

reactivity studies and variations in reactivity may be obtained with different batches of antibodies used in the canine kit, this assay could be used for canine leptin determination. Nevertheless, it would be desirable to decrease its limit of quantification to permit better detection of low concentrations of leptin. In addition, results of this study stress the importance of performing validation studies on every analytic kit before routine use in a veterinary laboratory.

- a. TapVal, Aquisel, Barcelona, Spain.
- b. Canine leptin ELISA kit, Millipore, Mo.
- c. Goat anti-canine leptin, BPB Biomedicals Inc, Franklin, Calif.
- d. Leptin EASIA, BioSource Europe SA, Nivelles, Belgium.
- e. Human Leptin ELISA, BioVendor-Laboratori Medicina AS, Modrice, Czech Republic.
- f. Mouse/rat enzyme immunoassay kit, SPIbio, Montigny le Bretonneux, France.
- g. PowerWave XS, Bio-Tek Instruments Inc, Winooski, Vt.
- h. GraphPad Prism, version 5 for Windows, GraphPad Software Inc, San Diego, Calif.

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Appendix

Characteristics of 5 ELISAs used for determination of leptin concentrations in various species.

Assay	Species	Antibodies	Measurement wavelength (nm)
A ^a	Canine	Polyclonal and biotinylated monoclonal anti-leptin	450–590
B ^c	Canine	Polyclonal anti-leptin*	450
C ^d	Human	Monoclonal and HRP-monoclonal anti-leptin	405–650
D ^e	Human	Polyclonal and HRP-polyclonal anti-leptin	450
E ^f	Mouse or rat	Biotinylated polyclonal and HRP-polyclonal anti-leptin	450

*This assay also uses biotinylated leptin and streptavidin-HRP as a measurement indicator.
HRP = Horseradish peroxidase.

3.2.3. Article 4

ORIGINAL ARTICLE

Validation of two ELISA assays for total ghrelin measurement in dogs

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Keywords

dog, ELISA, ghrelin, glucose, validation

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Summary

The aim of this study was to validate two commercially available ELISA assays for total ghrelin measurement in dogs: one canine-specific and one originally designed for measuring human ghrelin. The two assays showed intra-assay coefficient of variations (CVs) lower than 10%, while the inter-assay CVs exceeded the 15% limit. Sample dilutions resulted in linear regression equations with correlation coefficients close to 1. In order to compare methods and verify ability of the ghrelin assays to differentiate between low and high levels, ghrelin concentrations were measured in plasma samples obtained before and at different times after glucose administration in five Beagle dogs. A statistically significant changes in ghrelin after glucose administration was recorded only with assay B. In conclusion, the human ELISA validated in this study showed a good intra-assay precision, accuracy, and when applied to the glucose injection study, was better in distinguishing high and low canine ghrelin levels than the canine ELISA assay.

Introduction

Ghrelin is a 28-amino acid peptide that acts as the ligand for the growth hormone (GH) secretagogue receptor type 1a and is able to stimulate GH secretion from pituitary gland cells in many species such as rats, humans, goats and dogs (Kojima et al., 1999; Date et al., 2000; Hayashida et al., 2001; Bhatti et al., 2002; Hosoda et al., 2003). Ghrelin is a cardinal hormonal regulator of energy homeostasis, as it stimulates appetite, increases food intake, gastric acid secretion, gastric motor activity, induces adiposity and decreases fat use (Masuda et al., 2000; Tschöp et al., 2000; Gualillo et al., 2003; Hagemann et al., 2003; Tung et al., 2004). Because of its functions, ghrelin is highly studied in human patients suffering nutritional disorders such as hyperphagia, cancer anorexia, anorexia nervosa, and syndrom of Prader-Willi (Cummings et al., 2002; English et al., 2002; Tanaka et al., 2003). In the recent years, ghrelin is gaining importance in the human medicine due

to its orexigenic and gastric motility stimulating effects among others (Kojima and Kangawa, 2005) and also as a potential pharmacological compound for the treatment of obesity and anorexia (Yokoyama et al., 2005).

Recently, there is an increased interest in studies investigating the role of ghrelin in dogs, especially in relation to obesity and anorexia (Yokoyama et al., 2005). In addition, as ghrelin seems to have similar functions in the dog and the man and the distribution of ghrelin-immunoreactive cells in the canine stomach resembles that of the human stomach, the dog is considered as a suitable experimental model (Yokoyama et al., 2005).

Serum ghrelin in dogs has been measured in most published studies by radioimmunoassays (RIA) (Jeusette et al., 2005; Yokoyama et al., 2005; Bhatti et al., 2006; Yilmaz et al., 2007). The use of ELISA techniques has several advantages over RIA which include the lack of exposure to radiation, higher shelf-lives of reagents, no need for special facilities and

rapid turnaround time of results (Risch et al., 2006). Therefore, the use of ELISAs could contribute to a widespread use of ghrelin measurements in dogs. In addition, it has been shown that ELISA sandwich assays can distinguish different ghrelin responses better than RIAs (Prudom et al., 2010). Even so, to the authors' knowledge there are no complete validation studies published about the use of ELISA kits for the measurement of ghrelin in dogs. We postulated that a human ELISA assay could be used to quantify canine ghrelin, as ghrelin is highly homologous amongst different species. In addition, the canine and human ghrelins differ only in two amino acids, and the 10 amino acids in their NH₂ termini in particular, are identical (Tomasetto et al., 2001; Angeloni et al., 2004; Kojima and Kangawa, 2008). The aim of this study was to validate two commercially available ELISA assays: one canine-specific and one originally designed for measuring ghrelin in people. For the validation, the analytical performance of the assays was determined.

Materials and methods

Animals and samples

Plasma samples obtained from five University owned female Beagle dogs (age, 5.2 ± 0.5 years; BCS, 3/5; BW, 14.13 ± 0.7 kg) were used for the analytical validation of the assays. In order to obtain specimens with potentially different ghrelin concentration, the procedure of Mori et al. (2009) was followed: glucose solution (0.5 g/kg body weight) was injected intravenously for over 30 s to these dogs as a sterile 25% solution (50% solution was diluted by physiological saline). Blood samples from all animals were collected from the jugular vein prior to and 5, 10, 30, 60, 120, 180 min after glucose infusion.

Blood samples were placed into tubes on ice containing EDTA and aprotinin (100 µl/ml) (Aprotinin; Phoenix Pharmaceuticals, Burlingame, CA, USA) (Goodyear et al., 2010), and were immediately centrifuged, at 3500 *g* for 7 min at 4 °C, to minimise ghrelin degradation. After centrifugation, plasma aliquots were stored at -20 °C till analysis. On the day of analysis, samples were brought to room temperature and thoroughly vortexed prior to performing the assays.

Ghrelin analysis

Assay A

Canine ghrelin assay (Canine Ghrelin ELISA; Phoenix Pharmaceuticals) is a competitive ELISA assay for the quantitative measurement of ghrelin in

canine plasma samples. It employs microtitre wells coated with secondary antibodies, primary anti-canine ghrelin antibodies, biotinylated ghrelin and streptavidin-horseradish peroxidase. After the substrate 3,3',5,5'-tetramethylbenzidine is added, the enzymatic activity is measured spectrophotometrically from the absorbance at 450 nm of the formed products following their acidification with 2 N HCl.

Assay B

Human ghrelin assay (Human unacylated Ghrelin ELISA; BioVendor-Labartorni Medicina a.s., Modrice, Czech Republic) is a double-antibody sandwich ELISA assay for the quantitative measurement of ghrelin in human serum samples. It employs microtitre wells coated with monoclonal antibodies specific to the C-terminal part of ghrelin, acetylcholinesterase-Fab' conjugate and Ellman's reagent. Manufacturers of the assay B indicate that the absorbance should be read spectrophotometrically at 450 nm, 30–60 min after the addition of the substrate solution.

In order to verify possible changes due to different final incubation times, absorbance readings were performed at 30, 45 and 60 min after the substrate solution has been added. As no significant differences were detected between the three readings (data not shown), the 30-min incubation was performed in all cases.

Analytical validation

Preliminary tests

In order to verify the existence of reactivity between antibodies and canine ghrelin, two canine plasma samples were analysed five times each, while two other samples were serially diluted and analysed with both assays.

Analytical validation

For the analytical validation of the two methods, the following parameters were calculated:

- **Precision:** (i) Intra-assay coefficient of variation was calculated after analysis of two serum pools, five times in a single assay run; (ii) inter-assay coefficient of variation (CV) was determined by analysing the same samples in five separate runs carried out on five consecutive days.
- **Accuracy:** This evaluated indirectly by linearity after dilution and by recovery studies. To assess linearity after dilution, two canine serum samples with high ghrelin concentrations were serially diluted with the corresponding sample diluent

provided with each assay. To evaluate the ability of the assays to recover the amount of analyte added to baseline serum samples, a spiking recovery was performed (Guidance for Industry, 2010). For this purpose, two different samples were divided into five aliquots and each different aliquot was mixed with a known amount of purified canine ghrelin [Ghrelin (Canine) 100 µg; Phoenix Pharmaceuticals] (Table 2). Test recovery (%) was calculated for each dilution for comparison between the expected and the measured ghrelin.

- Lower (LLOQ) and upper (ULOQ) limit of quantification were calculated as the lowest and highest level of analyte that can be measured with an intra-assay variation under 20% (Tholen et al., 2003; Rauh et al., 2007). To that end, two plasma pools (one for each assay tested) were serially diluted in corresponding assay buffer and analysed in five replicates in the same analytical run. Then CV of each dilution was calculated and plotted as a function of ghrelin concentration.

All samples used for analysis were frozen in aliquots. In order to avoid possible sample deterioration due to repetitive thawing and freezing only vials needed for each run were used.

Agreement of methods and ability to differentiate ghrelin values

In order to compare methods and verify ability of the assays to detect different ghrelin levels, ghrelin concentrations were measured with both assays as described above in plasma samples obtained before and at different times after glucose administration in the five Beagle dogs.

Statistical methods

Arithmetic means, medians, intra-assay and inter-assay CVs were calculated using standard descriptive statistical procedures and software (GraphPad Prism; GraphPad Software, San Diego, CA, USA). Linearity under dilution was accomplished by ordinary linear regression analysis comparing the measured levels of ghrelin with the expected levels. Bland–Altman plots were used to evaluate the clinical agreement between results obtained with the two ELISA assays. Bias was computed as the mean difference between scores for the two methods. Values for the 95% limits of agreement (LOA) were computed as mean difference ± 1.96 SD. One-factorial repeated-measures ANOVA with Dunnetts multiple comparison post-test were used to compare ghrelin levels after glucose

administration. The significance level used in each case was $p < 0.05$.

Results

Assay characteristics

Preliminary tests

Preliminary performance of the assay A showed that all samples exceeded the absorbance detection limit of the assay and no linearity was observed. Adjustments based on different dilutions of the samples were performed in order to detect if any dilution could fit the range of absorbance measurement of the assay. It was found that a sample dilution of 1:8 showed major repeatability and the linearity under dilution resulted in a lineal regression equation.

When assay B was tested, good repeatability and linearity was observed.

Analytical validation

Assay A: Mean intra- and inter-assay CVs ranges were 8.5% and 43.8%, respectively (Table 1). The linearity after dilution of two canine samples is shown in Fig. 1. Dilution of serum samples with different ghrelin concentrations resulted in linear regression equations with correlation coefficients of 0.991 and 0.924. The recovery between observed and expected ghrelin concentrations ranged from 101% to 120% (Table 2). LLOQ and ULOQ were 0.52 and 17.31 ng/ml, respectively (Fig. 2).

Assay B: Mean intra- and inter-assay CVs were 4.6% and 17.4%, respectively (Table 1). The linearity after dilution of two canine samples is shown in Fig. 1. Dilution of serum samples with different ghrelin concentrations resulted in linear regression equations with correlation coefficients of 0.999 and

Table 1 Intra- and inter-assay variation in determinations of ghrelin concentration in canine plasma samples with canine and human ELISA assays

Method	Comparison	Pools	Ghrelin concentrations		
			Mean	SD	CV (%)
Assay A (ng/ml)	Intra-assay	1	5.05	0.5	10.0
		2	11.08	0.8	6.9
	Inter-assay	1	4.95	2.7	54.1
		2	9.27	3.1	33.5
Assay B (pg/ml)	Intra-assay	1	655.93	19.8	3.0
		2	4821.91	295.3	6.1
	Inter-assay	1	994.23	194.1	19.5
		2	5316.31	814.1	15.3

CV, coefficient of variation.

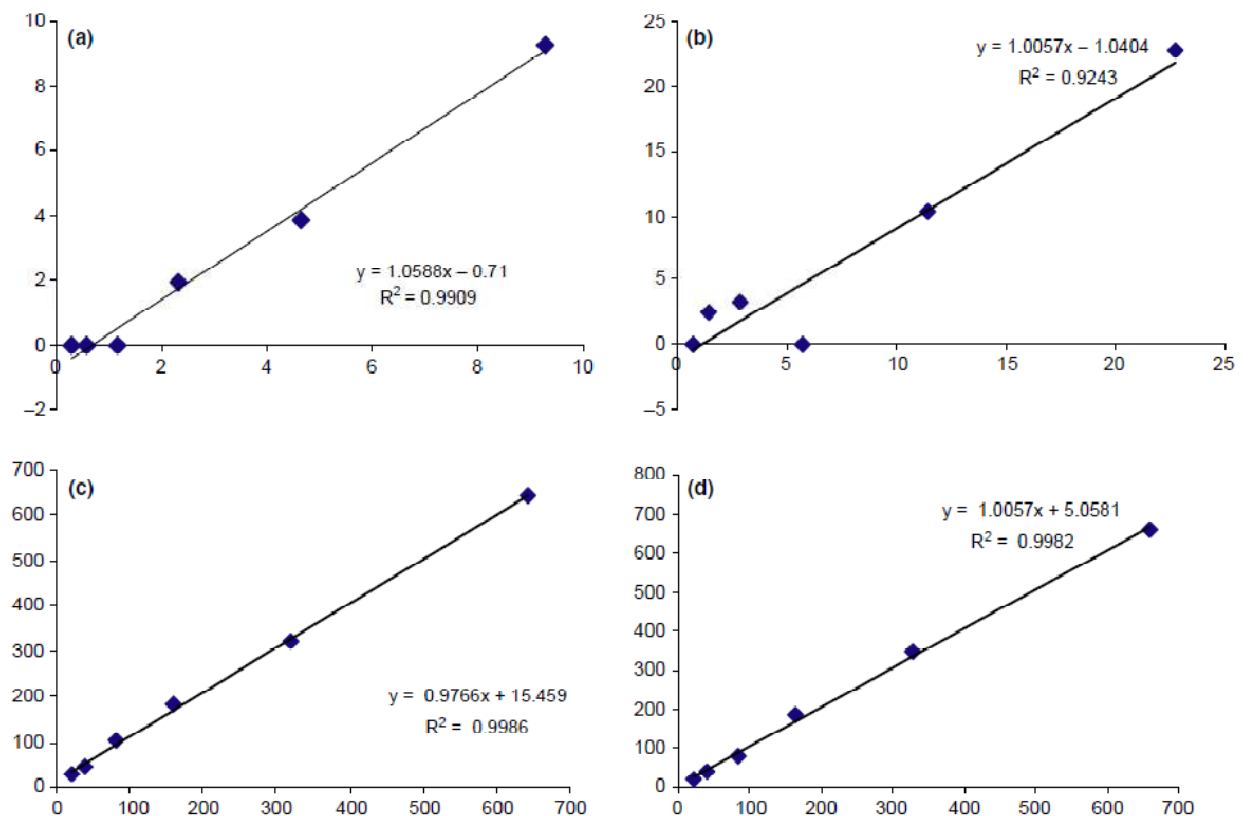


Fig. 1 Linearity under dilution of two canine serum samples with different ghrelin concentrations measured with assay A (a, b) and assay B (c, d).

0.998. The recovery between observed and expected ghrelin concentrations ranged from 83% to 96% (Table 2). LLOQ and ULOQ were 168 and 7720 pg/ml, respectively (Fig. 2).

Agreement of methods and ability to differentiate between high and low ghrelin values

When assaying the 35 plasma samples obtained after glucose administration, the range of ghrelin concentration obtained with assays A and B were 4.35–13.75 ng/ml and 435.62–6089.67 pg/ml, respectively. Bland–Altman plot of comparison between the two methods (A and B) showed a proportional error; the difference in ghrelin concentrations increased as the mean value increased (Fig. 3). The mean difference between the ghrelin levels obtained using canine and human ELISA assays was 5483.0 pg/ml (95% LOA, 3077.4–7888.6 pg/ml).

Changes in circulating levels of ghrelin after glucose administration determined with assays A and B are presented in Fig. 4.

In comparison with the basal values, a mean decrease of 17% and a mean increase of 19% in ghrelin concentrations were observed 5 and 120 min

after glucose administration, respectively, when analysed with assay A. However, no statistically significant changes were recorded.

When assay B was used, a decrease of over 30% in plasma ghrelin levels was detected 10 min after glucose administration ($p < 0.05$), followed by 50% increase in plasma ghrelin levels 120 min after glucose injection ($p < 0.05$) when compared with the basal levels.

Discussion

Ghrelin circulates in the bloodstream in two different forms: acylated and desacylated. The acylated form bears a unique post-translational octanoyl modification due to the esterification of a fatty acid on its third serine residue (Kojima et al., 1999) and it is presented in amounts far lower (only ~10% of total ghrelin) than desacylated form (Kojima and Kangawa, 2005). In this paper, we validated two ELISA kits for total ghrelin measurement. Both assays use antibodies that recognise an epitope common to the acylated and desacylated ghrelin, measuring in this way the total ghrelin (Liu et al.,

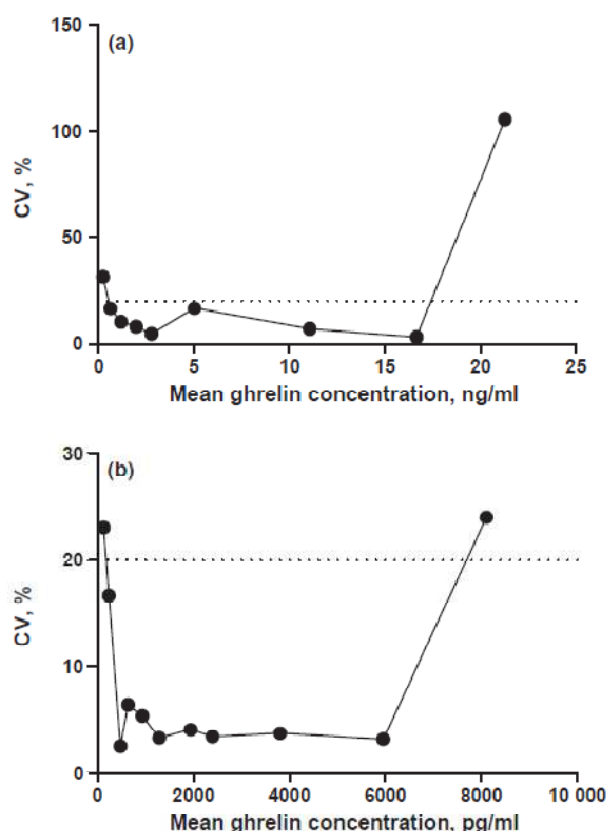


Fig. 2 Lower and upper limits of quantification. Different dilutions of two serum pools were assayed with assay A (a) and B (b) and the CVs (%) were plotted as a function of ghrelin concentration (ng/ml and pg/ml, respectively).

Table 2 Spiking recovery method. Different amounts of purified ghrelin were mixed with serum samples in which ghrelin concentrations were known

Method	Ghrelin concentration		Expected value	Observed value	Recovery (%)
	Sample	Purified			
Assay A (ng/ml)	6.3	25	15.7	18.9	120.5
		12.5	9.4	11.1	117.7
		6.25	6.3	6.3	101.1
Assay B (pg/ml)	435.6	10 000	5217.8	5030.8	96.4
		5000	2717.8	2410.5	88.7
		1000	717.8	595.9	83.0

2008). Although in humans, there are methods to separately measure the acylated and desacylated ghrelin, in dogs only quantification of total ghrelin is usually performed. The causes for that could be the inexistence of validated methods for measurements of acylated and desacylated ghrelin as well as the

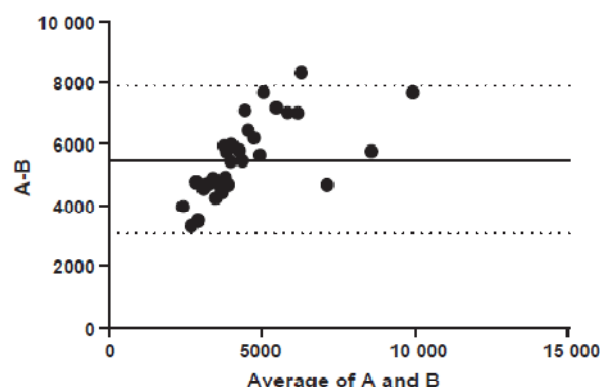


Fig. 3 Bland-Altman plot illustrating the 95% LOA for ghrelin values (pg/ml) determined by canine ELISA assay (A) and human ELISA assay (B) from 48 canine plasma samples. Top and bottom horizontal dotted lines represent 95% LOA values (calculated as mean difference $\pm 1.96 \times$ SD of the difference); middle horizontal line represents mean difference (bias).

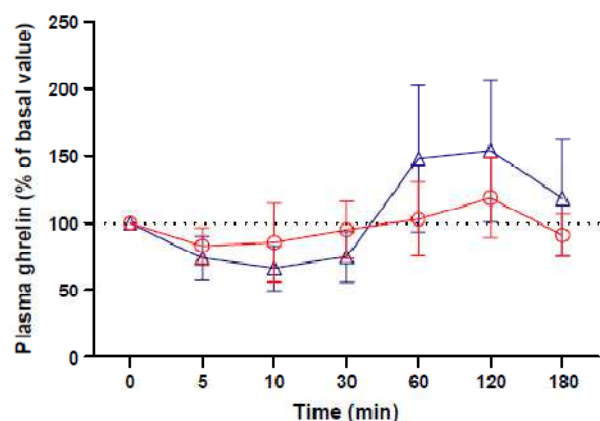


Fig. 4 Plasma ghrelin response to IV glucose (0.5/kg of body weight) injection in four Beagle dogs measured with assay A (O) and assay B (Δ).

instability of the particular antigenic portions of the acylated form (Jeusette et al., 2005). Although, it has been reported that total and acylated ghrelin are regulated in parallel (McCowen et al., 2002), it would be desirable in the future the development of assays that would allow the separate measurements of both forms in the dog. Such assays would be valuable in elucidating the physiological role of ghrelin as well to investigate the interconversion of ghrelin and desacyl-ghrelin in tissues and plasma (Rauh et al., 2007).

Validation studies must ensure that analytical methods are able to detect the corresponding analyte providing repetitive and accurate results (Teclès

et al., 2007). For this, precision, accuracy and limits of quantification. The intra-assay CV reported by the manufacturers of the assay B (4.3% at mean concentrations of 200 pg/ml) was similar to the one observed in this study, while the inter-assay CV reported by manufacturers (4.6% at mean concentrations of 200 pg/ml) was much lower in comparison with the one generated in this study. This discrepancy could be attributed to the different mean ghrelin concentrations (200 vs. 990 pg/ml) and the use of different species samples. Even so, the CVs of the assay B were below 20%. It is generally recommended that CVs must be lower than 10% for analytical determinations, but it is accepted that those CV% can reach values of 15–20% for immunological assays if concentrations of the analyte are low and close to the detection limit (Guidance for Industry: Bioanalytical Method Validation, US, 2001). The high inter-assay CVs (up to 54%) obtained with assay A could be influenced by the high inter-kit variability (data not shown). For this reason if assay A is employed, it would be recommended to analyse the samples in the same run and also with the same kit.

The accuracy of A and B ghrelin ELISA assays was indirectly evaluated by linearity under dilution and spiking recovery (Jensen, 2006). Regression analysis revealed that both assays were linear when measuring ghrelin in different dilution of canine serum samples. The recovery study showed that both assays were able to accurately measure the different concentrations of added ghrelin. These results were similar to those reported for human ghrelin RIA, where recoveries of 150 and 500 ng/l recombinant ghrelin (mean $\pm$ SD) in EDTA plasma were $99 \pm 12\%$ and $98 \pm 6\%$, respectively (Gröschl et al., 2002).

The range of detection reported by the manufacturers of the assay B was 6–250 pg/ml and was recommended in samples with concentrations higher than 250 pg/ml to repeat the analysis following dilution. LLOQ as well as ULOQ of the assay B in the present study were higher than those reported by the manufacturers. Unfortunately, manufacturers of the assay A did not include such data. In the current study, ghrelin levels obtained with the two assays were within LLOQ and ULOQ ranges. Similar data have been reported for human ELISAs, where less than 1% of the samples were outside the observed range (4.6–1000 pg/ml) of the assays (Prudom et al., 2010).

Despite a good correlation, Bland–Altman test revealed a mean difference in values obtained with the two assays higher than 5000 pg/ml. Variations in

ghrelin measurements using different assays have been also described in humans, where 10-fold differences have been reported when two commercial RIA kits were compared (Gröschl et al., 2004). In our case, the variation found could be attributed to possible affinity divergence of the antibodies to the canine ghrelin (species-specific and non-specific, assay A and B, respectively). Also it could be due to the different calibrators used (Gröschl et al., 2004). Ideally a calibrator of canine ghrelin with a known concentration measured by a validated or a reference method such as mass spectrometry should be used in the assays (Stöckl et al., 1997; Keevil et al., 2002; Fierens et al., 2003).

Glucose has been demonstrated to be the most potent inhibitor of systemic ghrelin levels in humans and rodents (McCowen et al., 2002; Haltia et al., 2010), suggesting that decreased plasma ghrelin levels during hyperglycaemia may represent a compensatory mechanism under condition of a positive energy balance (Nakagawa et al., 2002). Also in dogs, ghrelin is increased by short-term fasting and suppressed immediately after feeding (Bhatti et al., 2006). Based on these previous studies and in order to evaluate the ability of the assays in the present study to differentiate between different ghrelin levels, glucose was administered intravenously to the dogs and samples obtained at different times were analysed for ghrelin. Likewise as in humans and rats, ghrelin levels decreased after glucose infusion and returned to normal values within 3 h. The greater relative changes in ghrelin values were obtained with assay B in comparison with assay A. Differences in the ability to detect ghrelin in serum samples have been reported also in human when different ghrelin assays were compared (Prudom et al., 2010). Ghrelin is considered as a peptide difficult to quantify, as depending on the assay there are numerous factors that can affect ghrelin measurements. One of these factors is that due to its strong basic charge and hydrophobic acylation, ghrelin has a tendency to stick onto surfaces. Also ghrelin antisera differ in their ability to detect ghrelin bound to carrier proteins (Stöckl et al., 1997).

In conclusion, assay B showed a good precision, accuracy and was able to significantly detect changes in serum total ghrelin concentrations. We concluded that it could be used for measurement of total ghrelin in dogs and that due to its simplicity and lack of need for special equipment, could facilitate in the future the ghrelin measurements in this species. Our study also underlined the importance of an adequate validation of methods before their application in the

laboratory, as the two compared assays showed marked differences in precision and ability to detect different serum ghrelin concentrations, differences that could significantly alter the interpretation of the results.

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3.2.4. Article 5

Serum insulin-like growth factor-1 measurements in dogs: Performance characteristics of an automated assay and study of some sources of variation

Asta Tvarijonaviciute, Fernando Tecles, José M. Carillo, Monica Rubio, José J. Ceron

Abstract

The aim of this study was to evaluate the performance characteristics of an automated immunoassay for canine insulin-like growth factor 1 (IGF-1) measurement and to investigate the possible effects of some sources of variation, such as diurnal variations, feeding/fasting cycles, and glucocorticoid administration, in dogs. The immunoassay evaluated had an adequate analytical performance with intra- and inter-assay coefficients of variation (CVs) lower than 10%, linear regression equations with correlation coefficients of 0.9993 and 0.9988 after serial dilutions, and a limit of quantification of 7.1 ng/mL that was even lower than that reported by the manufacturer. The assay was significantly affected by hemolysis and lipemia producing a significant decrease in IGF-1 concentrations, but not by bilirubinemia.

Serum IGF-1 concentrations did not show significant diurnal changes in fed or fasted dogs and were not affected by glucocorticoid administration.

Résumé

L'objectif de la présente étude était d'évaluer les caractéristiques de performance d'une épreuve immuno-enzymatique automatisée pour la mesure du facteur de croissance 1 apparenté à l'insuline (IGF-1) et d'étudier chez les chiens les effets possibles de quelques sources de variation, telles que les variations diurnes, les cycles alimentation/jeûne, et l'administration de glucocorticoïdes. L'épreuve immuno-enzymatique évaluée avait une performance analytique adéquate avec des coefficients de variation (CV) intra- et inter-essais inférieur à 10 %, des équations de régression linéaire avec des coefficients de 0,9993 et 0,9988 après des dilutions sériées, et une limite de quantification de 7,1 ng/mL qui était inférieure à celle mentionnée par le fabricant. L'épreuve était affectée de manière significative par de l'hémolyse dans l'échantillon et une lipémie, qui entraînaient une réduction significative des concentrations d'IGF-1, mais pas par une bilirubinémie.

Les concentrations sériques d'IGF-1 n'ont pas montré de changements significatifs associés aux variations diurnes chez des chiens nourris ou mis au jeûne et n'étaient pas affectées par l'administration de glucocorticoïdes.

(Traduit par Docteur Serge Messier)

Insulin-like growth factor 1 (IGF-1) is a small polypeptide hormone consisting of 70 amino acids that is synthesized in the liver. It is involved in the regulation of growth and metabolism and mediates many of the anabolic effects of growth hormone (GH) in different tissues, such as bones, muscles, kidneys, spleen, heart, liver, or mammary gland (1). Serum IGF-1 measurements have garnered increased attention recently in dogs. It has been proposed as a marker of liver function with a diagnostic value nearly comparable with fasting bile acids (2). Also it has been reported to be higher in obese subjects and return to normal values after weight loss (3). Additionally, as in humans, the possible relationships between IGF-1 and neoplasia or cardiovascular disease have been studied, with a recently demonstrated a link having been found between serum IGF-1 concentrations and a worse prognosis in canine mammary cancers (4).

To facilitate studies on IGF-1 in dogs automated assays for determination of this factor need to be validated. Most of the studies published on the dog have used a multispecies radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) for IGF-1 quantification (2–4). The use of an automated solid-phase, enzyme-labelled chemiluminescent immunometric assay would have several advantages, such as easy integration into laboratory functions, high throughput, and rapid turnaround time. Secondly, knowledge about possible effects of some sources of variation, such as diurnal variations, feeding/fasting cycles, or corticoid administration on IGF-1, would allow proper interpretation of the values of this analyte. The aim of this study was to evaluate the performance characteristics of an automated immunoassay for canine IGF-1 measurement and to investigate the possible effects of some sources of variation, such

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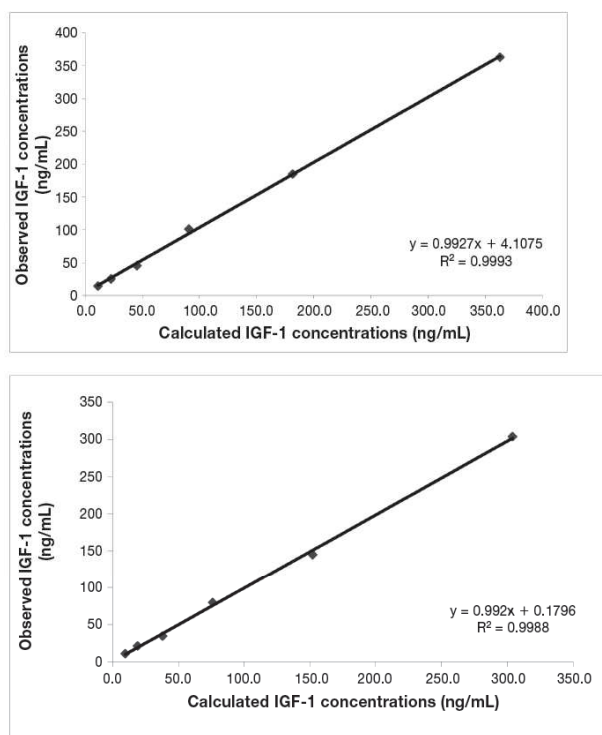


Figure 1. Linearity under dilution of 2 canine serum samples with different insulin-like growth factor (IGF-1) concentrations measured with an assay (IMMULITE 1000).

as diurnal variations, feeding/fasting cycles, and glucocorticoid administration in dogs.

Canine serum samples used for the validation study were obtained from 3 apparently healthy dogs of different sex, breeds, and ages that were presented at the Veterinary Teaching Hospital of Murcia University for routine check-ups. Dogs showed no remarkable findings on physical and clinical examination and in routine hematology and biochemistry analysis. In addition, samples were obtained from 3 dogs with chronic hepatitis, the diagnosis of hepatopathy was made as described by Neumann et al (2).

Eight young intact beagle dogs [4 females and 4 males; age range: 3.2 to 5.1 y; bodyweight (BW) range: 12.6 to 18.9 kg; body condition scores (BCS): 3 to 4], were included to investigate diurnal variations and influence of food intake on IGF-1. Fourteen young intact beagle dogs (8 males and 6 females; age range: 3.2 to 5.5 y; BW range: 10.1 to 17.9 kg; BCS: 3 to 4), were used to investigate possible effects of glucocorticoids on serum IGF-1. None of these dogs had ever received exogenous glucocorticoids. A single breed (beagle) within a narrow age range was used to avoid heterogeneity. All animals had a negative serological titer for *Leishmania infantum* and *Ehrlichia canis*. Animal care and procedures were in accordance with the guidelines of the University of Murcia, Spain. The assessment of the nutritional condition was based on a 5-scale BCS: 1 — thin; 2 — lean; 3 — optimal; 4 — obese; 5 — gross (5).

An automated solid-phase, enzyme-labelled chemiluminescent immunometric assay (Immulate 1000 IGF-1 assay; Diagnostic Products, Los Angeles, California, USA) was used. It was configured

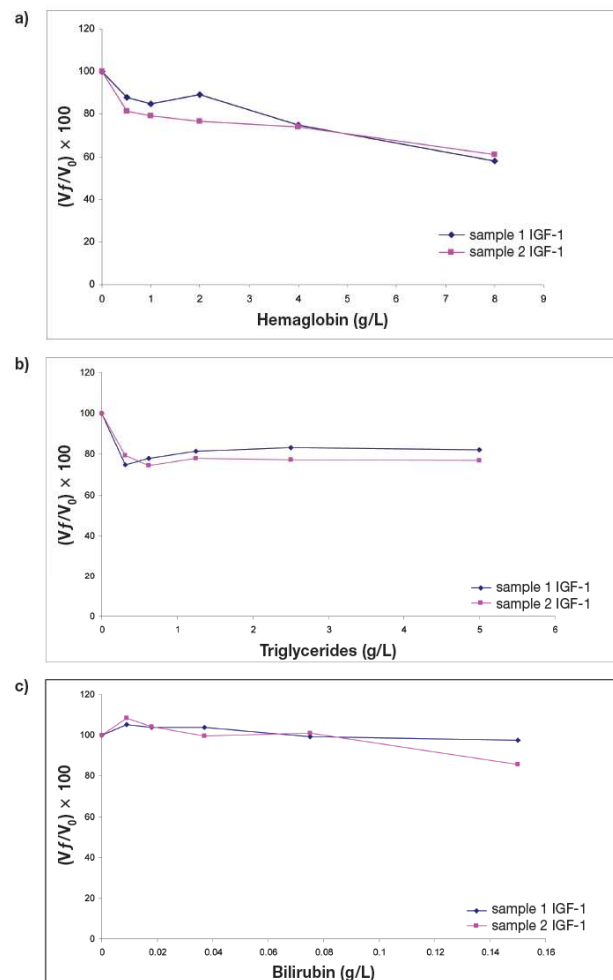


Figure 2. Interferograms corresponding to the effect of hemoglobin (a), triglycerides (b), and bilirubin (c) concentrations on insulin-like growth factor (IGF-1) determination by assay (IMMULITE 1000). Y-axes show percentage of change in IGF-1 $[(V_f/V_0) \times 100]$ for a given concentration of hemoglobin, triglycerides, or bilirubin, respectively. V_f , final value; V_0 , original value.

and calibrated on an instrument in our laboratory according to manufacturer's instructions. The assay has test units containing one bed coated with monoclonal murine anti-IGF-1, alkaline phosphatase (bovine calf intestine) conjugated to polyclonal rabbit anti-IGF-1 in buffer, chemiluminescent substrate (phosphate ester of adamantyl dioxetane in an AMP buffer with enhancer).

Cortisol was analyzed with using an automated chemiluminescent immunoassay (Immulate System; Siemens Health Diagnostics, Deerfield, Illinois, USA). Glucose was analyzed on an automated clinical chemistry analyser (Olympus AU2700; Olympus Diagnostica GmbH, Hamburg, Germany) as per the manufacturer's instructions.

For analytical validation of the method, the precision; accuracy; lower limit of quantification (LLOQ); and effects of hemolysis, lipemia, and bilirubinemia were investigated.

Two serum pools with different IGF-1 activities were prepared, one from the population of healthy dogs (mean IGF-1 close to

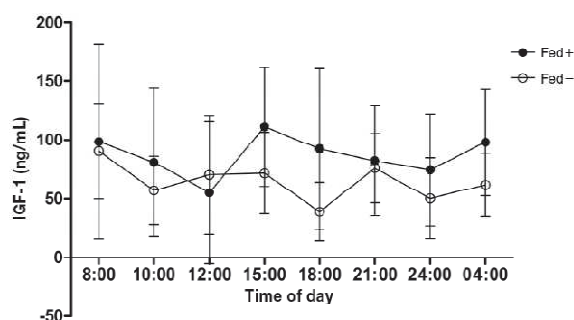


Figure 3. Mean $\pm$ standard deviation variation of serum insulin-like growth factor (IGF-1) concentrations. Four beagle dogs were fed at 09:00 h (Fed+) and 4 beagle dogs were fasted (Fed-) all day long.

315.20 ng/mL), and the other from dogs with chronic hepatopathies (mean IGF-1 close to 38.02 ng/mL) and were used for precision studies. Intra- and inter-assay coefficients of variation (CV) were calculated after analysis of these 2 serum pools 5 times in a single assay or on 5 consecutive days, respectively.

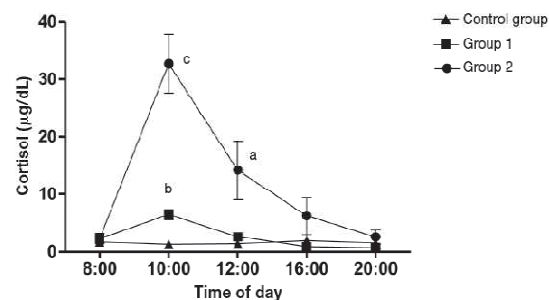
Accuracy of the assay was evaluated indirectly by linearity under dilution. This procedure is an alternative used in situations, as in this study, when no reference method is available and no commercially available certified reference materials exist (6).

To evaluate effect of hemolysis, lipemia, and bilirubinemia, 2 canine serum samples were mixed with different concentrations of hemoglobin, lipid, or bilirubin solution following previously described procedures (7) and each preparation was run in duplicate.

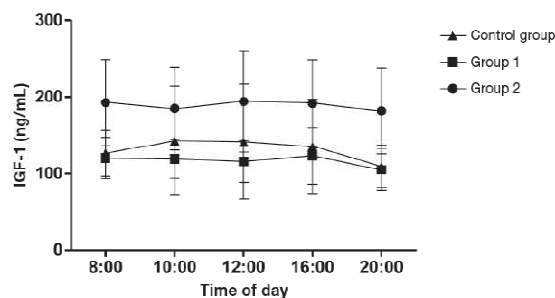
To investigate diurnal variation and possible effect of food intake on serum IGF-1, a previously described protocol (8) with some modifications was used. Briefly: 8 adult beagle dogs were divided into 2 groups of 4 dogs (2 males and 2 females in each). One group, defined as group Fed+, was fed at 09:00 as on previous days. The other group, defined as group Fed-, was fasted 24 h. Blood samples were taken prior to feeding at 08:00 and at intervals of 2, 4, 8, 10, 12, 16, and 20 h.

To study effects of glucocorticoids on serum IGF-1, 14 adult beagles were assigned to control ($n = 4$) and test groups ($n = 10$). Dogs in the control group (2 males and 2 females) were injected SC 0.9% NaCl (0.1 mL/kg). Dogs in the test group were divided into 2 subgroups: group 1 ($n = 5$, 3 males and 2 females) and group 2 ($n = 5$, 3 males and 2 females). Methylprednisolone (Urbason 40 mg; Aventis Pharma S.A., Alcorcon, Madrid, Spain) was injected once SC 1 mg/kg BW and 5 mg/kg BW in groups 1 and 2, respectively. The experiment was started at 08:00 after 12 h fasting in all groups. Blood samples were collected prior to treatment at 08:00 and at intervals of 2, 4, 8, 12, and 20 h. This protocol was chosen on the basis of data from previous studies (9,10).

Blood samples were collected by venipuncture in the cephalic vein into tubes containing clotting accelerator (TapVal; Aquisel, Barcelona, Spain). Samples were centrifuged at $2000 \times g$ for 10 min at room temperature to obtain serum, which was then stored in plastic vials at -20°C for less than 6 mo (11). Serum samples used for repetitive analysis were frozen in aliquots and only vials needed for each run were used, to avoid possible changes due to repetitive thawing and freezing.



b)



c)

Figure 4. Mean $\pm$ standard deviation plasma cortisol (a) and insulin-like growth factor (IGF-1) (b) response to subcutaneous (SC) bolus of methylprednisolone: 1 mg/kg BW (group 1; $n = 5$) and 5 mg/kg BW (group 2; $n = 5$). Four dogs in the control group were injected SC 0.9% NaCl (0.1 mL/kg). * Statistically significant ($P < 0.01$). ^b Statistically significant ($P < 0.001$). ^c Statistically significant ($P < 0.0005$).

Arithmetic means, medians, intra-assay and inter-assay CVs were calculated using routine descriptive statistical procedures and software (GraphPad Prism, GraphPad Software; San Diego, California, USA). Linearity under dilution was accomplished by using ordinary linear regression analysis comparing the measured levels of IGF-1 with the expected levels. Interferograms were prepared to show the differences in IGF-1 concentration when hemoglobin, triglycerides, or bilirubin were added. The influence of hemoglobin and triglycerides was investigated using a 1-way analysis of variance (ANOVA) and Dunnett's post test. Repeated measures ANOVA with Newman-Keuls multiple comparison post test was used to analyze the possible effects of feeding/fasting cycles and glucocorticoids on IGF-1 levels in dogs. A two-way ANOVA with Bonferroni post test was used to compare the biochemical parameters between fed and fasted animals, and between different groups of dogs in glucocorticoid experiment. The significance level used in each case was $P < 0.05$.

Intra-assay CVs were 7.4% and 4.1%, while inter-assay CVs of the same serum pools were 9.2% and 8.6%. In the present study, CV values from the assay (IMMULITE IGF-1) gave slightly higher intra- and inter-assay CVs than those reported by the manufacturers. Nevertheless, the assay appears to be suitable for detecting canine IGF-1, as CV values did not exceed 15%, the limit of the objective analytic performance standard for precision (12).

Dilution of 2 serum samples resulted in linear regression equations with correlation coefficients of 0.9993 and 0.9988 (Figure 1). The LLOQ was 7.1 ng/mL with the precision within 15% verified

by repeated analysis (13), and it was lower than that reported by manufacturers (20 ng/mL). This fact could help for detecting the low values of IGF-1 that can be expected in liver diseases.

Interferograms showing effects of hemolysis, lipemia, and bilirubinemia on determining IGF-1 concentrations are presented in Figure 2. On the graphs, X-axes show increasing concentrations of hemoglobin, triglycerides, or bilirubin while Y-axes show percentage change in IGF-1 $[(V_f/V_o) \times 100]$. A one-way ANOVA showed significant decrease in IGF-1 concentrations in presence of hemolysis ($P = 0.0015$) and lipemia ($P = 0.0043$), but not bilirubinemia ($P = 0.1$). Dunnett's post test showed a statistically significant decrease in IGF-1 concentrations when hemoglobin concentrations were 1, 2, 4, and 8 g/L ($P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.005$, respectively), and when any amount of triglycerides was added to the samples ($P < 0.01$, in all cases). These results would partly agree with ones reported by manufacturers, as they indicate the influence of low grade hemolysis on IGF-1 levels and that the presence of conjugated and unconjugated bilirubin in concentrations up to 200 mg/L had no effect on the results. But it is contradictory in the case of lipemia, as the manufacturers have reported that the presence of triglycerides, up to 3000 mg/dL, had no effect on the IGF-1 levels.

Figure 3 shows serum IGF-1 levels over a 20 h period in fed and fasted dogs. No consistent statistically significant diurnal patterns in total IGF-1 levels were observed in both groups. These data are in accordance with results published in human medicine, in which most studies agree that circulating IGF-1 levels remain relatively stable during the day and are unaffected by meal intake, as well as the type of meal (14–16). Conversely, in other studies on humans, there is evidence of a nocturnal decline in IGF-1 levels from midnight to 04:00 h, following which the levels return to baseline (16,17). This can be explained by shifts in plasma volumes (18).

Responses of cortisol and total IGF-1 to methylprednisolone, in comparison with placebo injection, are shown in Figure 4. Circulating cortisol levels increased 180.5% in group 1 ($P = 0.0008$) and 1318.9% in group 2 ($P = 0.0003$) 2 h after methylprednisolone injection. Four hours after methylprednisolone injection cortisol levels in group 1 returned to normal values ($P = 0.5$), while in group 2 cortisol levels were still significantly increased 36.4% ($P = 0.0089$) and returned to normal values 12 h after corticoid injection. Methylprednisolone 1 mg/kg BW and 5 mg/kg BW subcutaneously did not cause alterations in circulating IGF-1 levels, and no significant difference was found between the control group and groups that received glucocorticoids. Similar results were reported by Wolthers et al (19,20) as no changes in circulating IGF-1 levels in children with asthma treated with low doses of prednisolone (2.5 mg) or inhaled glucocorticoids were detected. In addition, normal IGF-1 levels have been reported in humans with Cushing's syndrome (21). Conversely, Miell et al (22) reported that treatment of men with dexamethasone (2.5 mg) for 3 d significantly stimulated total IGF-1 levels.

In conclusion, the automated method that was tested in the present study exhibited analytical characteristics validating its use in the laboratory, including adequate precision and linearity under dilution. Since it is robust, safe, and totally automated, this method will facilitate widespread studies and applications of IGF-1 measurements in dogs. Furthermore, we have demonstrated, that

canine IGF-1 was significantly influenced by hemolysis and lipemia, so hemolytic or lipemic samples should, ideally, be discarded or interpreted with caution. However it was not affected by meal, fasting, or methylprednisolone administration, so in practice no strict standardization of the sampling condition (time of the day, fasting versus non-fasting, no previous corticoid treatment) is required for collection of blood for IGF-1 measurements.

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3.2.5. Article 6



Analytical performance of commercially-available assays for feline insulin-like growth factor 1 (IGF-1), adiponectin and ghrelin measurements

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 Silvia Martínez-Subiela¹, Fernando Tecles¹ and Jose J Ceron¹

Abstract

The objective of this study was to evaluate three commercially-available human assays for the determination of adiponectin, ghrelin and insulin-like growth factor 1 (IGF-1) concentrations in feline serum samples. Intra- and interassay coefficients of variation were lower than 20%, 15% and 6% for adiponectin, ghrelin and IGF-1 assays, respectively. Dilutions of feline serum pools resulted in linear regression equations in all kits. Mean recovery of adiponectin, ghrelin and IGF-1 assays were 107%, 102% and 105%, respectively. Significant differences were detected in adiponectin and ghrelin concentrations between lean and obese cats ($P < 0.05$ in both cases), but there was no difference in IGF-1 concentrations ($P = 0.12$).

Accepted: 10 November 2011

Introduction

Obesity is an important disease in cats¹ and research interest in this topic is growing, driving a demand for more investigations in this field. Numerous studies have reported changes in different analytes that could proceed to obesity-related diseases such as diabetes mellitus and skin and oral pathologies, among others.^{2,3} Thus, accessible, fast, precise and accurate methods for evaluating the physiopathological changes associated with obesity and its related diseases are necessary in this species.⁴

Adiponectin, ghrelin and insulin-like growth factor 1 (IGF-1) are serum proteins associated with obesity and different metabolic diseases.^{5–7} In cats, adiponectin concentrations decrease in obesity and return to their initial values after weight loss.⁸ Additionally, low concentrations of adiponectin are associated with decreased insulin sensitivity and increased susceptibility to inflammation.⁹ Ghrelin is gaining importance in human medicine owing to its orexigenic and gastric motility stimulating effects,¹⁰ and also as a clinical pharmacological substrate in the treatment of obesity and anorexia.¹¹ IGF-1 is involved in the regulation of growth and metabolism, and also mediates many of the anabolic effects of growth hormone in different tissues.⁶

Currently, measurement of these analytes in cats is limited. Ghrelin and IGF-1 assays have usually been performed with radioimmunoassay (RIA) technology which requires special equipment and facilities^{12,13} and there is a paucity of commercially-available validated assays for adiponectin measurements in feline serum samples.^{8,14} Therefore, the objective of this study was to perform an analytical evaluation of commercially-available enzyme-linked immunosorbent assays (ELISAs) for adiponectin, ghrelin and IGF-1 on cat serum samples. Effects of lipaemia, haemolysis and bilirubinaemia on the determination of the three analytes were also investigated. In addition, overlap performance of the

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assays was studied in order to assess the ability of the assays to detect different concentrations of adiponectin, ghrelin and IGF-1 concentrations in obese and lean cats.

Materials and methods

Adiponectin, ghrelin and IGF-1 analysis

A human adiponectin ELISA (High Sensitivity Kit; BioVendor-Labartorni Medicina, Modrice, Czech Republic) was used for measurement of serum adiponectin. This assay employs microtitre wells coated with polyclonal antibody, horseradish peroxidase, hydrogen peroxide/3,3',5,5'-tetramethylbenzidine (TMB) substrate solution and acidic solution to stop the reaction. Fifteen minutes after substrate is added, the reaction is stopped by the addition of the acidic solution. The enzyme activity is measured spectrophotometrically at 450 nm in a microtitre plate reader (PowerWave XS; Bio-Tek Instruments, Winooski, VT, USA). A standard curve was constructed from the measurement of calibrators by plotting the absorbance values versus adiponectin concentrations of calibrators. Concentrations of unknown samples were then determined using this standard curve. All samples were diluted 1:300 with the dilution buffer provided in the kit prior to analysis, as recommended by the manufacturers. A dilution of 1:200 was also tested but gave poorer results in the linearity tests (data not shown).

A human ghrelin double-antibody sandwich ELISA (BioVendor-Labartorni Medicina) was used for measurement of circulating ghrelin concentration. It employs microtitre wells coated with monoclonal antibodies specific to the C-terminal of ghrelin, acetylcholinesterase-Fab' conjugate, and Ellman's reagent. Absorbance is measured spectrophotometrically at 450 nm, 30–60 min after adding the substrate solution in a microtitre plate reader (PowerWave XS; Bio-Tek Instruments). A standard curve was then constructed from measurement of calibrators by plotting absorbance values versus ghrelin concentrations of calibrators; concentrations of unknown samples were then determined using this standard curve.

In order to verify any changes that could occur with different final incubation times, absorbance readings were initially preformed at 30, 45 and 60 min after the substrate was added. As no significant differences were noted amongst the three time points (data not shown), all measurements were performed at 30 min after adding the substrate solution. Although the manufacturers of the ghrelin assay recommend diluting samples 1:10, preliminary analyses demonstrated that a 1:2 dilution gave more repeatable and accurate results for feline serum ghrelin (data not shown).

For IGF-1, the Immulite 1000 IGF-1 assay (Immulin 1000; Diagnostic Products, Los Angeles, USA), an automated solid-phase, enzyme-labelled chemiluminescent immunometric assay was used. The kit was configured and calibrated on an instrument in our laboratory

according to the manufacturer's instructions. It employs test units containing one bead coated with monoclonal murine anti-IGF-1, alkaline phosphatase (bovine calf intestine) conjugated to polyclonal rabbit anti-IGF-1 in buffer, chemiluminescent substrate [phosphate ester of adamantyl dioxetane in a 2-amino-2-methyl-1-propanol (AMP) buffer with enhancer]. A dilution of 1:5 was used, as recommended by the manufacturers.

Analytical performance

Feline serum samples used for the analytical performance study were obtained from six lean [body condition score (BCS), 4–5 based on a 9-point scale]¹⁵ and six obese (BCS, 7–9/9) cats of different sexes, breeds and ages presented to the Veterinary Teaching Hospital of Murcia University for routine evaluation. Cats had no abnormalities on physical examination (apart from obesity in six cats). Routine haematology (Advia 120; Siemens Healthcare Diagnostics, Bad Nauheim, Germany) and serum biochemistry (Olympus AU2700; Olympus Diagnostica, Melville, NY, USA) were within the reference intervals established for the cats.

Blood samples were collected by jugular or saphenous venepuncture in the morning, after an overnight fast of at least 12 h, and placed in tubes containing clotting accelerator (TapVal; Aquisel, Barcelona, Spain). The samples were then centrifuged at 2000 × g for 10 min at room temperature to obtain serum and were then stored in aliquots in plastic vials at –20°C until analysis. On the day of analysis, samples were brought to room temperature prior to measuring adiponectin, ghrelin and IGF-1 concentrations.

For analytical performance of the three methods, assay precision, accuracy and limit of detection were calculated. All samples used for repetitive analysis were frozen in aliquots and only vials needed for each assay run were used, to avoid possible changes caused by repetitive thawing and freezing.

For assay precision, three pools of sera with different concentrations of analytes that corresponded to low, mid-point and high ranges of the assays were prepared from serum samples obtained from obese and lean cats and used in each assay. For interassay precision, the pools were divided into aliquots and stored in plastic vials at –20 °C until analysis. Intra-assay coefficient of variation (CV) was calculated, after analysis of the three serum pools, five times in a single assay run. Inter-assay CV was determined by analysing the same samples in six separate runs carried out on different days.

The accuracy of the assays was evaluated indirectly by linearity under dilution and recovery studies.¹⁶ For linearity under dilution, the two feline serum pools were serially diluted with diluent provided with the kit (adiponectin, ghrelin, IGF-1) and analysed using the procedures previously described. The pools had the concentration of 7121.27 ng/ml and 4022.68 ng/ml of

adiponectin, 233.62 ng/ml and 120.18 ng/ml of ghrelin, and 315.00 ng/ml and 114.00 ng/ml of IGF-1. As certified species-specific reference material was not available, to perform a spiking recovery test, two samples with different amount of analytes were mixed in different ratios.^{16–18} Thus, samples with high adiponectin, ghrelin and IGF-1 concentrations (5553.40 ng/ml, 109.07 ng/ml and 1562.00 ng/ml, respectively) were mixed at different ratios with the serum samples with low adiponectin, ghrelin and IGF-1 concentrations (1788.79 ng/ml, 44.19 ng/ml and 205.00 ng/ml, respectively) to reach % dilutions of 100, 75, 62, 50, 37.5, 25 and 0%. Test recovery, expressed as a percentage, was calculated for each dilution for comparison of expected versus measured concentrations. Limit of detection was calculated on the basis of data from 20 replicate determinations of the zero standard (buffer of assays), as mean value plus three standard deviations.

To investigate the effects of haemolysis, lipaemia and bilirubinaemia, two feline serum pools were mixed with different concentrations of haemoglobin, lipid or bilirubin solution following previously-described procedures.¹⁹

Effects of haemolysis and lipaemia

To investigate the effects of lipids, a commercial fat emulsion (Lipofundina 20%; Braun Medical, Barcelona, Spain) with a triglyceride concentration of 200 g/l was serially diluted with sample diluent buffer; 10 µl of each dilution was added to two samples of 90 µl of feline serum. Sample homogeneity was then achieved by vortexing. The final triglyceride concentrations were 5, 2.50, 1.25, 0.625 and 0.3125 g/l. These triglyceride concentrations would correspond to slight lipaemia (0.3125 and 0.625 g/l), moderate lipaemia (1.25 and 2.5 g/l) and marked lipaemia (5 g/l).

To investigate the effect of haemolysis, a fresh haemolysate was prepared by addition of distilled water to packed, saline-washed, feline red cells from a healthy cat. The haemoglobin concentration in the haemolysate was determined by using a Veterinary Animal Blood Counter (Vet ABC; ABX Diagnostics, Montpellier, France) and adjusted to 200 g/l by adding assay buffer to produce a stock solution. This stock solution was serially diluted with sample buffer and 10 µl of each dilution was added to the samples of 90 µl of feline serum. The final haemoglobin concentrations were 8, 4, 2, 1, 0.5 and 0.0 g/l (10 µl of sample diluent buffer were added to the samples to give 0.0 g/l concentration). These haemoglobin concentrations would correspond to slight haemolysis (0.5 g/l), moderate haemolysis (1 and 2 g/l) and marked haemolysis (4 and 8 g/l).

To study the effects of hyperbilirubinaemia, 6 mg of bilirubin (Sigma Chemical, St Louis, MO, USA) were suspended in 1 ml of the sample diluent provided in the corresponding commercial kit for adiponectin, ghrelin and IGF-1 assays. This stock solution was serially

diluted with sample buffer and 10 µl of each dilution was added to the samples of 90 µl of feline serum. The final bilirubin concentrations were 0.15, 0.075, 0.037, 0.018, 0.009 and 0.0 g/l (10 µl of sample diluent buffer were added to the samples to give 0.0 g/l concentration). These bilirubin concentrations would correspond to slight bilirubinaemia (0.009 g/l), moderate bilirubinaemia (0.018 and 0.037 g/l) and marked bilirubinaemia (0.075 and 0.15 g/l).

Overlap performance

In order to evaluate whether the assays were able to detect differences in analyte concentrations between lean and obese individuals, samples from 20 client-owned cats were tested. Two groups were studied: group C1 comprised 10 overweight/obese neutered cats (BCS 7–9/9), all of which had been referred to the Royal Canin Weight Management Clinic, University of Liverpool (female/male 2/8; body weight range 5.8–10.7 kg; age range 5–10 years; mixed breeds); group C2 comprised 10 healthy normal weight entire cats (BCS 4–5/9), presented to the Veterinary Hospital, University of Murcia, Spain for routine check-ups or castration (female/male 2/8; body weight range 3.1–4.5 kg; age range 4.2–8.0 years; mixed breeds). Blood samples were collected by jugular or saphenous venepuncture in the morning after an overnight fast of at least 12 h. All cats were clinically normal (apart from obesity in 10 cats) and had normal haematology (Baker 9010 analyzer; Sero-Baker: Advia 120; Siemens Healthcare Diagnostics) and serum biochemistry (Kone Specific Supra biochemistry analyser; Thermo Fisher Scientific: Olympus AU2700; Olympus Diagnostica) results.

Statistical methods

Arithmetic means, medians, intra- and interassay CVs were calculated using routine descriptive statistical procedures and software (Excel, Microsoft, Redmond, WA, USA; GraphPad Prism, GraphPad Software, La Jolla, CA, USA). Linearity under dilution was accomplished by ordinary linear regression analysis comparing the measured concentrations of analyte with the expected levels. Interferograms were prepared to show the differences in analyte concentrations when triglycerides, haemoglobin or bilirubin were added, as previously described.¹⁹ The influence of haemoglobin, triglycerides or bilirubin was investigated using one-way repeated measures analysis of variance (ANOVA) and Tukey's multiple comparison post-test. Student's *t*-test for similar variances was used to evaluate the difference of adiponectin and IGF-1 as an *F* test revealed that the variances did not differ significantly, while Student's *t*-test for different variances was used to evaluate the difference of ghrelin between healthy and obese cats as an *F* test revealed that the variances differed significantly. The significance level used in each case was *P* < 0.05.

Table 1 Intra- and interassay coefficients of variation (CV) of adiponectin, ghrelin and IGF-1 assays in feline serum pools

Assay	Comparison	Pool	Mean	SD	CV(%)
Adiponectin ng/ml	Intra	Pool 1	249.8	16.2	6.5
		Pool 2	2070.6	102.6	5.0
		Pool 3	5969.7	89.3	1.5
	Inter	Pool 1	235.6	37.4	15.9
		Pool 2	1990.0	309.6	15.6
		Pool 3	6998.0	712.7	10.2
Ghrelin pg/ml	Intra	Pool 1	43.59	4.0	9.2
		Pool 2	76.5	5.6	7.3
		Pool 3	115.17	3.3	2.9
	Inter	Pool 1	41.68	5.5	13.2
		Pool 2	79.3	8.0	10.1
		Pool 3	123.98	10.0	8.1
IGF-1 ng/ml	Intra	Pool 1	58.0	0.5	0.9
		Pool 2	374.6	8.3	2.2
		Pool 3	1005.8	31.2	3.1
	Inter	Pool 1	58.6	2.7	4.6
		Pool 2	353.4	18.8	5.3
		Pool 3	1003.5	55.2	5.5

Table 2 Spiking recovery. Two samples with different amounts of analytes were mixed in different ratios and recovery, expressed as a percentage, was calculated for each dilution for comparison of expected versus measured concentrations

	Volume, %		Expected	Observed	%
	Sample 1	Sample 2			
Adiponectin	100	0	5553.40	5553.40	100
	75	25	4612.25	5134.75	111.3
	62.5	37.5	4141.67	4591.60	110.9
	50	50	3671.09	4095.93	111.6
	37.5	62.5	3200.52	3525.41	110.2
	25	75	2729.94	2951.53	108.1
	0	100	1788.79	1788.79	100
Ghrelin	100	0	109.07	109.07	100
	75	25	92.85	104.14	112.2
	62.5	37.5	84.74	76.92	90.8
	50	50	76.63	79.84	104.2
	37.5	62.5	68.52	73.16	106.8
	25	75	60.41	59.90	99.1
	0	100	44.19	44.19	100
IGF-1	100	0	1562.00	1562.00	100
	75	25	1222.75	1288.00	105.3
	62.5	37.5	1053.13	1155.00	109.7
	50	50	883.50	992.00	112.3
	37.5	62.5	713.88	747.00	104.6
	25	75	544.25	551.00	101.2
	0	100	205.00	205.00	100

Results

Assay characteristics

Intra- and interassay CVs of the three pools were below 7–16%, respectively (Table 1). The linearity under dilution of two feline serum pools is shown in Figure 1.

Dilution of feline serum samples with different adiponectin concentrations resulted in linear regression equations with correlation coefficient close to 1.0 in the mean range from 90–7000 ng/ml. The recovery between observed and expected adiponectin concentrations

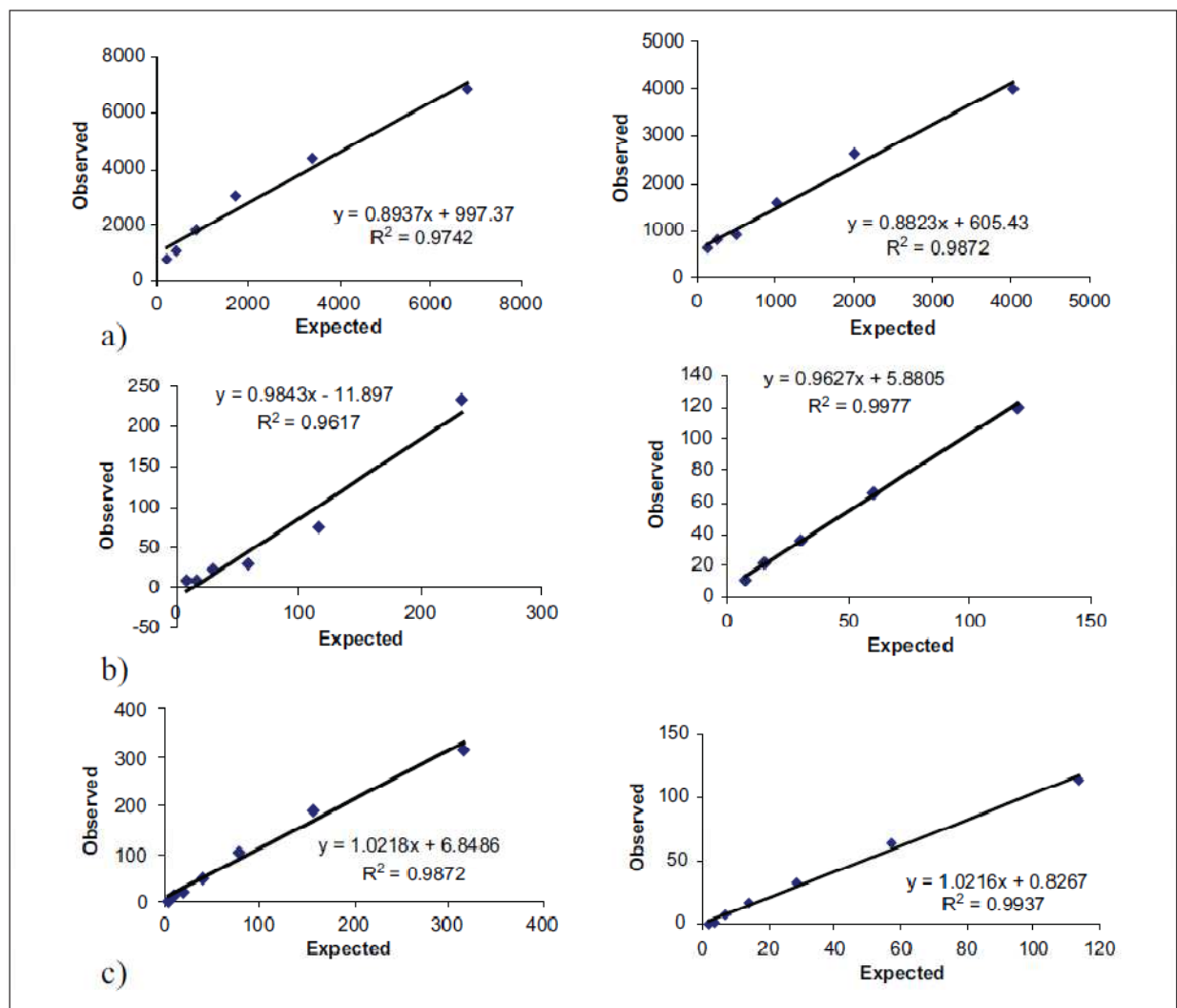


Figure 1 Linearity under dilution of two feline serum pools with different adiponectin (A), ghrelin (B) and IGF-1 (C) concentrations

ranged from 100–112%, with a mean of 107% (Table 2). The detection limit was 2.0 ng/ml [mean 0.2 ng/ml, standard deviation (SD) 0.6 ng/ml].

For ghrelin, intra- and interassay CVs of the three pools were below 15% in both cases (Table 1). The linearity under dilution of two feline serum pools is shown in Figure 1. Dilution of feline serum samples with different adiponectin concentrations resulted in linear regression equations with correlation coefficient close to 1.0 in the mean range from 30–130 pg/ml. The recovery between observed and expected ghrelin concentrations ranged from 91–112%, with a mean of 102% (Table 2). The detection limit was 9.6 pg/ml (mean 1.8 pg/ml, SD 2.6 pg/ml).

For IGF-1, intra- and interassay CVs of the three pools were below 3% and 6%, respectively (Table 1). The linearity under dilution of two feline serum pools is

shown in Figure 1. Dilution of feline serum samples with different adiponectin concentrations resulted in linear regression equations with correlation coefficient close to 1.0 in the mean range from 10–1600 ng/ml. The recovery between observed and expected IGF-1 concentrations ranged from 100–112%, with a mean of 105% (Table 2). The detection limit was lower than 0.01 ng/ml.

Interferograms showing effects of lipaemia, haemolysis and bilirubinaemia on adiponectin, ghrelin and IGF-1 concentrations determination are presented in Figures 2–4.

The different degrees of lipaemia, haemolysis and bilirubinaemia tested in this study did not affect the measured concentrations of adiponectin or ghrelin in the feline serum samples. Lipaemia with a lipid concentration up to 5 g/l and bilirubinaemia with a bilirubin concentration of up to 0.15 g/l did not affect IGF-1

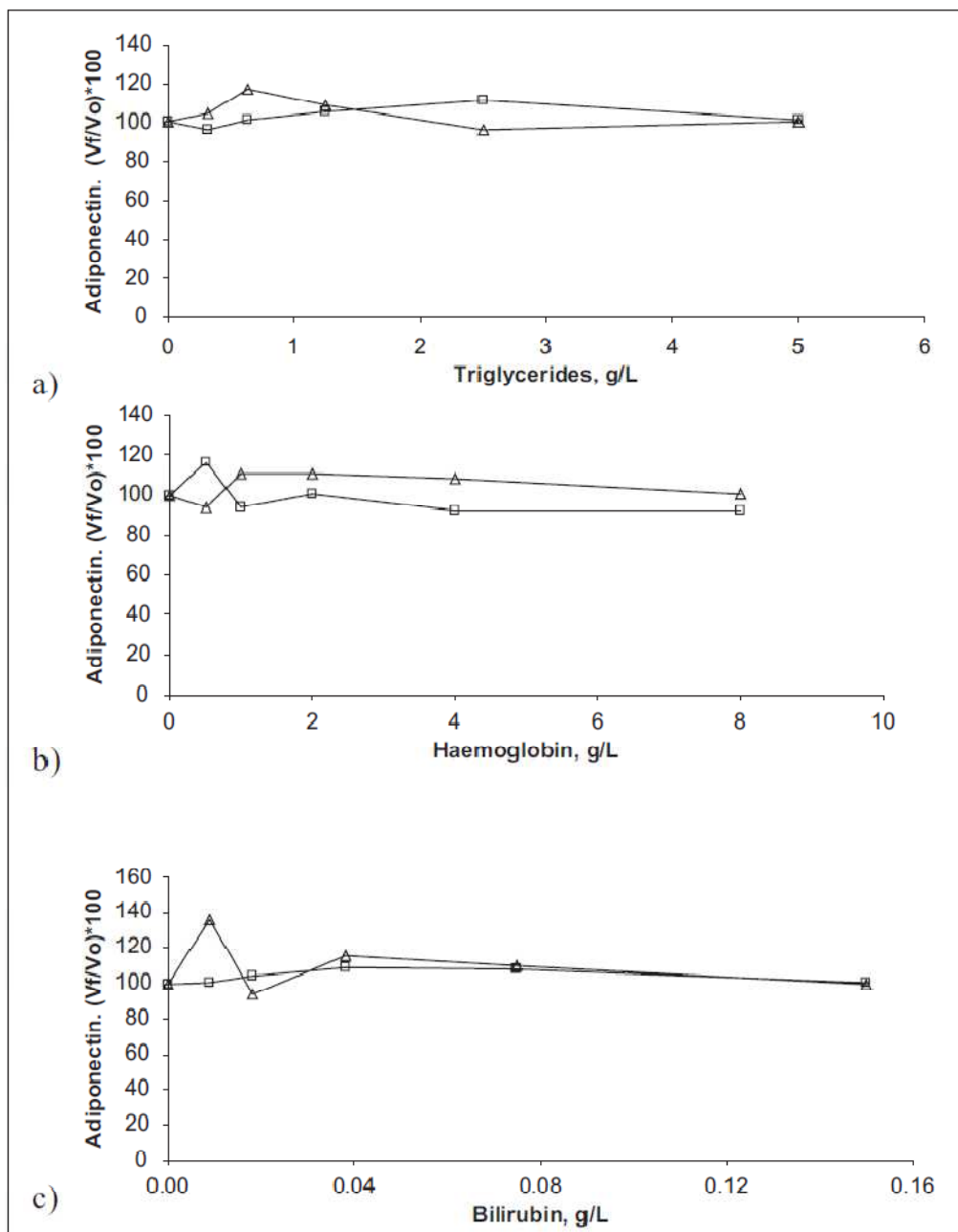


Figure 2 Interferograms corresponding to the effect of triglycerides (A), haemoglobin (B) and bilirubin (C) concentrations on adiponectin determination in two feline serum pools. In these graphs, results obtained with the two pools are presented with open boxes and with open triangles, respectively. X-axes show increasing concentrations of haemoglobin, triglycerides or bilirubin, whilst Y-axes show percentage of change in adiponectin $[(Vf/V_0) \times 100]$. Vf = final value; V_0 = original value

determination in feline serum. However, one-way ANOVA showed a significant decrease ($P < 0.05$) in IGF-1 concentrations in the presence of severe haemolysis with a haemoglobin concentration of 8 g/l.

Overlap performance

Serum adiponectin ($P = 0.01$) and ghrelin ($P = 0.03$) concentrations were lower in obese cats (group C1)

compared with normal weight cats (group C2), but there was no difference in IGF-1 concentrations between groups ($P = 0.12$) (Table 3).

Discussion

A primary function of any methodology is to minimise the amount of error so that test interpretation, patient care and consumer safety are not compromised.¹⁶

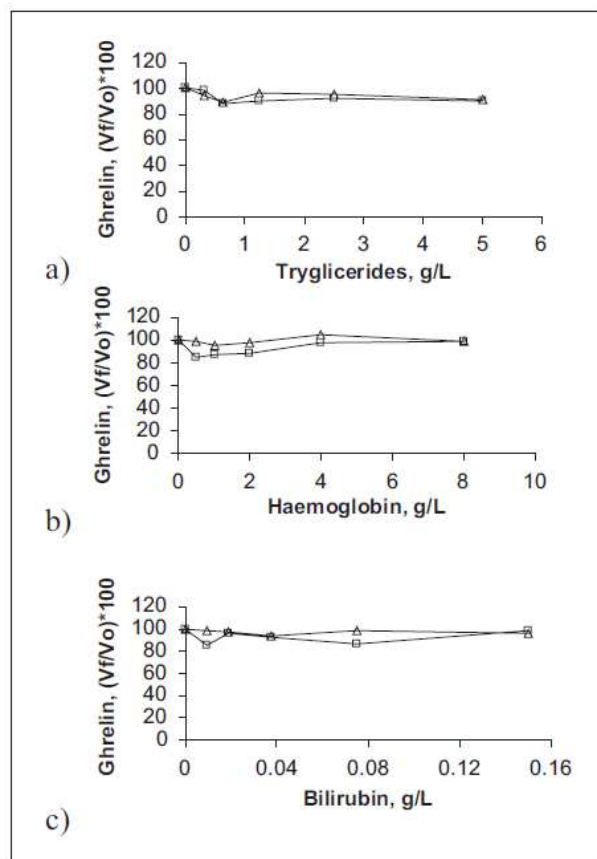


Figure 3 Interferograms corresponding to the effect of triglycerides (A), haemoglobin (B) and bilirubin (C) concentrations on ghrelin determination in two feline serum pools. In these graphs, results obtained with the two pools are presented with open boxes and with open triangles, respectively. X-axes show increasing concentrations of haemoglobin, triglycerides or bilirubin, whilst Y-axes show percentage of change in ghrelin $[(Vf/V_0) \times 100]$. V = final value; V_0 = original value

Therefore, a common task in clinical laboratories is to validate new tests, assuring that test results reflect the status of the animals more than they reflect variation caused by the laboratory itself. Furthermore, validation studies must ensure that analytical methods can detect the corresponding analyte and provide repeatedly accurate results.²⁰ The three assays evaluated in the present study showed adequate precision for adiponectin, ghrelin and IGF-1 measurements in feline serum samples with intra- and interassay CVs lower than 20%, the limit of the objective analytic performance standard for precision.²¹ However, interassay CVs for low and medium adiponectin concentrations were greater than 15%, which is considered to be poor by some authors. Therefore, when adiponectin is measured, analysing all samples in a single batch, if possible, would be

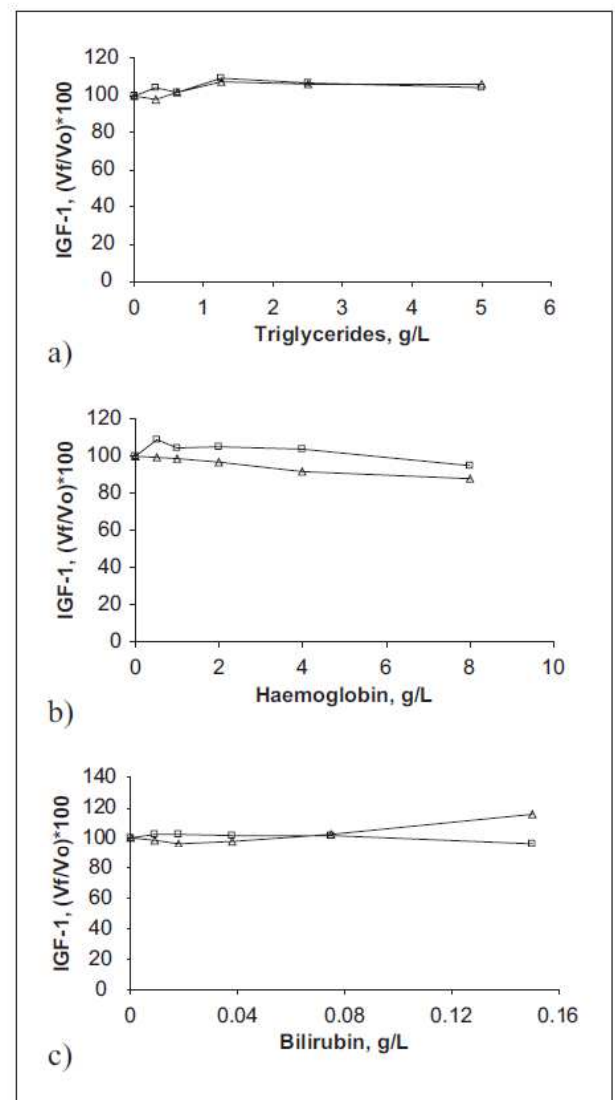


Figure 4 Interferograms corresponding to the effect of triglycerides (A), haemoglobin (B), and bilirubin (C) concentrations on IGF-1 determination in two feline serum pools. In these graphs, results obtained with the two pools are presented with open boxes and with open triangles, respectively. X-axes show increasing concentrations of haemoglobin, triglycerides, or bilirubin, whilst Y-axes show percentage of change in IGF-1 $[(Vf/V_0) \times 100]$. Vf = final value; V_0 = original value

recommended in order to minimise the effect of problems with interassay precision. Further, given such variability, it would not be advisable to use such assays for the purpose of clinical diagnosis, where assays with better precisions are preferred. The IGF-1 assay gave results with the best precision, possibly because it was automated. Automated assays are usually more robust and have the additional advantage of more rapid turnaround time.

Table 3 Serum adiponectin, ghrelin and IGF-1 in obese (C1) and normal weight (C2) cats

	Obese cats (group C1)		Lean cats (group C2)		<i>P</i>
	Mean	Range	Mean	Range	
Adiponectin pg/ml	875.85	(182.9–2822.3)	3567.58	(169.5–6578.9)	0.01
Ghrelin pg/ml	220.46	(130.21–306.6)	287.40	(196.0–332.9)	0.03
IGF-1 ng/ml	426.22	(293–570)	498.33	(291–998)	0.18

Similar results (CV <20%) were obtained when these three assays were validated in the authors' laboratory for use with canine serum samples.^{22–24}

Given that neither certified species-specific reference material nor a 'reference' method were available, accuracy was indirectly evaluated by linearity under dilution and spiking recovery,²⁵ performed by mixing different samples at different proportions. Ideally, the regression analysis of the relationship of the obtained and expected analyte values should result in a regression equation approximating R^2 to 1.0. For spiking recovery, percentages between 80% and 120%, should be recorded.¹⁶ In the present study, regression analysis revealed that the three assays were linear when measuring adiponectin, ghrelin and IGF-1, respectively, in different dilutions of feline serum samples with the regression equation R^2 close to 1.0. Additionally, given the lack of defined species-specific standards, the methods performed satisfactorily with the materials available for the recovery procedures, the recovery study showed that the three assays could accurately measure the different concentrations of adiponectin, ghrelin and IGF-1 when two samples were mixed at different ratios with a mean recovery close to 100% (107%, 102% and 105%, respectively). These findings confirm all three assays to be accurate when quantifying their respective analytes in feline serum samples.

The lower linearity ranges observed in evaluated tests were acceptable for measurement of adiponectin, ghrelin and IGF-1 in all studied cats. In some cases, serum from lean cats had analyte concentrations exceeding the linearity ranges of the assays. In such cases, the authors would recommend diluting the sample further and repeating the analysis.

In the present study, the three methods were performed with the human standards provided by the respective manufacturers because of a lack of feline reference material with known amounts of adiponectin, ghrelin and IGF-1, respectively. However, for the future, it would be desirable to use species-specific standards in order to achieve similar affinity of antiserum against standards and samples.²⁶

Adiponectin and ghrelin concentrations were significantly less in obese cats compared with normal weight cats, which is similar to findings from previous reports.^{8,12,14} Mean concentrations of adiponectin found in our study in

obese and normal weight cats are in an equivalent range to those found by Hoenig et al⁸ who reported concentrations of around 3 ng/ml (3000 pg/ml) in lean and 1 ng/ml (1000 pg/ml) in obese cats; however, they are less than those reported by Ishioka et al¹⁴ who found concentrations of 18000 ng/ml in lean and 7200 ng/ml in obese cats. Also, our concentrations for ghrelin are less than those reported by Backus et al¹² in cats, with values of around 2000 pg/ml. Differences in the standard used in the assays, as well as affinity in the antibodies, could be a factor that contributed to such diverse results. No difference in serum IGF-1 concentration has been observed between lean and obese cats in contrast to the findings in obese humans.^{27,28} This could be because of a high interindividual variability of the serum IGF-1 in cats¹³ or because of the study was performed comparing neutered and intact cats, as castration can affect IGF-1 concentrations.²⁹ It may be that differences in circulating IGF-1 levels would be evident between lean and obese cats either if larger groups were compared, or if IGF-1 was measured in the same group of cats before and after weight loss. However, mean concentrations found for IGF-1 in our study for lean cats were similar to the values previously reported by Alt et al.¹³ which reported mean IGF-1 concentrations of 452 ng/ml.

In conclusion, we have made a preliminary assessment of commercially-available assays for adiponectin, ghrelin and IGF-1 for use with feline serum samples. All methods exhibited acceptable analytical characteristics, allowing their use in the laboratory with an adequate precision, linearity under dilution and recovery. Further, each assay can detect different concentrations of its respective analyte in lean and obese cats, respectively. Use of these assays in future research studies may help to improve understanding of the pathogenesis of feline obesity and obesity-related diseases.

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Conflict of interest The authors declare that there is no conflict of interest.

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3.3. Variations in adiponectin and IGF-1 due to other but obesity factors

3.3.1. Article 7

SERUM ADIPONECTIN CONCENTRATION IN DOGS – ABSENCE OF DIURNAL VARIATION AND LACK OF EFFECT OF FEEDING AND METHYLPREDNISOLONE ADMINISTRATION

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The aim of the study was to determine the magnitude of diurnal variability of serum adiponectin in healthy beagle dogs, and the possible roles of feeding and glucocorticoids on adiponectin concentrations. For this, adiponectin was measured at 8:00, 10:00, 12:00, 16:00, 20:00, 24:00 and at 4:00 h in 4 beagle dogs that were fasted on the day of the experiment and in 4 dogs that were fed as usual at 9:00 h. Diurnal variability in serum adiponectin concentrations was negligible in both the fed and the fasted dogs. To study the possible effect of glucocorticoids on adiponectin, beagle dogs ($n = 14$) were assigned to one of three experimental groups. Dogs of the control group were injected with 0.1 ml/kg 0.9% NaCl subcutaneously, while dogs of Groups 1 and 2 were injected with 1 mg/kg and 5 mg/kg of methylprednisolone, respectively, and adiponectin was measured at 8:00, 10:00, 12:00, 16:00, and 20:00 h. Average serum adiponectin levels were not significantly different before and after methylprednisolone exposure at different time-points in the two treated groups. In conclusion, no evidence of postprandial changes in adiponectin level or effects of single-dose glucocorticoid administration on adiponectin were observed in the present study.

Key words: Adiponectin, diurnal variation, dog, glucocorticoids, methylprednisolone

Adiponectin is a secretory protein predominantly expressed by adipocytes and released at a high rate into circulation (Chandran et al., 2003). Unlike most other adipokines, serum levels of adiponectin are decreased in obesity (Chandran et al., 2003). Data from patients and experimental animals suggest that adiponectin has insulin-sensitising, antidiabetic, anti-inflammatory and cardioprotective properties (Weyer et al., 2001; Zoccali et al., 2002; Nishii et al., 2006). Furthermore, the ease with which the levels of adiponectin can be measured owing to its high abundance, small diurnal variation and high stability in plasma had made it a popular target for measurements in many human clinical studies

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(Chitalia et al., 2010). In dogs, adiponectin has gained attention over the past few years as it was reported that in controlled studies its average concentrations were lower for obese dogs than for dogs of normal weight (Ishioka et al., 2006; Brunson et al., 2007), although other studies did not find adiponectin concentration decreases in obese dogs (Verkest et al., 2011).

In previous studies another adipokine, leptin, has been investigated as a quantitative diagnostic marker of adiposity and obesity in dogs (Ishioka et al., 2002; Jeusette et al., 2005). It was demonstrated that leptin has diurnal changes in association with feeding–fasting cycles in dogs as well as in humans (Schoeller et al., 1997; Ishioka et al., 2005) and is influenced by endogenic and exogenic glucocorticoids (Masuzaki et al., 1997; Ishioka et al., 2002; Nishii et al., 2006; Yilmaz et al., 2007). However, no studies have been made about the influence of the above-mentioned factors on adiponectin in dogs, despite the interest that this knowledge may have for a proper interpretation of the values of this adipokine in veterinary diagnostic laboratories. So the aim of this study was to determine the magnitude of diurnal variability of serum adiponectin in healthy beagle dogs, and the possible roles of feeding and glucocorticoids on total adiponectin concentrations.

Materials and methods

Animals

Light young intact beagle dogs (4 females and 4 males; age range: 3.2–5.1 years; body weight [BW] range: 12.6–18.9 kg; body condition score [BCS]: 3–4) were included in Experiment 1. In Experiment 2, 14 young intact beagle dogs (8 females and 6 males; age range: 3.2–5.5 years; BW range: 10.1–17.9 kg; BCS: 3–4) were used. A single breed (beagle) within a narrow age range was used in order to avoid possible breed- and age-associated differences in adiponectin levels. All animals were housed in their usual kennels, one or two dogs per kennel, in a room with controlled temperature ($23 \pm 2^\circ\text{C}$) and a standard 12:12 light-dark cycle. The size of the kennel was 3×4 m. All dogs were fed a standard dry food (Premium Croc Adult, Affinity Petcare S.A., Barcelona, Spain), containing 24.0% crude protein, 16.1% crude fat, 2.5% crude fibre, 6% crude ash, 8% moisture, daily at 9:00 h. All dogs ate the food within 10 min after it was offered. Water was available *ad libitum*. General animal care was carried out by professional staff not associated with the research team. Prior to each experiment, general health examinations including complete blood count and biochemical profiles were performed to confirm normal health status and the absence of apparent abnormalities. All animals were serologically negative for *Leishmania infantum* and *Ehrlichia canis*. Animal care procedures were in accordance with the guidelines of the University of Murcia.

Experiment 1: Diurnal variation of serum adiponectin and food intake

A previously described protocol (Ishioka et al., 2005) with some modifications was used. Eight beagle dogs were assigned into two groups of four dogs (2 males and 2 females in each). One group, defined as Group Fed+, was fed at 9:00 a.m. as on the previous days. The other group, defined as Group Fed–, was fasted on the day of the experiment. Blood samples were taken at 8:00, 10:00, 12:00, 16:00, 20:00, 24:00 and at 4:00 h.

Experiment 2: Effects of glucocorticoids on serum adiponectin

Fourteen beagles were included in this experiment. None of these dogs had a history of exogenous glucocorticoid exposure. The dogs were assigned to a control (n = 4) and a test group (n = 10). Dogs in the control group (2 males and 2 females) were given 0.9% NaCl (0.1 ml/kg) by subcutaneous (s.c.) injection. The dogs in the test group were distributed to two subgroups: Group 1 (n = 5, 3 males and 2 females) and Group 2 (n = 5, 3 males and 2 females). To investigate the effects of glucocorticoids on serum adiponectin, methylprednisolone (Aventis Pharma S.A., Alcorcon, Madrid, Spain) was injected once s.c. in a dose of 1 mg/kg and 5 mg/kg in Groups 1 and 2, respectively. The experiment was started at 8:00 a.m. after 12-h fasting in all groups. Blood samples were collected prior to treatment at 8:00, 10:00, 12:00, 16:00, and at 20:00 h. The design of this experiment was based on protocols developed in previous studies (Martinez-Subiela et al., 2004; Yilmaz et al., 2007).

Samples

Blood samples for the biochemical assays of this study were collected from the cephalic vein by venipuncture into collection tubes containing clotting accelerator (TapVal, Aquisel, Barcelona, Spain). Samples were centrifuged at 2000 g for 10 min at room temperature to isolate serum from heavier cellular components. Serum samples were distributed to plastic vials and stored (for no more than two weeks) at –20 °C. Serum samples were thawed on ice in preparation for biochemical assays.

Assays of serum analytes

Total serum adiponectin concentration was determined by human high-sensitivity adiponectin ELISA (Human Adiponectin ELISA, High Sensitivity Kit, BioVendor-Laboratori Medicina, Modrice, Czech Republic), previously validated in our laboratory for use in dogs. It employs microtitre wells coated with polyclonal anti-human adiponectin antibody, horseradish peroxidase, hydrogen peroxide/TMB substrate solution, and an acidic solution to stop the reaction. The enzyme activity is measured spectrophotometrically at 450 nm in a mi-

crotitre plate reader (PowerWave XS, Bio-Tek Instruments Inc., Vermont USA). The absorbance is proportional to the concentration of adiponectin. A standard curve is constructed by plotting absorbance values against concentrations of canine serum standards, and concentrations of unknown samples are determined using this standard curve. Intra- and inter-assay coefficients of variation were below 11% in all cases. The assay was linear over the range of 0.8 to 26 µg/ml based on linear regression coefficients of 0.996–0.999. The limit of detection was of 0.74 µg/ml (TvariJonaviciute et al., 2010).

Cortisol concentrations were determined with the solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite System, Siemens Health Diagnostics, Deerfield, IL, USA), previously validated in dogs (Reimers et al., 1996). Glucose determination was performed using a hexokinase-based method on an automated clinical chemistry analyser (Olympus AU2700, Olympus Diagnostica GmbH) following the instructions of the manufacturer.

Data analysis

Data are presented for every dog and as the mean of the different groups of animals. The results for each parameter were evaluated for approximate normality of distribution by using the Kolmogorov-Smirnov statistics. Because the data were not distributed normally, data were *log* transformed to reduce non-normality. An unpaired *t*-test was used to compare BW and BCS between the different groups of dogs. Differences of adiponectin between individual dogs were analysed by one-way analysis of variance (ANOVA). Inter-individual coefficient of variation (CV) among the dogs with different BCS in Experiment 1 was calculated as SD divided by the average and multiplied by 100 (Faraj et al., 2008). One-way repeated measures ANOVA with Tukey's *post-hoc* test was used to evaluate possible changes of biochemical analytes in dogs in Experiment 1. Paired *t*-test was used to identify possible treatment-related differences of biochemical analytes in dogs in Experiment 2. Correlations between adiponectin, BW and BCS were estimated using Spearman's correlation coefficient. Values of $P < 0.05$ were considered significant.

Results

Experiment 1: Diurnal variation of serum adiponectin and food intake

Spearman's rank correlation test showed a statistically significant negative correlation between serum adiponectin concentrations and BW ($P < 0.05$; $r = -0.8333$), and BCS ($P < 0.01$, $r = -0.8452$) when data from the two groups were pooled. No statistically significant difference was recorded in serum adiponectin levels, BW or BCS between the two groups. No differences were observed between the two sexes in adiponectin levels (males vs. females, 11.69 ± 5.8 µg/ml

vs. 10.82 ± 3.9 $\mu\text{g/ml}$; $P = 0.4$) at 8:00 a.m. The inter-individual CV was 38% and 54% ($P < 0.0001$ in both cases) for serum adiponectin in dogs with BCS of 3/5 and 4/5, respectively.

Figure 1 shows mean $\pm$ SD serum adiponectin and glucose levels of fed and fasted dogs over a 20-h period. One-way ANOVA and Tukey's Multiple Comparison Test showed no consistent diurnal patterns in total adiponectin levels in the two groups. There were significant diurnal rhythms of serum glucose in fed and fasted dogs (Fig. 1).

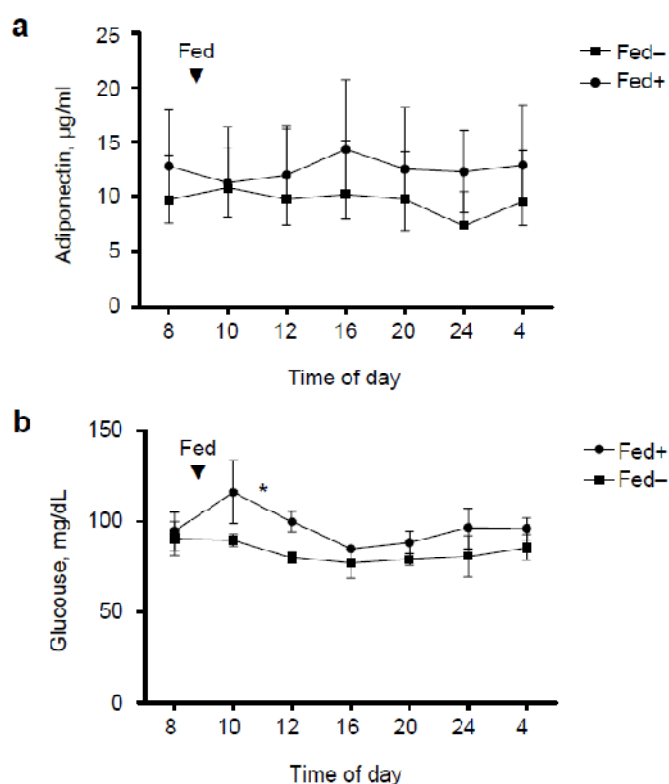


Fig. 1. Postprandial variation of serum adiponectin (a) and glucose (b) concentrations. Four beagle dogs were fed at 9:00 h and 4 beagle dogs were fasted all day long. * $P < 0.05$

Experiment 2: Effects of glucocorticoids on serum adiponectin

Spearman's correlation test showed a statistically significant negative correlation between serum adiponectin concentrations and BW ($P < 0.05$; $r = -0.5874$) as well as BCS ($P < 0.005$, $r = -0.7681$) when data from the three groups were pooled. No statistically significant differences were recorded in serum adiponectin levels, BW or BCS between the three groups. No differences were observed between the two sexes in adiponectin levels (males vs. females, 17.06 ± 11.1 $\mu\text{g/ml}$ vs. 11.47 ± 4.6 $\mu\text{g/ml}$; $P = 0.1$) at 8.00 a.m.

Individual and mean data of response of cortisol and total adiponectin levels to methylprednisolone in comparison with placebo injection are presented in Figs 2, 3 and 4. Circulating cortisol levels increased by 0180.5% in Group 1 ($P = 0.0008$) and 1318.9% in Group 2 ($P = 0.0003$) 2 h after methylprednisolone injection. Four h after methylprednisolone injection cortisol levels in Group 1 returned to normal values ($P = 0.5$), while in Group 2 cortisol levels were still significantly increased (by 36.4%, $P = 0.0089$) and returned to normal values 12 h after corticoid injection.

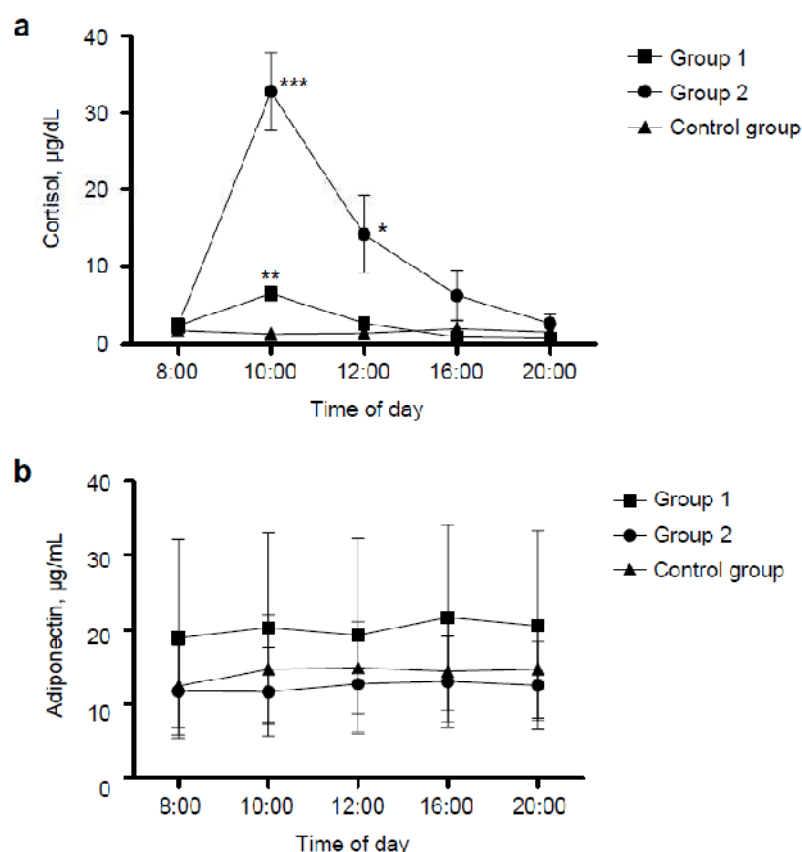


Fig. 2. Plasma cortisol (a) and serum adiponectin (b) response (mean + SD) to a subcutaneous bolus of methylprednisolone: Group 1 (1 mg/kg) and Group 2 (5 mg/kg). Dogs in the control group received 0.9% NaCl (0.1 ml/kg) by subcutaneous injection. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0005$

Methylprednisolone administered s.c. at a dose of 1 mg/kg and 5 mg/kg did not produce alterations in circulating adiponectin levels, and additionally no significant difference was found between the control group and the groups that received glucocorticoids at different time-points.

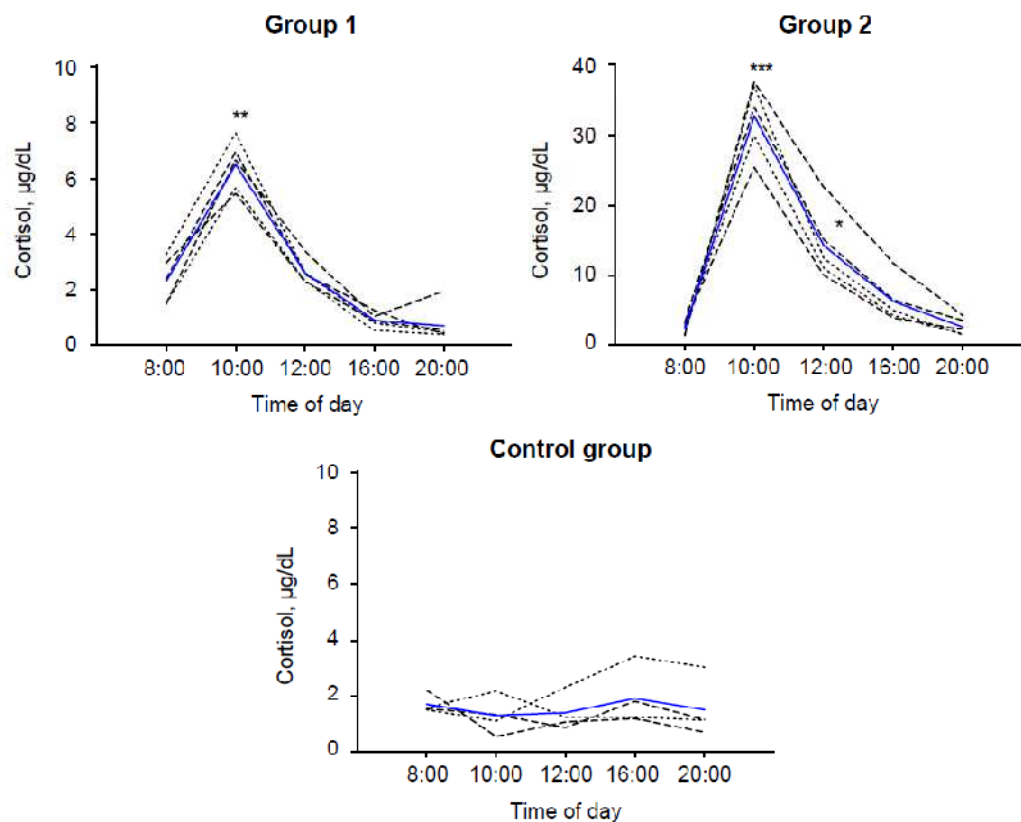


Fig. 3. Plasma cortisol response to a subcutaneous bolus of methylprednisolone: Group 1 (1 mg/kg) and Group 2 (5 mg/kg). Dogs in the control group received 0.9% NaCl (0.1 ml/kg) by subcutaneous injection. Discontinuous lines represent cortisol concentrations in individual dogs (males: dashed lines, females: dotted lines). Continuous line: mean cortisol concentration.

* $P < 0.01$; ** $P < 0.001$; *** $P < 0.0005$

Discussion

The objective of this study was to analyse the possible effects of different factors such as diurnal variations, feeding and glucocorticoids on total adiponectin concentrations in dogs. The results show that serum adiponectin levels were stable in individual animals. Inter-individual variability of serum adiponectin was high and statistically significant, in the same way as it has been described in humans (Gavrila et al., 2003). However, the variability in the present study could not be explained by time of day, meal timing or methylprednisolone treatment, as these factors had little impact on serum adiponectin levels, despite changes observed in glucose or cortisol levels. These results are consistent with the findings of some studies conducted in humans and animals that found no diurnal variation or postprandial alterations in adiponectin levels (Shand et al., 2006). However, it is not consistent with other studies. For example, Gavrila et al. (2003) showed a diurnal variation in serum adiponectin levels, characterised

by a nocturnal decline starting late in the evening and at night, reaching the nadir in the early morning; although in their study the variations of leptin and cortisol were more pronounced than the diurnal variability of adiponectin (Gavrila et al., 2003).

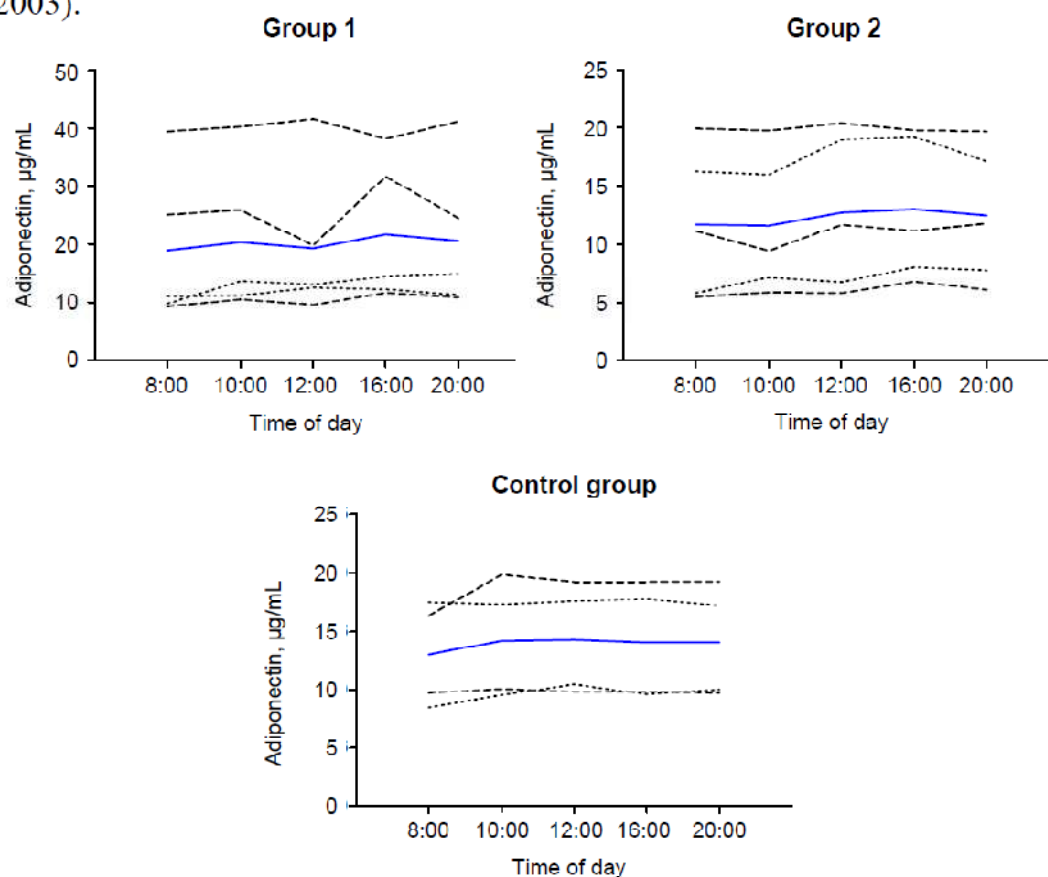


Fig. 4. Serum adiponectin response to a subcutaneous bolus of methylprednisolone: Group 1 (1 mg/kg) and Group 2 (5 mg/kg). Dogs in the control group received 0.9% NaCl (0.1 ml/kg) by subcutaneous injection. Discontinuous lines represent adiponectin concentration in individual dogs (males: dashed lines, females: dotted lines). Continuous line: mean adiponectin concentration

Basal adiponectin levels varied significantly between the individual animals. This could be influenced by different body weight/body condition score as described previously (Ishioka et al., 2006), although other studies did not find this effect (Verkest et al., 2011). However, the inter-individual variability was significant when it was calculated for different BCS separately. Despite the low number of animals used, gender – in the same way as reported previously reported (Ishioka et al., 2006; Verkest et al., 2011) – did not influence adiponectin values in our work. High inter-individual variability in adiponectin was also found in humans (Gavrila et al., 2003). These data suggest that adiponectin may not be a good diagnostic indicator of obesity when different individuals are compared and could partly explain the lack of variation in adiponectin between lean

and obese dogs found in previous studies (Verkest et al., 2011), but it still may be useful for individual monitoring of treatment-associated health effects. So baseline levels are important to be established in individual animals.

Methylprednisolone was administered in the present study as it is one of the glucocorticoids most frequently used in veterinary medicine. There are many variations in the route, dose and time of its administration: dose rates of 0.5 to 1 mg/kg per day are considered as anti-inflammatory, whereas doses of 2–4 or more mg/kg per day are prescribed for immunosuppressive treatments (Sturgess, 2002). In this study two protocols were tested: an anti-inflammatory protocol with a low dose of methylprednisolone (1 mg/kg), and an immunosuppressive protocol with a high dose of methylprednisolone (5 mg/kg), to cover the most common protocols using glucocorticoids in clinical canine practice (Martinez-Subiela et al., 2004; Yilmaz et al., 2007). In the present study, no consistent variations were observed in circulating adiponectin concentrations after methylprednisolone injection despite the increase in serum cortisol levels.

There are contradictory results in the literature regarding glucocorticoid effects on adiponectin. A direct inhibitory effect exerted by cortisol on adiponectin has been suggested, as an acute, about 25% decrease of adiponectin levels in response to i.v. exogenous hydrocortisone (25 mg/person) administration was observed in healthy human subjects (Fallo et al., 2004). Similarly, serum adiponectin levels were significantly decreased in both non-obese and obese rats after a 20-day administration of hydrocortisone at 5–15 mg/kg/day (Shi et al., 2010). It also has been shown that dexamethasone reduces adiponectin gene expression in murine 3T3-L1 adipocytes (Fasshauer et al., 2002), and that this glucocorticoid tended to inhibit adiponectin mRNA in human and rat adipose tissue (Halleux et al., 2001; Shi et al., 2010). However, other authors did not observe a difference in adiponectin levels in overweight and obese subjects after short-term oral dexamethasone (0.5 mg/person of dexamethasone every 6 h for 48 h) administrations (Lewandowski et al., 2006), while a third group of authors described increases in serum adiponectin levels after glucocorticoid administration. For example, increased circulating adiponectin levels were observed in neonatal rats after a 3-day tapering regimen of dexamethasone treatment from postnatal day (PD) 3–6 (Raff and Bruder, 2006), and in healthy humans when adiponectin was measured after treatment with dexamethasone at 4 mg daily for 4 days (Jang et al., 2008). Contradictory results obtained by distinct authors could be attributed to different corticoid, dose, route and time of administration.

Total adiponectin was measured without separating the different molecular forms of adiponectin. There are three forms of adiponectin in humans, which can be differentiated on the basis of molecular weight. The highest molecular weight (HMW) form is thought to be the most biologically active form (Leth et al., 2008). Although studies have demonstrated a correlation between HMW and total adiponectin in type 1 diabetes (Leth et al., 2008), in some situations such as for moni-

toring improvement of insulin sensitivity after treatment, HMW seems to be more sensitive than the measurement of total adiponectin (Suzuki et al., 2007). Also, the evaluation of HMW may strengthen the inverse relationship between adiponectin levels and chronic disease risk (Lara-Castro et al., 2006). Since the three isoforms of adiponectin have been characterised in the dog (Brunson et al., 2007), in the future it would be desirable to conduct studies that could comparatively analyse the behaviour of total adiponectin and the different subforms of adiponectin in different metabolic disorders, as well as to develop ELISA tests that would allow the measurement of different adiponectin subforms in an easy way in dogs.

A limitation of the present study could be the short sampling time after glucocorticoid treatment, although the study was designed to evaluate the possible effect of single-dose glucocorticoid administration on canine adiponectin as described elsewhere (Yilmaz et al., 2007). In the future, studies should be performed to evaluate the possible effects of long-term glucocorticoid administration in dogs, as many glucocorticoid-associated disturbances in metabolism are thought to be mediated via the downregulation of receptors (Jang et al., 2008; de Oliveira et al., 2011). This process can take months or years, and if receptor numbers are in excess, may not be apparent at all in functional assays. Also, prolonged glucocorticoid therapy might well contribute to the mechanism of obesity that could account for lower-than-normal adiponectin levels. Another limitation could be the low number of animals used for this study; however, it was in line with a previous study published about diurnal variations of the adipokine in dogs (Ishioka et al., 2005).

In conclusion, in the present study it was demonstrated that canine adiponectin was not affected by meal, fasting or single-dose methylprednisolone administration. These properties make it an attractive candidate marker for studies of obesity and obesity-related disease processes in dogs. Changes in serum adiponectin could potentially predict metabolic changes, in which case low levels would indicate the need for dietary changes. Also, serum adiponectin levels may be a useful quantitative marker of positive lifestyle changes or therapeutic effectiveness, interventions that would likely increase the average lifespan of dogs.

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3.3.2. Article 8

Effects of Orchidectomy in Selective Biochemical Analytes in Beagle Dogs

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Contents

The objective of this study was to evaluate the possible effects of orchidectomy and associate hormonal changes on circulating concentrations of acute-phase proteins (APPs) (CRP – C-reactive protein; Hp – haptoglobin; Cp – ceruloplasmin), adiponectin and IGF-1 in dogs. For this, a total of five adult Beagle dogs were subjected to orchidectomy. Blood samples were taken before neutering, during six consecutive days and on weeks 2, 4, 8 and 12 after surgery. Appropriate diet regime was maintained to keep stable body weight of the dogs. Concentrations of APPs significantly increased on days 2–3 for CRP and 2–7 for Hp and Cp. On days 3–4 after neutering, adiponectin levels were significantly lower than before surgery ($p < 0.05$ and < 0.01 , respectively). After this initial change, adiponectin did not show any significant alteration during the 3 months. Serum IGF-1 concentrations were significantly decreased at days 2–5 after neutering. In addition, on weeks 8 and 12 serum IGF-1 levels were significantly lower ($p < 0.001$ and < 0.01 respectively) in comparison with those before surgery. In conclusion, orchidectomy induced a short-term inflammatory process that was associated with the increase in serum levels of APPs and decrease in IGF-1 and adiponectin levels. However, orchidectomy did not result in long-term changes of circulating concentrations of APPs or adiponectin. Although a decrease in IGF-1 levels was recorded 2 months after surgery, possibly as a consequence of associated decrease in androgen levels or food restriction.

Introduction

In dogs, surgical sterilization is one of the most commonly procedures performed in veterinary practice (Howe 2006; Kustritz 2007), and is considered as a method of contraception to aid in the pet overpopulation problem, as well as to prevent diseases associated with the reproductive system, such as benign prostatic hyperplasia or behaviour problems (Schneider et al. 1969; Brendler et al. 1983; Olson et al. 1986). However, to the authors' knowledge, little is known about the possible physiopathological consequences of androgen deficiency in the male dog caused by sterilization procedure. In contrast, the question of an androgen deficiency of the ageing male syndrome (ADAM) is a matter of interest in human medicine (Morales et al. 2000). This syndrome is associated with muscle weakness, hypertrophy of adipose tissue and impaired neurotransmission among others clinical signs (Bain 2010). To elucidate this process, many studies have been undertaken in men and animals such as mice, cats or pigs to investigate the effects of decrease in androgen levels on biochemical parameters related with inflammation and body composition (Umapathy et al. 1997; Alexandersen and Christiansen 2004; Martin et al. 2006; Ohlson et al. 2006).

C-reactive protein (CRP), a major acute-phase protein (APP), seems to be partly regulated by androgens

(Alexandersen and Christiansen 2004). Studies in transgenic mice infected with *Streptococcus pneumoniae* have revealed the testosterone-dependent expression of CRP (Szalai et al. 1997). Furthermore, reports in human medicine indicate the significantly higher levels of CRP in women in comparison with men (Alexandersen and Christiansen 2004; Cartier et al. 2009; Khera et al. 2009).

Regarding biochemical analytes related with body composition, insulin-like growth factor-I (IGF-I) is a growth hormone (GH)-dependent peptide that plays an important role in growth and differentiation of many tissues (Florini et al. 1991). In boars, castration led to fall in serum GH and IGF-1 levels (Arbona et al. 1989; Dubreuil et al. 1989; Louveau et al. 1991). Although in rats, pre-pubertal castration led to increase in circulating IGF-1 levels (Handelsman et al. 1987). In human medicine, also controversial data have been reported according to the relationship between circulating levels of IGF-1 and testosterone, as some studies indicated that testosterone increases IGF-1 levels (Marin et al. 1992; Hobbs et al. 1993), whereas others report that testosterone alone does not have a stimulatory effect on levels of IGF-1 (Gibney et al. 2005).

Adiponectin is a cytokine produced in adipocytes that decreases in serum with body fat accumulation and it is considered a serum biomarker of obesity in humans and dogs (Arita et al. 1999; Ishioka et al. 2006). An inverse relation between adiponectin and testosterone in rodents and humans has been observed (Nishizawa et al. 2002; Lanfranco et al. 2004; Page et al. 2005), and human and mice females have elevated serum adiponectin levels compared with males (Arita et al. 1999; Combs et al. 2003).

Despite the above data showing the relationship between androgens and different biochemical analytes, no studies have been performed in dogs to investigate the possible effects of orchidectomy and associated decrease in androgen levels on markers of inflammation, such as APPs, or analytes associated with body composition, such as IGF-1 and adiponectin. So the objective of this study was to evaluate the possible effects of orchidectomy and associate hormonal changes on inflammatory markers and analytes associated with body composition. For this, circulating concentrations of APPs (CRP, Cp, Hp), IGF-1, and adiponectin were investigated in dogs that underwent orchidectomy.

Material and Methods

Animals and experimental set-up

A total of five male adult, university-owned beagle dogs (*Canis familiaris*) were used in this study [age range,

5.1–7.4 years; body weight range, 14.3–21.0 kg, body condition score (BCS) 3–4]. The assessment of the nutritional condition was based on a five-scale body condition score: 1, thin; 2, lean; 3, optimal; 4, obese; 5, gross (McGreevy et al. 2005). Dogs were maintained in their usual kennel (one dog per kennel) with controlled temperature ($23 \pm 2^\circ\text{C}$) and light (lights on at 08:00–20:00 hours). The size of each kennel was 3×4 m.

During the study, all dogs were fed a standard dry food (Premium Croc Adult, Affinity Petcare, Affinity Petcare S.A., Barcelona, Spain), containing 24.0% crude protein, 16.1% crude fat, 2.5% crude fibre, 6% crude ash, 8% moisture, metabolizable energy 4420 kJ/kg as fed. After surgery, the fed amount was reassessed each week and adjusted if necessary to maintain optimal BW by a reduction or an increase of 5% of the amount offered (Jeusette et al. 2004). Water was available *ad libitum*.

All dogs passed general health examinations weekly during the whole study and complete blood count and biochemical profiles were performed at the beginning of the study to confirm that there were no apparent abnormalities. All animals had a negative serological titre for *Leishmania infantum* and *Ehrlichia canis*.

Surgical procedures

The dogs were fasted for 24 h before surgery. All dogs were pre-medicated with 0.01 mg/kg IM medetomidine hydrochloride (0.1 mg/ml; Domitor, Pfizer Inc., New York, NY, USA) and 0.3 mg/kg IM butorfanol (10 mg/ml; Torbugesic, Fort Dodge, Iowa, USA), after 20 min anaesthesia was induced (5 mg/kg IV) and maintained [constant rate infusion (CRI) 1.5 mg/kg/h] with propofol (Propofol, Abbott animal health, Queenborough, UK) during all surgery. Open pre-escrotal castration method was performed as described by Fossum (2007). Each dog was treated with an antibiotic (amoxicillin LA, 15 mg/kg; Bivamox LA, Boehringer Ingelheim España, Barcelona, Spain). Post-operative care consisted of cleansing of surgical wounds and oral administration of 4 mg/kg/24 h Carprofen (Rimadyl Palatable 50 mg, Pfizer Inc.) for 5 days. No complications were detected in any of the dogs during the post-operative period.

Samples

Blood samples were collected on the morning after an overnight fasting of at least 12 h by venipuncture in the cephalic vein into tubes containing clotting accelerator (TapVal, Aquisel, Barcelona, Spain) for biochemical assays from all dogs before castration (D1), during six consecutive days (D2–D7) and on weeks 2, 4, 8 and 12 (W2–W12) after castration. Tubes for analytes assays were centrifuged at $2000 \times g$ for 10 min at room temperature to obtain serum, which was stored in plastic vials at -20°C until analysis. On the day of analysis, samples were brought to room temperature and thoroughly vortexed prior to measurements.

The experimental set-up was approved by the Animal Care Commission of Murcia University,

additionally housing and care was performed according to guidelines for the care and use of laboratory animals established by the European Union (Guidelines on the Care and Use of Animals for Scientific Purposes 2004).

Analysis

Testosterone was analysed with an automated solid-phase, enzyme-labelled chemiluminescent immunometric assay (Immulite System, Siemens Health Diagnostics, Deerfield, IL, USA). Preliminary validation of the method was performed in authors' laboratory for use in dog serum, showing intra- and inter-run CVs lower than 10% and a limit of detection of 34.5 ng/dl.

C-reactive protein concentration was measured using a human immunoturbidimetric assay (CRP OSR 6147 Olympus Life and Material Science Europe GmbH, Lismeehan, O'Callaghan's Mills, Co. Clare, Ireland), previously validated in dogs (Gentilini et al. 2005). This assay showed a correlation of 0.98 with a specific canine ELISA assay (Tridelta Phase range canine CRP kit, Tridelta Development Ltd, Brey, Ireland) which has been validated for use in dogs (Martínez-Subiela and Cerón 2005). The limit of detection for this method in our laboratory was 0.053 mg/l.

Hp concentration was measured by a commercially available colorimetric method (Tridelta Phase range haptoglobin kit, Tridelta Development Ltd) that was validated previously for use in dogs (Martínez-Subiela and Cerón 2005) with a limit of detection of 0.02 g/l.

C-reactive protein and Hp were measured in serum on an automated biochemistry analyser (Olympus 2700 Automatic Chemistry Analyzer, Olympus Europe GmbH, Hamburg, Germany) and had intra- and inter-run coefficients of variation $< 10\%$.

Serum concentration of ceruloplasmin was measured by use of a spectrophotometric method based on the *in vitro* oxidase activity of Cp with *p*-phenyldiamine validated for use in canine samples (Ceron and Martínez-Subiela 2004). Determinations were made by use of an automated biochemistry analyser (Cobas Mira Plus multiparametric autoanalyser, ABX Diagnostics, Montpellier, France) and results are reported as the change in absorbance per minute at 550 nm. This assay showed intra- and inter-run CVs lower than 10% and a limit of detection of 0.0007 $\Delta\text{abs}/\text{min}$.

Serum IGF-1 concentrations were determined with an automated solid-phase, enzyme-labelled chemiluminescent immunometric assay (Immulite System, Siemens Health Diagnostics, Deerfield, IL, USA) previously validated in our laboratory for use in dogs (Tvarijonavičiute et al. 2011). This assay showed intra- and inter-run CVs lower than 10% and a lower limit of quantification of 7.1 ng/ml.

Serum adiponectin concentration was determined by a human high sensitivity adiponectin ELISA (Human Adiponectin ELISA, High Sensitivity Kit, BioVendor-Laboratori medicina, Modrice, Czech Republic), previously validated in our laboratory for use in dogs (Tvarijonavičiute et al. 2009). This assay showed intra- and inter-run CVs lower than 11% and a limit of detection of 0.74 $\mu\text{g}/\text{ml}$.

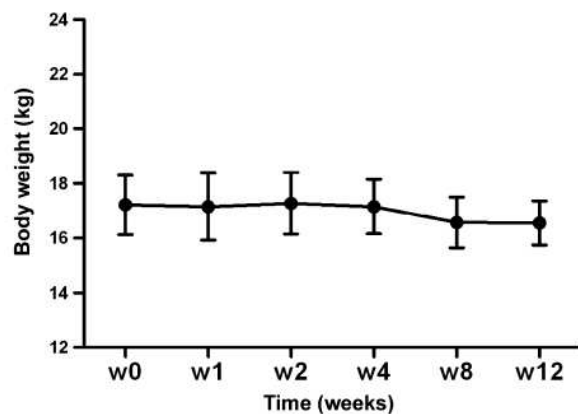


Fig. 1. Evolution of mean body weight (kg) of five Beagle male dogs during study period

Statistical analysis

Arithmetic medians, means and standard deviation (SD) were calculated using routine descriptive statistical procedures and software (SPSS statistical program; SPSS Inc., Chicago, IL, USA). The results for each parameter were evaluated for approximate normality of distribution by using the Kolmogorov–Smirnov statistics. Repeated measures ANOVA with Dunnett's Multiple

Comparison post-test were used to compare changes of BW and biochemical analytes in dogs. Values of $p < 0.05$ were considered significant.

Results

The mean BW of the dogs had no significant variations during all experimental period (Fig. 1). Before the castration, plasma testosterone levels of five adult male dogs varied widely from 112 to 800 ng/dl, with a median of 242.0 ng/dl. Subsequent days after castration, testosterone levels were below the lower quantification limit of the assay (34.5 ng/dl).

Repeated measures ANOVA test revealed a significant variation in circulating CRP, Hp, Cp, IGF-1 ($p < 0.0001$ in all cases), and adiponectin ($p < 0.05$) levels throughout the study (Fig. 2).

Dunnett post-test indicated that during the first 2 days after orchidectomy, CRP concentrations showed a significant increase of 17- and 10-fold respectively. CRP tended to decrease on subsequent days till got into similar ranges as those recorded before castration and no significant changes were observed during the rest of the study (Fig. 2a).

Hp concentrations began to increase on the second day after surgery and reached maximum on the day three with a twofold increase (Fig. 2b). On day 4, Hp concentrations began to decrease but remained

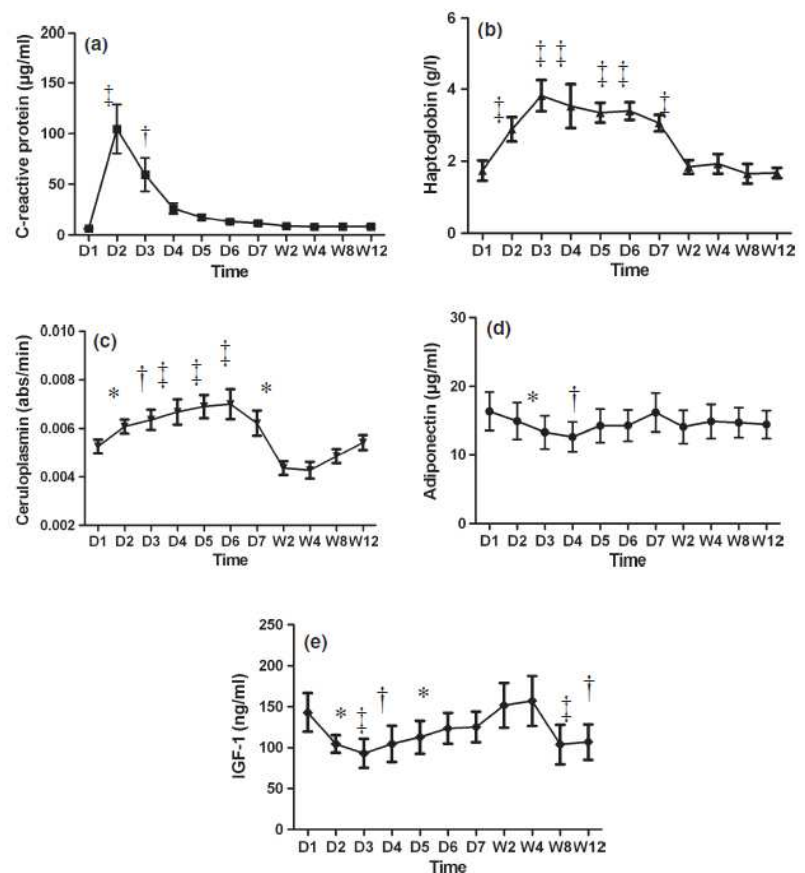


Fig. 2. Evolution of mean $\pm$ SD values of plasma C-reactive protein (a), haptoglobin (b), ceruloplasmin (c), adiponectin (d), and IGF-1 (e) during study period. * $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$

Table 1. Correlation coefficients between plasma C-reactive protein and other analytical variables in beagle dogs after orchiectomy

	n	p	r
Haptoglobin	55	<0.0001	0.6719
Ceruloplasmin	55	<0.0001	0.5025
IGF-1	55	0.0117	-0.4338
Adiponectin	55	0.01	-0.3446

significantly higher during the following 3 days compared with the results obtained before orchidectomy. Then reached values recorded before castration and showed no changes during the rest of the study period.

Serum Cp showed a statistically significant increase during seven consecutive days (~1.5-fold) after castration (Fig. 2c); then decreased and reached initial values.

Dunnet post-test showed a significant decrease in adiponectin levels on days 3 and 4 after orchidectomy ($p < 0.05$ and < 0.01 , respectively) (Fig. 2d). After this initial change, adiponectin returned to the initial levels, and did not show any significant alterations during the 3 months of the experimental period.

Serum IGF-1 concentrations were significantly decreased at days 2–5 after castration (Fig. 2e), then began to increase and showed similar values as recorded before castration on weeks 2 and 4 after surgery. However, on weeks 8 and 12 serum IGF-1 levels were 0.8-fold lower ($p < 0.001$ and < 0.01 , respectively) in comparison with ones recorded before castration.

Serum CRP levels showed positive correlation with the levels of CP and Hp, and negative correlation with IGF-1 and adiponectin (Table 1). Stronger correlation was detected between CRP and IGF-1 ($p = 0.0008$; $r = -0.9725$), and adiponectin ($p = 0.0059$; $r = -0.4563$) when only data of the first week after castration was compared. Spearman correlation analysis showed strong significant positive correlation between circulating levels of IGF-1 and adiponectin ($p < 0.0001$, $r = 0.7901$).

Discussion

Gonadectomy has been shown to have various effects on the release of hormones in dogs (Günzel-Apel et al. 2009). However, to the authors' knowledge, no studies have been reported about the possible effects of orchidectomy and associate hormonal changes on circulating concentrations of biochemical analytes related with inflammation, such as APPs, or related with body composition, such as IGF-1, and adiponectin in dogs. This is contradictory with the importance that these studies are having in human medicine as it has been reported possible implication of a testosterone deficit in the pathogenesis of different diseases (Allan and McLachlan 2010; Malkin et al. 2010; Vigano et al. 2010).

In present study, APPs, CRP, Hp and Cp, showed an initial rise in their circulating levels caused by the inflammation and within a week after surgery returned to the initial values. This finding agrees with the dynamics of APPs during injury (Ceron et al. 2005). In our case, the return to initial values of the APPs was

accompanied with the inexistence of any complication during the post-operative period. APPs can be used in the diagnosis of reproductive diseases such as pyometra and have been demonstrated to be useful markers for monitoring the post-operative period in bitches undergoing ovariohysterectomy and their determination facilitates early detection of post-operative complications (Dabrowski et al. 2009).

After this initial increase, no significant changes in APPs were recorded during the 3 months period after orchidectomy, situation in which inflammation associated with the surgery was finished and the circulating levels of testosterone were low. In human medicine, experimental studies investigating the effect of dehydroepiandrosterone and testosterone on inflammation have yielded divergent results. Some authors evidenced the androgen regulation of the inflammatory response due observations that testosterone down-regulates the cytokine interleukin 6 (IL-6), a potent stimulator of inflammation and CRP, and that plasma IL-6 concentrations were higher in women after menopause and in aging men (Ershler and Keller 2000). Also a study with 5504 patients indicated an inverse association between CRP and total and free testosterone in men (Kupelian et al. 2010). In contrast, in a study that included 715 middle-aged men (35–59 years), no association was observed between CRP and sex hormone levels, including total and free testosterone, sex hormone-binding globulin and oestradiol (Van Pottelbergh et al. 2003). Finally, data from testosterone replacement therapy among older men (age > 60 years) did not observe differences in CRP levels (Ng et al. 2002).

The decrease in IGF-1 levels in dogs during the first week after orchidectomy could be associated with inflammation as an inverse correlation was detected between serum levels of CRP and IGF-1. In humans and dogs, similar relationship between serum levels of IGF-1 and inflammation has been reported (Eivindson et al. 2005; Tvarijonavičiute et al. 2010).

Regarding to the second decrease of IGF-1 levels observed on weeks 8 and 12 post-castration in dogs in present study, it could be attributed to the direct effect of the decrease in androgens, similarly as reported in pigs (Arbona et al. 1989; Dubreuil et al. 1989), because expression of IGF-1 is through gonadotropins and GH through cAMP-signal transduction induced (Sirotkin 2005). This fact would explain also an increase in IGF-1 levels was observed in male calves during the onset of puberty (Renaville et al. 1996).

Although a previous study (Martin et al. 2006) indicates that castration in cats led to a further increase in body weight associated with the increased levels of IGF-1 and hypothesize that the increase in IGF-1 secretion could be partially due to the decrease in circulating testosterone, it could be postulated that this increase in IGF-1 would be more related with the enlargement in body fat, as it has been reported the increment of IGF-1 receptors and circulating IGF-1 levels in obese humans (Frystyk et al. 1995; Nam et al. 1997; Louveau and Gondret 2004). In contrary than in the cat study, in our work during all experimental period body weight of the dogs did not vary significantly, so the effects of castration on IGF-1 was investigated without

the influence of changes in body fat mass. However, on the other hand, reports in humans and different species of animals such as rats and pigs show that decrease in IGF-1 levels is susceptible to feed restriction (Prewitt et al. 1982; Spicer et al. 1992; Jull 2003). So the food restriction applied in the study to maintain stable body weight could also negatively affect circulating levels of IGF-1.

Controversial data have been published about the influence of testosterone on adiponectin levels. Recently, it was shown the inverse relation between adiponectin and testosterone in rodents and humans (Nishizawa et al. 2002; Lanfranco et al. 2004; Page et al. 2005). Castration in mice significantly increased the concentrations of plasma adiponectin, but subsequent testosterone injections reduced its levels (Nishizawa et al. 2002). In general, a sexual dimorphism for adiponectin has been reported in a number of investigations even after controlling for differences in body composition (Peake et al. 2005; Laughlin et al. 2006). However, on the other hand, one *in vitro* study with cultured cells reported that increasing concentrations of testosterone or oestradiol influenced neither adiponectin mRNA expression and secretion nor intracellular protein expression of high-, middle-, and low-molecular weight (HMW, MMW, LMW) adiponectin multimers (Horenburg et al. 2008). Moreover, Ishioka et al. (2006) in dogs found no difference in adiponectin levels related with sex. Our report would be in line with those studies, because serum adiponectin concentrations were not affected by the decrease in serum testosterone levels in dogs 3 months after castration.

The statistically significant decrease of adiponectin levels observed on the third and fourth days after orchidectomy was associated with an increase in CRP

levels. Some studies in human medicine found an inverse association between adiponectin and inflammatory markers such as TNF- α , interleukin 6, and CRP in normal subjects and in patients with metabolic syndrome (Engeli et al. 2003; Kern et al. 2003; Matsubara et al. 2003; Ouchi et al. 2003). In addition, it was demonstrated that expression of CRP was detected in adipose tissue and the expression of adiponectin is inversely associated with that of CRP (Ouchi et al. 2003). Furthermore, inverse association between serum adiponectin and APPs has been described in dogs by the authors (Tvarijonaviciute et al. 2010).

Although a limitation of this study could be the low number of dogs evaluated and ideally more dogs should be used to confirm these findings, in conclusion, the results obtained in present study indicate that orchidectomy induce a short-term inflammatory process caused by the surgical procedure that was associated with the increase in serum levels of APPs and decrease in serum levels of IGF-1 and adiponectin. However, orchidectomy did not result in long-term changes of circulating concentrations of APPs or adiponectin. Although, a decrease in IGF-1 level was recorded second and third months after surgery possibly as a consequence of associated decrease in androgen levels or food restriction.

Conflict of interest

None of the authors have any conflict of interest to declare.

Authorship statement

All listed authors have made substantial contributions to the research design, analysis and interpretation of data; and to drafting the paper and revising it critically.

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3.3.3. Article 9

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Serum and urinary adiponectin in dogs with renal disease from leishmaniasis

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The objective of this study was to perform an analytical validation of a commercially available ELISA kit (human adiponectin) for urinary adiponectin determination in dogs, and to evaluate urinary adiponectin in dogs with glomerular injury. For this purpose, urine samples from three healthy dogs and three dogs with diagnosed kidney disease were used for analytical validation of the method. In order to evaluate possible influence of kidney damage on urinary adiponectin, serum and urine samples from six healthy and 58 dogs with leishmaniasis were included. The diseased dogs were allocated to three groups according to their urine protein/creatinine (UPC) ratio as non-proteinuric (NP), borderline proteinuric (BP), and proteinuric (P). Intra- and inter-assay coefficients of variation (CV) were lower than 10 per cent and 12 per cent, respectively. Dilutions of canine urine samples resulted in linear regression equations close to 1. Mean recovery was of 112 per cent. The detection limit was 0.75 ng/ml. Urinary adiponectin and urinary adiponectin/creatinine (UAC) ratio showed significantly higher values in urine of P group dogs compared with healthy, NP and BP dogs. In conclusion, an ELISA kit can be used for precise and accurate urinary adiponectin measurement in dogs. Urinary adiponectin is increased in dogs with proteinuria suggesting its possible use as a marker of kidney damage.

Introduction

Chronic kidney disease is an important cause of morbidity and mortality in dogs with the prevalence of 15 per cent of dogs over 10 years old being affected (Polzin and others 1989). Early diagnosis may allow therapeutic intervention that prevents further damage and progressive decline of renal function. However, only a decrease of >75 per cent of renal functional mass will be detected by current diagnostic tests, such as serum urea nitrogen and serum creatinine concentrations (Braun and Lefebvre 2008). Because there is a high risk of progression to irreversible renal damage in patients with chronic kidney disease, there is a current need of biomarkers for early detection of kidney dysfunction in both humans and dogs (Maddens and others 2010). Urinary markers are considered to be more sensitive indicators of renal injury than the serum markers, and also have the potential to reflect the site and severity of damage. These markers are a group of proteins whose concentration in urine is determined by permeability of the glomerular barrier and/or by protein reuptake in renal proximal tubular cells, and the capacity of these cells to degrade the filtered proteins (D'amico and Bazzi 2003, Raila and others 2005). Thus, detection of certain urine proteins may provide a new approach for the diagnosis of particular pathological processes in the glomeruli (eg, urine albumin, urine protein:creatinine ratio (UPCR)), proximal tubules (retinol binding protein), or both (Forterre and others 2004, Raila and others 2010).

Adiponectin is a small (30 kDa) adipose-derived adipocytokine (Maeda and others 1996) involved in modulating whole-body metabolism and other vital functions related to inflammation and immune response (Berg and Scherer 2005). In dogs, similarly as in humans, adiponectin has been shown to be related to obesity and inflammation (Ishioka and others 2006, Tvarijonaviciute and others 2011). However, in recent years in human medicine, it was demonstrated that adiponectin is involved in the glomerular function (von Eynatten and others 2009) and it was suggested to be a marker of glomerulus injury (Koshimura and others 2004, von Eynatten and others 2009) being considered as an early marker of kidney damage in human systemic lupus erythematosus (Rovin and others 2005). However, no data about urine adiponectin have been published in dogs.

The objectives of the present study were (1) to perform an analytical validation of the ELISA kit originally designed for measuring human adiponectin, for urine adiponectin determination in dogs and (2) to investigate the possible influence of renal damage due to glomerular injury on urinary adiponectin in dogs. For this purpose, urinary and serum adiponectin were measured in healthy dogs and dogs with leishmania which presented different degrees of proteinuria.

Materials and methods

Western blot analysis of urinary adiponectin

Cross-reactivity between canine urinary adiponectin and the polyclonal goat anti-human adiponectin (Horse radish peroxidase (HRP)-labelled goat anti-human adiponectin antibody, BioVendor-Laboratori medicina, Modrice, Czech Republic) was evaluated and compared by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis, as previously described (von Eynatten and others 2009). Adiponectin standard 1 from ELISA kit (Human Adiponectin ELISA High Sensitivity Kit, BioVendor-Laboratori medicina) was used as a control. Samples (5 µl each) were separated in 10 per cent SDS-PAGE gels together with a colorimetric marker (Amersham

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full-range rainbow molecular weight markers (12–225 kDa), GE Healthcare Europe GmbH, Freiburg, Germany), and 5 µl human adiponectin standard 1 (150 ng/ml, BioVendor–Laboratori medicina). After gel electrophoresis, the separated proteins were electroblotted onto a nitrocellulose membrane with a Tris–glycine-based transfer buffer containing 15 per cent methanol. Tris buffered saline with 5 per cent milk powder was used to block non-specific binding sites on the blot. The membrane was incubated at room temperature during two hours with a 1/1000 diluted polyclonal goat anti-human adiponectin antibody conjugated with HRP. Proteins were visualised using a chemiluminescence substrate (ECL Plus Western Blotting detection reagents, RPN2132, Amersham Biosciences Europe GmbH, Freiburg, Germany), and blots were visualised using a scanner (Typhoon 9410, GE Healthcare, Piscataway, New Jersey, USA).

Adiponectin analysis

Serum and urine adiponectin concentrations were determined with a commercial sandwich enzyme immunoassay for the quantitative measurement of human adiponectin (Human Adiponectin ELISA, High Sensitivity Kit; BioVendor–Laboratori medicina) previously validated for use in dog serum using a canine species-specific standard (Tvarionaviciute and others 2010). Serum samples were diluted 1:300 with a dilution buffer provided with the kit as previously described, while urine samples were analysed without any dilutions. To perform the assay, standards, quality controls and samples were incubated in microplate wells pre-coated with polyclonal anti-human adiponectin antibody. After 60 minutes incubation and washing, polyclonal anti-human adiponectin antibody, conjugated with HRP was added to the wells and incubated for 60 minutes. Following another wash step, the remaining HRP conjugate was allowed to react with the substrate solution (TMB). The reaction was stopped by addition of acidic solution, and absorbance of the resulting yellow product is measured at 450 nm with reference wavelength 630 nm in a microtiter plate reader (PowerWave XS, Bio-Tek Instruments Inc., Vermont, USA). The absorbance is proportional to the concentration of adiponectin. A standard curve was constructed by plotting absorbance values against concentrations of standards using the four-parameter algorithm, and concentrations of unknown samples were determined using this standard curve.

Urinary adiponectin concentrations (ng/ml) were adjusted for urinary creatinine excretion (UAC) and expressed as micrograms per gram of creatinine for statistical analysis as previously described (von Eynatten and others 2009).

Analytical validation

Urine samples from three healthy dogs (complete blood count, biochemical analysis and urinalysis were unremarkable, and indirect immunofluorescence titres (IFI) for leishmania was negative) and three dogs with leishmania (positive IFI) and with serum creatinine >1.4 mg/dl and UPCR >0.5 (Elliot and Watson 2009), were diluted 1:2 with conservative solution (stabilisation solution for urine samples, reference number CS019, BioVendor–Laboratori medicina), and were used for analytical validation of the method for adiponectin quantification in urine; and the following parameters were calculated.

Precision

(1) Intra-assay CV was calculated after analysis of two urine pools (one was made by mixing urine samples obtained from three healthy dogs and the other by mixing urine samples obtained from three dogs with kidney disease), five times in a single assay run. (2) Inter-assay CV was determined by analysing the same pools in five separate runs carried out on five consecutive days. All samples used for repetitive analysis were frozen in aliquots and only the required aliquots for each run were thawed, to avoid possible changes due to repetitive thawing and freezing.

Accuracy

Accuracy of the assays was evaluated indirectly by linearity under dilution and by recovery studies. To assess linearity under dilution, two canine urine samples with high adiponectin concentrations that were obtained from dogs with kidney disease were serially

diluted with the sample diluent provided with assay. To evaluate the ability of the assays to recover the amount of analyte added to baseline urine samples, a spiking recovery was performed (Food and Drug Administration and others 2001). For this purpose, two samples with different amounts of adiponectin were mixed at different ratios (Table 1). Test recovery (in per cent) was calculated for each dilution for comparison of expected versus measured adiponectin.

Limit of detection

The detection limit was defined as the lowest concentration of adiponectin that could be distinguished from a specimen of zero value. For detection limit calculation we measured the blank (sample diluent buffer) 10 times, and as data were not normally distributed, the following formula was used: $Q3 + 1.5 \times IQR$, where $Q3$ = 75th percentile and IQR = interquartile range (Larson 2006).

Urinary adiponectin stability

In order to evaluate urinary adiponectin stability, four urine samples with expected high adiponectin concentrations were collected from dogs with serum creatinine >1.4 mg/dl and UPCR >0.5. Each urine sample was analysed on the day of urine extraction, and was then stored in different aliquots with (dil. 1:2) and without conservative solution (stabilisation solution for urine samples, reference number CS019, BioVendor–Laboratori medicina) in order to evaluate effect of storage at -20°C during one month, and of three freezing/thawing cycles on the stability of urinary adiponectin.

Study of adiponectin concentrations in dogs with kidney disease

The current study was performed at the Veterinary Medicine School, Murcia University. It was approved by the Local Ethical Committee and was performed in compliance with the laws RD32/2007 and RD1201/2005 related to animal experimentation in Spain.

Sixty-four dogs, 39 males and 25 females were included in this study. Six dogs, three males and three females, with ages between 3.83 and 10.92 years, body weight (BW) between 9 and 25 kg, and body condition score (BCS) of 3/5, were healthy dogs without any abnormality in the physical examination, and in the haematologic, biochemistry and urinary tests, and had negative IFI for leishmania, and had come to clinics for routine check-ups. The assessment of the nutritional condition was based on a five-scale BCS: 1, thin; 2, lean; 3, optimal; 4, obese; 5, gross (Edney and Smith 1986).

Fifty-eight dogs, 36 males and 22 females, with ages between 0.8 and 14.2 years, BW between 3 and 60 kg, and BCS between 2 and 5/5 were dogs with leishmaniasis that were staged based on urine protein/creatinine (UPC) ratio. The basis of the proteinuria classification was used similar to how it was defined in the IRIS scheme (Elliot and Watson 2009). Thus, the dogs were staged into three groups: as non-proteinuric group (NP; UPC ratio: <0.2; $n=9$), borderline proteinuric group (BP; UPC ratio: 0.2–0.5; $n=7$), or proteinuric group (P; UPC ratio: >0.5; $n=42$). Further, the P group was subdivided based on plasma creatinine concentrations in non-azotaemic dogs (subgroup P1; serum creatinine <1.4 mg/dl; $n=31$), and azotaemic dogs (subgroup P2; serum creatinine ≥ 1.4 mg/dl; $n=11$).

TABLE 1: Spiking recovery method

Sample volume (%)		Urine adiponectin (ng/ml)		
Sample 1	Sample 2	Expected	Observed	Recovery (%)
100	0	103.13	103.13	100.0
75	25	88.70	100.22	113.0
63	37	81.56	99.46	122.0
50	50	74.27	91.10	122.7
63	37	67.13	76.88	114.5
25	75	59.85	65.31	109.1
13	87	53.02	59.98	113.1
0	100	45.42	45.42	100.0

Two canine urine samples with different amounts of adiponectin were mixed in different ratios.

The dogs with leishmaniasis had clinical and/or analytical signs compatible with leishmaniasis (in case of group NP, only hyperglobulinemia in serum) and the diagnosis was confirmed by positive IFI to detect serum antibodies against the leishmania infection and/or visualisation of *Leishmania* spp. amastigotes in bone marrow samples. Some of the dogs did not show evident clinical signs, whereas, others presented weight loss, varying degrees of generalised lymphadenopathy, or skin lesions. Alterations found most commonly in haemograms and serum biochemical analyses were mild non-regenerative anaemia, hyperproteinemia with hypoalbuminemia and hyperglobulinemia.

All dogs (healthy and with leishmaniasis) were tested for presence of Canine Heartworm, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* and *Ehrlichia canis* antibodies using SNAP test (Canine SNAP 4Dx, IDEXX Laboratories, IDEXX Europe BV, Hoofddorp, The Netherlands). Fifteen dogs were excluded as they were suspected of having associated systemic or infectious diseases different from leishmaniasis, that could be a cause of glomerulopathy, or if active sediment in the urine was detected. Excluded dogs were out of the 64 dogs finally included in the study.

Blood samples were obtained from all dogs for routine serum biochemistry (Cobas Mira Plus Analyser, Horiba ABX, Montpellier, France) after at least a 12 hour fasting period. At the same time, a urine sample was obtained by cystocentesis (5 ml, 22 G needles) for sediment examination, and the urine protein and creatinine measurements. Using an automatic analyser (Cobas Mira Plus Analyser, I Horiba ABX), urine protein was determined by the pyrogallol method using a commercially available reagent (Protein u & csf, Spinreact SAU, Sant Esteve de Bas, Spain), while creatinine was measured using the modified Jaffe method using a commercially available reagent (Creatinine-J, Spinreact SAU) on samples diluted 1:20 with deionised water. The UPC ratio was then calculated with the formula: UPC ratio = protein (mg/dl)/creatinine (mg/dl). The urine samples were immediately diluted to 1:2 with a stabiliser solution (stabilisation solution for urine samples, reference number CS019, BioVendor-Laboratori medicina). Serum and urinary aliquots were stored at -20°C until analysis of adiponectin was performed.

Statistical analysis

Intra- and inter-assay CVs, recovery and detection limits were calculated using routine descriptive statistical procedures (Graph Pad Prism V.5 for Windows, Graph Pad software, San Diego, California, USA). Ordinary regression analysis was used to investigate linearity under dilution. Kolmogorov-Smirnov test was performed to assess the normality of data, giving a nonparametric distribution; data were then log transformed and one-way analysis of variance and Newman-Keuls multiple comparison post-test were used to compare values of different groups by using a statistical programme (Graph Pad Prism V.5 for Windows, Graph Pad software Inc). Data for healthy and leishmaniotic dogs is reported as median and 25-75 per cent percentile (IQR). A Spearman correlation analysis was used for the comparison of UPCr and serum creatinine with urine adiponectin and UAC. Receiver operating characteristic (ROC) curves of the urine adiponectin, UAC, and urine:serum adiponectin ratio were constructed to determine the optimal cut-off point for the diagnosis of proteinuria. Statistical significance was defined as a $P < 0.05$ on two-tailed testing.

Results

Western blot analysis of urinary adiponectin

Western blot analysis showed a high specificity of the polyclonal goat anti-human adiponectin antibody for canine urinary adiponectin (Fig 1). In canine urine, specific adiponectin oligomers were found at ~70 kDa, which represents the LMW trimer, two bands at ~140 and ~180 kDa, which represents the MMW hexamers, and the HMW complex at >300 kDa. No albumin-bound LMW trimers were observed in canine urine samples (~110 kDa).

Analytical validation of the assay for urine adiponectin measurements

Intra- and inter-assay CVs of the two urine pools from (1) healthy and (2) dogs with kidney disease were below 10 per cent and 12 per cent,

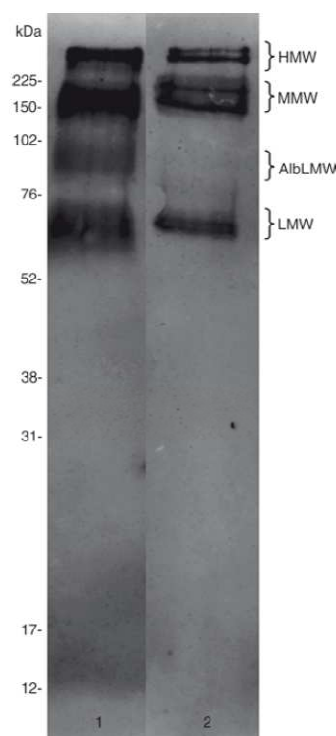


FIG 1: Western blot analysis showed a high specificity of the polyclonal goat anti-human adiponectin antibody for canine urinary adiponectin. Specific adiponectin oligomers were found at ~70 kDa, which represents the LMW trimer, a single band at approximately 110 kDa, which represents albumin-bound LMW trimer (albLMW), two bands at ~140 and ~180 kDa, which represents the MMW hexamers, and the HMW complex at >300 kDa, as described previously (Bobbitt and others 2005). (1) Human Adiponectin standard (5 µl, 150 ng/ml standard, Human Adiponectin ELISA High Sensitivity Kit, BioVendor-Laboratori medicina); (2) urine sample from dog with proteinuria (5 µl)

TABLE 2: Intra- and inter-assay variation in determinations of adiponectin concentration in canine urine pools with human adiponectin ELISA assay

		Adiponectin concentrations (ng/ml)		
Comparison	Pool	Mean	SD	CV (%)
Intra-assay	1	47.12	1.9	4.1
	2	148.65	14.3	9.6
Inter-assay	1	47.78	5.6	11.6
	2	146.43	14.0	9.6

CV Coefficient of variation

respectively (Table 2). The linearity under dilution of two canine urine samples is shown in Fig 2. Dilution of canine urine samples with adiponectin concentrations of 103 ng/ml and 165 ng/ml resulted in linear regression equations with correlation coefficient close to 1.0 in the mean range of 3-160 ng/ml. The recovery between observed and expected adiponectin concentrations ranged from 100 to 123 per cent, with a mean of 112 per cent (Table 1). The lower limit of detection was 0.75 ng/ml.

No significant changes in urinary adiponectin concentrations were detected in aliquots with stabilisation solution after three freeze/thaw cycles, and after one month of storage at -20°C. In all aliquots without stabilisation solution, a mean per cent decrease of 33 per cent

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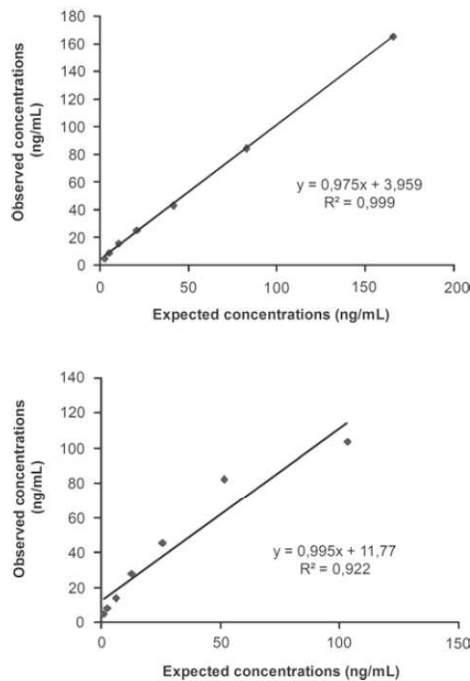


FIG 2: Linearity under dilution of two canine urine samples with different adiponectin concentrations

after the first freeze/thaw cycle ($P < 0.05$), and a mean per cent decrease of 41 per cent after one month of storage at -20°C in urine adiponectin was observed.

Adiponectin concentrations in dogs with renal damage

Mean (IQR) data of BCS, BW, age, UPCR and serum creatinine are presented in Table 3. No statistically significant differences were observed in BCS, BW, age or serum creatinine between different groups of dogs (with the exception of serum creatinine, being higher in subgroup P2 in comparison with the rest of the groups). UPCR was higher in P group when compared with healthy dogs, NP and BP groups ($P < 0.001$ in all cases).

Serum adiponectin, UPC ratio, urine adiponectin, UAC and urine:serum adiponectin ratio in different groups of dogs are presented in Figures 3 and 4. Median (IQR) serum adiponectin concentrations in the group of healthy dogs was 10.6 (8.3–11.6), in the NP group it was 13.9 (4.5–20.5), in the BP group it was 12.9 (8.8–19.1), and in the P group it was 8.6 (4.5–14.7) ($P > 0.5$). Urine adiponectin concentrations were higher in group P dogs (48.8 (10.7–76.8) ng/ml) when

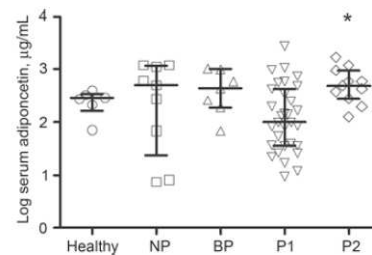


FIG 3: Serum adiponectin concentrations in healthy dogs and dogs with Leishmania without proteinuria, with borderline proteinuria, and proteinuria (P). The central lines present median and IQR. * $P < 0.05$ vs. P1 group

compared with healthy dogs (4.3 (3.4–5.4) ng/ml), and groups NP (4.7 (3.7–8.0) ng/ml) and BP (4.0 (2.4–9.4) ng/ml) ($P < 0.001$ in all cases). Also, higher UACs were observed in group P dogs (33.0 (10.5–93.0) $\mu\text{g/g}$ creatinine) when compared with healthy dogs (1.6 (0.8–2.8) $\mu\text{g/g}$ creatinine), and groups NP (2.8 (1.5–7.1) $\mu\text{g/g}$ creatinine) and BP (2.9 (1.3–5.4) $\mu\text{g/g}$ creatinine) ($P < 0.001$ in all cases). Higher urine:serum adiponectin ratio was observed in group P (4.4 (2.7–7.8)) when compared with healthy dogs (0.4 (0.4–0.5)), group NP (0.5 (0.3–1.9)), and group BP (0.5 (0.2–1.7)) ($P < 0.001$ in all cases).

When the group of dogs with proteinuria was subdivided into two subgroups according to the presence or not of azotaemia, serum adiponectin concentrations were higher in azotaemic dogs (subgroup P2; median (sd), 13.7 (10.6–18.7)) in comparison with non-azotaemic dogs (subgroup P1; median (sd), 6.4 (3.7–12.8)). Significantly higher UPC ratio, urine adiponectin concentrations, UAC ratio and urine:serum adiponectin ratio were observed in group P2 in comparison with healthy dogs, NP, BP and P1 groups. Also, significantly higher UPC ratio, urine adiponectin concentrations, UAC ratio and urine:serum adiponectin ratio were observed in group P1 in comparison with healthy dogs, NP and BP groups.

When data from all groups were pooled, serum creatinine was positively correlated with serum ($r = 0.306$, $P < 0.05$) and urinary ($r = 0.420$, $P < 0.01$) adiponectin, UPCR ($r = 0.304$, $P < 0.05$), UAC ($r = 0.376$, $P < 0.01$), and urine:serum adiponectin ratio ($r = 0.252$; $P = 0.05$). Positive correlation was observed between UPC ratio and urine adiponectin ($r = 0.822$, $P < 0.001$), UAC ($r = 0.875$, $P < 0.001$), and urine:serum adiponectin ratio ($r = 0.819$, $P < 0.001$). No significant correlation was observed between serum adiponectin and urine adiponectin, UPC or UAC ratios.

The ROC curve analysis indicated that the optimal cut-off point for urinary adiponectin for detecting proteinuria is 7.0 ng/ml with an area under the ROC curve of 0.93 (95 per cent CI, 0.866 to 0.991), a sensitivity of 93 per cent and a specificity of 80 per cent (Fig. 5a). The optimal cut-off point for UAC is 5.0 with an area under the ROC curve of 0.94 (95 per cent CI, 0.888 to 0.998), a sensitivity of

TABLE 3: Median (IQR) body weight (BW), body condition score (BCS), age, urine protein:creatinine ratio (UPCR), serum creatinine and the IRIS stage of the groups of dogs included in the study

	Group				Subgroup	
	Healthy	NP	BP	P	P1	P2
Number of animals	6	9	7	42	31	11
BW, kg	23.3 (21.5–25.0)	29.0 (18.0–36.2)	18.6 (17.0–25.0)	25.0 (10.6–37.0)	25.0 (8.6–38.0)	24.4 (15.3–37.5)
BCS	3 (3–3)	3.5 (2.5–4.8)	3 (2–3)	3.5 (2–5)	3 (2–5)	2 (2.0–3.5)
Age, years	6.2 (3.8–10.9)	3.4 (2.0–14.2)	6.6 (2.8–12.0)	4.6 (0.8–11.0)	4.8 (2.9–7.2)	3.5 (3.0–7.2)
UPCR	0.06 (0.03–0.11)	0.13 (0.06–0.18)	0.36 (0.28–0.44)	2.19 (1.06–3.65)	1.71 (0.93–3.12)	6.83 (2.39–14.76)
Serum Creatinine, mg/dl	0.8 (0.6–0.9)	0.8 (0.7–0.8)	0.9 (0.7–1.2)	0.9 (0.7–1.7)	0.8 (0.7–1.0)	2.3 (1.9–5.4)
IRIS stage (number of animals per stage)	–	–	1 (7)	1 (31), 2 (3), 3 (5), 4 (3)	1 (31)	2 (3), 3 (5), 4 (3)

IQR Interquartile range, NP Group of dogs without proteinuria (UPC ratio, < 0.2), BP Group of dogs with borderline proteinuria (UPC ratio, 0.2–0.5), P Group of dogs with proteinuria (UPC ratio, > 0.5), P1 Group of dogs with proteinuria without azotaemia (UPCR > 0.5 ; serum creatinine < 1.4 mg/dl), P2 Group of dogs with proteinuria and azotaemia (UPCR > 0.5 ; serum creatinine ≥ 1.4 mg/dl).

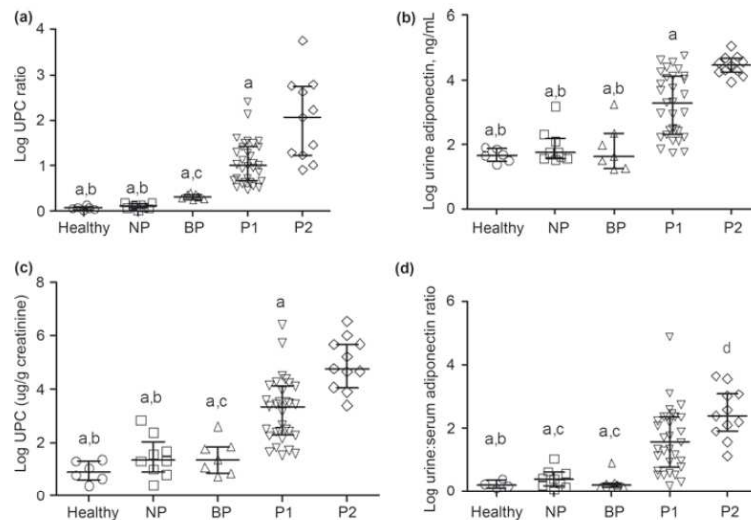


FIG 4: Urine protein:creatinine ratio (a), urine adiponectin (b), urine adiponectin corrected by urinary creatinine ($\mu\text{g/g}$ creatinine) (c), and urine:serum adiponectin ratio (d) in healthy dogs and dogs with *Leishmania* without proteinuria, with borderline proteinuria, and proteinuria (P). The central lines present median and IQR. (a) $P < 0.001$ vs. P2 group of dogs; (b) $P < 0.001$ vs. P1 group of dogs; (c) $P < 0.01$ vs. P2 group of dogs; (d) $P < 0.05$ vs. P1 group of dogs

90 per cent and a specificity of 80 per cent (Fig. 5b). The optimal cut-off point for urine:serum adiponectin ratio is 1.2 with an area under the ROC curve of 0.94 (95 per cent CI, 0.883 to 0.998), a sensitivity of 93 per cent, and a specificity of 85 per cent (Fig. 5c).

Discussion

The first objective of the present study was to evaluate the use of an ELISA kit designed to measure adiponectin in human serum samples for urine adiponectin measurements in dogs. Western blotting experiment showed that (a) the goat anti-human adiponectin antibody included in ELISA kit cross-reacted with canine adiponectin and (b) that these antibodies did not cross-react with urine proteins other than adiponectin. Stability results revealed urine adiponectin is unstable when stored at -20°C unless a stabilisation solution is used. Thus, in order to avoid the adiponectin degradation in urine, use of conservative solution (dil. 1:2) would be recommended.

The analytical validation data indicated that the assay was precise and accurate when measuring this adipocytokine in canine urine samples. Intra- and inter-assay CVs were below 20 per cent, the limit of the objective analytic performance standard for precision (Food and Drug Administration and others 2001). Additionally, linearity under dilution and recovery studies revealed the high accuracy of the assay in detecting adiponectin in canine urine samples. Ideally, the regression analysis, of the relationship of the obtained and expected analyte values should result in a regression equation approximating 1.0 (Jensen and Kjølgaard-Hansen 2010). In the present study, a regression equation approximating 1.0 (0.999) was observed when one urine sample was serially diluted. However, the dilution of a second sample resulted in a more curvilinear line, and the regression correlation coefficient was 0.922. It could be postulated that some dogs could present interfering substances in the urine that could slightly modify the concentrations of adiponectin, although this variation is of limited importance since no interactions of adiponectin antibody were observed in Western blotting analysis. The recovery study showed that the assay slightly over-estimated adiponectin concentrations when intermediate adiponectin values were measured in urine; however, percentages between 80 per cent and 120 per cent for spiking recovery are acceptable to confirm the correct accuracy of the assays (Jensen and Kjølgaard-Hansen 2010). These findings confirm that the evaluated assay is accurate when quantifying adiponectin in canine urine samples.

The second aim was to investigate the possible influence of proteinuria and renal damage on serum and urinary adiponectin in dogs. For this purpose, only dogs with leishmaniasis were included in the study mainly because of two reasons. (1) Canine leishmaniasis was considered as a natural model of kidney disease (Cortadellas and others 2009), as it causes different degrees of glomerular disease that can lead to chronic kidney disease (Guy and others 1993). The immune complexes originated due to the infection cause a secondary inflammatory reaction and a reduction in the perfusion of the peritubular capillaries that leads to tubular and interstitial damage (Nieto and others 1992, Ciaramella and others 1997). (2) Canine leishmaniasis is an endemic disease in the Mediterranean area with a prevalence of 67–80 per cent (Berrahal and others 1996, Cabral and others 1998, Solano-Gallego and others 2001), what allowed us to obtain disease-homogenous population of dogs.

In the present study, higher serum adiponectin levels were detected in subgroups of dogs with proteinuria and azotaemia (P2) in comparison with those with proteinuria but without azotaemia (P1), while no significant differences were observed between the rest of the groups. These results could explain the positive correlation between serum adiponectin and serum creatinine observed in our work. In humans, it has been described that serum adiponectin concentrations are increased in presence of severe renal damage cases (Koshimura and others 2004, Shen and others 2007), and it was hypothesised that these results, at least in part, could be attributed to the decreased BW in patients with kidney disease (Koshimura and others 2004). However, in our study, although the median BCS was lower in the P2 subgroup in comparison with P1, there was no statistical significance. This could be attributed to a relatively low number of animals in the subgroups, and high inter-individual variability of BCS in our groups of animals. Furthermore, the different degrees of inflammation associated with leishmaniasis and kidney failure, are factors that could influence serum adiponectin concentrations. Longitudinal studies, or work with a more homogenous BCS population, would be required to clarify the mechanisms that could regulate the serum adiponectin concentrations in dogs with kidney disease.

The results of this study indicate that adiponectin in urine is increased in dogs with proteinuria, similarly as described in humans (Koshimura and others 2004, Rovin and others 2005, von Eynatten and others 2009). In human medicine, two principal hypotheses were formulated in order to explain this finding. (1) The increase in urinary

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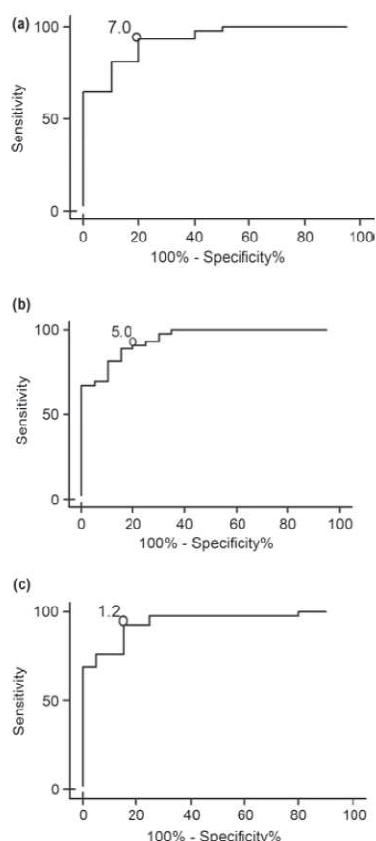


FIG 5: Receiver-operator characteristic (ROC) curves of urinary adiponeptin (a), urinary creatinine excretion (UAC) (b) and urine: serum adiponeptin ratio (c) to differentiate dogs with (urine protein:creatinine ratio (UPCR) >0.5) and without (UPCR <0.5) proteinuria. The true-positive rate (sensitivity (y axis)) is plotted against the false-positive rate (100-specificity (x axis)). The white circle on the ROC curve indicates the cut-off level of 7.0 ng/ml, 5.0, and 1.2 for best sensitivity (93 per cent, 94 per cent, 94 per cent) and specificity (80 per cent, 80 per cent, 85 per cent) for urinary adiponeptin, UAC, and urine: serum adiponeptin ratio, respectively

adiponeptin appears to be due to glomerular capillary leak as a result of a glomerular damage (Rovin and others 2005). This hypothesis is supported by the strong correlation between UPC ratio and urinary adiponeptin, as well as UAC ratio, similarly as described elsewhere (Koshimura and others 2004). (2) On the other hand, von Eynatten and others (2009) found that adiponeptin in normal kidneys is lining glomerular capillaries and intrarenal arterioles, and hypothesised that binding of adiponeptin to the glomerular endothelium could be essential for kidney homeostasis, and that subsequent increase of adiponeptin in the urine come from the adiponeptin lining glomerular vessels reflecting earlier vascular damage than the vascular leakage detected by albuminuria (von Eynatten and others 2009). This hypothesis, at least in part, could explain the high concentrations of urine adiponeptin of some leishmaniotic dogs without proteinuria (groups NP and BP) had when compared with the healthy dogs (although no statistically significant differences were found between the groups). This suggested that these dogs probably already had some renal injury, undetected by UPC ratio, as has been indicated in human systemic lupus erythematosus (Rovin and others 2005). However, other studies with more animals, and including histopathologic analysis, should be done to confirm this aspect.

ROC curve analysis revealed a high diagnostic accuracy of the urine:serum adiponeptin ratio, as well as urine adiponeptin and

UAC, to distinguish dogs with and without evident proteinuria. Furthermore, it is worth mentioning that in the present study, the three evaluated variables (urine adiponeptin, UAC and urine:serum adiponeptin ratio) showed similar results, suggesting the possible utility of the three of them for glomerulus damage evaluation in dogs.

Conclusion

The commercial ELISA assay evaluated in this study can be used for precise and accurate urinary adiponeptin measurement in dogs. Urinary adiponeptin was increased in dogs with proteinuria suggesting its possible use as a tool for glomerular injury evaluation.

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3.3.5. Article 10



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Short communication

Adiponectin and IGF-1 are negative acute phase proteins in a dog model of acute endotoxaemia

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ABSTRACT

The objective of this study was to evaluate the influence of an experimentally induced acute inflammation on serum adiponectin and insulin-like growth factor 1 (IGF-1) levels in the dog, and to compare their evolution with other well-established acute phase proteins (APPs) such as C-reactive protein (CRP), and haptoglobin (Hp). Therefore levels of adiponectin, IGF-1 and a profile of APPs were measured in healthy dogs after intravenous administration of *E. coli* LPS (0.02 mg/kg) and compared with dogs injected with saline solution (0.2 mL/kg). Adiponectin and IGF-1 were both decreased in response to endotoxins in the dog. Significant positive correlations were found between adiponectin and IGF-1 ($r = 0.31$; $p < 0.05$). Adiponectin had also a significant negative correlation with CRP ($r = -0.39$; $p < 0.05$) and Hp ($r = -0.27$; $p < 0.05$), whereas IGF-1 had significant negative correlation with CRP ($r = -0.52$; $p < 0.001$).

The results obtained in the present study indicate that adiponectin and IGF-1 behave as negative acute phase proteins after acute inflammatory stimulus in dogs.

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1. Introduction

Adiponectin is a peptide hormone produced by the adipose tissue that has gained increased attention in the last years due to its involvement in glucose metabolism and insulin sensitization. Adiponectin decreases lipid synthesis and glucose production in the liver and causes decrease in glucose and free fatty acid concentrations in the blood. Decreased adiponectin concentrations are associated with insulin resistance and hyperinsulinemia (Meier and Gressner, 2004). Also a negative correlation between obesity and circulating adiponectin has been established in the dog (Ishioka et al., 2006).

A relationship between adiponectin and some components of the inflammatory reaction has been described. The cytokine TNF- α suppresses adiponectin production in

adipose tissue (Stumvoll, 2003), and on the other hand adiponectin has showed to have an anti-inflammatory effect since it reduces the secretion of TNF- α from monocytes, macrophages and foam cells, and also attenuates the biological effects induced by this cytokine (Fernandez-Real et al., 2003).

Insulin-like growth factor 1 (IGF-1), also known as somatomedin C, is a peptide hormone, which is synthesized in the liver. It is involved in the regulation of growth and metabolism and mediates many of the anabolic effects of growth hormone (GH) in different tissues (Klapper et al., 1983). Overall IGF-1 is considered a true multi-potent growth factor, controlling cell proliferation, differentiation and apoptosis, tissue growth and organ-specific functions throughout the body (Frystyk et al., 2010). During inflammation, IGF-1 synthesis in the liver and the levels in serum are decreased (Priego et al., 2003). Furthermore, the decrease in circulating IGF-1 during inflammation occurs despite an increase in GH levels, inducing a situation of GH resistance that contributes to direct amino acids away from

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growth and skeletal muscle accretion towards the hepatic synthesis of acute-phase proteins (Frost and Lang, 2004).

Despite the influence that inflammation can have in both adiponectin and IGF-1, to the author's knowledge there is no data published about the behaviour of adiponectin and IGF-1 in inflammation in the dog, and no studies were reported having evaluated simultaneously both hormones in an inflammatory situation. The objective of this study was to evaluate the influence of an experimentally induced acute inflammation on adiponectin and IGF-1 levels, and to compare their evolution with other well-established acute phase proteins (APPs) such as C-reactive protein (CRP), and haptoglobin (Hp). Therefore, levels of adiponectin, IGF-1 and a profile of APPs were measured in healthy dogs after intravenous administration of *E. coli* LPS and compared with dogs injected with saline solution. This canine model of endotoxaemia has proven to be useful in replicating the signs and laboratory findings observed in canine and human sepsis/endotoxaemia (Ilcol et al., 2005; Yilmaz et al., 2006).

2. Material and methods

2.1. Animal and general procedures

A total of 10 adult healthy mongrel dogs (6 male and 4 female) were used in this study. The dogs weighed 14.2–31.3 kg (mean $\pm$ SD, 24.3 ± 4.6 kg) and ranged from 2 to 5 years (mean $\pm$ SD, 3.2 ± 1.4 years). The animals had a body condition score (BCS) of 3 in all cases. BCS was evaluated in accordance with a 5-point scale (McGreevy et al., 2005).

The dogs were housed in individual cages in a controlled room (18–24°C and 12/12 h light/dark cycle) for 3 days before the experiment at the Veterinary Teaching Hospital, Uludag University, Bursa, Turkey. All dogs were kept in similar conditions, provided with ad libitum water, and were fed twice daily with an extruded diet (IAMS-Propet, Istanbul, Turkey). The dogs were clinically healthy based on normal clinical examinations and results of CBC and biochemistry, including C-reactive protein, were within reference limits.

The experimental protocol was approved by the Animal Care and Use Committee of the University of Uludag.

2.2. Experiments

The dogs were assigned to the control ($n = 5$; 2 male and 3 female) and LPS group ($n = 5$; 2 male and 3 female).

Dogs in the control group received an intravenous vehicle (0.9% NaCl, 0.2 mL/kg), whereas endotoxin was injected intravenously once at 0.02 mg/kg to the dogs of LPS group.

The experiment was initiated at 9:00 am, after 14 h of fasting in both groups of dogs. All dogs were monitored clinically for 48 h after the treatment. During this period, dogs were fed four times (at 12, 24, 36 and 48 h just after blood sampling).

2.3. Sample collection and measurements

Venous blood samples were collected into tubes with and without EDTA (Vacutest EDTA K₃, BD, 3 mL, and

Vacutest, BD, 10 mL, Hema & Tube Tic. Ltd. Sti., Ankara-Turkey) before (baseline) and at 0.5, 1, 4, 24 and 48 h post-treatment.

Total serum adiponectin concentration was determined by human high sensitivity adiponectin ELISA (Human Adiponectin ELISA, High Sensitivity Kit, BioVendor-Laboratori medicina, Modrice, Czech Republic), previously validated in our laboratory for use in dogs. Intra- and inter-assay coefficients of variation were below 11% in all cases, linearity under dilution resulted in lineal regression equations with correlation coefficients of 0.9989 and 0.9958 and the limit of detection was of 0.74 μ g/mL (Tvarijonaviciute et al., 2010).

IGF-1 was analyzed with an automated solid-phase, enzyme labelled chemiluminescent immunometric assay (Immulite System, Siemens Health Diagnostics, Deerfield, IL, USA). This immunoassay had an adequate analytical performance with intra- and inter-assay CVs ranges lower than 10%, linear regression equations with correlation coefficients of 0.9993 and 0.9988 after serial dilutions, and lower limit of quantification of 7.1 ng/mL (Tvarijonaviciute et al., in press).

CRP concentration was measured using a human immunoturbidimetric assay (CRP OSR 6147 Olympus Life and Material Science Europe GmbH, Lismeehan, O'Callaghan's Mills, Co. Clare, Ireland) that showed a correlation of 0.98 with a specific canine ELISA assay (Tridelta Phase range canine CRP kit, Tridelta Development Ltd., Brey, Ireland) which has been validated for use in dogs (Martinez-Subiela and Ceron, 2005). A pooled canine serum sample with high concentration of CRP measured by a canine specific time resolved fluorometric immunoassay was used as standard.

Hp concentration was measured by a commercially available colorimetric method (Tridelta Phase range haptoglobin kit, Tridelta Development Ltd.) that was validated previously for use in dogs (Martinez-Subiela and Ceron, 2005).

CRP and Hp were measured in serum on an automated biochemistry analyzer (Olympus 2700 Automatic Chemistry Analyzer, Olympus Europe GmbH, Hamburg, Germany) and had intra-run and inter-run coefficients of variation <10%.

2.4. Chemicals

Endotoxin (lipopolysaccharide, *Escherichia coli* serotype 055:B5, purity >97%) was purchased from Sigma Chemical (St. Louis, MO); it was dissolved in sterile saline solution (0.9% NaCl) immediately before the experiment. The volume of solution injected intravenously was 0.2 mL/kg.

2.5. Statistical analysis

Summarized data is shown as mean $\pm$ SD. The results for each parameter were evaluated for approximate normality of distribution by using the Kolmogorov–Smirnov statistics. Because the data was not distributed normally, data was *log* transformed to assess normality. Repeated measures ANOVA with Dunnett's Multiple Comparison post test was used to evaluate changes of proteins in dogs'.

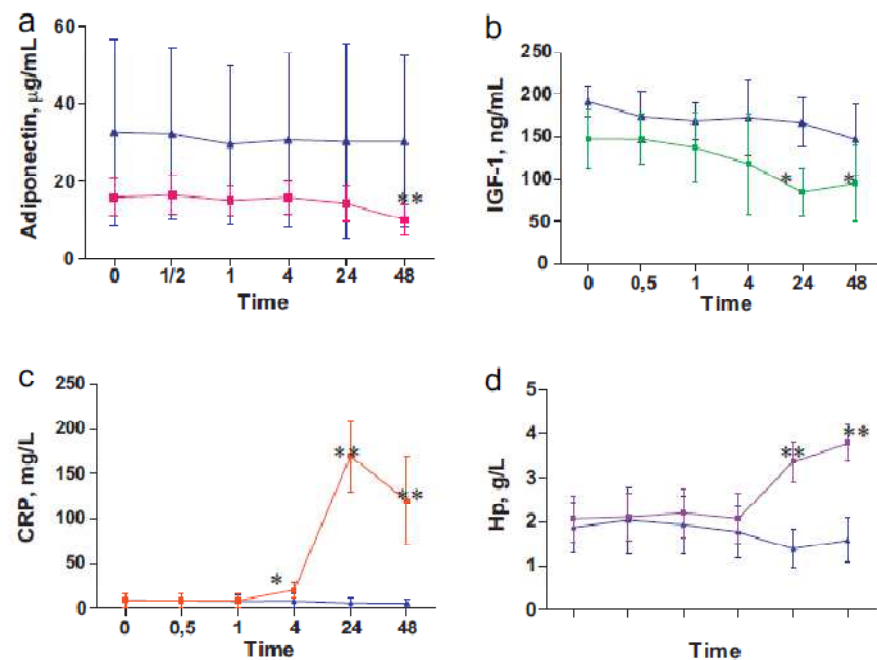


Fig. 1. Mean $\pm$ SD changes of adiponectin (a), IGF-1 (b), CRP (c), and Hp (d) levels during experimental period in dogs treated with 0.9% NaCl sol. (Control group, $\blacktriangle$) and in dogs that were injected LPS (Test group, $\blacksquare$). * $p < 0.05$; ** $p < 0.01$ vs. 0 h.

sera in different groups. Pearson correlation test was proceeded in order to verify the possible correlations between adiponectin, IGF-1 and APPs. Values of $p < 0.05$ were considered significant.

3. Results and discussion

Perturbations in adiponectin concentrations have been generally associated with obesity and metabolic syndrome. However, data about the role and behaviour of adiponectin in inflammation and sepsis is limited even in human medicine (Venkatesh et al., 2009) and studies about the mechanisms that could clarify the relation between adiponectin and inflammation are currently demanded (Owecki, 2009). In our work we describe for the first time a decrease in serum adiponectin (of 30% at 48 h post-treatment) and IGF-1 (of 42% at 24 h and of 35% at 48 h post-treatment) levels after experimentally induced endotoxaemia by LPS administration in dogs (Fig. 1). Similarly to our study a significant decrease in plasma adiponectin concentrations was detected in rats when endotoxaemia was induced by cecal ligation and puncture (Tsuchihashi et al., 2006). Our results would also agree with the down-regulation of adiponectin expression in adipose tissue induced by LPS that have been demonstrated both “in vivo” after injection of LPS in mice (Leuwer et al., 2009) and in “in vitro” studies on canine adipocytes incubated with LPS (Ryan et al., 2010). However, these findings are in contrast with a previous report in humans in which inflammation associated with endotoxaemia did not change plasma concentrations of adiponectin (Keller et al., 2003). These divergences could be attributed to the different doses and/or types of endotoxins used.

The reasons for the decrease in adiponectin levels after induction of endotoxaemia in dogs observed in the present study are not totally clear. (1) Some authors postulated that the decrease in adiponectin levels is due to the binding of adiponectin to LPS in order to produce LPS neutralization (Tsuchihashi et al., 2006). (2) In addition, based on the results of our study it could be hypothesised that the decrease in adiponectin could be related with the low levels of IGF-1 that was detected after the endotoxin administration. Although it has not been described in the dog, the decrease in IGF-1 after an inflammatory-infectious stimulus has been reported in other species such as rats, mice, pigs and steers (Frost and Lang, 2004) being caused in part due to the inhibitory effect of proinflammatory cytokines on the IGF-1 expression in the liver (Colson et al., 2003). IGF-1 has been demonstrated to increase adiponectin synthesis in adipose tissue (Meier and Gressner, 2004), so the decrease in IGF-1 that appears in the acute phase response could produce an associated lower production of adiponectin and decreased serum adiponectin levels. (3) Finally the decrease in adiponectin could be associated with the increase in TNF-alpha that has been described to be produced in an endotoxin injection in a similar experimental model that was used in this study (Ilcol et al., 2005) and that suppresses adiponectin production in adipose tissue (Stumvoll, 2003).

A limitation of this study would be the measurement of total adiponectin without separating the different molecular forms of adiponectin. Although the three isoforms of adiponectin have been characterized in the dog (Brunson et al., 2007), to the authors' knowledge, no methods for comparative analysis of the total and different subforms of adiponectin in dogs are validated in this specie. The measurement of the three different

subforms of adiponectin (the high-molecular-weight (HMW), the medium-molecular-weight (MMW) and the low-molecular-weight (LMW) forms) have gained attention in human medicine in previous years. The HMW subform is believed to be the primary biologically active form (Leth et al., 2008), although studies in humans have demonstrated the existence of the correlation between HMW and total adiponectin (Leth et al., 2008).

Since adiponectin has an anti-inflammatory effect, a significant fall in adiponectin may add to the upregulation of inflammatory cytokines that is produced after an inflammatory stimulus in leading to a major and severe pro-inflammatory response that can have a negative impact on the organism (Leuwer et al., 2009). Overall adiponectin was considered as a protective factor (Zoccali et al., 2003), so it could be postulated that the decrease observed in adiponectin levels during inflammation should not be too severe in order to preserve an adequate anti-inflammatory response of the organism. There are some practical evidences that would support this theory: obesity is a risk factor for intensive care unit mortality in patients with sepsis (Yaegashi et al., 2005). In these cases, the reduced adiponectin levels that appear in obesity would lead to an impaired neutralization of LPS activity and a higher inflammatory response (Tsuchihashi et al., 2006). In humans critical illness was associated with lower adiponectin concentrations as compared with controls (Venkatesh et al., 2009). Administration of rosiglitazone, which increases plasma adiponectin concentrations, significantly lowers inflammatory mediators in plasma and, might improve management of patients with sepsis (Uji et al., 2010).

A controversy exists about the relation between adiponectin and inflammation, since some autoimmune and chronic inflammatory conditions such as rheumatoid arthritis, systemic lupus or inflammatory bowel disease showed increased serum concentrations of adiponectin. The explanation could be that increases in adiponectin can help survival during periods of catabolism secondary to chronic inflammation, since adiponectin decreases energy expenditure and limits the amount of adipose tissue loss during fasting (Fantuzzi, 2008). Further studies should be undertaken to elucidate the differences in the behaviour of adiponectin between acute and chronic inflammatory conditions.

The increase of CRP after LPS injection that reached significance at 4 h and peaked (22-fold) at 24 h and the increase in Hp that reached significance and peaked later with 2-fold increment (at 24 and 48 h, respectively) (Fig. 1), indicated that an inflammatory acute phase response was produced in our experimental model, as evidenced by the behaviour of these two proteins which are major (CRP) and moderate (Hp) positive acute phase proteins (Ceron et al., 2005). The APP is a blood protein which, influenced by the interaction of the cytokines (produced at the site of the lesion) with the tissue in which the APP is produced, increase or decrease its circulating levels after an inflammatory stimulus. Positive acute phase proteins increase their concentrations whereas negative APPs show a decrease in serum levels (Ceron et al., 2005). Taking this concept in consideration, adiponectin and IGF-1 behaved as

Table 1

Correlations between adiponectin, IGF-1 and APPs.

	Adiponectin		IGF-1	
	R	P	R	P
CRP	−0.3903	0.004	−0.5155	<0.001
Hp	−0.2745	0.046	−0.1737	0.21
Adiponectin			−0.3098	0.024

CRP—C-reactive protein; Hp—haptoglobin.

negative acute phase proteins in our experimental model in that they showed a decrease in serum concentrations, probably induced at least in part by the effects of TNF- α or other cytokines in their tissues of production (adipose tissue and liver, respectively). A weak although significant correlation was found between the values of CRP, the most sensitive and specific acute phase protein, adiponectin and IGF-1 (Table 1). Similarly, correlation between CRP and adiponectin has been previously described in humans (Xydakis et al., 2004). This data would support the hypothesis that both adiponectin and IGF-1 are negative acute phase proteins in the dog.

The present study demonstrated that adiponectin and IGF-1 are both decreased in response to endotoxins in the dog. Further studies should be performed in order to clarify the roles of these decreases in adiponectin and IGF-1 in the physiopathological mechanisms during endotoxaemia as well the clinical implications that both proteins could have in the management, treatment and prognosis of inflammatory diseases and sepsis.

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3.4. Variations due to obesity and obesity related diseases

3.4.1. Article 11



Serum acute phase proteins concentrations in dogs during experimentally short-term induced overweight. A preliminary study

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ABSTRACT

The purpose of the study was to analyse serum concentrations of four different positive acute phase proteins (APPs): C-reactive protein, haptoglobin, serum amyloid A and ceruloplasmin in a model of experimentally short-term developed obesity in Beagle dogs. All APPs were quantified by commercially available ELISA methods and C-reactive protein was also determined by a highly sensitive time-resolved fluorometric assay. There were no significant differences between APPs concentrations at the beginning and the end of the study in groups of dogs that increased their body condition scores. In addition, dogs with body condition scores of 4 and 5 did not shown significant differences for any of the acute phase proteins studied compared with control dogs of BCS of 3, with exception of a decrease in haptoglobin. It was concluded that overweight induced in the experimental conditions of this study does not produce a significant change in acute phase proteins.

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1. Introduction

Obesity is one of the most important nutritional problems in dogs; approximately 25–40% of dogs receiving veterinary care in western countries are overweight to grossly obese (Mc Greevy et al., 2005). The dog has been used as an animal model for the study of the physiological and genetic basis of obesity, and it is even expected in the near future an expansion in activity using the dog as an animal model, as the epidemic of human obesity continues and spreads geographically (Speakman et al., 2008).

Positive acute phase proteins (APPs) such as C-reactive protein (CRP), haptoglobin (Hp), serum amyloid A (SAA) and ceruloplasmin (Cp) increase its rate of synthesis and release when an inflammation occurs (Ceron et al., 2005; Dabrowski et al., 2009; Eckersall, 2000). Measurements of APPs can be used in dogs as screening test for systemic response to an inflammatory stimulus and are considered as the most accurate markers of inflammation (Ceron et al., 2008). However, there are some aspects of the APP response in dogs that have not been widely evaluated such as the possible variations in obesity. Regarding this aspect, although reports indicating that serum CRP is increased in obese humans have been published (Park et al., 2005; Zagorski et al., 2005), a recent paper have postulated that CRP production can be inhibited in obese dogs (Veiga et al., 2008).

The main objective of the present study was to evaluate influence of overweight in the acute phase proteins in dogs. For this purpose CRP, SAA, Hp and Cp were measured in Beagle dogs with different weight and body conditions obtained after an experimental short-term induced overweight trial.

2. Material and methods

2.1. Animals and experimental set-up

A total of fourteen adult intact, university-owned beagle dogs (*Canis familiaris*) were used in this study (6 females and 8 males; age range, 5.1–7.4 years; body weight range, 12.4–18.6 kg). Dogs were maintained in their usual kennel (one or two dogs per kennel) with controlled temperature (23 ± 2 °C) and light (lights on at 08:00–20:00). The size of each kennel was 3×4 m.

To obtain a wide range of weights and body conditions, a protocol previously described (Sagawa et al., 2002) with some modifications was used (Tvarijonaviciute et al., 2009). Dogs were separated into two groups and maintained for 10 weeks as follows: group A (3 dogs, 2 males and 1 female) was fed a standard dry food (Premium Croc Adult, Affinity Petcare), containing 24.0% crude protein, 16.1% crude fat, 2.5% crude fibre, 6% crude ash, 8% moisture. Group B (11 dogs, 6 males and 5 females) was fed a high-energy diet (Advance Performance, Affinity Petcare) containing 31.0% crude protein, 21.0% crude fat, 2.0% crude fibre, 6.5% crude ash, 8% moisture. For all dogs food and water was available *ad libitum*. The assessment of the nutritional condition was based on a five-

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scale body condition score (BCS): 1, thin; 2, lean; 3, optimal; 4, obese; 5, gross (Mc Greevy et al., 2005). At the beginning of the study all dogs in group A (2 males/1 female) had optimal weight (BCS 3); and in group B, 7 dogs (4 males/3 females) had optimal weight (BCS 3) and 4 dogs (2 males/2 females) were obese (BCS 4).

All dogs passed general health examinations weekly during the whole study and complete blood count and biochemical profiles were performed at the beginning of the study to confirm that there were no apparent abnormalities (except obesity). Female dogs were in anestrus during study period, although oestrus cycle has no effect on APPs (Ulutas et al., 2009). All animals had a negative serological titre for *Leishmania infantum* and *Ehrlichia canis*.

Blood samples were collected on the morning after an overnight fasting of at least 12 h by venipuncture in the cephalic vein into EDTA tubes for CBCs and tubes containing clotting accelerator (TapVal, Aquisel, Barcelona, Spain) for APP assays. Samples were taken from all dogs at the beginning (T1) and the end (T2) of the experimental period. Tubes for APP assays were centrifuged at 2000g for 10 min at room temperature to obtain serum, which was stored in plastic vials at -20°C until analysis. On the day of analysis, samples were brought to room temperature and thoroughly vortexed prior to measurements.

The experimental set-up was approved by the Animal Care Commission of Murcia University, additionally housing and care was performed according to guidelines for the care and use of laboratory animals established by the European Union (European Economic Community, 1986).

2.2. Analysis

Concentrations of CRP were analyzed by two methods:

- A solid-phase sandwich immunoassay specific for canine CRP (Tridelta Ltd., Brey, Ireland) in accordance with directions provided by the manufacturer. Final absorbance of samples was measured by use of a microtiter plate reader (PowerWave XS, Bio-Tek Instruments Inc., Vermont USA) at 450 nm. This method was validated in the laboratory of the authors for canine serum (Martinez-Subiela and Cerón, 2005).
- A time-resolved immunofluorometric assay (TRFIA) previously validated for canine samples using goat anticanine polyclonal antibodies (Parra et al., 2006). The fluorescence, proportional to the concentration of CRP in the sample, was measured in a VICTOR 1420 multilabel counter (Perkin-Elmer Lifesciences, Wallac Oy; Turku, Finland). This method has a lower limit of detection and a lower between-run imprecision compared with other commercial kits for CRP analysis (Parra et al., 2006).

Serum concentrations of haptoglobin were determined by use of the hemoglobin-binding method using a commercial kit (Tridelta Ltd., Brey, Ireland) and an automated biochemistry analyzer (Cobas Mira Plus multiparametric autoanalyser, ABX Diagnostics, Montpellier, France). Concentrations of SAA were determined by use of a solid-phase sandwich ELISA. The ELISA was designed for use in determining concentrations of SAA in different animal species such as dogs. Final absorbance of samples was measured by use of a microtiter plate reader at 450 nm. Both methods were validated in the laboratory of the authors for canine serum (Martinez-Subiela and Cerón, 2005).

Serum concentration of ceruloplasmin was measured by use of a spectrophotometric method based on the *in vitro* oxidase activity of Cp with *p*-phenyldiamine based validated for use in canine samples (Ceron and Martinez-Subiela, 2004). Determinations were made by use of an automated biochemistry analyzer (Cobas Mira Plus multiparametric autoanalyser, ABX Diagnostics, Montpellier,

France) and results were reported as the change in absorbance per minute at 550 nm.

CBC was determined by an impedance hematological analyser (Vet Abc, ABX Diagnostics, Montpellier, France).

2.3. Statistical analysis

Data are shown as mean $\pm$ SD that were calculated using routine descriptive statistical procedures and software (SPSS statistical program, SPSS Inc., Chicago, IL, USA). Data were log transformed to assess normality. Two-way ANOVA of repeated measures was used to compare concentrations of the different APPs at the beginning (T1) and the end of the study (T2) between different groups. Paired *t* test was used to compare concentrations of the different APPs at the beginning (T1) and the end of the study (T2) within the same group. Values of $p < 0.05$ were considered significant.

3. Results

The protocol of feeding used in this study produced a wide range of weight gain and BCS in the dogs. Dogs in group A maintained their BCS of 3 as did not change body weight (BW). At the end of fattening period dogs from group B were divided into two groups based on their BCS: group B1 had 5 dogs (3 males/2 females) with BCS 4, and group B2 had 6 dogs (3 males/3 females) with BCS 5.

Table 1 shows the values of the body weight and APPs of the different groups of dogs at the beginning and the end of the trial. When compared the different groups for the BW and APPs no significant differences were found at T1; however, BW statistically increased at T2 in group B2 if compared with group A ($p < 0.05$).

Comparing T1 and T2 within each group, mean BW statistically increased in groups B1 and B2 ($p < 0.05$ and $p < 0.005$, respectively).

Obtained SAA concentrations were below detection limit (Martinez-Subiela and Cerón, 2005) in one animal at T1 and in six animals at T2, so statistical study could not be performed for this acute phase protein. When serum concentration of hs-CRP, CRP and ceruloplasmin were compared between the group of dogs of optimal weight and both groups of overweight dogs at any time, no statistically significant differences were detected. Hp showed a statistically significant decrease in the group B2 compared with the groups A ($p < 0.01$) and B1 ($p < 0.001$) at T2.

When serum APPs concentrations were also assessed between T1 and T2 within each group, statistically significant differences were only observed for ceruloplasmin in group A (from 8.10 to 6.60×10^{-3} $\Delta\text{abs}/\text{min}$, respectively; $p < 0.05$) and for Hp in group B2 (from 1.17 to 0.71 g/L, respectively; $p < 0.05$).

4. Discussion

The objective of this study was to evaluate serum concentrations of positive APPs in animals with different overweight degrees experimentally induced in order to elucidate the possible role of APPs in canine obesity. For this reason two major, CRP and SAA, and two moderate APPs, Hp and Cp, were evaluated. Major APPs have an early and marked high rise following by a rapid decline in concentration, while the moderate ones require more time to increase in concentration, show increases of less magnitude and have slower decline (Ceron et al., 2005).

In present study, APPs showed no significant differences before and after fattening period. When data was analysed in individual animals, they were inside the limits of the critical differences (differences produced by individual variation) described for CRP, Hp and Cp (Martinez-Subiela et al., 2003). The critical differences

Table 1

Values of body weight and the different acute phase proteins of the groups A, B1 and B2 at the beginning (T1) and the end of the study (T2).

	Animal	BCS		BW (kg)		Ceruleplasmin (Δ abs/min)		hs-CRP (mg/L)		CRP (mg/L)		SAA (mg/L)		Hp (g/L)	
		T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Group A	A1	3	3	12.8	13.5	0.0085	0.0074	4.63	3.78	3.90	4.30	0.84	1.16	1.33	1.22
	A2	3	3	15.8	16.5	0.0078	0.0059	4.11	0.68	5.00	2.50	ND	1.42	1.14	1.03
	A3	3	3	13.9	13.6	0.0080	0.0065	1.87	0.30	3.20	0.60	1.42	ND	1.30	1.34
	Mean			14.17	14.53	0.00810	0.00660 [†]	3.54	1.58	4.03	2.47	1.13	1.29	1.26	1.20
	sd			1.52	1.70	0.00036	0.00075	1.47	1.91	0.91	1.85	0.41	0.19	0.10	0.16
Group B1	A1	3	4	15.6	17.4	0.0086	0.0065	2.88	3.80	1.70	3.60	2.48	ND	1.48	1.22
	A2	3	4	13.4	14.4	0.0080	0.0051	8.52	0.89	7.20	2.80	0.94	ND	1.68	1.03
	A3	3	4	12.7	15.2	0.0068	0.0062	2.45	2.62	3.20	3.90	0.94	1.00	1.15	1.03
	A4	3	4	15	15.5	0.0086	0.0108	8.85	1.24	10.20	3.20	2.32	ND	1.51	1.44
	A5	3	4	12.8	15.8	0.0057	0.0046	2.98	0.88	3.90	2.50	1.05	ND	1.32	1.15
	Mean			13.90	15.66 [†]	0.00754	0.00664	5.13	1.89	5.24	3.20	1.55	1.00	1.43	1.17
	sd			1.32	1.10	0.00126	0.00245	3.25	1.29	3.43	0.57	0.78		0.20	0.17
Group B2	A1	3	5	14.1	22.1	0.0102	0.0051	3.20	1.43	3.40	4.50	4.93	2.80	1.44	0.56
	A2	3	5	18.6	24	0.0086	0.0129	4.87	7.75	5.00	2.80	2.54	2.22	1.19	1.17
	A3	4	5	12.7	16.4	0.0087	0.0063	2.37	3.49	5.80	1.00	6.43	7.87	1.23	0.62
	A4	4	5	12.9	15	0.0076	0.0065	2.90	0.38	3.20	3.60	1.16	ND	1.19	0.74
	A5	4	5	15.9	19.8	0.0105	0.0064	2.27	1.40	2.11	0.60	4.24	2.96	0.92	0.52
	A6	4	5	12.4	17.6	0.0104	0.0049	5.85	2.10	7.60	2.10	15.04	9.21	1.07	0.65
	Mean			14.43	19.15 ^{†,*}	0.00933	0.00702	3.57	2.76	4.52	2.43	5.72	5.01	1.17	0.71 ^{†,*,***}
	sd			2.41	3.46	0.00120	0.00296	1.45	2.65	2.01	1.50	4.92	3.27	0.17	0.24

BCS – body condition score; BW – body weight; hs-CRP – high sensitivity C-reactive protein; CRP – C-reactive protein; SAA – serum amyloid A; Hp – haptoglobin; ND – not detected.

* $p < 0.05$ vs. group A.** $p < 0.01$ vs. group A.*** $p < 0.001$ vs. group B1.† $p < 0.05$, T1 vs. T2.‡ $p < 0.005$, T1 vs. T2.

could also influence the differences in CRP previously described between lean and obese dogs in a clinical work (2.72 vs. 0.76 mg/L) (Veiga et al., 2008). Future studies are necessary to assess if the difference in the APPs between lean and obese dogs could be due to individual variations and not due to the proper effect of obesity.

These results suggest that short-term induced obesity in experimental conditions, does not produce a significant increase of APPs contrarily to the findings described in humans for CRP (Park et al., 2005; Zagorski et al., 2005) and ceruloplasmin (Cignarelli et al., 1996). Increase in cytokines such as TNF (Gayet et al., 2004) and also variations in adipokines such as adiponectin and leptin have been described in the obese dogs (Sagawa et al., 2002). From a practical point of view and using the same experimental model that was in this report, we have found that other serum biomarkers such as butyrylcholinesterase or adiponectin were more sensitive to detect obesity in dogs in these conditions (Tvarijonavičiute et al., 2009).

In the case of CRP, it could be postulated that the lack of differences found in obese dogs with the commercial ELISA used in our study would be due to not using ultrasensitive methodology (highly sensitivity CRP) as is made in human medicine. In addition the values obtained for CRP in our study were very close to the limit of detection of the ELISA method used, which also can have a high imprecision at low CRP levels. In order to solve these limitations we also analyzed CRP in all samples by a TRFIA which is much more sensitive than the current commercial ELISAs (Parra et al., 2006) and we did not find significant differences in CRP between obese and lean dogs. So in the case of CRP there is not much method influence in the values obtained in our study.

One of the limitations of experimental procedure used in this study is that short-term induced overweight does not reflect real clinical situation, especially in those chronic obesity cases. However, this experimental protocol avoids different variations that can occur when studies are performed in a clinical environment such as the use of different diets and environmental conditions,

different lengths in the onset of obesity, medications, and possible secondary diseases, that could have some effect on APPs (such as diabetes, pancreatitis, hypothyroidism, dyslipidemia, osteoarthritis, and altered kidney function or respiratory distress). Anyway it should be desirable in the future to undertake studies in order to clarify APPs evolution in chronic obesity.

Other limitation could be the low number of animals used. This was mainly due to ethical reasons. However, the number of animals is in line with previously published studies about induced obesity in dogs (Jeusette et al., 2006; Briand et al., 2008). In the future it would be desirable to perform long-scale studies with high number of animals as studies in human are made.

In conclusion short-term induced overweight in the experimental conditions of this study does not produce significant changes in positive acute phase proteins in the dog. This fact should be taken in consideration if this species is used as a model for the study of inflammatory changes associated with obesity and also when acute phase proteins are interpreted in obese dogs.

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3.4.2. Article 12



Short Communication

Effect of weight loss on inflammatory biomarkers in obese dogs

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ABSTRACT

The objective of this study was to evaluate the effects of weight loss on selected serum inflammatory biomarkers in obese dogs. An experimentally induced bodyweight reduction of approximately 2.5%/week was accompanied by significant decreases in metabolic markers of obesity (lipidic profile, fructosamine, and insulin-like growth factor-1). The concentrations of acute phase proteins and of selected cytokines remained within reference ranges in obese dogs during weight loss, suggesting that significant inflammation was not a major component of this experimental model. However, adiponectin concentrations increased following the period of weight loss suggesting reduced susceptibility of these animals to obesity-related inflammation.

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Obesity is a very common nutritional disorder in dogs and a major risk factor for a number of diseases (German et al., 2010). Although there is increasing evidence of relationships between obesity and inflammation, the exact nature and details of these links remain to be determined. The objective of this study was to evaluate the effects of weight loss on a profile of serum inflammatory biomarkers in obese dogs. The study was approved by the Ethics Committee of The School of Veterinary Medicine, Murcia University.

Six obese, intact female beagles, aged 3.2–6.1 years were used in the study. The animals were obese, >30% of bodyweight (BW) with body condition scores (BCSs) of 5/5, and remained on the study for 3 months by which time their BCS had reduced to 3/5, a score more compatible with an ideal BW (Yamka et al., 2006). All dogs remained in anoestrus during the weight loss period and were given a strictly controlled amount of a hypo-energetic commercial diet (Obesity Management, Royal Canin) once daily (see Appendix A, Supplementary material). The quantity of food offered was progressively adjusted to induce a rapid weight loss (2–3% of BW/week), yet providing minimal protein requirements (Blanchard et al., 2004). Drinking water was available ad libitum.

BCS and BW were assessed weekly and a measure of body fat mass (FM) was obtained prior to and after weight loss using dilution of a single dose of deuterium oxide (2/5 mg/kg) (Son et al., 1998). Blood samples were collected by cephalic venepuncture in the morning following overnight fasting at study commencement (obese state, T0) and then monthly (T1–T3) during the weight loss period. Fasting plasma glucose, fructosamine, total HDL- and LDL-

cholesterol, triglycerides, and immunoglobulin (Ig) G, M, and A were measured using an automated analyser (Olympus AU2700, Olympus Diagnostica). Insulin-like growth factor (IGF)-1 was measured using an automated chemiluminescent immunometric assay (Immulite System, Siemens Health Diagnostics). Fasting plasma insulin concentrations were calculated using a canine insulin ELISA (Mercodia). As an indirect assessment of insulin resistance, insulin:glucose (I:G) ratios were calculated (German et al., 2009). C-reactive protein (CRP), haptoglobin (Hp), ceruloplasmin (Cp), and adiponectin were measured as previously described (Tvarijonaviciute et al., 2011a).

Results are shown as medians (with ranges) unless otherwise stated and were calculated using routine descriptive statistical procedures and software (Graph Pad Prism, Version 5). The Kolmogorov–Smirnov test was used to assess the normality of the data. When not normally distributed, the data were log transformed for further analysis. A paired Student's *t* test was used to compare changes in BW and in the selected analytes prior to, and during the weight loss period. Values of *P* < 0.05 were considered significant. The mean BW and FM reductions in the dogs in the study were considered satisfactory (approximately 2.5% and 3.7%/week, respectively) (Table 1). Weight loss was accompanied by significant decreases in the following serum analytes: 'lipidic profile'; fructosamine; insulin; I:G ratio; and IGF-1 (Table 2). These changes were consistent with the findings of previous studies (Yamka et al., 2006; German et al., 2009).

Changes in the concentrations of adiponectin, acute phase proteins (APPS), and Igs at different time points are illustrated in Fig. 1. Serum interleukin (IL)-6 values remained below the detection limit in all dogs during the experiment. No significant changes were

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Table 1

Bodyweight and body fat mass (FM) measurements of six obese beagles before, and after, a 3 month period of experimentally-induced weight loss.

Beagle no.	Bodyweight (kg)		Body FM (%)	
	Before	After	Before	After
1	24.7	16.0	46.8	23.8
2	19.7	13.4	40.3	33.3
3	18.1	13.0	55.8	20.3
4	20.1	13.4	41.4	18.4
5	20.4	14.8	49.4	30.0
6	17.6	14.0	45.9	26.5
Mean $\pm$ SD	20.1 $\pm$ 2.5	14.1 $\pm$ 1.1 ^b	46.6 $\pm$ 5.7	25.4 $\pm$ 5.7 ^a

^a $P < 0.05$ vs. 'before'.

^b $P < 0.001$ vs. 'before'.

observed in APPs after weight loss, and the concentrations of this biomarker in our obese dogs remained within the reference ranges used by our laboratory.

Previous research has provided contradictory data as regards alterations in the concentrations of CRP in obese dogs. Increases in APP concentrations were observed in obese animals by German et al. (2009), with these levels decreasing following weight loss.

Table 2

Concentrations of recognised biomarkers of obesity in six obese beagles before, and after, a 3 month period of experimentally-induced weight loss. HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; IGF-1, insulin-like growth factor 1.

Biomarker	Before Median (range)	After Median (range)	P
Serum total cholesterol (μ g/dL)	233.00 (184.20–279.90)	178.55 (115.10–231.50)	0.005
Serum HDL-C (μ g/dL)	172.15 (151.00–190.80)	143.30 (91.50–163.30)	0.018
Serum LDL-C (μ g/dL)	89.10 (55.80–112.70)	52.80 (34.00–73.80)	0.001
Serum TG (μ g/dL)	48.65 (45.00–76.80)	37.10 (24.90–62.40)	0.101
Serum fructosamine (μ mol/L)	726.30 (476.10–946.10)	422.20 (290.60–566.20)	0.010
Serum IGF-1 (ng/mL)	159.00 (117.00–201.00)	105.00 (91.40–148.00)	0.030
Serum glucose (mg/dL)	89.10 (85.70–108.80)	98.05 (59.90–107.70)	0.693
Serum insulin (μ UI/mL)	23.76 (4.80–101.51)	4.80 (4.80–68.37)	0.046
Insulin:glucose (μ l/mg)	27.43 (4.55–115.22)	6.98 (4.46–71.14)	0.049

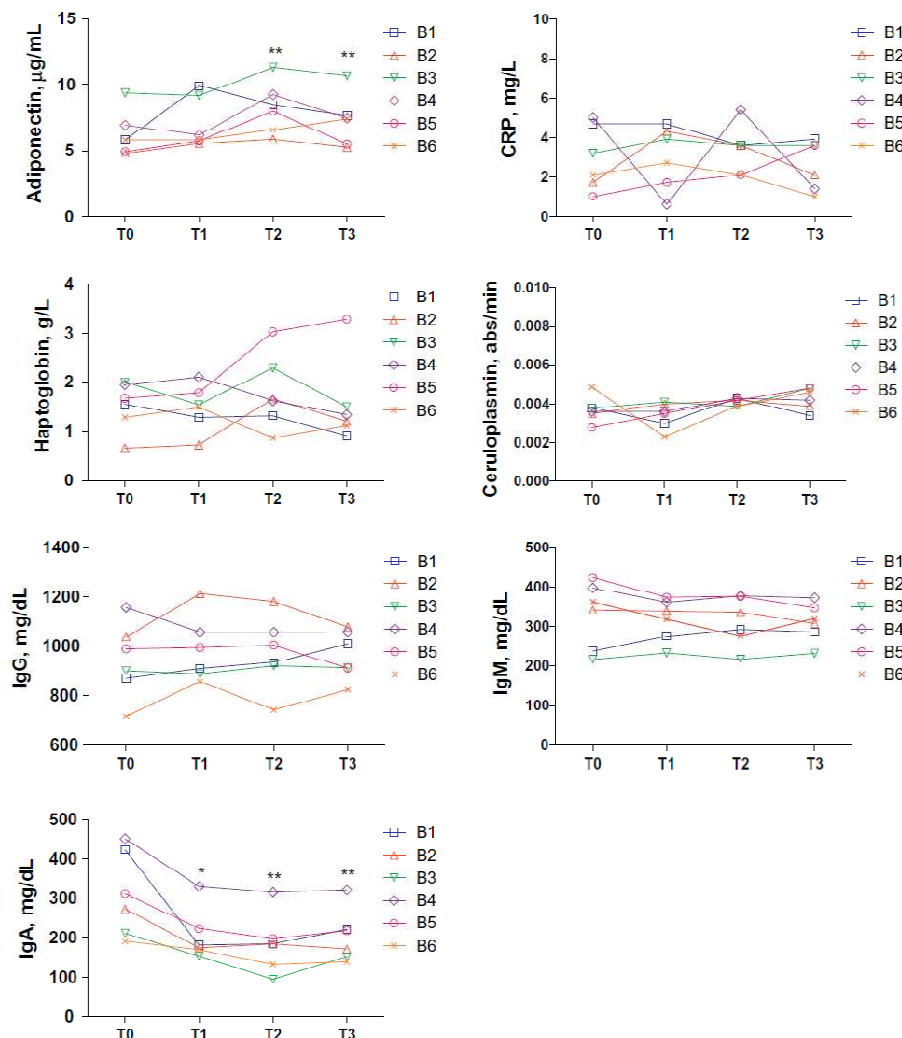


Fig. 1. Changes in the concentrations of the serum inflammatory biomarkers adiponectin, C-reactive protein (CRP), haptoglobin, ceruloplasmin, immunoglobulin (Ig) G, M and A in six obese beagles (B1–B6) at monthly intervals (T0–T3) over a 3 month period of experimentally-induced weight loss. *, $P < 0.05$; **, $P < 0.01$ vs. T0, respectively.

However, Tvarijonavičiute et al. (2011b) did not observe significant differences in the levels of APPs during experimentally induced obesity, and Veiga et al. (2008) reported decreased CRP in obese dogs. It is noteworthy, that in these studies, APP concentrations in both obese and control dogs did not reflect an inflammatory state. In cases of human obesity, CRP is usually found at intermediate concentrations lower than 10 mg/L, limit for clinically significant inflammation, but higher than 3 mg/dL, limit for normal values. It has been postulated that these intermediate values could be due to genetic, demographic, behavioural or dietary factors (Kushner et al., 2006). Since IL-6 and APP concentrations were within the reference ranges in our obese dogs, and did not vary after weight loss, the results support the hypothesis that inflammation is not a significant component in this particular model of canine obesity.

A decrease in IgA was observed in dogs following the period of weight loss, whereas no changes were recorded in the concentrations of either IgG or M. Why IgA levels increased remains unclear and requires further study. Adiponectin concentrations increased following the weight loss period. In humans, adiponectin is considered anti-inflammatory molecule with low levels associated with an increased risk of inflammation (Zoccali et al., 2003). A significant fall in adiponectin concentrations may thus contribute to a deleterious up-regulation of pro-inflammatory cytokines (Leuwer et al., 2009). Taken together, these findings suggest that, similar to humans, weight loss in dogs could result in an adiponectin-mediated decreased risk of obesity-related inflammation (German, 2010).

Our results demonstrate that short term weight loss in obese dogs improves their metabolic status (lipidic profile, insulin and IGF-1 concentrations, and I:G ratio). The fact that the concentrations of the selected inflammatory biomarkers remained within reference ranges suggests that significant inflammation was not induced in this model of canine obesity. The increased adiponectin concentrations observed following the period of weight loss could be beneficial in reducing the risk of obesity-related inflammation.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tvjl.2012.02.015>.

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3.4.3. Article 13

Evaluation of automated assays for immunoglobulin G, M, and A measurements in dog and cat serum

Running head: Immunoglobulin G, M, and A measurements in dog and cat serum

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Keywords: Cat; Dog; Immunoglobulins; Turbidimetric Immunoassay; Validation

Abstract

Background: Measurements of immunoglobulins (Igs) in companion animals can be useful to detect immunodeficiencies of the humoral system that can be associated with secondary infections, immune-mediated disorders, or neoplasms of B cells.

Objective: The purpose of this study was to evaluate human automated commercially available immunoturbidimetric assays for Igs G, M, and A measurements in canine and feline serum using species specific calibrators.

Methods: Canine and feline serum samples with different concentrations of Igs were used for the analytical validation of the assays. Intra- and inter-assay precision, linearity under dilution, spiking recovery and limit of detection were determined. In addition effects of lipaemia, haemolysis, and bilirubinaemia were evaluated. For overlap performance concentrations of Igs in groups of healthy and diseased dogs and cats were quantified.

Results: Spiking recovery and linearity under dilution tests showed that the assays were able to measure Igs in canine and feline serum samples in an accurate way. Intra and inter assay imprecision were lower than 15% in all cases. Significantly higher IgG, IgM and IgA levels were observed in dogs with Leishmania, while dogs with pyometra showed a statistically significant increase in IgM and IgA in comparison with healthy ones. Significantly higher IgG and IgM levels were observed in cats with FIV compared with healthy ones.

Conclusions: The methods for Igs determination in dogs and cats, evaluated in present study, showed an adequate precision and accuracy. In addition they were able to discriminate between different concentrations of Igs that appear in healthy and diseased animals.

Introduction

Measurements of immunoglobulins (Igs) in companion animals can be useful to detect immunodeficiencies of the humoral system that can be associated with secondary infections,¹ as well as increases in Igs that occur in immune-mediated disorders, neoplasms of B cells or infectious diseases.²⁻⁴ Additionally, in cat hypergammaglobulinaemia has been observed in feline immunodeficiency virus (FIV) that was extensively studied for its similarity to human immunodeficiency virus.⁵

Previous studies quantifying canine or feline Igs have generally been performed using single radial immunodiffusion (SRID)⁶ or ELISA.^{5,7-12} SRID and ELISA are performed manually and this procedure could influence assay precision and implies prolonged turn-around time for samples analysis. Turbidimetric immunoassays (TIAs) can be easily automated and are based on the principle of immunologic agglutination and light scattering of the agglutination products. The use of automated analysers for Igs quantification could improve assay precision and decrease the time needed for analysis in each sample. TIAs for human immunoglobulin quantification that can be performed on automated chemistry analyzers have been developed. In addition, an automated TIA initially designed for humans has been validated for Igs determination in foals.¹³ However, to the authors' knowledge no automated TIA has been evaluated for Igs measurements in dogs and cats. Therefore, the aim of the current study was to validate automated human TIAs for immunoglobulin G (IgG), immunoglobulin M (IgM), and immunoglobulin A (IgA) measurement in canine and feline serum.

Material and methods

Samples

The current study was approved by the Local Ethical Committee of the Veterinary Medicine School, Murcia University. In addition it was performed in compliance with the laws RD32/2007 and RD1201/2005 about animal experimentation in Spain.

Canine and feline serum samples with different IgG, IgM and IgA concentrations were used for the evaluation of the analytical performance of the assays. Dogs and cats were of different sex, breeds and ages presented at teaching veterinary hospital of University of Murcia, Spain and San Marco veterinary hospital, Padua, Italy, between June and December of 2010. High IgG, IgM and IgA concentration specimens were obtained from dogs with untreated *Leishmania* and cats with feline immunodeficiency virus (FIV) infection. In addition, specimens with expected normal IgG, IgM and IgA concentrations were obtained from healthy dogs and cats, presented to the clinics for routine check-ups or elective surgery that showed no remarkable findings on physical and clinical examination, or in routine haematology and biochemistry analysis.

Blood samples were collected in the morning after an overnight fast of at least 12 hours by venipuncture in the saphenous vein using a 21G needle and placed into 5 mL tubes containing a clotting accelerator (TapVal, Aquisel). None of the samples was lipaemic or haemolytic. No later than fifteen minutes after collection, samples were centrifuged at 2000g for 10 min at room temperature to obtain serum and were then stored in aliquots in plastic vials at -20°C until analysis. Duration of storage was less than 6 months in all cases. It was observed that Igs were stable for at least one year at -20 °C (data not shown).

Pools of samples were used for the analytical performance procedures, as none of the samples contained sufficient volume of serum for repeated analyses.¹⁴ Therefore serum samples were pooled on the basis of expected Igs concentrations: four serum samples from diseased animals were used to prepare pools with potentially high Igs concentrations; and five serum samples from healthy animals were used to prepare pools with potentially normal Igs concentrations.

IgG, IgM, IgA analysis

Serum IgG (Code number OSR6145, Olympus Europe GMBH), IgM (Code number OSR6146, Olympus Europe GMBH), and IgA (Code number OSR1171, Olympus Europe GmbH) levels were measured by using of commercial kits with an automated analyser (OLYMPUS AU600, Olympus Europe GmbH) following the instructions of the manufacturer. The general procedure of the all methods is: 1) sample is mixed with buffer (R1) and antiserum solution (R2), containing polyclonal goat anti-human IgG, IgM or IgA antibodies, to yield insoluble aggregates; 2) immune complexes formed in solution scatter light in proportion to their size, shape, and concentration; 3) turbidimeter measure the reduction of incidence light due to reflection, absorption or scatter, which is proportional to Ig concentration in the sample.

The methods were calibrated with their own species specific calibrator. Canine calibrator (Bethyl dog reference serum, Catalog Number RS10-105, Bethyl Laboratories, Inc.) presented 31000 mg/L of IgG, 2500 mg/L of IgM, and 1500 mg/L of IgA. Feline calibrator (cat reference serum, Catalog Number RS10-106, Bethyl Laboratories, Inc.) presented 5400 mg/L of IgG, 550 mg/L of IgM, and 220 mg/L of IgA. The manufacturers indicate that Igs classes of the calibrators were quantitated by RID and ELISA using highly purified normal dog/cat immunoglobulins for reference. Five dilutions of each calibrator were prepared (Table 1) and a five-point non-linear (polygonal) calibration curve (option available from the Olympus AU600 software) was constructed from measurement of these five dilutions of calibrators by plotting absorbance values versus Igs concentrations of the 5 calibrators, and concentrations of unknown samples are then determined using this standard curve. Feline calibrator had a very low concentration of IgA (220 mg/L), so neither calibration nor programming could be done for this immunoglobulin measurement in cats. Programming data for canine and feline Igs are presented in Table 2.

Cross-reactivity of antigen

Ouchterlony procedure¹⁵ was performed in a 0.7% agarose gel. Antiserum solution (R2) with polyclonal goat anti-human IgG, IgM or IgA antibodies from the assay kits were placed in the centre wells. Human serum was used as a positive control, and canine and feline serum samples from healthy and diseased animals were placed in adjacent wells. Samples were used without any previous dilution.

Analytical performance

The following parameters were calculated for analytical performance of methods:

➤ Accuracy was evaluated by spiking recovery and linearity under dilution studies. To evaluate the ability of the assays to recover the amount of analyte added to baseline serum samples, a spiking recovery test was performed following a previously described procedure.¹⁶ A recovery between 80% and 120% will be considered as acceptable as previously described.¹⁷ For this purpose, two commercially available reference serum with known amounts of the different Igs, for dogs (Bethyl dog reference serum) and cats (Bethyl cat reference serum) were used. For each species and Ig, the reference serum was mixed at five different concentrations with five aliquots of a serum pool of known Ig concentration (1:1) and Igs were measured with the methods evaluated in present study. Test recovery (in percent) was calculated for each dilution for comparison of expected versus measured Igs. To assess linearity under dilution, 2 canine/feline serum samples with different IgG, IgM or IgA concentrations were serially diluted with distilled water. The linearity test will be considered as appropriate if correlation coefficient not differ significantly from 1, the confidence intervals of slope include 1, confidence interval of intercept include 0, and Runs-test show not significant deviation from linearity.¹⁷

➤ Precision: 1) Intra-assay coefficient of variation (CV) was calculated after analysis of 2 serum pools with high and low IgG, IgM or IgA concentrations, ten times in a single assay run. 2) Inter-assay coefficient of variation was determined by analysing the same serum pools in ten separate runs carried out on different days. The precision of the assay will be considered as acceptable with the CV <15% as reported elsewhere.¹⁶

All pools used for repetitive analysis were frozen in aliquots and in order to avoid possible changes due to repetitive thawing and freezing only vials needed for each run were thawed at room temperature immediately before analysis.

➤ Lower and upper limits of quantification (LLOQ and ULOQ) were calculated as the maximum and minimum amount of analyte that can be measured with an intra-assay variation under 15%.¹⁸ To that end, different dilutions of three canine and three feline serum pools were prepared and each dilution was analysed five times. Then % CV for each dilution was calculated and plotted against its concentration.

➤ Effects of haemolysis, lipaemia, and bilirubinaemia. Two canine and two feline serum pools were mixed with different concentrations of haemoglobin, lipid (Lipofundina 20%, Braun Medical SA, Barcelona, Spain), or bilirubin (Bilirubin, Sigma Co., St. Luis, MO, USA) solution following previously described procedures.¹⁹ The clinically relevant interferences will be considered when ANOVA test show significant difference in concentrations of measured analytes in aliquots with different concentrations of haemoglobin, lipids, or bilirubin.

Overlap performance

Samples from 50 client-owned dogs and 20 client-owned cats were included in the study. Dogs were divided into three groups: group D1 was formed of 14 healthy dogs (female/male, 9/5; body weight range, 5 to 20.1 kg; age range, 0.5 to 12.0 years; different breeds); group D2 was formed of 21 dogs with Leishmania (female/male, 9/12; body weight range, 3.0 to 48.5 kg; age range, 1 to 10

years; different breeds); and group D3 was formed of 15 female dogs with pyometra (body weight range, 5.3 to 48.5 kg; age range, 4.5 to 12.5 years; different breeds).

Similarly all cats were divided into 2 groups: group C1 was formed of 10 healthy cats (female/male, 6/4; body weight range, 2.3 to 4.5 kg; age range, 0.5 to 8.5 years; different breeds), and group C2 was formed of 10 cats with FIV (female/male, 3/7; body weight range, 2.0 to 3.7 kg; age range, 1 to 10 years; different breeds).

The dogs with leishmaniasis had clinical signs consistent with the disease and the diagnosis was confirmed by positive IFI titres against the leishmania infection and visualization of *Leishmania* spp. amastigotes in bone marrow samples. In case of pyometra, case history and physical examination data were registered and the diagnosis pyometra confirmed by identification of a pus-filled enlarged uterus during ovariohysterectomy. Cats with FIV presented symptoms consistent with the disease that was confirmed by a PCR test.

Statistical methods

Arithmetic means, medians, intra-assay and inter-assay CVs were calculated using routine descriptive statistical procedures and software (GrafPad Prism 5.00, GraphPad Software). Runs-test was performed to determine whether data deviated significantly from the linearity. Interferograms were prepared to show the differences in immunoglobulin concentration when haemoglobin, triglycerides, or bilirubin were added. On the graphs, X-axes show increasing concentrations of haemoglobin, triglycerides, or bilirubin while Y-axes show percentage change in IgG, IgM, or IgA $[(V_f/V_o)*100]$. The influence of haemoglobin, triglycerides, or bilirubin was investigated using repeated measures ANOVA and Dunnett's post test. Comparison of IgG, IgM and IgA concentration between different groups of animals was determined by use of One Way ANOVA with Newman-Keuls Multiple Comparison Test, since Gaussian distribution of the data was confirmed by D'Agostino & Pearson

omnibus normality test. Ordinary linear regression analysis comparing measured and expected concentrations of IgG, IgM and IgA was used to evaluate the linearity under dilution. The significance level used in each case was $p < 0.05$.

Results

Cross-reactivity of antigens

Ouchterlony procedure indicated that goat anti-human IgG and IgM antibodies used in the TIA were able to precipitate with canine and feline serum samples (Fig. 1). Canine Igs showed a reaction of partial identity with human Igs, as canine serum precipitation line fused completely with human serum precipitation line, whereas the latter (human) extended a spur beyond the site of coalescence with canine serum. Similarly, feline Igs showed a reaction of partial identity with human Igs, as feline serum precipitation line fused completely with human serum precipitation line, whereas the latter (human) extended a spur beyond the site of coalescence with feline serum. Bands that were generated from diseased animals were more intense than those from healthy animals. IgA antibodies formed only weak bands of precipitation with canine serum.

IgG, IgM, and IgA assays characteristics

Dogs. The recovery between observed and expected Igs concentrations ranged from 104 to 121% for IgG, from 102 to 110% for IgM, and from 75 to 118% for IgA (Table 3). Dilution of canine serum pools with different Igs concentrations resulted in linear regression equations (Table 4) in which correlation coefficients did not differ from 1, confidence intervals of the slope and intercept included 1 and 0, respectively and Runs test revealed no deviation from linearity ($P > 0.1$).

Intra- and inter-assay CVs were below 1 % and 4 % respectively for IgG, below 2 % and 15 % respectively for IgM, and below 5 % and 15 % respectively for IgA (Table 5). LLOQ for IgG, IgM, and IgA were 353 mg/L, 35 mg/L, and 206 mg/L, respectively (Fig. 2).

Cats. The recovery between observed and expected Igs concentrations ranged from 98 to 118% for IgG and from 98 to 121% for IgM (Table 3). Dilution of feline serum pools with different immunoglobulins concentrations resulted in linear regression equations (Table 4) in which correlation coefficients did not differ from 1, confidence intervals of the slope and intercept included 1 and 0, respectively, and Runs test revealed no deviation from linearity ($P>0.1$).

Intra- and inter-assay CVs were below 4% and 6 %, respectively for IgG (Table 5). Intra- and inter-assay CVs were below 5 % and 10 %, respectively for IgM (Table 5). LLOQ for IgG and IgM were 380 and 105 mg/L, respectively (Fig. 3).

Upper limits of quantification of all Igs in dog and cat serum samples were not possible to determine because of high precision of the methods. The authors observed that even when the Ig value was outside the calibration curve the CV was lower than 15%.

Effects of lipaemia, haemolysis, and bilirubinaemia

Interferograms showing effects of haemolysis, lipaemia, and bilirubinaemia on IgG, IgM, and IgA concentrations in canine and feline serum pools are presented in Figs. 4 and 5.

Dogs. A statistically significant decrease in IgM levels appeared in samples with triglyceride concentrations of 0.625 g/L ($p<0.05$), 1.25 g/L ($p<0.01$), 2.5 g/L ($p<0.01$), and 5 g/L ($p<0.001$), and

with haemoglobin concentrations of 8 g/L ($p<0.05$). A statistically significant increase in IgA levels appeared in samples with triglycerides at a concentration of 2.5 g/L ($p<0.05$), and 5 g/L ($p<0.01$).

Cats. A statistically significant increase in IgG levels appeared in serum pools with triglyceride concentrations of 5 g/L ($p<0.05$). IgM levels were decreased in samples with concentrations of triglyceride of 5 g/L ($p<0.01$) and bilirubin of 0.15 g/L ($p<0.05$).

Overlap performance of the assays

Immunoglobulin G, M, and A levels in different groups of dogs are presented in Fig. 6. Three of 14 serum samples collected from control dogs gave IgA result below LLOQ (206 mg/L) (for further statistical analysis IgA concentrations of these samples were calculated as 206 mg/L). A statistically significant increase in IgG ($p<0.001$), IgM ($p<0.001$), and IgA ($p<0.01$) concentrations was detected in dogs with Leishmania when compared with healthy control dogs. When immunoglobulin concentrations were compared in dogs with pyometra vs. control dogs, a statistically significant increase in IgM and IgA was detected ($p<0.05$ in both cases). Higher IgG levels were recorded in dogs with Leishmania vs. dogs with pyometra ($p<0.001$).

IgG and IgM levels in different groups of cats are presented in Fig. 7. IgG and IgM levels were significantly higher in cats with FIV when compared with healthy ones ($p<0.001$ and $p<0.05$, respectively).

Discussion

Changes in immunoglobulin levels in dogs and cats are associated with different pathologies such as infection or immunodeficiency and could be used as an aid for the diagnosis and monitoring of treatment of immune diseases.^{4,20} However, immunoglobulin determination in routine practice in

companion animals is quite unusual and one of the reasons could be the absence of precise and fast methods for their measurements. The present study demonstrates the utility of the automated human TIA for the measurement of immunoglobulins G, M and A in dog and IgG and IgM in feline sera. The advantages of the methods evaluated in present study over SRID or ELISA include automation, a shorter turnaround time (<1 hour after submission to the laboratory), and elimination of human error in measurement of the precipitin ring diameter or pipetting.¹³

Although the assays used in this study were initially designed for use in humans, the Ouchterlony procedure showed a significant precipitation in wells that contained human, canine, and feline serums with IgG and IgM antibodies. Igs of different species gave the reaction of partial identity indicating that the antibodies used may recognize fewer epitopes of canine and feline Igs in comparison with epitopes recognized in human Igs, resulting in lower signal. An evident although weak precipitation was observed between IgA antibodies and serum of diseased dog; this weak precipitation could be the reason of the reduced ability of the assay to recognize low values of IgA. However, the precipitations were more intense when serum samples from diseased animals with higher Igs concentrations were used. Additionally, the spiking recovery and linearity under dilution tests indicated that the assays could also detect and accurately measure the different canine and feline Igs. In order to assure that measurements are in linear ranges, it would be recommend to dilute the sample when Igs levels exceed the upper limit of calibration curve in a first assay (authors did not observe prozone effect in samples with very high concentrations of Igs). Ideally for the accuracy study, a comparison with a “gold standard” assay should have been done. However, the only commercially available species-specific assay that has been validated is an ELISA that showed imprecision that could reach up to 25%¹¹ and should not be considered as a “gold standard” for the assay. Therefore we evaluated accuracy by spiking recovery and linearity under dilution as recommended in cases in which a reference method is not available.¹⁷

Use of species-specific standards is highly recommended to achieve similar affinity of antiserum against standards and samples.²¹ For this reason the human calibrators supplied by manufacturers were replaced by canine/feline specific ones.

The methods showed good intra and inter-assay repeatability with CVs lower than 15%¹⁶ and were similar to those reported by manufacturers for human serum samples. Higher intra- and inter-assay CVs (up to 25%) were reported for canine and feline ELISA for Igs determination.^{11,12} The lower CVs observed in present study could be due to the automation of the methods that decrease pipetting imprecision.

The lower limits of quantification for immunoglobulin G, and M were slightly higher for cats than dogs. However, LLOQs of canine and feline IgG and IgM observed in present study were low enough to be used for measuring Igs in healthy animals. Also these LLOQs were low enough to detect the values reported till now in dogs with immunodeficiencies (IgG, 1200 mg/L - 14000 mg/L; IgM, 210 mg/L - 3500 mg/L).^{4,22,23} The LLOQ for canine IgA determination was 206 mg/dL, being the main limitation of this method as some healthy animals in present study showed lower IgA values. This must be taken in account when analysing sera from healthy dogs or dogs with immunodeficiencies, as IgA values lower than 206 mg/dL were observed in these dogs.²²⁻²⁴

In dogs and cats, haemolysis, lipaemia, and bilirubinaemia can be attributed to different diseases, difficulties at the time of blood sampling, recent meal, or, in contrast, starving.²⁵ Haemolysis and bilirubinaemia significantly only affected IgM in our study, although manufacturers

indicate that haemoglobin and bilirubin produce interferences also in IgG and IgA. Regarding lipaemia, even low concentrations of triglycerides decreased IgM in canine serum samples, reaching up to a 25 % reduction at a concentration of 5 g/L of triglycerides, while a significant decrease in feline IgM was observed only at the highest concentration of triglycerides. The presence of lipaemia can interfere with many clinical chemistry tests by different mechanisms, the most frequent mechanism is due to the scattering of light rays by the lipids (mainly chylomicrons and very low density lipoproteins).²⁶

It should be pointed out that although human methods can be conveniently modified for use in veterinary practice, but, especially when immunological assay are used, as in this case, methods must be validated for each new species (e.g. canine and feline) using a species-specific standards if available,²⁷ and analytical validation must be undertaken with each lot and every time the antibody batch is changed.²⁸

In this study IgG, IgM, and IgA levels in healthy dogs were similar to those reported by different authors (Table 6). IgG ad IgM levels observed in healthy cats in this work were 2 fold lower than those reported by other authors (Table 6). These discrepancies may be explained by several factors. The animals used in the different studies were of different breeds, ages, and sex, factors that have been shown to influence circulating Igs levels.^{20,29,30} Furthermore, divergences in the obtained results could be attributed to the different methods used for immunoglobulin determination.

When assays were applied to serum samples from dogs with different inflammatory diseases, a 2.5 fold significant increase in IgM was observed in dogs with pyometra, whereas dogs with Leishmania showed a significant 5 fold increase in IgG and IgM. IgM is considered by some authors to

be an immunological marker for the early phase of infections as its levels increases relatively rapid, while increase in circulating levels of IgG is generally observed later than this of IgM but lasts long after infection.^{31,32} The results of present work are in line with these studies, as significantly higher IgM, but not IgG, levels were detected in dogs with pyometra, an acute inflammatory disease. However, when the dogs were affected by a chronic disease as Leishmania, statistically significant increases were observed in both Igs, similarly as described in experimental studies.^{33,34}

In the present study cats with FIV showed significantly higher IgG (1.5 fold) and IgM (2 fold) levels than healthy ones. Increases in IgG have been described by other authors in cats with FIV.^{5,35} Although the pathogenesis of the increment of Igs in cats with FIV is not totally clear, it has been postulated that B-cell abnormalities associated with this disease would result in decreased specific antibody responses to many non-FIV antigens but an overall increase in the total levels of serum immunoglobulins. The excess immunoglobulins apparently would reflect the response to FIV itself and not to unrelated antigens, as described in HIV-infected people.³⁶

Overall, measurements of circulating Igs provide an insight into humoral immune response. Although Igs are very sensitive to detect the activation or deficiency of this humoral immune response, they are highly unspecific in provide information of the cause of this activation or deficiency.

In conclusion, the automated assays for Igs determination in dogs and cats that were evaluated in present study, with the exception of IgA measurement in cats, exhibit analytical characteristics allowing their use in the laboratory with an adequate precision, accuracy, and ability to discriminate between different concentrations of Igs that appear in healthy and diseased animals. It is expected

that the TIAs evaluated in this study will increase the analytical options of veterinary laboratories for Igs measurements in dogs and cats, and will contribute to a wider use of these analytes in these species.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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455 passive transfer of immunity to neonatal kittens. J Feline Med Surg 2006;8:184-191.

456 **Table 1. Immunoglobulins G, M, and A programming data for biochemical analyser OLYMPUS AU600.**

Specie	Analyte	Sample vol., µL	Sample Dil. Vol., µL	R1 vol., µL	R1 Dil. Vol., µL	R2 vol., µL	R2 Dil. Vol., µL	Wave-length, nm		Method	Reaction	Point 1, s		Point 2, s	
								Pri.	Sec.			Fst	Lst	Fst	Lst
Dog	IgG	2.0	0	110	190	100	40	600		END	+	0	23	0	10
	IgM	2.5	0	50	100	50	15	340	800	END	+	0	27	0	10
	IgA	4.5	0	110	190	100	40	600	800	END	+	0	27	0	10
Cat	IgG	2.0	0	110	190	100	40	600		END	+	0	23	0	10
	IgM	2.5	0	50	100	50	15	340	800	END	+	0	27	0	10

457 Sample vol., sample volume; Sample Dil. Vol., sample dilution volume; R1 vol., volume of reactant 1 (buffer solution); R1 Dil. Vol., reactant 1 dilution volume;
 458 R2 vol., volume of reactant 2 (antiserum solution); R2 Dil. Vol., reactant 2 dilution volume; Pri., primary wave-length, nm; Sec., secondary wave-length, nm;
 459 END, an end-point assay method (self blank method) that uses reagent blank absorbance as the reference for measurement data at each photometric point;
 460 Reaction (+), positive reaction (OD at first measurement < OD at last measurement); Point 1 and 2, sec, the time in seconds of the first (Fst) and last (Lst)
 461 measurements of the point 1 and point 2.

462 The optical density (OD) value in this assay is calculated by the following expression: OD value = ([OD value at the specific point after dispensing 2nd reagent
 463 (Point1, Lst)] - [OD value at Point 1, Fst]) - ([OD value at the specific point before dispensing 2nd reagent (Point 2, Lst)] - [OD value at Point 2, Fst]).

464 IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A.

Table 2. Immunoglobulins G, M, and A calibration data.

Standard Dil.	Dog			Cat	
	IgG*, mg/L	IgM*, mg/L	IgA*, mg/L	IgG*, mg/L	IgM*, mg/L
1:20	1550	125	-	-	-
1:10	3100	250	150	540	55
3:10	9300	750	450	1620	165
6:10	18600	1500	900	3240	330
8:10	24800	2000	1200	4320	440
-	-	-	1500	5400	550

Standard Dil. – reference serum dilution.

*, the values of immunoglobulins presented in this table were calculated and provided to the instrument for the purpose of calibration.

Table 3. Spiking recovery method. Different amounts of Dog/Cat reference serum were mixed with serum pools in which Igs concentrations were known.

Species	Immunoglobulin	Ig concentration, mg/L		Expected value, mg/L	Observed value, mg/L	Recovery (%)
		Pool	Dog/Cat reference serum			
Dog	IgG	5163	1550	3356.5	3488	104
			3100	4131.5	4307	104
			9300	7231.5	7951	110
			18600	11881.5	13728	116
			24800	14981.5	18174	121
	IgM	3955	125	2040.0	2189	107
			250	2102.5	2235	106
			750	2352.5	2544	108
			1500	2727.5	3001	110
			2000	2977.5	3049	102
	IgA	1418	150	784	589	75
			450	934	913	98
			900	1159	1223	106
			1200	1309	1302	100
			1500	1459	1720	118
Cat	IgG	2784	540	1662	1624	98
			1620	2202	2290	104
			3240	3012	3213	107
			4320	3552	3843	108
			5400	4092	4838	118
	IgM	344	55	199.5	196	98
			165	255	268	105
			330	337	366	109
			440	392	417	106
			550	447	541	121

471 **Table 4. Linear regression analysis data of canine and feline serum pools.**

	Ig	Regression equation			Runs test
		Slope (95% Confidence Intervals)	Intercept (95% Confidence Intervals)	R ²	
Dog	IgG	1.019 (0.974-1.065)	-117.5 (-508.1-273.0)	0.998	NS
		0.992 (0.969-1.016)	159.3 (-40.88-359.5)	0.999	NS
	IgM	1.036 (0.981-1.091)	-43.16 (-93.68-7.34)	0.999	NS
		1.007 (0.976-1.040)	-15.45 (-40.56-9.66)	0.999	NS
	IgA	1.114 (0.876-1.353)	-157.5 (-374.8-59.76)	0.986	NS
		1.049 (0.692-1.406)	-3.34 (-165.1-158.1)	0.966	NS
Cat	IgG	1.031 (0.976-1.087)	-140.4 (-283.6-2.637)	0.999	NS
		1.045 (0.966-1.126)	-163.9 (-367.1-39.18)	0.998	NS
	IgM	1.008 (0.969-1.048)	-4.16 (-13.22-4.88)	0.999	NS
		1.006 (0.866-1.146)	-9.16 (-15.41-29.12)	0.994	NS

472 NS, not significant (P>0.1)

473

474 **Table 5. Intra- and inter-assay variation in determination IgG and IgM concentrations in**
475 **canine and feline serum pools.**

Specie	Analyte	Comparison	Sample			CV (%)
				Mean (mg/L)	SD	
Dog	IgG	Intra-assay	Pool A	19735	150	0.8
			Pool B	8481	56	0.7
		Inter-assay	Pool A	20293	783	3.9
			Pool B	8415	172	2.0
	IgM	Intra-assay	Pool A	1862	17	0.9
			Pool B	932	12	1.2
		Inter-assay	Pool A	1855	97	5.2
			Pool B	836	118	14.1
	IgA	Intra-assay	Pool A	1748	24	1.4
			Pool B	541	26	4.7
		Inter-assay	Pool A	1892	133	7.0
			Pool B	593	85	14.3
Cat	IgG	Intra-assay	Pool A	7153	122	1.7
			Pool B	2080	68	3.3
		Inter-assay	Pool A	7391	309	4.2
			Pool B	2120	115	5.4
	IgM	Intra-assay	Pool A	1039	28	2.7
			Pool B	213	9	4.2
		Inter-assay	Pool A	1028	31	3.0
			Pool B	192	17	9.1

476 CV, coefficient of variation.

477 **Table 6. Comparative data from the literature for IgG, IgM, and IgA concentrations in healthy dog and cat serum.**

Specie	Author	Immunoglobulin, mg/L			Method
		IgG	IgM	IgA	
Dog	Present study	10840±2975	2080±777	899±688	<u>TIA</u>
	Heddle and Rowley ²⁴	9800 (5200-17300)	1700 (700-2700)	500 (200-1200)	RID
	Schreiber et al. ⁷	4350-12290	190-12290	180-7230	ELISA
	German et al. ¹¹	17200	15000	1000	ELISA
	Provost et al. ³⁰	7720-9921	2420-2495	-	RID
	Foale et al. ⁴	10000-20000	1000-2000	400-1600	
	Watson et al. ²²	10000-20000	1000-2000	200-1600	RID
Cat	Present study	4863±1464	486±257		<u>TIA</u>
	Ackley et al. ⁵	15100±6600	900±170	1500±100	RID IgG; ELISA IgA and IgM
	Poli et al. ³⁵	21900±5500	900±200	-	RID
	Casal et al. ³⁷	-	3700-6400	-	RID
	Harley et al. ¹²	19080±5400	2040±900	2600±2200	RID
	Claus et al. ³⁸	15000±5400	-	1900±1400	RID IgG; ELISA IgA

478 TIA, turbidimetric immunoassay; RID, radial immunodiffusion; ELISA, enzyme-linked immunosorbent assay.

Figures

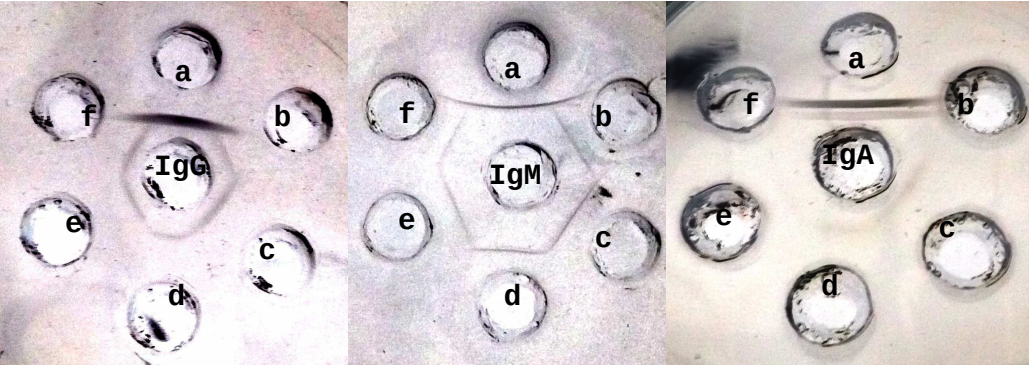
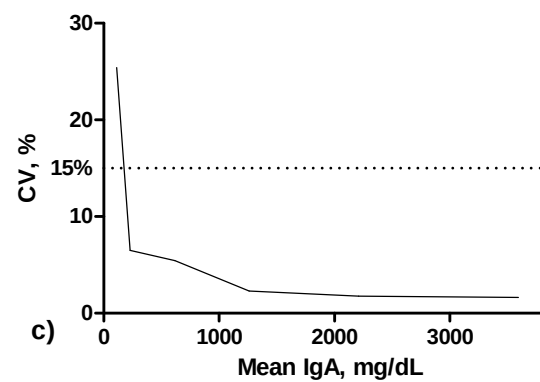
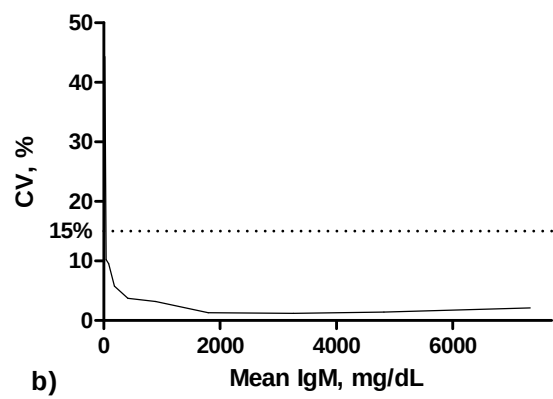
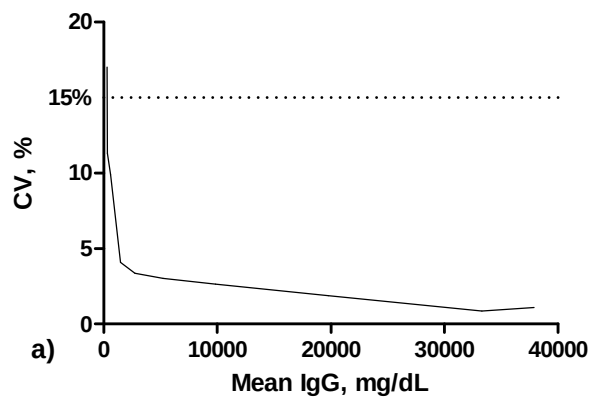


Fig. 1. Ouchterlony procedure for visualisation of cross-reactivity between goat anti-human IgG, IgM, and IgA antibodies and serum of the dog (c, diseased dog; d, healthy dog) and cat (b,e, diseased cat; f, healthy cat). As a control human serum was used (a). IgG, goat antihuman IgG antiserum; IgM, goat antihuman IgM antiserum; IgA, goat antihuman IgA antiserum.



486

487 Fig. 2. Lower limit of quantification for canine serum samples. a - IgG; b – IgM, c - IgA.

488

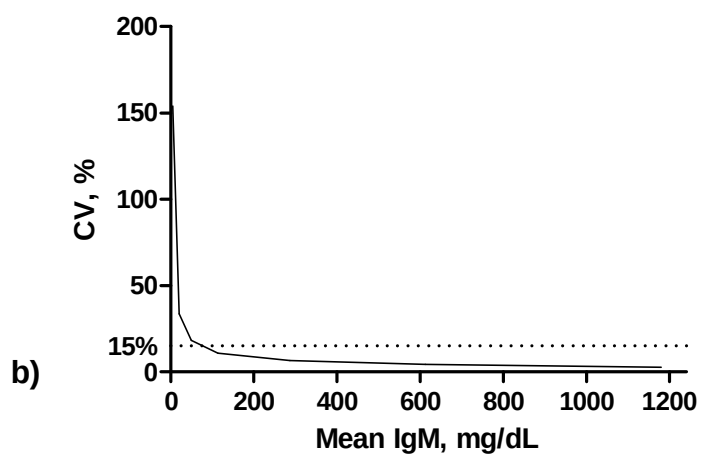
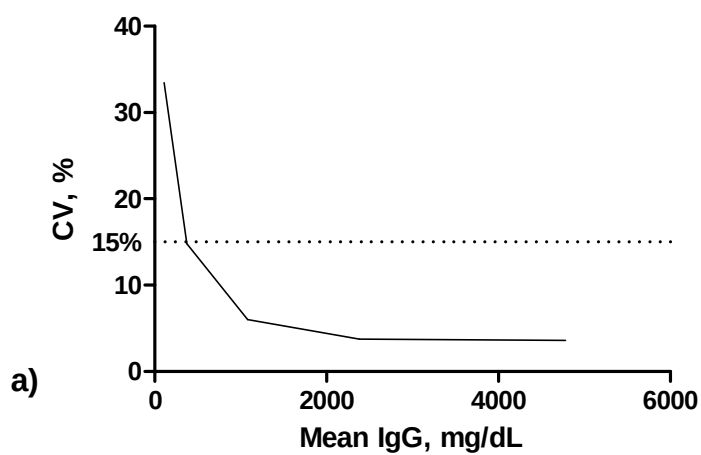


Fig. 3. Lower limit of quantification for feline serum samples. a - IgG; b - IgM.

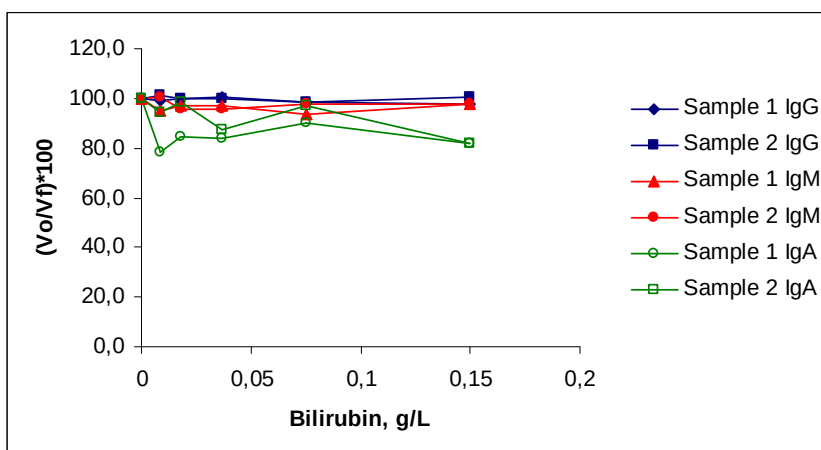
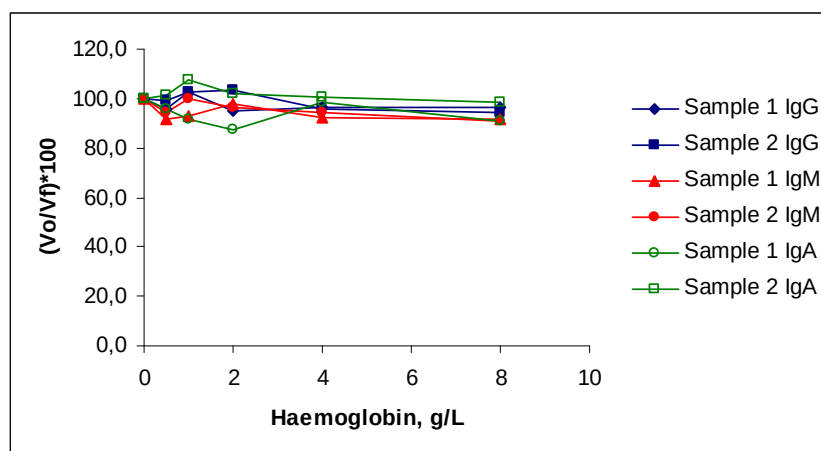
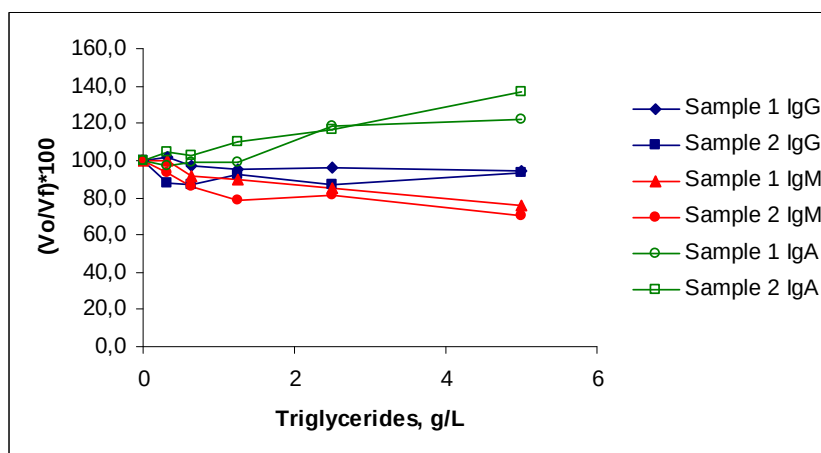


Fig. 4. Interferences of lipids (a), haemoglobin (b), and bilirubin (c) on IgG, IgM, and IgA determination in two canine serum samples. A statistically significant decrease in IgM levels was detected in samples with triglyceride concentrations of 0.625 g/L ($p<0.05$), 1.25 g/L ($p<0.01$), 2.5 g/L ($p<0.01$), and 5 g/L ($p<0.001$), and with haemoglobin concentrations of 8 g/L ($p<0.05$). A statistically significant increase in IgA levels appeared in samples with triglycerides at a concentration of 2.5 g/L ($p<0.05$), and 5 g/L ($p<0.01$).

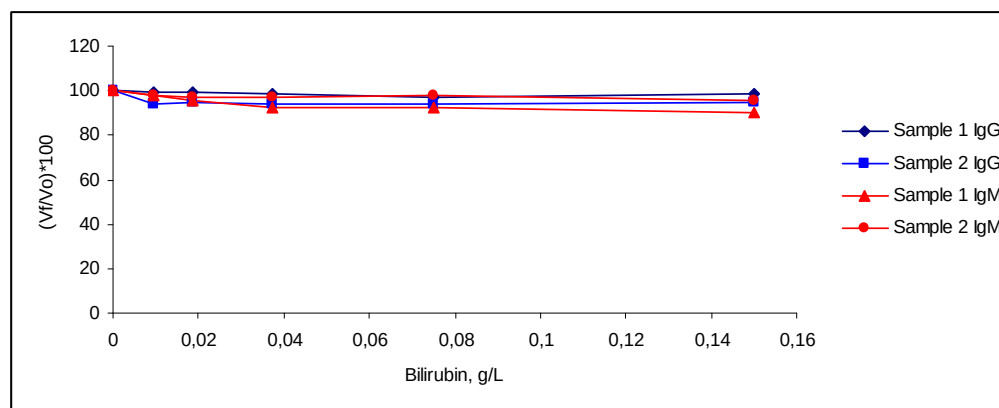
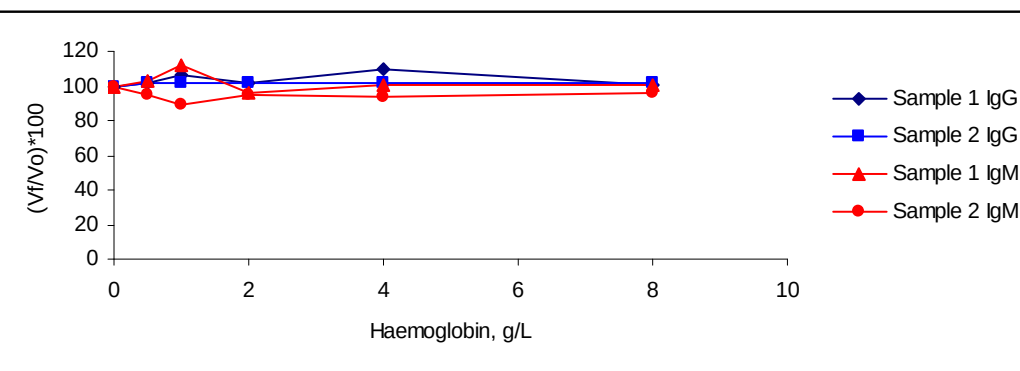
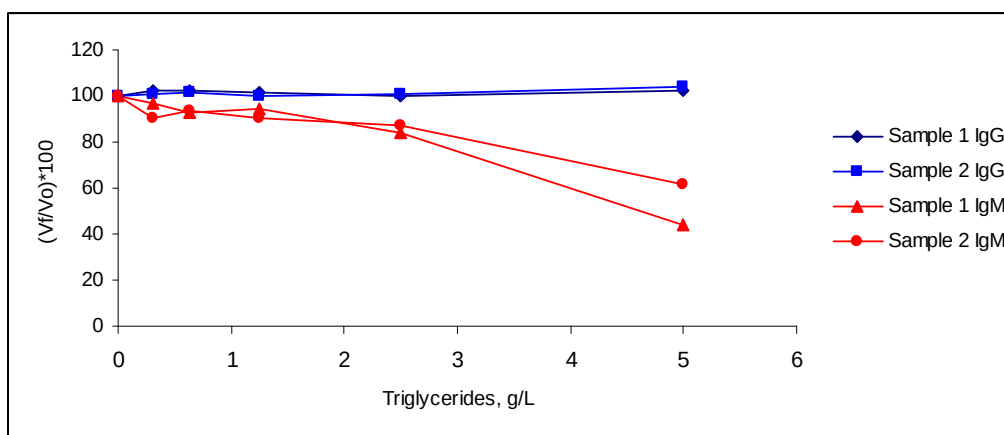


Fig. 5. Interferences of lipids (a), haemoglobin (b), and bilirubin (c) on IgG and IgM determination in two feline serum samples. A statistically significant increase in IgG levels appeared in serum pools with triglyceride concentrations of 5 g/L ($p < 0.05$). IgM levels were decreased in samples with concentrations of triglyceride of 5 g/L ($p < 0.01$) and bilirubin of 0.15 g/L ($p < 0.05$).

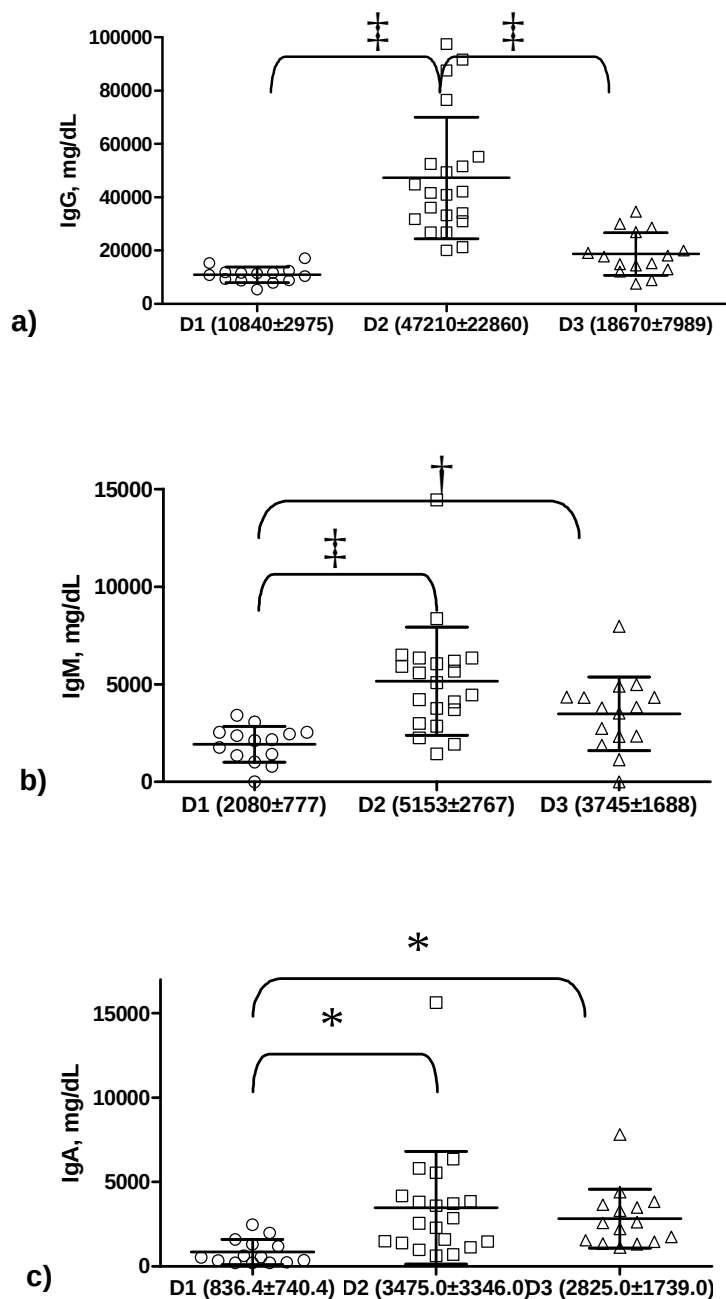
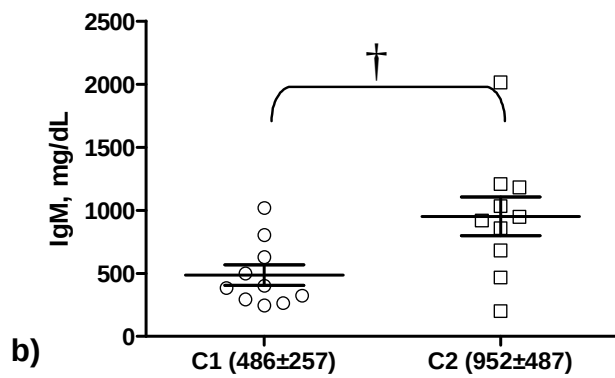
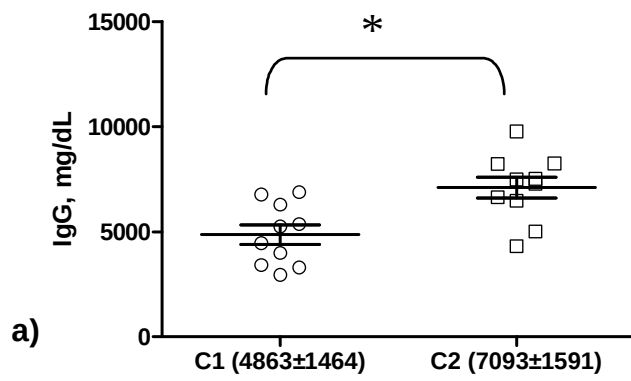


Fig. 6. Immunoglobulin G (a), M (b), and A (c) levels in healthy dogs [D1 (mean±SD)], dogs with Leishmania [D2 (mean±SD)], and pyometra [D3 (mean±SD)]. Horizontal lines represent mean±SD.

*, p<0.05; †, p<0.01; ‡, p<0.001.



516

517 Fig. 7. Immunoglobulin G (a) and M (b) levels in healthy cats [C1 (mean±SD)] and cats with FIV
 518 [C2 (mean±SD)]. Horizontal lines represent mean±SD.

519 *, $p < 0.05$; †, $p < 0.001$.

09-Sep-2012

Dear Dr. Ceron:

I am pleased to inform you that your manuscript entitled "Evaluation of automated assays for immunoglobulin G, M, and A measurements in dog and cat serum" is accepted for publication in Veterinary Clinical Pathology pending editorial review. You may consider your manuscript as "in press". Your manuscript will be edited prior to publication and any additional reviewer comments can be addressed at that time.

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3.4.4. Article 14

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Obesity-related metabolic dysfunction in dogs: a comparison with human metabolic syndrome

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Obesity-related metabolic dysfunction in dogs: a comparison with human metabolic syndrome

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Abstract

Background

Recently, metabolic syndrome (MS) has gained attention in human metabolic medicine given its associations with development of type 2 diabetes mellitus and cardiovascular disease.

Canine obesity is associated with the development of insulin resistance, dyslipidaemia, and mild hypertension, but the authors are not aware of any existing studies examining the existence or prevalence of MS in obese dogs.

Thirty-five obese dogs were assessed before and after weight loss (median percentage loss 29%, range 10-44%). The diagnostic criteria of the International Diabetes Federation were modified in order to define canine obesity-related metabolic dysfunction (ORMD), which included a measure of adiposity (using a 9-point body condition score [BCS]), systolic blood pressure, fasting plasma cholesterol, plasma triglyceride, and fasting plasma glucose. By way of comparison, total body fat mass was measured by dual-energy X-ray absorptiometry, whilst total adiponectin, fasting insulin, and high-sensitivity C-reactive protein (hsCRP) were measured using validated assays.

Results

Systolic blood pressure ($P=0.008$), cholesterol ($P=0.003$), triglyceride ($P=0.018$), and fasting insulin ($P<0.001$) all decreased after weight loss, whilst plasma total adiponectin increased ($P=0.001$). However, hsCRP did not change with weight loss. Prior to weight loss, 7 dogs were defined as having ORMD, and there was no difference in total fat mass between these dogs and those who did not meet the criteria for ORMD. However, plasma adiponectin concentration was less ($P=0.031$), and plasma insulin concentration was greater ($P=0.030$) in ORMD dogs.

Conclusions

In this study, approximately 20% of obese dogs suffer from ORMD, and this is characterized by hypo adiponectinaemia and hyperinsulinaemia. These studies can form the basis of further investigations to determine path genetic mechanisms and the health significance for dogs, in terms of disease associations and outcomes of weight loss.

Keywords

Canine, Insulin resistance, Weight loss, Adiponectin

Background

Recently, metabolic syndrome (MS) has gained attention in human medicine given its associations with development of diabetes mellitus and cardiovascular diseases [1]. Central obesity (determined by waist circumference) is critical to its definition, in conjunction with dyslipidaemia, hypertension and glucose intolerance. This suggests that MS is a complex cluster of metabolic risk factors that together may predispose to development of secondary diseases [1]. In humans, MS doubles the risk of cardiovascular disease whilst the risk of diabetes mellitus is increased five-fold [2]. More recently, proteinuria (or microalbuminuria) has been observed in patients with MS, suggesting altered renal function [3-5].

In veterinary medicine, equine MS is well described [6-8], and known to be a risk factor for laminitis amongst other pathologies [9,10]. Canine obesity is associated with the development of insulin resistance, altered lipid profiles, and mild hypertension, which are

ameliorated by weight loss [11-13]. Furthermore, overweight dogs are more likely to suffer from diabetes mellitus [14], whilst lifelong overfeeding leads to being overweight, metabolic disturbances and decreased lifespan [15]. Although laboratory dogs, rendered obese by overfeeding, have many features that resemble human MS [13], the authors are not aware of any studies examining the existence of MS in obese dogs. Therefore, the objective of the present study was to determine whether canine obesity-related metabolic dysfunction (ORMD, using a definition modified from that used for human MS) could be identified in pet dogs with naturally-occurring obesity, and whether it correlated with specific patient characteristics (e.g. signalment, body fat content), metabolic (e.g. adiponectin, insulin) and inflammatory (e.g. high-sensitivity C-reactive protein; hsCRP) biomarkers. We also determined the effect of weight loss on MS and its consequences.

Methods

Animals

Dogs were referred to the Royal Canin Weight Management Clinic, University of Liverpool UK, for investigation and management of obesity and associated metabolic disorders. Sixty-five dogs were recruited between February 2005 and August 2010, and those successfully losing weight had completed by January 2011. Eligibility criteria included confirmation of obesity (e.g. body fat mass >35%, as measured by dual-energy X-ray absorptiometry; DEXA; [16]), completing a weight loss programme, and having sufficient surplus plasma to enable analyses to be completed.

Ultimately, a group of 35 dogs fulfilled the inclusion criteria. The median age was 72mo (12 to 132mo); twenty dogs were male (19 neutered) and 15 were female (13 neutered). Nine of the dogs were Labrador retrievers, and a range of other breeds were also represented including Akita, Border Collie, Cairn Terrier, CKCS (3), Cocker Spaniel, Corgi, Dachshund, Doberman (2), English Bull Terrier, Golden Retriever, Irish Setter, Lhasa Apso, Miniature Schnauzer, Mixed Breed (3), Pug (3), Samoyed, Schipperke, and Yorkshire Terrier (2). None of the dogs enrolled participated in a previous study examining metabolic effects of obesity [12], but many participated in a separate study examining renal biomarkers [17].

The study protocol adhered to the University of Liverpool Animal Ethics Guidelines, and was approved by both the University of Liverpool Research Ethics Committee and the WALTHAM ethical review committee. Owners of all participating animals gave informed written consent.

Weight loss regimen

Full details of the weight loss regimen have been previously described [12,18]. Briefly, dogs were determined to be systemically well, and without significant abnormalities on complete blood count, serum biochemical analysis and urinalysis. Throughout weight loss, patients were weighed on electronic weigh scales (Soehnle Professional), which were regularly calibrated using test weights (Blake and Boughton Ltd). The degree of adiposity was estimated, clinically, using a 9-integer body condition score (BCS) system [19].

The weight management protocol has been previously described in detail [12,18], and involved using either a high protein high fiber (Satiety Support, Royal Canin; 34 dogs) or a

high protein moderate fiber (Obesity Management, Royal Canin; 1 dog) weight loss diet (Table 1). The initial food allocation for weight loss was determined by first estimating maintenance energy requirement ($\text{MER} = 440 \text{ kJ [105Kcal]} \times \text{body weight [kg]}^{0.75}/\text{day}$; [20] using the estimated target weight. The exact level of restriction for each dog was then individualized based upon gender and other factors (i.e. presence of associated diseases), and was typically between 50-60% of MER at target weight [18]. Owners also implemented lifestyle and activity alterations to assist in weight loss. Dogs were reweighed every 7-21 days and changes made to the dietary plan if necessary, until their target weight was reached [12,18].

Table 1 Composition of diets used for weight loss

<i>Criterion</i>	HPHF diet		HPMF diet	
<i>ME content</i> [*]	2900 Kcal/kg		3275 Kcal/kg	
<i>Moisture</i>	9.0		9.0	
	Per 100 g DM	g/1000 Kcal (ME)	Per 100 g DM	g/1000 Kcal (ME)
<i>Moisture</i>	8	28	9	27
<i>Crude protein</i>	30	103	34	104
<i>Crude fat</i>	10	34	10	31
<i>Crude fibre</i>	17.5	60	11.5	35
<i>Total dietary fibre</i>	28	97	18.5	56
<i>Ash</i>	5.3	18	7.9	24
<i>Fibre sources</i>	Cellulose, beet pulp, FOS, psyllium husk, diet cereals		Cellulose, beet pulp, diet cereals	

HPHF = High protein high fibre diet^d. *HPMF* = High protein medium fibre^e. *ME* = Metabolisable energy content, as measured by animal trials according to the American Association of American Feed Control Officials protocols; *DM* = dry matter; *FOS* = fructo-oligo-saccharides

Body composition analysis

Body composition was analyzed before and after the weight loss regime in all dogs, using fan-beam DEXA (Lunar Prodigy Advance; GE Lunar). Pre-weight loss total body composition results were used to estimate target weight [18,21]. Further, by comparing pre- and post-weight loss DEXA scan results, change in fat and lean mass could be estimated [12,18].

Definition of metabolic syndrome

The guidelines of the International Diabetes Federation [22] were modified in order to produce an accessible system for dogs. Accordingly, we defined ORMD, using the either the upper limit of the reference range for the laboratory used (e.g. cholesterol, triglyceride, and glucose), or based upon internationally accepted criteria defining borderline results (e.g. SBP) [23]. Therefore, the final definition was as follows:

- a) BCS 7-9/9

AND any two of the following:

1. Triglycerides >200 mg/dL, (2.3 mmol/L).
2. Total cholesterol > 300 mg/dL (7.8 mmol/L).
- b) 3. Systolic blood pressure >160 mmHg.
4. Fasting plasma glucose >100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes mellitus.

The prevalence of metabolic syndrome before and after weight loss was assessed based upon these criteria.

Indirect blood pressure measurement

Blood pressure was measured non-invasively using an oscillometric method (Cordell Veterinary Monitor 9401BP; Paragon Medical). All dogs were fully conscious, and either in a sitting position or in dorsal recumbence. A cuff, of appropriate size (~40% width of the leg) was used, and placed on the right forelimb. Once the dog was calm and not moving, at least five readings were taken and averaged to produce a result for systolic blood pressure (SBP).

Clinical sampling and biochemical parameters

Blood samples were collected by jugular venepuncture prior to and after the weight loss period. All blood samples were taken after a fast of at least 16 h. Plasma cholesterol, triglycerides, and fasting plasma glucose concentrations were performed in an automated clinical chemistry analyzer (Olympus AU2700, Olympus Diagnostic GmbH) with intra and inter-assay CVs <2% for all the analyses.

Plasma adiponectin and insulin concentrations were determined using ELISA kits (Human Adiponectin ELISA, High Sensitivity Kit, BioVendor-Labartorni Medicine for adiponectin; and Insulin, Canine ELISA, Mercodia AB, Uppsala, Sweden for insulin). Intra and inter-assay coefficient of variations (CVs) were <11% and <8% for adiponectin and insulin, respectively [24,25]. High sensitivity CRP (hs-CRP) was measured with a time-resolved immuno fluorometric assay with intra and inter-assay CVs of <14% [26].

Assessment of the consequences of ORMD in dogs

In order to assess the significance of ORMD, comparisons were made between groups of dogs defined as having and not having metabolic syndrome. The parameters assessed included, signalment data, starting body weight, pre-weight loss body fat percentage, pre-weight loss plasma metabolic biomarkers (e.g. insulin, CRP, and adiponectin), rate of weight loss, energy intake required for weight loss, and change in lean tissue mass during weight loss.

Statistical analysis

Data are expressed as median (range) except where indicated. Statistical analyses were performed with computer software (Stats Direct version 2.6.8; Stats Direct Ltd.), with the level of significance set at $P < 0.05$ for two-sided analyses. The Shapiro-Walk test was first used to assess whether or not data were normally distributed and, given that the majority of

datasets were not normally distributed, non-parametric tests were chosen for all analyses. Differences in the concentrations of the various biomarkers, prior to and after weight loss, were assessed with the Wilcoxon signed rank sums test, whilst differences in the number of dogs affected by ORMD pre- and post-weight loss were compared with an exact test for matched pairs [27]. Comparisons between groups of dogs with and without ORMD were made with the Mann–Whitney test. Finally, a possible association between pre-weight loss adiponectin and insulin concentrations was tested with Kendall’s rank correlation.

Results

Baseline characteristics and outcomes of weight loss

Median body weight prior to weight loss was 32.9 kg (5.4 to 77.0 kg), and decreased to 25.6 kg (4.4 to 51.4 kg) after weight loss. Median BCS decreased from 8 (7-9) prior to weight loss to 5 (4 to 6) after weight loss. Weight loss took a median of 259 days (91 to 674 days), and a median weekly rate of 0.8%/week (0.2 to 1.4%). As a result, median percentage weight loss was 29% (9% to 44%). The choice of weight loss diet had no effect on the results obtained (data not shown).

Changes in body composition and plasma metabolic biomarkers with weight loss

Median total body fat percentage decreased significantly upon weight loss (median change - 53%, range -16 to -78%, $P < 0.001$). Although some dogs gained lean tissue mass during the process, most lost lean tissue, and the median difference was estimated to be -7% (range - 21% to 9%, $P < 0.001$).

BCS ($P < 0.001$), SBP ($P = 0.008$), plasma cholesterol concentration ($P = 0.003$), plasma triglyceride concentration ($P = 0.018$), plasma insulin concentration ($P < 0.001$), and UPCr ($P = 0.034$) all decreased after weight loss (Table 2), whilst plasma adiponectin concentration increased ($P = 0.001$). Plasma hs-CRP ($P = 0.822$) and plasma glucose ($P = 0.166$) concentrations did not change with weight loss.

Table 2 Pre- and post-weight-loss metabolic biomarkers in the study dogs

Biomarker	Pre-weight loss	Post-weight loss	P^1
BCS ¹	8 (7-9)	5 (4-6)	<0.001
SBP ² (mmHg)	155 (108-220)	130 (105-180)	0.008
Cholesterol (mmol/L)	5.6 (2.5-9.3)	5.0 (1.9-7.7)	0.003
Triglycerides (mmol/L)	1.2 (0.6-5.3)	0.9 (0.1-2.1)	0.018
Glucose (mmol/L)	5.4 (3.5-8.7)	5.2 (3.0-7.4)	0.166
Insulin (pmol/L)	256 (36-687)	135 (24-626)	<0.001
Adiponectin (µg/mL)	7.8 (0.8-19.5)	8.0 (1.1-34.9)	0.001
hsCRP (nmol/L)	9.1 (0.1-225.5)	9.1 (0.0-193.6)	0.822

Results are expressed as median (range). ¹ Body condition score assessed with a 9-integer scale [19]. ² Systolic blood pressure, measured indirectly by an oscillometric technique. ³ Urine protein:creatinine ratio. For UPCr, due to lack of availability of urine samples, was measured on 30/35 dogs. All results are expressed as mean, median (range) & number above

upper limit; the exception is except for body condition score (BCS), where the mean values are not reported as the data were categorical. hsCRP: high-sensitivity C-reactive protein

Identification of dogs with ORMD

When different ORMD criteria were studied individually, all dogs had a BCS 7-9/9 prior to weight loss, whereas the BCS range after weight loss was 4-6/9 (Table 3). Although, after weight loss, the BCS of 6 dogs was 6/9 (i.e. above the optimal range for healthy dogs of BCS 4-5/9, [15], there was no association with persistence of abnormal metabolic parameters (data not shown). Prior to weight loss, occasional results, above respective upper reference limits, were noted for cholesterol (4/35) and triglycerides (3/35), but these were no longer evident after weight loss. In contrast, increases in SBP (10/35) and plasma glucose (11/35) were more common prior to weight loss, and many remained above the upper limit after weight loss (SBP 8/35 and glucose 7/35) (Table 3). When BCS and 4 parameters (i.e. triglycerides, cholesterol, SBP, and glucose) were used in the definition of ORMD, 7/35 fulfilled the criteria before weight loss (Table 3).

Table 3 The number of animals with BCS, SBP, cholesterol, triglyceride, and glucose above the upper reference limit pre- and post-weight-loss

Biomarker	Upper limit ¹	Pre-weight loss	Post-weight loss
Body condition score ²	>6/9	35	0
Cholesterol	>7.8 mmol/L	4	0
Triglycerides	>0.23 mmol/L	3	0
SBP ³	>160 mmHg	10	8
Glucose	>5.5 mmol/L	11	8

¹ Upper limit: the upper reference range limit used for the definition of metabolic syndrome. ² Body condition score assessed with a 9-integer scale [19]. ³ Systolic blood pressure, measured indirectly by an oscillometric technique. All results are expressed as mean, median (range) & number above upper limit; the exception is except for body condition score (BCS), where the mean values are not reported as the data were categorical

Comparison of obese dogs with and without ORMD pre-weight loss

To determine factors associated with ORMD baseline data, pre-weight loss body composition analysis results, weight loss outcomes and plasma metabolic biomarker concentrations were compared between dogs that either did or did not fit the definition. Plasma adiponectin concentration was less in dogs with metabolic syndrome ($P=0.031$), whilst plasma insulin concentration was greater ($P=0.030$; Table 4), and they were negatively correlated with one another (Kendall's tau -0.29, $P=0.016$). However, none of the other parameters, including assessment of total and regional body fat or weight loss rate differed between groups ($P>0.1$ for all).

Table 4 Comparison pre-weight loss parameters in obese dogs with and without metabolic syndrome

Biomarker	Metabolic syndrome	No metabolic syndrome	<i>P</i>
Age mo	98 (31 to 132)	66 (12 to 132)	0.335
Sex ¹	5 NM, 2 NF	1 M, 14 NM, 2 F, 11 NF	0.835

Breed²	Dachshund, Doberman, English Bull Terrier, Irish Setter, Labrador, Lhasa Apso, Mixed Breed	Akita, Border Collie, Cairn Terrier, CKCS (3), Cocker Spaniel, Corgi, Doberman, Golden Retriever, Labrador (8), Miniature Schnauzer, Mixed Breed (2), Pug (3), Samoyed, Schipperke, Yorkshire Terrier (2)	0.321
Start weight kg	42.8 (8.5 to 56.3)	28.6 (5.4 to 77.0)	0.479
Total BF³ %	47 (37 to 51)	44 (30 to 55)	0.424
Weight loss %	28 (16 to 44)	28 (9 to 38)	0.531
Weight loss rate %/wk	0.7 (0.2 to 1.4)	0.8 (0.3 to 1.4)	0.642
EI during weight loss⁴	62 (53 to 74)	60 (44 to 74)	0.672
Change in lean mass %	-7 (-21 to -2)	-7 (-20 to 10)	0.537
Adiponectin µg/mL	4.1 (2.4 to 8.1)	8.4 (0.8 to 19.5)	0.031
Insulin pmol/L	428 (125 to 686)	222 (36 to 524)	
µIU/mL	62 (18 to 99)	32 (5 to 75)	0.030
hs-CRP nmol/L	7.0 (0.1 to 56.6)	11.1 (4.0 to 214.8)	0.719

¹ For sex, *F*: female, *NF*: neutered female, *NM*: neutered male. Upper limit: the upper reference range limit used for the definition of metabolic syndrome. ² For Breed, CKCS: Cavalier King Charles spaniel. ³ Total body fat measured by dual-energy X-ray absorptiometry. ⁴ Energy intake for weight loss is the median value for the whole of weight loss, expressed as energy (in Kcal) per kg metabolic body weight ($\text{kg}^{0.75}$)/day. All results are expressed as median (range)

Discussion

Human MS is now well recognized and predisposes to cardiovascular diseases and type 2 diabetes mellitus [1]. In equine MS, obesity, regional adiposity, insulin resistance, hypertriglyceridaemia and hyperleptinaemia are part of the definition, and it is a risk factor for laminitis, altered reproductive function, and seasonal alterations in arterial blood pressure [6,9]. In the current study, we classified pet dogs with naturally-occurring obesity on the basis of presence of ORMD (using a modification of criteria used for human metabolic syndrome). We then examined the effect of weight loss on these metabolic criteria, and sought to identify factors that were potentially associated with the syndrome. The criteria for ORMD were met in approximately 20% of obese patients pre-weight loss. The presence of ORMD was not associated with total fat mass, as measured by DEXA, but was associated with increased and decreased plasma insulin and adiponectin concentrations, respectively. These findings suggest that defining obese dogs on the basis of their metabolic status may have some merit, although further work is now required to determine the true significance of ORMD in terms of disease risk and outcome. Weight loss was associated with decreased body fat mass, BCS, SBP, circulating lipid concentrations, and plasma insulin concentration, whilst circulating adiponectin increased. These findings are similar to those reported in many

studies of canine obesity [11-13,28], and suggest that the study population was representative. Nonetheless, the study was small and extending the work further studies would now be desirable.

We chose to base our ORMD definition on the guidelines of the International Diabetes Federation [22], although other guidelines are available including those of the World Health Organization [29], the European Group for the Study of Insulin Resistance [30], the National Cholesterol Education Program [31], and a combined statement from the American Heart Association and National Heart, Lung, and Blood Institute [32]. The main reason for this was that the criteria used could readily be adapted to produce a practical method in dogs. For each criterion used, and in a similar manner to humans, either the upper limit of the respective laboratory reference ranges (e.g. cholesterol, triglyceride, and glucose), or internationally accepted criteria, above which the parameter is borderline (e.g. SBP) [23] was chosen as the cut-off. Whilst many of the criteria were identical to those used in the human system, others were substituted for similar parameters thought to be more relevant for dogs. The main advantages of such an approach were that all chosen parameters are already in widespread clinical use and techniques for measurement are better validated, thereby making the whole system more accessible for practicing veterinarians. For example, we replaced waist circumference (a human measure of central obesity), with BCS, since the significance of central obesity has not been studied in dogs. Given differences in canine anatomy, the human measures of central adiposity are not likely to be appropriate for dogs, and developing a clinical measure of abdominal obesity in dogs (e.g. waist circumference) would be challenging given the wide variability in size and shape amongst breeds. In contrast, differences in BCS are associated with both disease risk and decreased longevity [14,15]. Nonetheless, it may be necessary to modify these criteria in the future, if a practical method of measuring central obesity can be validated.

In a similar manner, total plasma cholesterol concentration was used in place of HDL-cholesterol. The main reason for this modification was the fact that lipid profiles differ between dogs and humans, with humans demonstrating an 'LDL pattern', whilst HDL is the dominant cholesterol type in dogs [33]. Further, alterations in lipoprotein profiles differ in human and canine obesity: obese humans display increased LDL-cholesterol and decreased HDL-cholesterol [1], whereas the circulating concentrations of both LDL and HDL-cholesterol are increased in obese dogs [11,34]. Thus, the use of total cholesterol, rather than cholesterol fractions, is more logical for the latter species.

Based upon definitions of 4 (i.e. SBP, cholesterol, triglyceride and glucose) parameters in addition to BCS, approximately 20% of the obese dogs of the study were classified with ORMD, respectively. This suggests that the prevalence of MS in obese dogs is somewhat less than for humans where prevalence is typically 22-28% and 50-60% in overweight and obese patients, respectively [35]. The reasons for such a difference are not known, and further elucidation of the underlying mechanisms of ORMD is recommended for comparative purposes.

In order to determine the significance of ORMD, we chose to assess a variety of other parameters including fasting insulin concentration, plasma adiponectin concentration, CRP and adiposity, as determined by DEXA. When the obese dogs of the present study were subdivided on presence or absence of ORMD prior to weight loss, only plasma adiponectin and insulin concentrations differed. Adiponectin was approximately twofold less, and insulin approximately twofold greater, in the ORMD group, and both were negatively correlated with

one another. This finding is similar to that described in the human literature on MS [36]. In man, one of the major obesity and MS outcomes is insulin resistance and type 2 diabetes mellitus [1], and the risk of this condition is increased fivefold when MS is present [2]. The exact pathogenetic link between obesity and insulin resistance has not yet been fully elucidated [37]. However, adiponectin is known to have an insulin-sensitizing effect, acting through the AMP-activated protein kinase [38], so that the association with MS may (at least in part) be explained by the decreased adiponectin concentration that accompanies obesity. Adiponectin may also have anti-inflammatory effects, such that decreased adiponectin concentrations are associated with the increased risk of inflammation [39]. Thus, hypoadiponectinaemia observed in human MS may be responsible for development of secondary diseases due to increased susceptibility to inflammation and insulin resistance. Interestingly, no difference was noted in hsCRP, either when comparisons were made before and after weight loss, or when obese dogs were categorized as either having or not having concurrent ORMD. This may suggest that associations amongst adiponectin, MS and obesity are independent of the function of this particular acute phase protein. Further, these findings are different from some previous studies examining CRP concentrations in obese dogs, where increased [12,40] or decreased [13] concentrations have been seen. The reasons for such differences are not entirely clear, but may have resulted from differences in the test populations and assay used. Most notably, our work utilized a high-sensitivity assay recently validated for dogs [26], and such assays are thought to be more reliable in humans [41]. The findings regarding adiponectin concentrations in the current study are different from some [12,42] but not other [43] studies. Again, the reasons for this are not clear but similar explanations would be feasible, namely that this related to population differences or assay type. In the present study, we used a high sensitivity human adiponectin ELISA assay previously validated for use in dogs [34]. In this assay, human calibrators were substituted for species-specific standards, in order to achieve similar affinity of antiserum against standards; this ensures that better differentiation between samples with greater and lesser adiponectin concentrations [34]. Nonetheless, high and low molecular weight adiponectin species were not measured in the current study, and these may differ in importance in development of obesity-associated consequences [44]. Thus, the true significance of these findings requires further study.

The presence of increased plasma insulin concentration, and decreased plasma adiponectin concentration implies physiological consequences to ORMD. However, in order to determine its true significance, further investigations would be needed examining other biomarkers and also clinical consequences. For example human MS is associated with dysregulated fatty acid metabolism [45], cardiac and vascular functional derangements [46], and hepatic manifestations such as non-alcoholic fatty liver disease [47]. Therefore, future studies could assess alterations in a variety of biomarkers in a prospective population of dogs. Epidemiological studies could also be considered, as a means of determining potential disease associations and, ultimately, risk of death in ORMD.

Another interesting observation from the current study was the fact that no differences in fat mass were identified between dogs classified with or without MS. This implies that canine obesity does not inevitably lead to metabolic dysfunction, and is similar to findings in man, where some obese individuals are determined to be metabolically healthy [48]. These patients are not insulin resistant, are normotensive, and have normal plasma concentrations of triglyceride, glucose, high-sensitivity C-reactive protein (hsCRP), and high-density lipoprotein (HDL) cholesterol. The reasons why some dogs may be protected from developing metabolic derangements of obesity are not known and require further study.

Possible explanations, not examined in the current study include the age of onset obesity, time taken to become obese, duration of obesity prior to weight loss, and the type of diet fed during the obese stage. As a result, further work is required to determine the mechanisms involved in the development of ORMD.

As is often the case, this study has limitations that should be considered. The main study limitation was that client-owner out bred dogs were used, which undoubtedly added to study variability. For instance, environment, diet, exercise and husbandry were variable. Most notable is the issue of diet, since the amount of food eaten, and type of diet could have influenced the pre-weight-loss metabolic parameters, particularly cholesterol and triglycerides. Unfortunately, the information obtained from the owner regarding diet fed before enrolment was vague and incomplete. A range of foods was fed, including commercial pet food, treats, and human food. Further, owners frequently fed many different diets, rarely measured the amount fed out accurately, and did not record the extra food fed. As a result, it was not possible to generate a meaningful record of pre-weight-loss feeding that could be used in this study. Further, information was unclear as to the exact duration of obesity in many cases and, again, this may have influenced the results obtained. Moreover, ethical limitations meant that we were unable to perform more invasive but gold standard assessments of insulin sensitivity such as hyperinsulinaemic, euglycaemic clamps or minimal model analysis to determine insulin sensitivity [49,50]. That said, the obesity had developed naturally and been longstanding (e.g. >12 months) in most cases, which is arguably more representative of the at-risk population of interest. Nonetheless, it would be sensible to consider further studies as a means of elucidating the underlying mechanisms of ORMD more precisely.

Conclusions

In conclusion, this study has described that up to a third of obese dogs do suffer from ORMD, which is characterized by hypoadiponectinaemia and hyperinsulinaemia. This study can form the basis of further investigations to determine pathogenetic mechanisms and the health significance for dogs, in terms of disease associations and outcomes of weight loss.

Competing interests

The following conflicts of interest apply: AJG's Senior Lectureship is funded by Royal Canin; the diet used in this study is manufactured by Royal Canin; PJM is an employee of WALTHAM, whilst VB is employed by Royal Canin.

Authors' contributions

AT – performed laboratory assays, drafted manuscript, and reviewed the manuscript; JJC – performed laboratory assays, drafted the manuscript, and reviewed the manuscript; SLH – collected clinical data, and reviewed manuscript; DJC – Assisted with results interpretation, and reviewed the manuscript; PJM – designed the study, reviewed the results, and reviewed the manuscript; VB – designed the study, reviewed the results, and reviewed the manuscript; AJG – designed the study, collected the clinical data, analyzed the results, and drafted the manuscript. All authors have approved the final article.

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3.4.5. Article 15

Effect of weight loss in obese dogs on a range of renal biomarkers

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Short title: Renal biomarkers during weight loss

Keywords: Clusterin; Cystatin C; Homocysteine;

Abbreviations:

BCS body condition score

BW body weight

DEXA dual-energy X-ray absorptiometry

UAC urine albumin corrected by creatinine

21	UPCR	urine protein:creatinine ratio
22	USG	urine specific gravity
23	Hcy	homocysteine
24	CKD	chronic kidney disease
25	CysC	Cystatin C
26	MER	maintenance energy requirement
27	CLU	Clusterin.

28

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39 manuscript, reviewed manuscript SLH – collected clinical data, reviewed manuscript; PJM –
40 designed study, reviewed results, reviewed manuscript; VB – designed study, reviewed results,
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45

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47 Lectureship is funded by Royal Canin; the diet used in this study is manufactured by Royal
48 Canin; PJM is an employee of WALTHAM, whilst VB is employed by Royal Canin.

49

Abstract

Background: Obesity is a common medical disorder in dogs, and may predispose to a number of associated diseases. Recently, human obesity has been recognized as a risk factor for the development and progression of chronic kidney disease.

Objectives: To investigate the possible influence of weight loss on biomarkers of renal status.

Animals: 37 obese dogs that successfully lost weight were included in the study.

Methods: three novel biomarkers of renal functional impairment and/or pathology (homocysteine, cystatin C, and clusterin), in addition to traditional markers of chronic renal failure (serum urea and creatinine, urine specific gravity [USG], urine protein-creatinine ratio [UPCR], and urine albumin corrected by creatinine [UAC]) before and after weight loss in dogs with naturally-occurring obesity were investigated.

Results: Urea ($P=0.043$) and USG ($P=0.012$) were both greater post-weight loss than pre-weight loss, whilst UPCR, UAC, and creatinine were less post-weight loss ($P=0.032$, $P=0.006$, and $P=0.026$, respectively). Homocysteine ($P<0.001$), Cystatin C ($P<0.001$) and Clusterin ($P<0.001$) all decreased upon weight loss. Multiple linear regression analysis suggested associations between percentage weight loss (greater weight loss, more lean tissue loss; $r=-0.67$, $r^2=0.45$, $P<0.001$) and pre-weight-loss plasma clusterin concentration (greater clusterin, more lean tissue loss; $r=0.48$, $r^2=0.23$, $P=0.003$).

Conclusion and clinical importance: These results suggest possible subclinical alterations in renal function in canine obesity, which improve with weight loss. Further work is required to determine the nature of these alterations and, most notably, the reason for the association between pre-weight loss plasma clusterin and subsequent lean tissue loss during weight management.

Chronic kidney disease (CKD) is a major cause of morbidity and mortality in dogs and, given that it typically diagnosed relatively late in the course of the disease, dogs with advanced pathology represent only a fraction of all dogs with CKD.^{1,2} Recently, human obesity has been recognized as a risk factor for the development and progression of CKD,³ and reducing body fat mass, either through dietary energy restriction or bariatric surgery, can reverse many of the associated clinical and nephropathologic manifestations.⁴

Obesity is a common medical disorder in dogs, and may predispose to associated diseases such as osteoarthritis, respiratory disease, neoplasia and insulin resistance.⁵ However, it is less clear as to whether there is an association between excess adiposity and renal functional or pathological alterations in this species. When monitoring renal disease, clinicians rely primarily on serum creatinine and urea concentrations, urine specific gravity (USG), and urine protein:creatinine ratio (UPCR). Further, microalbuminuria is considered to be an excellent marker of early kidney disease,⁶ being a sign of mildly altered glomerular permeability.⁷ However, all of these tests are insensitive, with experimental studies suggesting that azotemia and impaired urine concentrating ability are not seen until functional nephron mass is reduced by at least two thirds.⁸ Non-invasive and simple methods that have the ability to detect renal damage in CKD prior to functional nephron impairment (e.g. inadequate urine concentrating ability or azotaemia) are limited. Given that there is a high risk of progression to irreversible renal damage in patients with CKD, there is a current need both to develop markers that enable early detection of renal dysfunction,⁹ as well as to identify causal factors that might predispose to such dysfunction. In response, new serum biomarkers for early renal injury have started to gain attention in human and veterinary medicine, and examples include homocysteine (Hcy), cystatin C (CysC), and clusterin (Clu).¹⁰

Homocysteine is an amino acid the majority of which (98%) circulates in an oxidized form bound to protein.¹¹ In humans, total plasmahomocysteine (tHcy) concentration is inversely correlated with the glomerular filtration rate (GFR),¹² and positively correlated with circulating creatinine concentration.¹³ In dogs, significantly greater tHcy concentrations are reported in dogs with cardiac and renal diseases.¹⁴ CysC is a 13 kDa, 122-amino acid, cysteine protease inhibitor, and is produced at a constant rate by all nucleated cells in the body.^{15,16} As a result, circulating concentrations correlate with GFR.¹⁷ Dogs appear to be similar to humans, where by circulating CysC concentration may be a better marker of renal functional impairment than serum creatinine concentration.^{10,18,19} Clu is a glycoprotein which is composed of two 40 kD subunits (NA1, NA2) bound by disulfide groups.²⁰ Reports in human medicine indicate that Clu is upregulated and released into the urine after nephron damage.^{21,22}

The aim of this study was to investigate the possible influence of weight loss, in dogs with naturally-occurring obesity, on biomarkers of renal status, as described in humans.⁴ As a result, we chose to investigate the behavior of three novel biomarkers of renal functional impairment and/or pathology (tHcy, CysC, and CLU), in addition to traditional markers of CKD (serum urea and creatinine, USG, UPCR, and urine albumin:creatinine [UAC]) before and after weight loss in dogs with naturally-occurring obesity.

Material and methods

Animals

Dogs were referred to the Royal Canin Weight Management Clinic, University of Liverpool UK, for investigation and management of obesity and associated disorders.

Sixty-five dogs were recruited between February 2005 and August 2010, and those successfully losing weight had completed by January 2011. Eligibility criteria included confirmation of obesity (based upon body fat measurement by dual-energy X-ray absorptiometry; DEXA) and availability of sufficient surplus plasma and urine for analysis. The study protocol adhered to the University of Liverpool Animal Ethics Guidelines, and was approved by both the University of Liverpool Research Ethics Committee and the WALTHAM ethical review committee. Owners of all participating animals gave informed written consent.

Weight loss regimen

Full details of the weight loss regimen have been previously described.^{23,24} Briefly, dogs were determined to be systemically well, and without significant abnormalities on complete blood count, serum biochemical analysis and urinalysis. Throughout weight loss, patients were weighed on electronic weigh scales,^a which were regularly calibrated using test weights.^b Body composition was analysed by fan-beam DEXA,^c and results used to estimate target weight.^{24,25}

A weight management protocol was then instigated,^{23,24} using either a high protein high fiber^d or high protein moderate fiber^e weight loss diet (Table 1). The initial food allocation for weight loss was determined by first estimating maintenance energy requirement ($MER = 440 \text{ kJ [105 Kcal]} \times \text{body weight [kg]}^{0.75} / \text{day}^{26}$) using the estimated target weight. The exact level of restriction for each dog was then individualized based upon gender and other factors (i.e. presence of associated diseases), and was typically between 50-60% of MER at target weight.²⁴ Owners also implemented lifestyle and

activity alterations to assist in weight loss. Dogs were reweighed every 7-21 days and changes made to the dietary plan if necessary.^{23,24}

Analysis

THcy concentration was measured with a commercial kit^f and an automated analyser^g following the instructions of the manufacturer. CysC and Clu concentrations were measured with species-specific commercially-available ELISAs assays^h. The three assays had intra- and inter-assay coefficients of variation less than 15%; and the dilution of canine serum samples resulted in linear regression equations with correlation coefficient close to 1.0.

Urine specific gravity was measured by a hand refractometer.ⁱ Serum urea and creatinine and urine protein and creatinine assays were performed on the automated clinical chemistry analyzer.^g In the cases of serum urea and creatinine, commercially available reagents were used.^g Urine protein was determined by the pyrogallol method using a commercially available reagent^j, while creatinine was measured using the modified Jaffe method using a commercially available reagent^k on samples diluted 1:20 with deionized water. The UPCR was then calculated with the formula: $UPCR = \text{protein (mg/dL)} / \text{creatinine (mg/dL)}$. Urine albumin was determined by human immunoturbidometric assay^l in an automated clinical chemistry analyzer^g previously validated for use in canine urine samples.²⁷ Urinary albumin levels (mg/L) were adjusted for urinary creatinine excretion (UAC) and expressed as micrograms per gram of creatinine for statistical analysis as previously described.²⁸

Statistical analysis

Data are expressed as median (range) except where indicated. Statistical analyses were performed with computer software (Stats Direct version 2.6.8; Stats Direct Ltd.), with the level of significance set at $P<0.05$ for two-sided analyses. The Shapiro-Wilk test was first used to assess whether or not data were normally distributed, and either parametric or non-parametric tests were used, as appropriate. These included Fisher's exact test, simple and multiple linear regression, paired and unpaired Student's t test, Mann-Whitney U test, and Kendall's rank correlation.

Differences in the concentrations of the various biomarkers, prior to and after weight loss, were assessed with either the paired Student's t test or the Wilcoxon signed rank sums test. Linear regression was used to determine factors associated with outcomes of weight loss (i.e. percentage change in lean tissue and rate of weight loss). Initially, simple regression was used to determine associations between with outcome variables and both pre-weight-loss parameters (e.g. age at enrolment, sex, percentage body fat) and pre-weight loss plasma biomarker concentrations. A multiple linear regression model was then constructed, which initially included any variables identified as $P<0.2$ on univariable analysis. The model was subsequently refined by backwards-stepwise elimination of the least significant variable at each round. Variables were retained in the final model, either if they were significant ($P<0.05$) or if removal resulted in substantial ($\geq 10\%$) change to the effect of other variables.

Results

Baseline characteristics of the dogs

Full details of the baseline characteristics of both groups are given in Table 1. The median age of the 37 dogs was 72mo (12 to 132mo), and a range of breeds was represented (Table 1); 21

were male (20 neutered) and 16 were female (14 neutered). Median body weight prior to weight loss was 35.0 kg (5.4 to 77.0 kg), and body fat mass was 45.0% (30.0 to 54.7%).

Associations between baseline parameters and pre-weight-loss renal biomarkers

There was no association between baseline parameters (including age, gender, breed, neuter status, body weight and body fat percentage) and pre-weight-loss renal biomarker (plasma Hcy concentration $P=0.082$, all other results $P>0.2$).

Changes in renal biomarkers with weight loss

The results for all renal biomarkers are shown in Table 2. Urea ($P=0.043$) and USG ($P=0.012$) were both greater post-weight loss than pre-weight loss, whilst UPCr, UAC, and creatinine were less post-weight loss ($P=0.032$, $P=0.006$, and $P=0.026$, respectively). Based upon, accepted international criteria,²⁹ increased UPCr (>0.5) was present in 8 dogs before and in 1 dog after weight loss ($P=0.012$). UAC >30 mg/g (a cut point that has been used to define microalbuminuria in humans³⁰) was present in 8 dogs before and 3 dogs after weight loss. Hcy ($P<0.001$), CysC ($P<0.001$) and Clu ($P<0.001$) all decreased upon weight loss.

Associations between baseline parameters and pre-weight-loss renal biomarkers with the outcomes of weight loss

Both simple and multiple linear regression analyses were used to determine the effect of baseline parameters (e.g. age, sex, start weight, and body fat mass) and plasma biomarkers on two weight loss outcomes (e.g. change in lean tissue mass and rate of weight loss). For rate of weight loss, simple linear regression did not reveal any significantly associated factors ($P>0.2$),

and a multivariable regression model including all factors was also not significant ($P=0.522$). In contrast, simple linear regression (Table 3) suggested associations between a number of factors and change in lean tissue mass including breed (retriever vs. non-retriever, $P=0.006$), duration of weight loss ($P=0.008$), percentage weight loss ($P<0.001$), body fat mass pre-weight loss ($P=0.018$), creatinine ($P=0.122$) and Clu ($P=0.080$). However, the only factors remaining on multiple regression were percentage weight loss (greater weight loss, more lean tissue loss; $r=-0.67$, $r^2=0.45$, $P<0.001$) and pre-weight-loss plasma Clu concentration (greater clusterin, more lean tissue loss; $r=0.48$, $r^2=0.23$, $P=0.003$).

Discussion

The current study has investigated putative renal biomarkers in obese dogs undergoing a weight management program, that lead to a marked reduction in body fat mass. Both conventional biomarkers in current clinical use (e.g. serum urea, serum creatinine, USG, UAC, and UPCR) and novel biomarkers (e.g. tCcy, CysC, and Clu) were assessed before and after weight loss. Whilst it is tempting to speculate that the results may be the result of altered renal function, alternative explanations may be possible for many of the changes noted. Most notably, the differences identified might have been the result of other alterations occurring concurrently during the weight loss program. As a result, further studies would be required to confirm these findings and determine their significance.

The observed changes in urine biomarkers used in routine clinical practice (USG, UPCR, and UAC) with weight loss could imply improved renal function, through an increase in tubular concentrating ability (increased USG) and a decrease in protein filtered by the glomerulus (decreased UPCR and UAC). Experimentally-induced obesity in dogs is known to alter renal

function (e.g. glomerular hyperfiltration with an associated increase in GFR) and cause histologic changes such as expansion of Bowman's capsule, cell proliferation in the glomeruli, thickening of glomerular and tubular basement membranes, and increased mesangial matrix.³¹ Similar changes could be the reason why USG was less (e.g. due to an increase in GFR) and UPCR and UAC more commonly abnormal (e.g. due to the glomerular lesions causing protein leakage) for the dogs of the current study, when in an obese state. Other diagnostic modalities such as kidney biopsy could have helped to determine the significance of the changes in this study. However, since this study was done in clinical conditions it was not ethically possible to perform invasive procedures such as serial kidney biopsy in client-owned dogs.

Although the concentrations of urea and creatinine were within laboratory reference intervals, an increase in serum urea and a decrease in serum creatinine were observed after weight loss. The increase in plasma urea concentration in obese dogs undergoing weight loss has been previously reported in some,³² but not all³³ previous studies. Although the reason for such an increase is not clear, it might either have resulted from a decrease in GFR as a result of weight loss, or from feeding a high-protein weight loss diet. Increased post-weight loss plasma urea concentration has also been described in obese humans consuming a moderate protein diet for weight loss, but not those receiving a high carbohydrate diet.^{34,35} Measurement of GFR pre- and post-weight loss might have helped to differentiate between these possibilities. The decrease in serum creatinine concentration is contradictory to previous studies in obese dogs, whereby increased serum creatinine concentration have been documented following weight loss.^{32,33} This decrease could be the result of loss of muscle mass during weight management. If a genuine decrease in GFR were to have occurred with weight loss, as suggested by the increase in urea concentration, it could counteract the effect of lean tissue loss on serum creatinine concentration.

266

267 The current study also investigated three novel biomarkers putatively associated with
268 renal functional impairment or pathology: tHcy, CysC, and Clu. Increased tHcy concentration
269 has been associated with renal disease in dogs¹⁴ and humans¹¹ whilst, in humans, moderate
270 hyperhomocysteinaemia has been noted in early stages of chronic renal failure, becoming
271 more prominent as renal function deteriorates.¹¹ Furthermore, other human studies have
272 identified greater tHcy concentrations in overweight and obese patients when compared with
273 normal weight patients.³⁶ Thus, the post-weight-loss decrease in tHcy concentration in the
274 obese dogs of the current study, might indicate altered renal structure or function in the obese
275 state with subsequent improvement with weight loss. However, given that dietary folate
276 intake can influence plasma tHcy concentration, folate should ideally have been measured in
277 all study dogs. This is a limitation of the current study and further assessment is required.
278 However, in a previous human study, changes in folate concentration did not influence serum
279 tHcy concentration in weight loss programs.³⁷

280

281 Dogs are similar to humans in that circulating CysC concentration is reportedly a better
282 and more accurate marker of renal function than creatinine or creatinine-based equations.
283 This is thought to be because the influence of non-renal factors (such as body composition,
284 age, gender, or dietary protein intake) on circulating CysC concentration is less than for
285 creatinine concentration.^{10,15,16,19,38,39} Further, given that increases in circulating CysC
286 concentration with progressive renal compromise, parallel one another in obese and non-
287 obese humans, changes in CysC concentration are thought to reflect renal function whatever
288 the degree of obesity.^{40,41} However, circulating CysC is consistently increased in obese subjects
289 independent of GFR, and adipose tissue expression of CysC is increased in the obese state.⁴¹
290 This suggests that adipose tissue may contribute directly to circulating CysC concentration

through increased adipose tissue synthesis in the obese state. Given that it is not currently known whether canine adipose tissue can synthesise CysC, it is feasible that the decrease in plasma CysC concentration is either the result of improved renal function or to decreased synthesis of CysC by adipose tissue after weight loss.

Weight loss also resulted in a decrease in plasma Clu concentration, which could have been the result of two different mechanisms: (1) improvement in renal injury, given that increased serum Clu concentration is seen after nephron damage in humans;^{21,22} (2) improvement in lipid profile after successful weight loss, since circulating Clu reportedly acts as an apolipoprotein, by partially associating with high density lipoprotein (HDL).⁴² In dogs, HDL is the predominant lipoprotein⁴³ and this decreases after weight loss.⁴⁴ Furthermore, a positive correlation between changes of Clu with body fat mass has been described in humans independent of age, gender, HbA1c and fasting plasma insulin concentrations.⁴⁵ It is again advisable to consider further studies to determine the true significance of this finding.

Preservation of lean tissue mass during weight loss is a key outcome for successful obesity management and, in humans, loss of skeletal muscle mass is correlated with physical impairment and disability⁴⁶ as well as being associated with an increased incidence of death.⁴⁷ In the present study, pre-weight-loss Clu concentration correlated with amount of lean tissue lost during subsequent weight management (greater Clu, more lean tissue loss). The reasons for this association are not clear but, in humans, widespread upregulation of Clu gene expression and protein synthesis is seen in diseases where either abnormal cell death or proliferation occurs, including atherosclerosis, myocardial infarction,

and muscle damage.^{48,49} Thus, it could be hypothesized that Clu acts as a surrogate marker of deranged metabolic function in canine obesity, and this improves after successful weight loss. Whatever the reason, this intriguing study finding raises the possibility that pre-weight loss Clu concentration could be used as a biomarker to identify those dogs at risk of excessive loss of lean tissue mass.

The main limitation of this study would be that the studied animals were a population of client-owned dogs with variable living conditions, family environment, husbandry and medical care. This made it impossible to evaluate influence of changes in diet composition on studied analytes, to perform GFR measurements, or to perform renal biopsies in order to evaluate the weight loss on histologic changes in the kidneys. Nonetheless, the results are arguably more representative of the true clinical picture since the obesity is naturally-occurring.

In summary, the current study has demonstrated changes in a variety of renal function biomarkers in obese dogs undergoing weight loss. Although these results might suggest possible subclinical alterations in renal function in canine obesity, other explanations are possible for many of the changes observed. Therefore, further studies are now necessary to determine whether renal functional changes or injury occur in canine obesity and whether or not they improve after weight loss. Finally, the reason for the association between pre-weight plasma clusterin and subsequent lean tissue loss during weight management is intriguing, and warrants additional investigation.

Footnotes

- a. Soehnle Professional GmbH & Co. KG, Backnang, Germany.
- b. Blake and Boughton Ltd, Norfolk, UK.
- c. DEXA, Lunar Prodigy Advance; GE Lunar, GE Medical Systems, Madison, WI.
- d. Satiety Support, Royal Canin, Aimargues, France.
- e. Obesity Management, Royal Canin, Aimargues, France.
- f. Diazyme Laboratories, Diazyme Europe GMBH, Dresden, Deutschland.
- g. biochemistry analyzer, Olympus AU2700, Olympus Diagnostica GmbH.
- h. Canine Cystatin C ELISA Kit and Canine Clusterin ELISA Kit; BioVendor–Laboratorni medicina, Brno, Czech Republic.
- i. ATAGO Company Ltd, Tokyo 173-0001, Japan.
- j. Protein u&csf, Spinreact SAU, Sant Esteve de Bas, Spain.
- k. Creatinine-J, Spinreact SAU, Sant Esteve de Bas, Spain.
- l. Microalbumin OSR6167, Olympus system reagent, Olympus Diagnostica GmbH.

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Table 1

Summary of weight loss in the study dogs.

<i>Criterion</i>	<i>Result</i>
<i>Age</i>	72mo (12 to 132)
<i>Sex</i>	1 M, 20 NM, 2 F, 14 NF
<i>Breed</i>	Akita, Border Collie, Cairn Terrier, CKCS (3), Cocker Spaniel, Corgi, Dachshund, Doberman, English Bull Terrier, Golden Retriever, Irish Setter, Labrador (10), Lhasa Apso, Miniature Schnauzer, Mixed Breed (5), Pug (3), Samoyed, Schipperke, Yorkshire Terrier (2)
<i>Body weight PRE</i>	35.0 kg (5.4 to 77.0)
<i>Body weight POST</i>	25.8 kg (4.4 to 51.4)
<i>Body fat mass PRE</i>	13400g (1600 to 37700); 45% (30 to 55)
<i>Body fat mass POST</i>	6700g (700 to 37700); 29% (11 to 45)
<i>Lean tissue mass PRE</i>	17700g (3500 to 36600); 52% (43 to 68)
<i>Lean tissue mass POST</i>	17700g (3300 to 33400); 68% (53 to 86)
<i>Duration</i>	250 days (91 to 674)
<i>Rate of weight loss¹</i>	0.8%/week (0.2 to 1.4)
<i>Body weight change²</i>	-28 % (-10 to -44)
<i>Change in fat mass²</i>	-52 % (-78 to -18)
<i>Change in lean mass²</i>	-7 % (-21 to 14)
<i>EI during weight loss³</i>	251 (184 to 310) [60 (44 to 74)]

All data are expressed as median (range). M: male; NM: neutered male; F: female; NF: neutered female; CKCS: Cavalier King Charles Spaniel. ¹ Rate of weight loss expressed as percentage of starting body weight lost per week. ² Refers to the percentage change in starting mass calculated as follows: $([\text{start mass} - \text{end mass}] \div \text{start mass}) \times 100\%$. ³ EI: energy intake expressed as metabolisable energy (in kJ [Kcal]) per kg of metabolic body weight ($\text{BW}^{0.75}$) per day.

Table 2

Pre- and post-weight-loss renal biomarkers in the study dogs.

Analyte	Pre-weight loss	Post-weight loss	<i>P</i>
Urea (mmol/L)	5.3 (1.6-30.6)	5.5 (3.1-8.9)	0.043
Creatinine (μmol/L)	84 (37-123)	76 (8-122)	0.026
USG	1.033 (1.011-1.058)	1.037 (1.017-1.052)	0.012
UPCR	0.50 (0.03-5.57)	0.28 (0.05-3.30)	0.032
UPCR>0.5	Yes: 8; No: 19	Yes: 1; No: 26	0.015
UAC (mg/g)	54.6 (0.3-555.8)	13.8 (0.6-157.4)	0.006
UAC>30mg/g	Yes: 8; No: 19	Yes: 3; No: 24	
Hcy (μmol/L)	10.8 (5.7-23.2)	7.8 (1.1-18.1)	<0.001
CysC (mg/L)	1.6 (1.0-2.5)	1.3 (0.7-2.0)	<0.001
Clu (μg/mL)	91.4 (47.6-108.70)	72.2 (38.9-122.0)	<0.001

Results are expressed as mean (range). USG: urine specific gravity; UPCR: urine protein creatinine ratio; UAC: urine albumin micrograms per gram of creatinine; Hcy: homocysteine; CysC: Cystatin C; CLU:clusterin.

Table 3

Associations between baseline parameters and pre-weight-loss renal biomarkers with the change in lean tissue mass.

Simple regression	<i>r</i>	<i>r</i> ²	<i>P</i>
Age	0.102	0.010	0.540
Sex	-0.062	0.004	0.713
Neuter status	-0.066	0.004	0.696
Breed ¹	0.438	0.192	0.006
Starting weight	0.121	0.015	0.469
Body fat percentage	-0.380	0.145	0.018
Duration of weight loss	-0.424	0.180	0.008
Rate of weight loss	0.126	0.016	0.449
Percentage weight loss	0.589	0.344	<0.001
Energy intake during weight loss	0.112	0.013	0.487
Serum urea ²	-0.051	0.002	0.763
Serum creatinine ²	0.255	0.065	0.122
Urine specific gravity ²	-0.188	0.035	0.294
Urine protein:creatinine ratio ²	0.044	0.002	0.828
Plasma homocysteine ²	-0.147	0.022	0.385
Plasma cystatin C ²	0.010	0.000	0.950
Plasma clusterin ²	-0.291	0.085	0.08
Multiple regression	<i>r</i>	<i>r</i> ²	<i>P</i>
Final model	0.708	0.501	<0.001
Percentage weight loss	-0.674	0.454	<0.001
Plasma clusterin	-0.484	0.234	0.003

¹ Breed based upon a dummy variable where dogs of retriever breed were assigned a value of 1, and dogs of other breeds were assigned a value of 0; ² refers to pre-weight loss biomarker results.

17-Aug-2012

Dear Dr. German:

Manuscript ID JVIM-SA-12-130.R1 entitled "Effect of weight loss in obese dogs on a range of renal biomarkers" has been reviewed by experts in the field whose concerns are detailed in the attached review. The manuscript may be acceptable for publication provided that you address the reviewers' concerns to the editors' satisfaction. We encourage you to revise and resubmit this manuscript after carefully considering the enclosed reviewers' comments.

Please submit the revised manuscript within 30 days. In your response to the reviewers' comments, which must be separate to the revised manuscript, please address each point separately and specifically.

Once again, thank you for submitting your manuscript to the Journal of Veterinary Internal Medicine and I look forward to receiving your revision.

Sincerely,

Prof. Kenneth Hinchcliff

Editor in Chief, Journal of Veterinary Internal Medicine

hkw@unimelb.edu.au

3.4.6. Article 16



Effects of weight loss in obese cats on biochemical analytes related to inflammation and glucose homeostasis

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Abstract

The aim of the current study was to measure circulating metabolic and inflammation-related biochemical analytes in obese cats before and after weight loss. Thirty-seven overweight neutered cats were studied, median body weight 6.85 kg (range, 4.70 to 10.30 kg), representing a range of ages and both sexes. An individualized weight-loss program was devised for each cat and monitored until completion. Body fat mass was determined by dual-energy x-ray absorptiometry, whereas plasma concentrations of acute-phase proteins (APPs; eg, haptoglobin and serum amyloid A), hormones (eg, insulin, IGF-1, and adiponectin), and enzymes (eg, butyrylcholinesterase and paraoxonase type 1 [PON-1]) associated with inflammation and metabolic compounds (eg, glucose) were also measured. No significant changes were found in APPs after weight loss ($P > 0.3$), but significant increases in plasma adiponectin ($P = 0.021$) and IGF-1 ($P = 0.036$) were seen, whereas insulin ($P < 0.001$) and homeostasis model assessment ($P = 0.005$) decreased significantly. Plasma concentrations before weight loss of PON-1 ($P = 0.004$), adiponectin ($P = 0.02$), and IGF-1 ($P = 0.048$) were less in cats that failed to complete weight loss than cats that were successful, whereas glucose concentration was greater. Finally, multivariable linear regression analysis showed that lean tissue loss during weight management was associated with percentage weight loss (greater weight loss, greater lean tissue loss; $R = 0.71$, $P < 0.001$) and plasma adiponectin concentration before weight loss (lesser adiponectin, more lean tissue loss; $R = -0.52$, $P = 0.023$). In conclusion, various metabolic abnormalities occur in feline obesity, and these can be linked to outcomes of weight-loss programs. The changes that occur with weight loss suggest an improved metabolic status.

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Keywords: Adipose tissue; Acute phase protein; Obesity; Diabetes mellitus; Feline

1. Introduction

Feline obesity is a common disorder with recent epidemiologic studies reporting 27% to 36% of domes-

tic cats to be above their ideal body weight [1]. Similar to humans, there is circumstantial evidence that obesity may be associated with other medical disorders, including insulin resistance and diabetes mellitus [2]. Human obesity is associated with a chronic, low-grade systemic inflammation, which can be a key factor in the development of many of the obesity-associated diseases [3]. Most notably, “positive” acute-phase proteins

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(APPs), such as C-reactive protein (CRP) and haptoglobin (Hp), increase in humans with increasing adiposity [4]. Some studies suggest that obese dogs have normal to mildly increased APP concentrations (consistent with subclinical inflammation), which decrease on successful weight loss [5], although other work has not supported these findings [6]. Currently, no information is available as to the situation in cats.

Other inflammation-related biochemical analytes are known to change with obesity in some mammalian species. For instance, butyrylcholinesterase (BChE) activity is increased in both human and canine obesity [7,8]. Although its biological role has not been clearly established [9], this marker has been associated both with inflammation and different parameters of adiposity [10,11]. Paraoxonase type 1 (PON-1) is an enzyme that is synthesized in the liver and transported in the plasma in association with high-density lipoprotein [12]. In humans, it has antioxidant functions, namely protecting both low-density and high-density lipoprotein particles from oxidative stress by promoting degradation of lipid peroxides contained in cholesteryl esters and phospholipids [13]. Paraoxonase type 1 also has anti-inflammatory properties [14], and its circulating concentration is decreased in human obesity [15]. To our knowledge, no studies have examined PON-1 expression in feline obesity, although evidence of increased oxidative stress was seen in one experimental study in this species [16].

Adiponectin and IGF-1 have also been associated with inflammation in other species. Adiponectin, a peptide hormone produced by white adipose tissue, is recognized to be a key adipokine in humans; circulating concentrations decline in human obesity, and this has been implicated in the development of insulin resistance, a major pathophysiological factor in the development of type 2 diabetes mellitus [17]. Adiponectin concentrations are decreased in obese cats, and these increase on subsequent weight loss [18]. A recent canine study identified adiponectin as a negative APP, suggesting an association between this adipokine and inflammation [19]. Insulin-like growth factor 1 is a hormone associated with abdominal fat mass [20]. Decreased concentrations of IGF-1 have been noted in both humans [21] and cats [22] with insulin resistance, although a causal link has not been definitively established. Nutrition also plays an important role in the regulation of IGF-1, with both caloric (protein held constant) and protein restriction known to decrease IGF-1 concentration in humans [23]. As with adiponec-

tin, IGF-1 is a negative APP in the dog [19], but its significance in cats is not yet known.

In contrast to dogs and humans, in which several circulating factors are known to be associated with either obesity or inflammation or both, information in cats is more limited. Therefore, the first aim of this study was to evaluate a variety of circulating inflammatory and metabolic biochemical analytes in obese cats both before and after weight loss. Analytes measured included APPs (eg, Hp and serum amyloid A [SAA]), hormones (eg, insulin, IGF-1, and adiponectin), enzymes (eg, BChE and PON-1) related to inflammation, and metabolic compounds (eg, glucose). This study also examined whether concentrations of the same metabolites before weight loss could influence outcomes of a weight-loss program. The ultimate purpose of the study was to improve the understanding of the pathophysiological mechanisms of feline obesity, their link to inflammation, and their association with comorbidities, such as insulin resistance.

2. Materials and methods

2.1. Study animals

Thirty-seven cats participated, all referred to the Royal Canin Weight Management Clinic, University of Liverpool, United Kingdom, for the investigation and management of obesity or obesity-related disorders. The study protocol adhered to the University of Liverpool Animal Ethics Guidelines and was approved by both the University of Liverpool Research Ethics Committee and the WALTHAM ethical review committee. The owners of participating animals gave informed written consent.

2.2. Weight-loss regimen

Full details of the regimen used for weight loss have been previously described [5]. Initially, complete blood count, serum biochemical analysis, and urinalysis were performed to determine overall health status and whether any concurrent diseases were present. All cats were weighed with electronic scales (Soehnle Professional, Murrhardt, Germany), which were calibrated regularly with test weights (2 kg, 5 kg, 10 kg, and 50 kg; guaranteed accuracy $\leq 0.5\%$; Blake and Boughton, Ltd, Thetford, UK). A BCS was assigned to each patient with the use of a 9-integer system as previously described [24]. Body composition was analyzed by fan-beam dual-energy x-ray absorptiometry (DEXA; Lunar Prodigy Advance; GE Healthcare, Buckingham-

Table 1
Average analysis of the diets used in the study.

Criterion	High protein (dry) ^a		High fiber (dry) ^b		High protein (moist) ^c	
	Per 100 g AF ^d	Per 1000 kcal (ME) ^e	Per 100 g AF ^d	Per 1000 kcal (ME) ^e	Per 100 g AF ^d	Per 1000 kcal (ME) ^e
Crude protein	42.0	120	7.5	124	34.0	104
Crude fat	10.0	29	2.0	33	9.0	27
Carbohydrate	18.8	54	3.1	51	18.7	57
Crude fiber	6.8	19	1.5	25	14.1	26
Total dietary fiber	14.3	41			23.0	43
Ash			1.9	32	8.3	25

ME was measured, on a panel of seven healthy cats, at the Royal Canin Research Centre, according to the American Association of American Feed Control Officials protocol [51].

^a Obesity Management DP 42 (Dry); Royal Canin, Aimargues, France. The ME content was 3500 kcal/kg as fed with moisture of 7.0.

^b Satiety Support (Dry); Royal Canin. The ME content was 3274 kcal/kg as fed with moisture of 7.0.

^c Obesity Management S/O (Moist); Royal Canin. The ME content was 604 kcal/kg with moisture of 84.0.

^d Nutrients expressed as g/100 g as fed (AF).

^e All nutrients expressed as g/1000 kcal ME.

shire, UK) and associated computer software (enCORE 2004, 8.70.005; GE Healthcare), as previously described [25]. Target body weight (TBW) was estimated with reference to DEXA results, and an individually tailored weight-management protocol was instigated, using caloric energy restriction [5].

All cats received commercially available purpose-formulated weight-loss diets (Table 1). Twenty cats were fed the high-protein dry diet (Obesity Management DP 42; Royal Canin, Aimargues, France), 4 cats received a high-fiber dry diet (Satiety Support; Royal Canin), 1 cat received a high-protein moist diet (Obesity Management; Royal Canin), and the remaining 12 cats received a combination of the diets. Diets were fed according to the preferences of the owners. The initial energy allocation was calculated as 35 to 40 kcal ME $\times$ estimated TBW (in kg). Further adjustments were then made, based on other factors, for example, ability to exercise, current energy allocation (in cases referred after failure to lose weight at referring veterinary practice), and owners' request for a gradual rather than sudden acclimatization to the weight-loss program [5]. In addition to dietary energy restriction, owners were counseled on the lifestyle and activity alterations, which would be required to assist in weight loss, again, as previously described [5]. The type of exercise varied and could include play sessions and feeding games (eg, use of a puzzle feeder).

All cats were reassessed every 7 to 28 d, depending on the availability of the owner. Body weight measurements were taken, and changes were made to the dietary plan, if necessary. A detailed evaluation was then conducted after the period of weight loss. Cats were confirmed to have remained healthy on the basis of

physical examination, routine hematologic 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 before and after the weight-loss period. All blood samples were taken after a fast of ≥ 16 h. Immediately after collection into heparinized tubes, samples were centrifuged at room temperature for 150 sec at 12,000g. The plasma was then harvested and frozen at -20°C . Samples were subsequently shipped, on dry ice, to the Hospital Clínico Veterinario, University of Murcia. Immediately on arrival, samples were returned to a freezer and subsequently analyzed in batches.

2.4. Plasma analysis of insulin, glucose, and homeostasis model assessment

Fasting plasma glucose concentration was measured immediately after sample collection with the use of a handheld glucometer validated for use in cats (Alpha-Trak glucose meter; Abbott Laboratories, North Chicago, IL) [26]. The reported limit of detection is 20.0 mg/dL (1.1 mmol/L). Fasting plasma insulin concentration was measured by feline insulin ELISA (Merckodia, Inc, Winston Salem, NC), according to the manufacturer's instructions. Intra- and interassay CVs were $< 5\%$ and $< 9\%$. The limit of detection was 0.15 IU/mL (5 ng/L). As an indirect assessment of insulin sensitivity, homeostasis model assessment (HOMA) values were calculated, as previously described [27], with the following formula:

$$\text{HOMA} = (\text{fasting insulin } [\mu\text{U/mL}] \times \text{fasting glucose } [\text{mg/dL}]) \div 22.5$$

2.5. Analysis of plasma adiponectin and IGF-1 concentrations

Total plasma adiponectin concentration was determined by human high-sensitivity adiponectin ELISA (Human adiponectin ELISA, High Sensitivity Kit; BioVendor-Laboratornmedicina, Modrice, Czech Republic). This assay has recently been validated in cats [28]. Briefly, linearity under dilution of feline samples was confirmed (regression equations close to 1.0), with acceptable results on spiking recovery. Intra- and interassay CVs were <5% and <16%, and the limit of detection was 2.0 pg/mL. Total IGF-1 was analyzed with an automated solid-phase, enzyme-labeled chemiluminescent immunometric assay (Immulate System; Siemens Health Diagnostics, Deerfield, IL). This assay has also been validated recently for feline samples [28]. Briefly, linearity under dilution of feline samples was confirmed (regression equations close to 1.0), with acceptable results on spiking recovery. The limit of detection of the assay for feline samples was 20 ng/mL, and intra- and interassay coefficients of variation were <3% and <6%.

2.6. Analysis of plasma Hp and SAA

Plasma concentrations of Hp were determined by use of the hemoglobin-binding method with the use of a commercial kit (Tridelta Ltd, Brey, Ireland) and an automated biochemistry analyzer (COBAS, Mira plus multiparametric autoanalyzer; ABX Diagnostics, Montpellier, France). This assay has been validated in the laboratory of two of the authors (A.T and J.J.C., unpublished data). Linearity under dilution of feline samples and spiking recovery gave acceptable results. Intra- and interassay CVs were <6% and <10%, and while the limit of detection was 0.0088 g/L. Serum amyloid A concentrations were determined (from plasma samples) with a human turbidimetric immunoassay (LZ-SAA; Eiken Chemical, Co, Tokyo, Japan), and analyses were performed on an automated analyzer (Olympus AU2700; Olympus Diagnostica, GmbH, Freiburg, Germany). This assay has been validated in a previous study [29]. Briefly, linearity under dilution of feline samples was confirmed, intra- and interassay CVs were <10% and <13%, respectively, and the limit of detection was 0.4 mg/L [29].

2.7. Analysis of BChE activity and PON-1 activity

Activity of BChE was measured by previously reported method [30] and adapted for an automated analyzer (Olympus AU2700; Olympus Diagnostica, GmbH). Intra- and interassay CVs were <4% and <5%, and the limit of detection was 0.2 UI/mL. Plasma PON-1 activity was determined measuring arylesterase activity following a previously described method [31] with some modifications [32]. Intra- and interassay CVs were <5% and <7%. The limit of detection was 0.3 UI/mL.

2.8. Statistical analysis

Data are expressed as median (range) except where indicated. Statistical analyses were performed with computer software (StatsDirect version 2.6.8; Stats Direct, Ltd, Cheshire, UK), with the level of significance set at $P < 0.05$ for two-sided analyses. The Shapiro–Wilk test was first used to assess whether data were normally distributed, and either parametric or nonparametric tests were used, as appropriate. These included the χ^2 test, Fisher's exact test, univariable and multivariable linear regression, paired and unpaired Student *t*-test, Mann–Whitney *U*-test, and Kendall's rank correlation.

Differences in the concentrations of the various biomarkers before weight loss were also compared between cats successfully completing weight loss and reaching target and cats failing to complete; tests used in this part of the study included the two-sample Student *t*-test or the Mann–Whitney *U*-test. Differences in the concentrations of the various biomarkers, before and after weight loss, were assessed with either the paired Student *t*-test or the Wilcoxon signed rank sums test. Linear regression was used to determine factors associated with outcomes of weight loss (ie, percentage change in lean tissue, mean energy intake during weight loss, and rate of weight loss). Initially, univariable regression was used to determine associations between outcome variables and both parameters before weight loss (eg, age at enrollment, sex, percentage of body fat) and plasma biomarker concentrations before weight loss. Sex was considered a fixed effect, and the remaining variables were considered random effects. A multivariable linear regression model was then constructed, which initially included any variables identified as $P < 0.1$ on univariable analysis. The model was subsequently refined by backward-stepwise elimination of the least significant variable at each round. Variables were retained in the final model, either if they were significant ($P < 0.05$) or if

removal resulted in substantial ($\geq 10\%$) change to the effect of other variables.

3. Results

3.1. Baseline characteristics of the cats

The median age of the 37 cats in the study was 84 mo (16 to 156 mo); 23 were neutered male and 14 were neutered female. Besides one Selkirk Rex, all cats were domestic shorthair. Median body weight before weight loss was 6.85 kg (4.70 to 10.30 kg), body fat mass was 34.6% (17.3% to 45.5%), and BCS was 8 (6 to 9). Of this population of cats, 22 successfully completed weight loss and reached their target weight. The remaining 15 cats did not complete their program; in 14 cases, progress had been slow, and the owner decided to stop (11 cats), refused to be contacted (2 cats), or put the cat up for adoption (1 cat). The final case was diagnosed with diabetes mellitus at the time of enrollment; insulin therapy and weight management were instigated, but progress was slow, and the owner subsequently decided not to continue. The data from this cat were not excluded because the presence of clinical diabetes mellitus was not an *a priori* exclusion criterion, and removal of plasma analyte measurements made no difference to any of the findings (data not shown).

Full details of the baseline characteristics of both groups are given in Table 2. None of the baseline characteristics differed significantly between groups

($P > 0.6$ for all), and there were also no differences in diet type ($P = 0.15$).

3.2. Weight-loss parameters

Details of the outcomes of weight loss, for the 22 cats successfully reaching target weight, are given in Table 2. Percentage of weight lost was 20% (7% to 37%) of starting body weight (SBW), mean rate of weight loss was 0.6% SBW/wk (0.2% to 1.3% SBW/wk), and mean energy intake during weight loss was 32 kcal/kg TBW/d (21% to 42 kcal/kg TBW/d). The median change in body fat mass was -59% (-83% to -44%), and median change in lean tissue mass was -6% (-17% [loss] to 21% [gain]).

3.3. Plasma analyte concentrations

The results for all plasma biomarkers are shown in Table 3. Results were obtained for all analytes except for SAA, whereby concentrations in most samples were below the detectable limit of the assay.

3.3.1. Effect of baseline parameters on plasma biomarker concentrations

Plasma BChE activity before weight loss was greater in neutered female cats (neutered female 3.2 kU/L [2.3 to 3.9 kU/L] vs neutered male 2.6 kU/L [1.7 to 5.1 kU/L]; $P = 0.042$). However, no other sex effects were noted ($P > 0.2$ for all). Plasma biomarker concentrations before weight loss did not differ significantly among cats fed different diets ($P > 0.2$ for all), and diet type had no

Table 2
Summary of weight loss in the study cats.

Criterion	Cats not completing	Cats completing	
		Before weight loss	After weight loss
Age, median (range), mo	82 (24–140)	84 (16–156)	
Sex	9 NM, 6 NF	14 NM, 8 NF	
Breed	15 DSH	21 DSH, 1 Selkirk Rex	
Body weight, median (range), kg	6.90 (4.70–8.50)	6.75 (5.45–10.30)	5.25 (3.95–6.50)
BCS, median (range)	7 (6–9)	8 (6–9)	5 (5–5)
Body fat mass, median (range)			
Grams	2200 (1200–3600)	2000 (900–4200)	1000 (300–1700)
%	36 (27–45)	33 (17–45)	18 (6–31)
Lean tissue mass, median (range)			
Grams	3900 (2900–4700)	4000 (3100–5500)	3900 (2900–4500)
%	61 (52–71)	65 (53–80)	79 (67–91)
Duration, median (range), d	—	243 (91–796)	
Rate of weight loss, median (range)	—	0.6 (0.2–1.3)	
Body weight change, median (range), %	—	-20 (-7 to -37)	
Change in fat mass, median (range), %	—	-59 (-83 to -44)	
Change in lean mass, median (range), %	—	-6 (-17 to 21)	
EI during weight loss, median (range)	—	32 (21–42)	

Abbreviations: NM, neutered male; NF, neutered female; DSH, domestic shorthair; EI, energy intake.

Table 3
Plasma biomarkers in the study cats.

Criterion	Cats not completing	Cats completing	
		Before weight loss	After weight loss
Glucose, mg/100 mL	127.8 (84.6–385.2)	80.1 (59.4–108.0)	82.8 (59.4–126.0)
Insulin, μ U/L	4.5 (0.2–14.5)	5.8 (1.1–18.6)	2.3 (0.6–11.8) ^a
HOMA	20.4 (3.1–81.0)	15.9 (3.1–56.9)	8.9 (4.8–37.2) ^b
Haptoglobin, g/L	2.0 (0.1–4.4)	1.7 (0.2–3.1)	1.8 (0.2–2.9)
SAA, mg/L	0.38 (0.38–0.80)	0.38 (0.38–0.38)	0.38 (0.38–0.38)
BChE, kU/L	2.8 (1.8–3.9)	2.9 (1.7–5.1)	2.8 (1.7–4.3)
PON-1, kU/L	4.6 (3.1–6.9) ^b	5.5 (3.8–7.3)	5.6 (3.3–7.4)
IGF-1, ng/mL	357 (233–451) ^c	422 (329–1,081)	465 (140–959) ^c
Adiponectin, pg/mL	2,137 (557–3456) ^c	2,661 (978–6039)	3,174 (668–7821) ^c

All data are expressed as median (range).

Abbreviations: HOMA, homeostasis model assessment; SAA, serum amyloid A; BChE, butyrylcholinesterase; PON-1, paraoxonase type 1.

^a $P < 0.001$ versus successful cats before weight loss.

^b $P < 0.01$ versus successful cats before weight loss.

^c $P < 0.05$ versus successful cats before weight loss.

significant effect on changes in plasma biochemical analyte concentration ($P > 0.2$ for all). Age was positively correlated, albeit weakly, with both IGF-1 (Kendall's τ , 0.27, $P = 0.028$) and Hp (Kendall's τ , 0.26, $P = 0.03$) concentrations but was not significantly correlated with any other biomarker ($P > 0.2$ for all).

3.3.2. Differences in plasma biomarker concentrations before weight loss between cats completing and failing to complete a weight-loss program

Before weight-loss PON-1 activity ($P = 0.004$), adiponectin concentration ($P = 0.02$), and IGF-1 concentration ($P = 0.048$) were less in cats that failed to complete weight loss than in cats that were successful, whereas glucose concentration ($P < 0.001$) was greater (Fig. 1). No differences were noted for insulin ($P = 0.72$) and HOMA ($P = 0.680$; Fig. 1), and no differences were noted for the other biomarkers measured (BChE, $P = 0.777$; Hp, $P = 0.346$; SAA, $P = 0.315$).

3.3.3. Changes in plasma analytes and enzyme activity with weight loss

Weight loss in obese cats lead to increases in adiponectin ($P = 0.021$) and IGF-1 ($P = 0.036$) concentrations, whereas insulin ($P < 0.001$) and HOMA ($P = 0.005$) both decreased (Fig. 2). However, no differences were observed in glucose concentration ($P = 0.791$) or in the results of any other measured biomarkers (BChE, $P = 0.737$; PON-1, $P = 0.278$; Hp, $P = 0.891$; SAA, $P = 0.999$).

Circulating adiponectin concentrations increased in 18 of the cats that lost weight and decreased in the other 4 cats, whereas median percentage change was 20% (range, –43% to 158%). Circulating IGF-1 concentra-

tions increased in 16 of the cats that lost weight and decreased in the other 6 cats, with a median percentage change of 13% (range, –57% to 50%). Finally, insulin concentrations and HOMA both declined in all but two (insulin) or one (HOMA) of the cats, where the concentrations increased. Median change in insulin concentration was –49% (–79% to 66%), and the median change for HOMA was –46% (–82% to 15%).

3.3.4. Associations between baseline parameters, biochemical analyte concentrations before weight loss, or enzyme activity before weight loss with weight-loss outcomes

Both univariable and multivariable linear regression analyses were used to determine the effect of baseline parameters (eg, age, sex, start weight, and body fat mass) and plasma biomarkers on three weight-loss outcomes (eg, change in lean tissue mass, rate of weight loss, and energy intake during weight loss) (Fig. 2). With the use of simple linear regression, no significant effects were identified for any parameter on energy intake during weight loss ($P > 0.1$ for all). For rate of weight loss, simple linear regression did not indicate any significant associated factors, and a multivariable regression model, including all factors, was also not significant ($P = 0.89$). In contrast, univariable linear regression suggested associations between several factors and lean tissue loss, including insulin ($P = 0.09$), Hp ($P = 0.024$), adiponectin ($P = 0.031$), percentage of weight loss ($P < 0.001$), body fat mass before weight loss ($P = 0.017$), and age ($P = 0.099$). However, the only factors remaining on multivariable regression were percentage of weight loss (greater weight loss, greater lean tissue loss; $R = 0.71$, $R^2 = 0.50$, $P <$

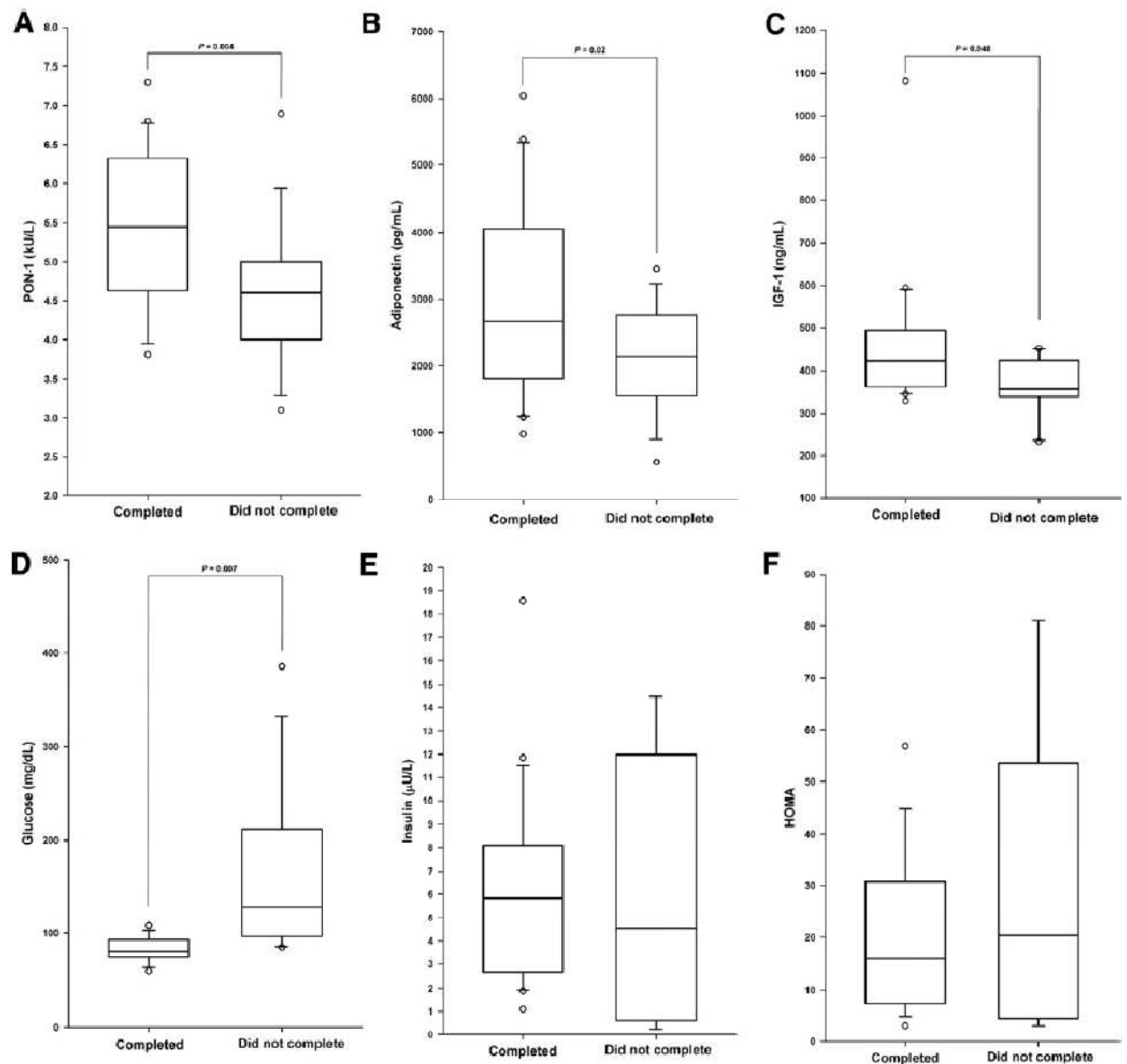


Fig. 1. Comparison of plasma biomarker concentrations before weight loss between cats completing and failing to complete a weight-loss program. Box and whisker plots show plasma (A) paraoxonase type 1 (PON-1), (B) adiponectin, (C) IGF-1, (D) glucose, (E) insulin, and (F) homeostasis glucose measurement (HOMA) in groups of cats successfully completing or failing to complete a weight-loss program. The boxes depict median (horizontal line) and interquartile range (top and bottom of box), the whiskers show the 10% to 90% range, and outliers are shown as separate points. Before weight loss values of PON-1 ($P = 0.004$), adiponectin ($P = 0.02$), and IGF-1 ($P = 0.048$) were less in cats that failed to complete weight-loss programs than cats that were successful, whereas glucose ($P = 0.007$) concentration was greater.

0.001) and plasma adiponectin concentration before weight loss (less adiponectin, more lean tissue loss; $R = -0.52$, $R^2 = 0.27$, $P = 0.023$).

4. Discussion

The current study has examined the effect of obesity in cats and subsequent weight loss on a variety of

biomarkers related to inflammation, glucose homeostasis, and insulin sensitivity. Cats that successfully lost weight had decreased insulin concentration and HOMA. These findings, taken from a clinical study that involved pet cats with naturally occurring obesity, are consistent with previous studies performed experimentally in cats [33].

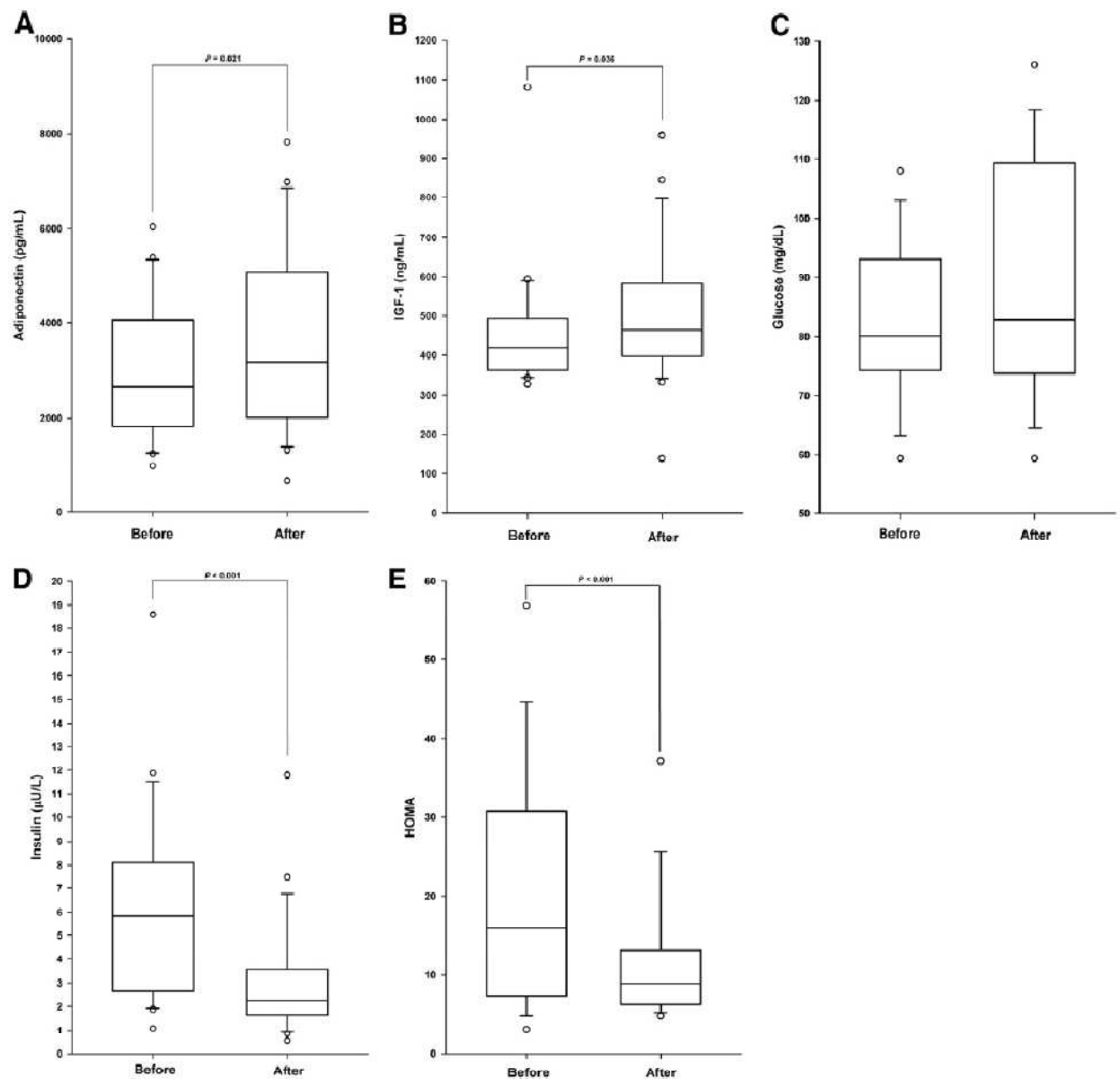


Fig. 2. Changes in plasma analytes and enzyme activity with weight loss. Box and whisker plots show plasma (A) adiponectin, (B) IGF-1, (C) glucose, (D) insulin, and (E) homeostasis glucose measurement (HOMA) before and after weight loss in 22 cats successfully completing a weight-loss program. The boxes depict median (horizontal line) and interquartile range (top and bottom of box), the whiskers show the 10% to 90% range, and outliers are shown as separate points. Weight loss in obese cats lead to significant increases in adiponectin ($P = 0.021$) and IGF-1 ($P = 0.036$) concentrations, whereas insulin concentration ($P < 0.001$) and HOMA ($P < 0.001$) both decreased significantly.

Acute-phase proteins are the most sensitive markers of inflammation [34], and increased circulating APPs are seen in obese persons [4], which decrease with weight loss [35]. However, such increases are typically mild and may remain within assay reference ranges. In the current study, both a major (SAA) and a moderate (Hp) feline APP were assessed. Before weight loss, circulating concentrations of these APPs were within

laboratory reference ranges (SAA < 20 mg/L; Hp < 3.84 g/L), and no changes occurred with weight loss. This finding is noteworthy because feline adipocytes can synthesize APPs *in vitro* [36]. Further studies are needed to clarify the role of inflammation in feline obesity, probably through *in vitro* assessments of how feline adipocytes respond to inflammation, in terms of APP gene expression and protein secretion. In addition,

the use of more-sensitive assays for APP quantification may enable more subtle alterations to be detected. For example, human high-sensitivity CRP assays can measure lesser concentrations of circulating CRP than traditional assays and can therefore more sensitively predict vascular disease [37].

Activity of BChE is increased in obese dogs [8] and humans [7], whereas circulating PON-1 activity is decreased in obese humans [15]. In contrast, no changes were observed for either enzyme in the obese cats of the current study both before and after weight loss. Although these data would not support the hypothesis that feline obesity is associated with systemic inflammation, the findings are preliminary, and further studies are recommended to clarify if either BChE or PON-1 activity is changed in overweight cats.

In contrast to the measured enzyme activities, weight loss in obese cats lead to significant increases in IGF-1 concentrations. This finding is in line with one study in humans [38] but contrasts with other reports in which greater free/total IGF-1 concentrations were observed in obese patients compared with patients of ideal weight [39]. Lesser concentrations have also been recorded in humans with type 1 and 2 diabetes [40]. Further, IGF-1 concentrations are dependent on the degree of glycemic control, with near-normal IGF-1 concentrations in well-controlled diabetics but lesser concentrations in poorly controlled persons [21]; this suggests a possible association between IGF-1 concentration and insulin activity. Similarly, in cats, IGF-1 concentrations are less in untreated diabetic cats than in diabetic cats treated with insulin [22]. Therefore, the lesser total circulating IGF-1 concentrations seen in the obese cats of the current study provide further evidence for aberrant glucose homeostasis, which can be ameliorated on a successful weight-loss program. Nevertheless, the association between IGF-1 and insulin is clearly complex and needs further clarification in the cat.

Increases in plasma adiponectin concentration were also seen after weight loss in obese cats. This finding mirrors that seen with circulating adiponectin concentrations in obese humans after successful weight loss [17,38] and agrees with previous feline studies [18]. A main effect of adiponectin is thought to be as an insulin sensitizer and, in monkeys and humans, lesser adiponectin concentrations are correlated with insulin resistance and precede the onset of type 2 diabetes mellitus [41]. Further, results from a mouse model have recently suggested that an obesity-induced reduction in adiponectin concentration may have causal roles in

both mitochondrial dysfunction and the insulin resistance seen in diabetes mellitus [42]. Nonetheless, the results of the current study suggest that similar associations between adiponectin status and insulin sensitivity may exist in the obese cat, which can revert after successful weight loss. However, the significance of this association is not known. In humans, adiponectin concentration is decreased by inflammation [4], and adiponectin may have anti-inflammatory properties [43]. This may suggest direct links between these hormonal changes, the development of the subclinical inflammatory state that accompanies human obesity [3], and end effects, such as insulin resistance and diabetes mellitus. In contrast, the absence of both changes in APPs and enzyme activities (PON-1 and cholinesterase) in the cats of the current study suggest that the adiponectin and IGF-1 changes may be associated with the metabolic abnormalities via noninflammatory (eg, metabolic) pathways.

A second aim of the study was to determine whether the status of pro-inflammatory factors before weight loss could predict success of the subsequent weight-loss regime. In this respect, PON-1, adiponectin, and IGF-1 before weight loss were less in the unsuccessful cats, whereas glucose concentration was greater. In human medicine, adiponectin concentration before gastric bypass surgery is predictive of the extent of subsequent weight loss [44]. To our knowledge, this is the first time a study has identified circulating biochemical factors that may be able to predict the success of a weight-loss regime in veterinary species. The reason for such associations and whether they are causal or incidental are unclear. However, it would be tempting to speculate that the genetic background of cats, which ultimately fail to lose weight, may predispose to development of an aberrant metabolic state that then either slows or complicates the weight-loss phase. Nonetheless, the ultimate explanation as to how such metabolic changes might be associated with failure is not known. Given that the owners of these cats generally made the decision to stop the weight program, it is possible that the metabolic abnormalities produced negative behavioral effects in their cat (ie, excessive hunger and begging activity), with which they could not cope and thereby provoking discontinuation. Alternatively, the aberrant metabolic state may have produced poor demeanor (leading to perception of poor quality of life from the owner) or the cats may have developed associated illnesses (ie, insulin resistance could predispose to other diseases). Another possibility is that the metabolic abnormalities could actually be surrogate markers of other

unmeasured factors, such that their association with failure to lose weight is incidental. It is also possible that failure was the result of poor compliance with the weight-loss program (ie, feeding treats and inappropriate foodstuffs), and similar bad owner practices may also have led to the metabolic abnormalities before weight loss. Whatever the nature of the association between aberrant metabolism and failure, it may be that these factors could be suitable biomarkers, in that measurement before weight loss will help the clinician to predict success. In time, alternative strategies could be developed to help at-risk cats.

No differences were observed in PON-1 activity before and after weight loss, although an initial lesser PON-1 activity was related to failure of the weight-loss program. A decrease in PON-1 has been described in human obesity, but PON-1 did not differ between obese and lean humans in a recent study [45]. However, in the aforementioned study, persons with a particular polymorphism of PON-1 were at increased risk of developing obesity. Therefore, further studies in cats are necessary to evaluate if PON-1 polymorphisms are associated with increased risk of developing obesity and a failure to lose weight.

The fact that circulating blood glucose concentrations were greater in the unsuccessful cats is worthy of additional comment. One cat in the unsuccessful group was diagnosed with diabetes mellitus at the time of initial assessment, based on the concurrence of hyperglycemia, glycosuria, and elevated fructosamine concentration. Other cats in this group also had glucose concentrations above the reference range, although other findings were not typical of clinical diabetes mellitus (eg, no glycosuria, normal fructosamine concentration). This borderline hyperglycemia could imply that some individuals in this group were in a prediabetic state, and it may have been this that was associated with subsequent failure to lose weight. To our knowledge, no previous studies have reported such a tendency. Alternatively, these changes could be the result of stress hyperglycemia, and being prone to stress could also be an explanation for cats failing to lose weight, that is, if the demeanor of the cat was such that it created problems with the owner–animal bond. Stress might also have been the result of other factors that again complicated weight loss; that is, the presence of concurrent diseases known to be associated with obesity, such as osteoarthritis, dermatologic disease, and lower urinary tract disease [1].

Muscle wasting is a serious complication of some conditions, and loss of muscle mass is an independent

predictor of mortality in elderly humans with chronic disease [46]. Whole-body protein catabolism is increased in obese humans and in persons with type 2 diabetes mellitus [47]. As a result, preservation of lean tissue mass during weight loss is a key outcome for successful obesity management. A final key study finding was the fact that, even in the cats that successfully lost weight, adiponectin concentration before weight loss was negatively correlated with the amount of lean tissue lost (ie, those with least circulating adiponectin lose more lean tissue during weight loss). Interestingly, recent murine studies have identified that increased muscle loss in obesity-related insulin resistance occurs because of down-regulation of the insulin receptor substrate-1/phosphatidylinositol-3-kinase Akt pathway [47], and that adiponectin itself can inhibit accelerated muscle degradation through this pathway [46]. The findings of the current study suggest that similar mechanisms may occur in cats and that the lesser circulating adiponectin concentration is directly responsible for excessive muscle protein catabolism and lean tissue loss. Nonetheless, further work would be required to confirm this preliminary observation. Additional work would also be required to determine whether excessive lean tissue loss during weight management is associated with longer term consequences. A final question is whether such cats are more likely to rebound because of both their abnormal metabolism and lean tissue loss.

Both age-related (Hp and IGF-1) and sex-related (BChE) effects were identified in the current study. First, plasma BChE activity was greater in female than in male cats, and similar findings have been noted previously in rats [48]. Further, positive linear associations with age were noted for both Hp and IGF-1. Although we are unaware of previous studies reported age-related effects on Hp concentration in any species, circulating IGF-1 concentration in humans increases during childhood until puberty and then declines thereafter [49]. Although these findings might imply species-related differences, interpretation must be cautious because the assessing age changes was not a primary aim of the study and, thus, did not take account of other potential confounding effects (ie, level of obesity). To assess age effects more appropriately, a larger population, which includes growing cats, would be necessary.

The study has a few limitations that should be considered. First, the animals studied were a population of client-owned cats with variable living conditions, family environment, husbandry, and medical care. The degree of adiposity was also variable (eg, $\leq 45\%$ overweight), and duration of obesity (which could not

reliably be determined from information provided by the owners) probably varied among cats. This diversity may well have increased between-subject variability, with the probable effect of blunting the differences before compared with after weight loss. Nonetheless, the results are arguably more representative of the true clinical picture because the obesity is naturally occurring. Second, samples were stored for variable periods, and it is not known what effect this may have on the measurements made, especially on enzyme activities. A third limitation is that, given the use of client-owned cats, it was not ethically possible to assess global insulin sensitivity either by euglycemic-hyperinsulinemic clamping or minimal model analysis [50]. These methods are used in laboratory studies and are more reliable, but they are more invasive and not practical for use in a clinical setting [27]. Instead, plasma insulin concentration and HOMA were used, and both methods correlate well with both glucose clamping and minimal model analysis in cats, thus providing useful indirect markers of insulin resistance in clinical studies [27]. Fourth, the use of client-owned cats meant that it was not ethically acceptable to include a healthy lean control population that could undergo the same procedures, such as blood sampling and body composition analysis. It was also not possible to treat some of the clinical cases with a placebo (ie, no weight loss). Despite this limitation, the fact that cats could be sampled both before and after weight loss meant that each cat could act as its own control. Further, it was also possible to compare successful cats with cats failing to complete a weight-loss program, all of which had had a similar program. Therefore, although the current findings should rightly be interpreted with caution, we still believe the findings to be of importance.

5. Conclusion

In conclusion, during obesity in cats the traditional biomarkers of inflammation, such as APPs are not increased, despite changes in other biomarkers, suggestive of insulin resistance, such as adiponectin and IGF-1. The situation is reversed during weight loss, and this is consistent with improving metabolic status. Further, concentrations before weight loss of some factors (including PON-1 activity, adiponectin concentration, and IGF-1 concentration) were associated with outcome, in terms of success of the overall program. Finally, plasma adiponectin concentrations were negatively correlated with lean tissue loss, a further and important marker of outcome during weight loss. These

findings provide insights into pathogenesis of obesity-related disease and causes of poor performance in some cats on a feline weight-management program.

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3.4.7. Article 17



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Effects of thyroxin therapy on different analytes related to obesity and inflammation in dogs with hypothyroidism

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ABSTRACT

Hypothyroidism in dogs is accompanied by changes in intermediary metabolism including alterations in bodyweight (BW), insulin resistance, and lipid profile. In this study, changes in selected adipokines (adiponectin, leptin), butyrylcholinesterase (BChE), and acute phase proteins, including C-reactive protein, haptoglobin (Hp) and serum amyloid A (SAA), were studied in dogs with hypothyroidism under thyroxin therapy. Blood samples were collected when hypothyroidism was diagnosed (before treatment) and after treatment with thyroxin.

Twenty-eight of 39 dogs exhibited a good therapeutic response (group A), whereas the remainder were considered to have been insufficiently treated (group B). Following treatment, group A dogs demonstrated a statistically significant decrease in canine thyroid stimulating hormone (c-TSH) ($P < 0.001$) and an increase in free thyroxine (fT4) ($P < 0.001$) concentrations, associated with a significant decrease in BW ($P < 0.05$), leptin ($P < 0.01$), and adiponectin, ($P < 0.001$) and an increase in BChE ($P < 0.01$) and Hp ($P < 0.05$). Group B dogs showed no statistically significant changes in c-TSH, but had a significant increase in fT4 ($P < 0.001$) accompanied by a significant decrease in adiponectin ($P < 0.05$) of lower magnitude than group A. No significant changes in the mean circulating levels of APPs were observed in both groups, with the exception of an increase in Hp ($P < 0.05$) in group A. In summary, the successful treatment of hypothyroidism reduces circulating levels of adiponectin and leptin, while increasing BChE activity in dogs. The mean increase in Hp values and decrease in SAA for some of the dogs after treatment warrants further investigation.

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Introduction

Thyroid abnormalities, hyperthyroidism and hypothyroidism are accompanied by changes in intermediary metabolism, including alterations in bodyweight (BW), insulin resistance, and plasma lipid profile in humans (Heimberg et al., 1985; Pucci et al., 2000) and dogs (Dixon et al., 2002; Hofer-Inteeworn et al., 2012). Hypothyroidism is associated with decreased concentrations of triiodothyronine (T3), thyroxine (T4), and increased thyroid stimulating hormone (TSH). These changes lead to increased BW with increased plasma lipids and lipoproteins, and are associated with alterations in glucose and insulin metabolism (Diekmann et al., 2000; Pucci et al., 2000; Hofer-Inteeworn et al., 2012).

Adipose tissue secretes a variety of active biological substances called adipocytokines, such as adiponectin and leptin, which among others are involved in appetite control, thermogenesis and thyroid function. Butyrylcholinesterase (BChE) is a non-specific choline

esterase and although its biological role has yet to be clearly established (Kutty et al., 1995), BChE has been associated both with different parameters of both adiposity and inflammation in humans, rats and dogs (Antopol et al., 1973; Jain et al., 1983; Magarian and Dietz, 1987; Tvarijonaviciute et al., 2011b). Measurements of acute phase proteins (APPs) can be used in dogs as a screening test for gauging the systemic response to an inflammatory stimulus and are considered as the most accurate markers of inflammation. Positive APPs, such as C-reactive protein (CRP), haptoglobin (Hp) and serum amyloid A (SAA) increase their rate of synthesis in the liver and are released into the blood when inflammation occurs (Eckersall, 2000).

Some adipocytokines and inflammatory proteins have been studied in human hypothyroidism (Lee et al., 2004; Yu et al., 2006; Kokkinos et al., 2007) but information about canine hypothyroidism is more limited (Dixon et al., 2002; Mazaki-Tovi et al., 2010; Jaillardon et al., 2011). Moreover, to the best of our knowledge, no data have been reported about the possible influence of thyroxin therapy on analytes related to obesity and inflammation in dogs with hypothyroidism. Therefore, the aim of the present study was to study the concentration of selected adipokines (such

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as adiponectin and leptin), BChE, and various APPs (such as CRP, SAA, and Hp) in dogs with hypothyroidism under thyroxin therapy.

Material and methods

Animals

Hypothyroid client-owned dogs of different breeds ($n = 39$) presented at the Veterinary University Hospital in Nantes were included in the study. The median age of the 39 dogs in the study was 5 years (range, 2.5–10 years); 19 of the dogs were females (10 spayed) and 20 were males (7 castrated). Median BW prior to treatment was 37 kg (range, 6–69 kg). Primary hypothyroidism was diagnosed based on clinical and laboratorial signs of primary hypothyroidism, i.e. lethargy, BW gain, dermatologic signs, high cholesterol (>6.5 mmol/L), high c-TSH (>0.5 ng/mL) and low free T4 (fT4) values (≤ 12 pmol/L) (Tyler, 2007). All dogs were subjected to thyroxin therapy (mean $\pm$ standard deviation, 14.4 ± 5.7 μ g/kg/day) for 3 months. Good therapeutic response was considered if the dogs exhibited a significant clinical improvement (including BW loss, increase activity, improvement of the skin) and at least a 30% decrease in c-TSH values.

Blood samples were collected by jugular venepuncture when hypothyroidism was diagnosed (before treatment, T1) and after treatment with thyroxin over 3 months (T2). All blood samples were taken after a fast of at least 12 h, and in T2 4 h after thyroxin administration. Immediately after collection, samples were centrifuged and the plasma was harvested and frozen at -20°C . Samples were subsequently shipped on dry ice to the University of Murcia. Immediately upon arrival, samples were transferred to a freezer at -20°C and subsequently analysed in batches.

Assays

fT4 was measured by radioimmunoassay (Immunotech kit 1363) and c-TSH by chemiluminescence (Siemens Immulite CanineTSH) (Jaillard et al., 2011). Leptin concentrations were measured with a canine leptin enzyme-linked immunosorbent assay (ELISA) (Millipore) (Tvarijonavičiute et al., 2011a). Adiponectin concentration was determined by ELISA (Human Adiponectin ELISA, High Sensitivity Kit, BioVendor-Laboratori medicina) (Tvarijonavičiute et al., 2010a). BChE activity was measured as previously reported (Tecles et al., 2000) and adapted for an automated analyser (Olympus AU2700, Olympus Diagnostica).

High sensitivity CRP (hs-CRP) was measured with a time-resolved immunofluorometric assay (TRFIA) previously validated for canine samples using goat anti-canine polyclonal antibodies (Parra et al., 2006). Haptoglobin (Hp) concentration was measured by a commercially available colorimetric method (Tridelta Phase range haptoglobin kit, Tridelta Development). SAA concentrations were measured using the Tridelta Phase Range assay. Final absorbance of the samples was measured by use of a microtitre plate reader (Powerwave XS, Biotek Instruments) at 450 nm using 630 nm as the reference (Martinez-Subiela and Cerón, 2005).

Statistical analysis

Results are expressed as the median (range) except where indicated. The D'Agostino and Pearson omnibus normality test was used to assess normality. A paired Student's t test was used to compare changes for data that were normally distributed and Wilcoxon signed rank test was used to compare changes for data that were not normally distributed. Analytes that were shown to be more significantly affected by hypothyroidism treatment (leptin, adiponectin, and BChE) were used to set a predictive model by binary logistic regression analysis and to perform a Spearman correlation test. Statistical significance was defined as $P < 0.05$ on two-tailed testing for all tests.

Results

Twenty-eight of 39 dogs exhibited a good therapeutic response with a decrease of more than 30% in c-TSH value, which was associated with a satisfactory clinical improvement. These dogs were assigned to group A, whereas the remainder of the dogs ($n = 11$) were considered to have been insufficiently treated and were assigned to group B. No significant differences were observed in baseline BW between the two groups of dogs. However, dogs in group A (5.75 years; range, 3–10 years) were significantly older than group B dogs (3.50 years; range, 2.5–6 years) ($P = 0.005$).

After treatment, group A dogs demonstrated a statistically significant decrease in c-TSH ($>30\%$) and an increase in fT4 levels ($\sim 60\%$), whereas group B dogs demonstrated a significant increase in fT4 ($\sim 40\%$), and no statistically significant changes were observed in c-TSH (Tables 1 and 2). In addition, group A dogs showed

Table 1

Median (range) data of variables of successfully treated dogs (group A).

	T1	T2	P
BW, kg	35.50 (6.00–69.00)	33.45 (15.00–70.00)	0.023
c-TSH, ng/mL	1.55 (0.60–8.30)	0.40 (0.03–4.80)	<0.0001
fT4, pmol/L	10.00 (≤ 2.4 –16.00)	16.50 (10.00–29.00)	<0.0001
Leptin, ng/mL	14.20 (≤ 3.6 –92.80)	6.90 (≤ 3.6 –21.80)	0.003
Adiponectin, μ g/mL	13.30 (5.23–23.78)	9.48 (0.43–27.92)	0.0006
BChE, μ mol/mL/min	5.20 (2.90–9.50)	5.65 (2.70–10.70)	0.009
hs-CRP, mg/L	1.17 (0.00–29.00)	1.16 (0.00–17.35)	NS
Hp, g/L	2.15 (0.09–7.4)	2.4 (0.28–9.20)	0.0393
SAA, μ g/mL	5.04 (0.68–116.00)	1.83 (0.20–24.88)	NS

BW, bodyweight; c-TSH, canine thyroid stimulating hormone; fT4, free thyroxine; BChE, butyrylcholinesterase; hs-CRP, high sensitivity C-reactive protein; Hp, haptoglobin; SAA, serum amyloid A.

Table 2

Median (range) data of variables of unsuccessfully treated dogs (group B).

	T1	T2	P
BW	39.00 (26.00–54.00)	38.50 (26.00–51.00)	NS
c-TSH, ng/mL	1.00 (0.60–4.30)	0.90 (0.50–4.20)	NS
fT4, pmol/L	10.00 (7.00–11.00)	14.00 (12.00–17.00)	<0.0001
Leptin, ng/mL	6.60 (≤ 3.60 –26.30)	5.50 (≤ 3.60 –31.70)	NS
Adiponectin, μ g/mL	16.97 (8.02–29.87)	14.53 (5.92–23.64)	0.0383
BChE, μ mol/mL/min	6.80 (1.90–12.30)	6.10 (2.40–10.60)	NS
hs-CRP, mg/L	1.13 (0.00–10.54)	1.18 (0.00–4.95)	NS
Hp, g/L	2.11 (0.05–4.03)	2.17 (0.20–3.72)	NS
SAA, μ g/mL	2.21 (0.20–90.60)	3.57 (0.84–33.95)	NS

BW, bodyweight; c-TSH, canine thyroid stimulating hormone; fT4, free thyroxine; BChE, butyrylcholinesterase; hs-CRP, high sensitivity C-reactive protein; Hp, haptoglobin; SAA, serum amyloid A.

a decrease in BW, leptin, and adiponectin concentrations ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively), and an increase in BChE activity ($P < 0.01$) and in Hp concentrations ($P < 0.05$). In contrast, group B dogs showed only a significant decrease ($P < 0.05$) in adiponectin, which was of lower magnitude than that of group A dogs (14% vs. 30%).

Although no statistically significant changes were observed, CRP concentrations were >20 mg/L in two dogs, while SAA > 5 mg/L was observed in 14 dogs before treatment; with the exception of two animals, all of these dogs showed a decrease in these APPs after therapy.

When initial values of the different analytes in both groups were compared, group A had higher leptin and lower adiponectin values than group B ($P < 0.05$ for both parameters). No significant differences were found in BChE and APPs between the two groups.

Binary logistic regression analysis indicated that pre-treatment adiponectin levels can be a predictor of successful/failed response to treatment (lower pre-treatment adiponectin = more likely to obtain successful treatment), whereas pre-treatment leptin levels showed only a trend (higher pre-treatment leptin = more likely to obtain successful treatment) (Table 3).

Table 3

Coefficients of the model of binary logistic regression analysis.

Variable	B	P	Exp(B)	CI 95.0% for Exp(B)	
				Lower	Higher
Adiponectin	−0.164	0.049	0.849	0.72	1.001
BChE	−0.376	0.131	0.687	0.422	1.118
Leptin	0.116	0.071	1.123	0.99	1.275
Constant	4.174	0.05	65.001		

B, coefficient of the logistic regression model; Exp(B), exponential B (odd ratio); CI, confidence intervals of exponential B.

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When all data (before and after treatment) for both groups were pooled, leptin was negatively correlated with fT4 ($r = -0.289$, $P = 0.001$), and positively correlated with c-TSH ($r = 0.284$, $P = 0.012$), SAA ($r = 0.300$, $P = 0.028$), and age ($r = 0.547$, $P < 0.001$). Adiponectin was negatively correlated with fT4 ($r = -0.373$, $P = 0.001$) and Hp ($r = -0.277$, $P = 0.016$), and positively with BW ($r = 0.298$, $P = 0.01$). No correlation was found between BChE and other variables. No significant correlations were observed between the variations before vs. after treatment in BW (Δ BW) and Δ leptin, Δ adiponectin, or Δ BChE.

Discussion

The current study examined the effect of hypothyroidism therapy with thyroxine on a variety of obesity and inflammation related biomarkers. A majority (72%) of dogs responded adequately to the therapy, as evidenced by a 30% decrease in c-TSH value, which is associated with satisfactory clinical improvement. These data are in accordance with previously published studies indicating that $\geq 60\%$ of dogs show an adequate response to hypothyroidism therapy (Nesbitt et al., 1980).

The criterion for a good therapeutic response was chosen based on the finding of satisfactory clinical response, accompanied by a decrease of c-TSH $> 30\%$. Intra-assay coefficient of variability (CV) for our c-TSH assay was 9.4%. Thus, the cut-off of 30% was established in order to be higher than the expected CV for two TSH measurements ($9.4\% \times 2$). However, 3 months after treatment, some of the dogs in this group still had concentrations of TSH > 0.5 ng/mL, the upper limit for healthy dogs. This could be due to the fact that in some cases, a decrease in TSH concentrations < 0.5 ng/mL can take years (Siliart et al., 2009).

A significant decrease of about 50% was observed in leptin levels in successfully treated dogs. These data are in accordance with what has been reported in humans, in which hypothyroidism treatment has been associated with reduction in plasma leptin levels ($\sim 40\%$) without changes in body mass index (BMI) (Pinkney et al., 1998, 2000). Moreover, reductions in plasma leptin concentrations, as well as leptin mRNA expression, have been observed in response to T3 treatment in rats (Escobar-Morreale et al., 1997; Fain et al., 1997). An hypothesis, which might explain the observed decrease in leptin in treated dogs, is that leptin has been suggested to stimulate the hypothalamic–pituitary–thyroid axis in response to low thyroid hormone concentrations (Mazaki-Tovi et al., 2010). Therefore, when hypothyroidism is successfully treated, there is no longer any need to stimulate the hypothalamic–pituitary–thyroid axis, resulting in a reduction of leptin level.

Adiponectin shares some physiological actions with thyroid hormones, such as reduction of body fat by increasing thermogenesis and lipid oxidation (Ahima et al., 2006). A direct positive correlation between T4 and adiponectin has been described in healthy humans (Fernandez-Real et al., 2003). However, adiponectin data in humans suffering from hypothyroidism, before and after therapy, remain controversial. Some studies performed failed to find any changes in adiponectin levels after treatment in hypothyroid patients (Iglesias et al., 2003; Santini et al., 2004). In contrast, other studies (as in the present study) have reported a significant decrease in adiponectin levels after therapy (Caixàs et al., 2009; Ozdemir et al., 2010). In our study, a negative correlation was observed between adiponectin and T4, which contrasts to reports in healthy human subjects, but is similar to what has been described in haemodialysis patients (Malyszko et al., 2006). Recently, Luvizotto et al. (2011) reported that triiodothyronine treatment inhibits adiponectin expression in obese rats under caloric restriction. As such, there is a need for larger scale studies to better elucidate the relationship between T4 and adiponectin in

dogs with hypothyroidism subjected to thyroxine therapy, as has been suggested in humans (Ozdemir et al., 2010).

Interestingly, although a low number of animals were used in the present study, pre-treatment adiponectin could be used as predictor, while pre-treatment leptin showed a trend to be a predictor ($P = 0.07$) of successful or failed response to treatment. Therefore, it seems that the finding of high values of adiponectin and/or low values of leptin in a hypothyroid dog could indicate the need to use a higher dose of thyroxine. However, the data should be interpreted with caution as a high inter-individual variability of circulating adiponectin levels exists in dogs. These data should be further explored in the future with a large population of dogs analysing the duration of clinical signs and possible secondary pathologies.

The successful treatment of hypothyroid dogs was also accompanied by an increase in serum BChE activity, as described in humans (Popovic et al., 1998). Increase of BChE activity may be involved in the hydrolysis of triglycerides (Popovic et al., 1998), resulting in lipid profile improvement, as described in hypothyroid dogs after thyroxine therapy (Dixon et al., 2002).

In healthy dogs, leptin, adiponectin, and BChE have all been shown to correlate with BCS (Ishioka et al., 2002; Sagawa et al., 2002; Jeusette et al., 2005; Tvarijonavičiute et al., 2010b). However, some controversy exists about the association of adiponectin with obesity in dogs (Ricci and Bevilacqua, 2012; Verkest and Bjornvad, 2012), since some studies have failed to demonstrate any association (Verkest et al., 2011a; Grant et al., 2011). Although a significant decrease in BW was observed after hypothyroidism therapy in the present study, no significant correlations between the change in BW and the changes in leptin, adiponectin, or BChE were detected. Similarly, in humans, no statistically relevant correlations between leptin or adiponectin and BMI or BW pre- and post-therapy have been observed in patients with hypothyroidism (Pinkney et al., 1998, 2000; Iglesias et al., 2003). Our data indicate that alterations in adiponectin, leptin and BChE levels in hypothyroid dogs after treatment are not due to variations in body mass, but may instead be directly attributed to the influence of thyroid hormones, as has been suggested in humans (Iglesias et al., 2003). Further studies in dogs measuring body fat and fat-free mass using dilution of a single dose of deuterium oxide or dual-energy X-ray absorptiometry would be warranted to confirm these findings.

In our study, there was no significant difference in mean circulating levels of APPs before and after treatment with the exception of a significant increase in Hp concentrations in successfully treated dogs, although these values were within the range observed in healthy animals (Mylonakis et al., 2011). Interestingly, CRP concentrations were elevated (> 20 mg/L) only in two dogs, while SAA values higher than the limit of our laboratory reference range (< 5 mg/L) were found in 14 dogs before treatment. These dogs (with the exception of two) showed a decrease in these APPs after the therapy. Higher SAA, but not CRP concentrations have been reported in humans with Hashimoto thyroiditis (Erden et al., 2008). Moreover, our CRP results are in agreement with previous reports, indicating that disturbances in thyroid status do not produce evident increases in CRP in dogs (Nakamura et al., 2008) and humans (Pérez et al., 2004; Caixàs et al., 2009). However, some reports indicate that low-grade systemic inflammation in humans with hypothyroidism may be associated with increases in hs-CRP (Tuzcu et al., 2005; Nagasaki et al., 2007; Erden et al., 2008). Our hs-CRP data failed to find significant differences before and after therapy.

Total adiponectin is known to be an anti-inflammatory molecule (Tilg and Wolf, 2005). For this reason, low levels of adiponectin have been associated with an increased inflammatory risk and high levels of adiponectin inhibit inflammation. In contrast, in our study some successfully treated dogs showed a decrease in

inflammatory markers despite decreased adiponectin levels. More studies are needed to evaluate the levels of the different isoforms of adiponectin, since distinct adiponectin isoforms may have different roles in inflammation (Verkest et al., 2011b; Mori et al., 2012).

The study has several limitations. Firstly, the animals used were a population of client-owned dogs with variable living conditions, family environment, husbandry and medical care, which likely had the effect of blunting pre- vs. post-treatment differences. However, the results are arguably more representative of the true clinical picture. Secondly, although a control group should ideally have been included, the use of client-owned dogs meant that it was not ethically acceptable to include control populations with healthy dogs receiving T4 treatment and dogs with hypothyroidism receiving placebo treatment. However, we sampled dogs both before and after hypothyroidism treatment that meant that each individual animal was acting as its own control. Thirdly, body fat mass was not determined by a precise method, such as dilution of a single dose of deuterium oxide or dual-energy X-ray absorptiometry (Son et al., 1998; Raffan et al., 2006), which could have been of interest to evaluate possible correlations between changes in serum analytes related to obesity (such as leptin, adiponectin, and BChE) and changes in body fat mass after treatment. Lastly, fT4 should ideally have been measured by the equilibrium dialysis method (SED) (Peterson et al., 1997; Dixon and Mooney, 1999), although no significant differences in fT4 values have been observed when different methods were compared in hypothyroid dogs (SED vs. modified equilibrium dialysis vs. different commercial radioimmunoassays) (Schachter et al., 2004; Martin et al., 2006).

Conclusions

The data from the present study indicate that the successful treatment of hypothyroidism in dogs results in reduced circulating levels of adiponectin and leptin, and increases in BChE activity. No significant changes were observed in hs-CRP levels before and after therapy but there was an increase in the mean values of Hp after successful treatment, and there was an increase in SAA in some animals with hypothyroidism, which was reduced after the therapy. Further studies are required to clarify the significance of these findings.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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3.5. Study and identification of new potential serum biomarkers involved/linked to obesity

3.5.1. PON1 and BChE

3.5.1.1. Article 18

Validation of spectrophotometric assays for serum paraoxonase type-1 measurement in dogs

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Objective—To evaluate and validate 3 spectrophotometric assays for measuring serum activity of paraoxonase type-1 (PON1), an enzyme associated with high-density lipoproteins, in dogs.

Animals—22 healthy adult dogs and 10 dogs with eccentricity.

Procedures—2 methods were adapted for use in 96-well microplates with phenyl acetate and 5-thiobutyl butyrolactonase as substrates, and 1 was adapted for use in an automated analyzer with p-nitrophenyl acetate as substrate. Blood samples were collected from all dogs, serum was harvested, and serum PON1 activity was measured with each method.

Results—Imprecision was low for all 3 methods, with the exception of interassay imprecision for 5-thiobutyl butyrolactonase, and results were linear across serial sample dilutions. The 3 methods were able to detect low PON1 activity when EDTA was used for blood sample collection, yielded lower PON1 values in sick dogs with eccentricity than in healthy dogs, and yielded highly correlated results.

Conclusions and Clinical Relevance—The methods described here may allow a wider use of PON1 activity as a biomarker of oxidative stress in dogs in clinical and research settings. Results of each method were robust and precise (with the exception of the interassay values for the lactonase method), and the methods were easy to set up in a laboratory. (*Am J Vet Res* 2012;73:34–41)

Serum paraoxonase type-1 is an enzyme associated with high-density lipoproteins. Synthesized by the liver, PON1 protects low-density lipoprotein and high-density lipoprotein particles from oxidative stress by promoting degradation of lipid peroxides contained in cholesteryl esters and phospholipids.¹ This enzyme has received increasing attention in human medicine, largely because alterations in circulating PON1 are associated with various diseases involving oxidative stress, such as renal or liver disease or neoplasia.^{2–4} In addition, PON1 has anti-inflammatory properties.⁵ The activity of PON1 is reportedly low in humans with type 2 diabetes mellitus⁶ and obesity,^{7,8} and low activity may contribute to the development of cardiovascular disease.⁹ Because of the important role PON may play in lipid metabolism, there is a need for reliable PON assays for clinical and epidemiological studies.¹⁰

Activity of PON1 can be assessed by use of various substrates, such as phenyl acetate and its derivatives, paraoxon, and butyrolactones, which can be used to measure the arylesterase, triesterase, and lactonase activities of this enzyme, respectively.^{7,11,12} Use of the paraoxon method in clinical practice is limited be-

ABBREVIATIONS	
CV	Coefficient of variation
PON	Serum paraoxonase
TAC	Total antioxidant capacity
TBBL	5-thiobutyl butyrolactonase

cause of the extreme toxic effects of paraoxon and the strong influence of PON1 gene polymorphisms on the enzyme's activity.² Measurements of PON1 lactonase and arylesterase activities are free of these limitations. Although lactonase measurements would better reflect the main activity of PON1, correlation between arylesterase and lactonase activities has been observed in human studies.⁷

Serum PON1 activity has been measured in dogs with a manual method that involves paraoxon as substrate.¹³ To the authors' knowledge, no methods for measuring PON1 activity in canine serum by use of substrates other than the toxic paraoxon or through automated or semiautomated assays have been described. The purpose of the study reported here was to evaluate and validate 3 spectrophotometric assays of PON1 activity in canine serum: 2 adapted to a 96-well microplate format with phenyl acetate and TBBL as substrates, and 1 adapted to an automated analyzer with p-nitrophenyl acetate as substrate. The development of facile enzymatic assays with nontoxic substrates that are suitable for high-throughput screening tests could be useful for evaluating the role of PON1 in dogs.

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Materials and Methods

Animals—Sixteen 6- to 8-year-old healthy adult dogs (5 sexually intact females, 3 spayed females, 7 sexually intact males, and 1 castrated male) were evaluated at San Marco Veterinary Hospital and included in the study. Breeds represented included mixed ($n = 9$), Golden Retriever (2), American Cocker Spaniel (2), Yorkshire Terrier (2), and German Shepherd Dog (1). All dogs had no evident acute or chronic disease. Results of physical and clinical examinations were unremarkable, as were results of routine hematologic and serum biochemical analyses. Also included were 6 healthy 6- to 8-year-old Beagles (3 sexually intact females and 3 sexually intact males) from the Murcia Animal Resources Centres.

In addition to the healthy dogs, ten 4- to 15-year old sick dogs (4 sexually intact females, 1 spayed female, 3 sexually intact males, and 2 castrated males) were included in which eccentrocytosis (an indicator of oxidative cell damage) was diagnosed at the San Marco Veterinary Hospital during 2009. These dogs were of various breeds and had a broad range of hematologic abnormalities. Diagnoses were as follows: diabetes mellitus ($n = 3$), onion intoxication (2), acute leukemia (2), rodenticide intoxication (1), hemangioma (1), and brain tumor (1).

All procedures involving the Beagles from Murcia Animal Resources Centres were performed in accordance with the animal care guidelines of the University of Murcia. Consent for study participation was obtained from owners of the 26 dogs that were evaluated at San Marco Veterinary Hospital.

Sample collection and processing—After food was withheld from the dogs for at least 12 hours overnight, blood samples were collected from each via jugular or lateral saphenous venipuncture into tubes containing EDTA for hematologic analysis and tubes with clotting accelerator^a for serum biochemical analysis. Samples were centrifuged at $2,000 \times g$ for 10 minutes at room temperature (20° to 22°C), and serum was harvested. The CBCs and serum biochemical analyses were performed with automatic analyzers.^{b,c}

Total antioxidant capacity was determined as described elsewhere.¹⁴ The method used is based on 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)^d decolorization by antioxidants according to their concentrations and antioxidant capacities. The color change is measured as a change in light absorbance at 660 nm. For the process, an automated analyzer^e was used, and the assay was calibrated with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.^f

Biochemical measurement of PON1 activity—Serum lactonase activity was analyzed by measuring the hydrolysis of TBBL (assay A). The method involves use of a chromogenic lactone that structurally resembles the proposed natural lipolactone substrates.² Serum samples were prepared in sample buffer consisting of 50mM Tris^g and 1mM CaCl_2 ^h (pH, 8.0) in a 20-fold dilution. One microliter of 100mM 5,5'-dithio-bis-2-nitrobenzoic acidⁱ in dimethyl sulfoxide,^j 45 μL of 4% acetonitrile^k solution in sample buffer, 5 μL of diluted serum, and 50 μL of sample buffer then were added to

the wells of a 96-well microplate.¹ Finally, 100 μL of freshly made substrate buffer containing 0.4mM TBBL^m solution in sample buffer was added to the wells to initiate the reaction. Two minutes after TBBL addition, the reaction was monitored at 412 nm in an automated microplate readerⁿ at 37°C . The nonenzymatic hydrolysis of TBBL, which was based on the hydrolysis rate in the absence of serum, was subtracted from the total hydrolysis rate. Lactonase activity was expressed as units per milliliter of serum, in which 1 unit equals 1 mmol of TBBL hydrolyzed/min. The molar extinction coefficient used to calculate the rate of hydrolysis was $7,000 \text{ M}^{-1} \text{ cm}^{-1}$. A path-length correction was applied for the use of microtiter plates.

Serum arylesterase activity was analyzed by measuring the hydrolysis of phenyl acetate^o into phenol (assay B) as described elsewhere.¹¹ Serum samples were prepared in sample buffer consisting of 20mM Tris^g and 1mM CaCl_2 ^h (pH, 8.0) in a 40-fold dilution. Five microliters of diluted serum was added to 200 μL of freshly made substrate buffer containing 20mM Tris, 1mM CaCl_2 , and 1mM phenyl acetate (pH, 8.0). The reaction was monitored on a microtiter plate¹ at 260 nm in an automated microplate readerⁿ at 37°C . The nonenzymatic hydrolysis of phenyl acetate, which was based on the hydrolysis rate in the absence of serum, was subtracted from the total hydrolysis rate. Arylesterase activity is expressed as units per milliliter of serum, in which 1 U equals 1 μmol of phenyl acetate hydrolyzed/min. The molar extinction coefficient used to calculate the rate of hydrolysis was $1,310 \text{ M}^{-1} \text{ cm}^{-1}$. A path-length correction was applied for the use of microtiter plates.

Serum arylesterase activity was also analyzed by measuring the hydrolysis of p-nitrophenyl acetate to p-nitrophenol as described elsewhere¹⁵ but with a modification to remove substrate from the working reagent buffer and prepare it in water as a separate starting reagent that remained colorless (assay C).¹⁶ This modification was made because p-nitrophenylacetate is subject to considerable spontaneous hydrolysis in the reagent buffer system originally described, and this hydrolysis was grossly apparent in the yellow color of the reagent. The starting reagent was added to initiate the kinetic reaction.

Because p-nitrophenyl acetate^p is insoluble in water, 63 mg of this compound was dissolved in 10 mL of methanol^q and stored at 2° to 8°C . In our experience, this stock solution can be kept for approximately 1 week with only a small increase in free p-nitrophenol. Afterward, 1 mL of this solution was slowly added to 20 mL of distilled water with strong agitation to prevent precipitation. The aqueous solution was freshly prepared each day.

To perform assay C, serum samples were each mixed with 307 μL of buffer containing 50mM Tris and 1mM CaCl_2 (pH, 8.0) and then freshly made substrate containing 2.5mM p-nitrophenyl acetate in distilled water was added. After 100 seconds, the reaction was monitored at 405 nm at 37°C for 210 seconds in an automated biochemistry analyzer.^r The nonenzymatic hydrolysis of phenyl acetate, which was based on the hydrolysis rate in the absence of serum, was subtracted from the total hydrolysis rate. The activity was expressed as units per milliliter of serum, in which 1 U

equals 1 μmol of phenyl acetate hydrolyzed/min. The molar extinction coefficient used to calculate the rate of hydrolysis was $14,000 \text{ M}^{-1} \text{ cm}^{-1}$.

Assay validation—For analytic validation of the 3 methods used for PON1 activity measurement, the following characteristics were calculated: precision, intra-assay CV, accuracy, and limit of detection. Two pools of sera with different PON1 activities were prepared from the group of healthy dogs and the group of dogs with eccentricity and used for precision evaluations. To determine interassay precision, these pools were divided in aliquots and stored in plastic vials at -20°C until analysis. On the day of analysis, samples were brought to room temperature prior to PON1 measurement. Intra-assay CV was calculated after analysis of the 2 serum pools 6 times in 1 assay run. Interassay CV was determined by analyzing the same pools in 6 separate runs performed on different days.

Because of the lack of a criterion-referenced standard or commercially available certified reference material for canine PON activity, assay accuracy was evaluated indirectly through assessment of dilutional linearity. For this process, 2 canine serum samples were serially diluted with saline (0.9% NaCl) solution and analyzed by use of the procedures previously described. Limit of detection was calculated on the basis of data from 20 replicate determinations of the zero standard (buffer of assays) as mean value plus 2 SDs.

To determine whether results of the 3 assays were correlated, serum samples from all study dogs were evaluated with each assay. All samples used for repetitive analysis were frozen in aliquots, and to avoid possible changes due to repetitive thawing and freezing, only vials needed for each run were used.

Effects of hemolysis and lipemia—To investigate effect of hemolysis and lipemia on results of the 3 assays for PON1 activity, 3 serum samples from 3 healthy Beagles were mixed with various concentrations of hemoglobin or lipid solution as described,¹⁷ and each preparation was run in duplicate. For hemolysis investigation, a fresh hemolysate was prepared by addition of distilled water to packed, saline solution-washed RBCs from 1 Beagle. Hemoglobin concentration in hemolysate was determined by use of a veterinary animal blood cell counter^b and adjusted to 200 g/L by adding assay buffer to create a stock solution. The stock solution was serially diluted with sample buffer, and 10 μL of each dilution was added to three 90- μL samples of canine serum. The final hemoglobin concentrations were 8, 4, 2, 1, 0.5, and 0.0 g/L. The 0.0 g/L concentration was achieved by adding 10 μL of sample diluent buffer. These hemoglobin concentrations were intended to correspond to slight hemolysis (0.5 g/L), moderate hemolysis (1 and 2 g/L), and marked hemolysis (4 and 8 g/L). Prepared samples were used to measure PON1 activity with the 3 assays.

For the lipids investigation, commercial fat emulsion^c with a triglycerides concentration of 200 g/L was serially diluted with sample diluent buffer and 10 μL of each dilution was added to two 90- μL samples of canine serum. Homogeneity was achieved by mixing with

a vortex device. The final triglycerides concentrations were 5, 2.50, 1.25, 0.625, and 0.3125 g/L, which were intended to correspond to slight lipemia (0.3125 and 0.625 g/L), moderate lipemia (1.25 and 2.5 g/L), and marked lipemia (5 g/L). Prepared samples were used to measure PON1 activity with the 3 assays.

Effects of EDTA—Blood samples (8 mL each) from 6 Beagles were obtained via jugular venipuncture by use of a disposable syringe and 21-gauge needle. Aliquots of the samples were placed in 2 types of tubes: one containing 0.072 mL of 7.5% tripotassium methylenediaminetetraacetic acid (EDTA K3)^d for EDTA-treated plasma and the other containing clot activator (5-mL tube^e) for serum. All samples were centrifuged at $2,000 \times g$ for 10 minutes, and serum and EDTA-treated plasma were immediately separated prior to analysis on the same day. The interval between collection and centrifugation was approximately 30 minutes. All plasma and serum samples were analyzed by use of the 3 study methods to measure PON1 activity.

Statistical analysis—Arithmetic means, medians, and intra-assay and interassay CVs were calculated by use of routine descriptive statistical procedures and computer software.^g Dilutional linearity was evaluated through ordinary linear regression analysis in which the measured activities of PON1 were compared with the expected activities. Correlation among results obtained with the 3 methods was assessed by use of linear regression. Interferograms were prepared to show the differences in PON1 activities when hemoglobin or triglycerides were added. The influence of hemoglobin or triglycerides on PON1 activity was investigated by use of 1-way ANOVA and Dunnett posttests. Comparisons of the results for serum and EDTA-treated plasma were made by use of a Student *t* test for repeated measurements. A Student *t* test was used to evaluate the difference of TAC and PON1 activities between healthy dogs and dogs with eccentricity. The correlation between PON1 activity and TAC was evaluated by calculation of the Spearman correlation coefficient (ρ). Values of $P < 0.05$ were considered significant for all analyses.

Results

Assays—For assay A (serum lactonase activity by use of microplates), intra-assay and interassay CV ranges were 5.5% to 6.7% and 11.3% to 17.7%, respectively (Table 1). Serial dilution of 2 serum samples resulted in linear regression equations with correlation coefficients close to 1 ($r = 0.997$ and 0.953 ; Figure 1). The assay detection limit was 1.0 U/mL (mean $\pm$ SD, 0.4 ± 0.2 U/mL). For assay B, intra-assay and interassay CV ranges were 4.0% to 4.8% and 5.4% to 9.5%, respectively. Serial dilution of 2 serum samples resulted in linear regression equations with correlation coefficients close to 1 ($r = 0.999$ and 0.996). The assay detection limit was 9.1 U/mL (mean $\pm$ SD, 1.8 ± 2.5 U/mL). For assay C, intra- and interassay CV ranges were 4.5% to 5.6% and 7.3% to 8.2%. Serial dilution of 2 serum samples resulted in linear regression equations with correlation coefficients close to 1 ($r = 0.997$ and 0.995). The assay detection limit was 0.6 U/mL (mean $\pm$ SD, 0.49 ± 0.05 U/mL).

When results for 38 serum samples analyzed with the 3 assays were used, linear regression analysis revealed significant correlations between the results of assays A and B ($P < 0.001$; $r = 0.852$), A and C ($P < 0.001$; $r = 0.850$), and B and C ($P < 0.001$; $r = 0.870$).

Table 1—Intra-assay and interassay variation in PON1 activity in 2 pools of canine serum samples (healthy dogs [pool 1] and dogs with eccentricity [pool 2]) as measured by use of lactonase activity via measurement of TBBL hydrolysis (assay A), arylesterase activity via measurement of phenyl acetate hydrolysis (assay B), and arylesterase activity via measurement of p-nitrophenyl acetate hydrolysis (assay C).

Assay	Comparison	Pool	Mean $\pm$ SD activity (U/mL)	Mean CV (%)
A	Intra-assay	1	4.3 ± 0.2	5.5
		2	1.0 ± 0.2	6.7
	Interassay	1	2.0 ± 0.9	17.7
		2	4.4 ± 0.5	11.3
B	Intra-assay	1	20.1 ± 0.8	4.0
		2	15.7 ± 0.8	4.8
	Interassay	1	21.7 ± 1.2	5.4
		2	17.5 ± 1.7	9.5
C	Intra-assay	1	5.1 ± 0.2	4.5
		2	2.6 ± 0.2	5.6
	Interassay	1	5.5 ± 0.4	7.3
		2	3.1 ± 0.3	8.2

Effects of hemolysis and lipemia on assay results—For assay A, a significant decrease in PON1 activities was evident when hemoglobin was added to the serum samples at a concentration of 8 g/L or when triglycerides were added at a concentration of 5 g/L (Figures 2 and 3). For assay B, a significant decrease in PON1 activities appeared when triglycerides at a concentration of 5 g/L were added to the samples. No substantial interference in PON1 activities by hemoglobin was evident at concentrations < 8 g/L. For assay C, a significant decrease in PON1 activities was evident when triglycerides at a concentration of ≥ 1.25 g/L were added to the samples. No significant interference by hemoglobin was evident at concentrations < 8 g/L.

Effects of EDTA on assay results—Results obtained when serum and EDTA-treated plasma samples were evaluated by use of the 3 methods were summarized (Table 2). Significant ($P < 0.001$) decreases in PON1 activity were detected in EDTA-treated plasma samples versus serum samples for each assay.

Assay discrimination—Activities of PON1 as well as TAC in dogs with eccentricity were significantly lower than those in healthy dogs when analyzed with

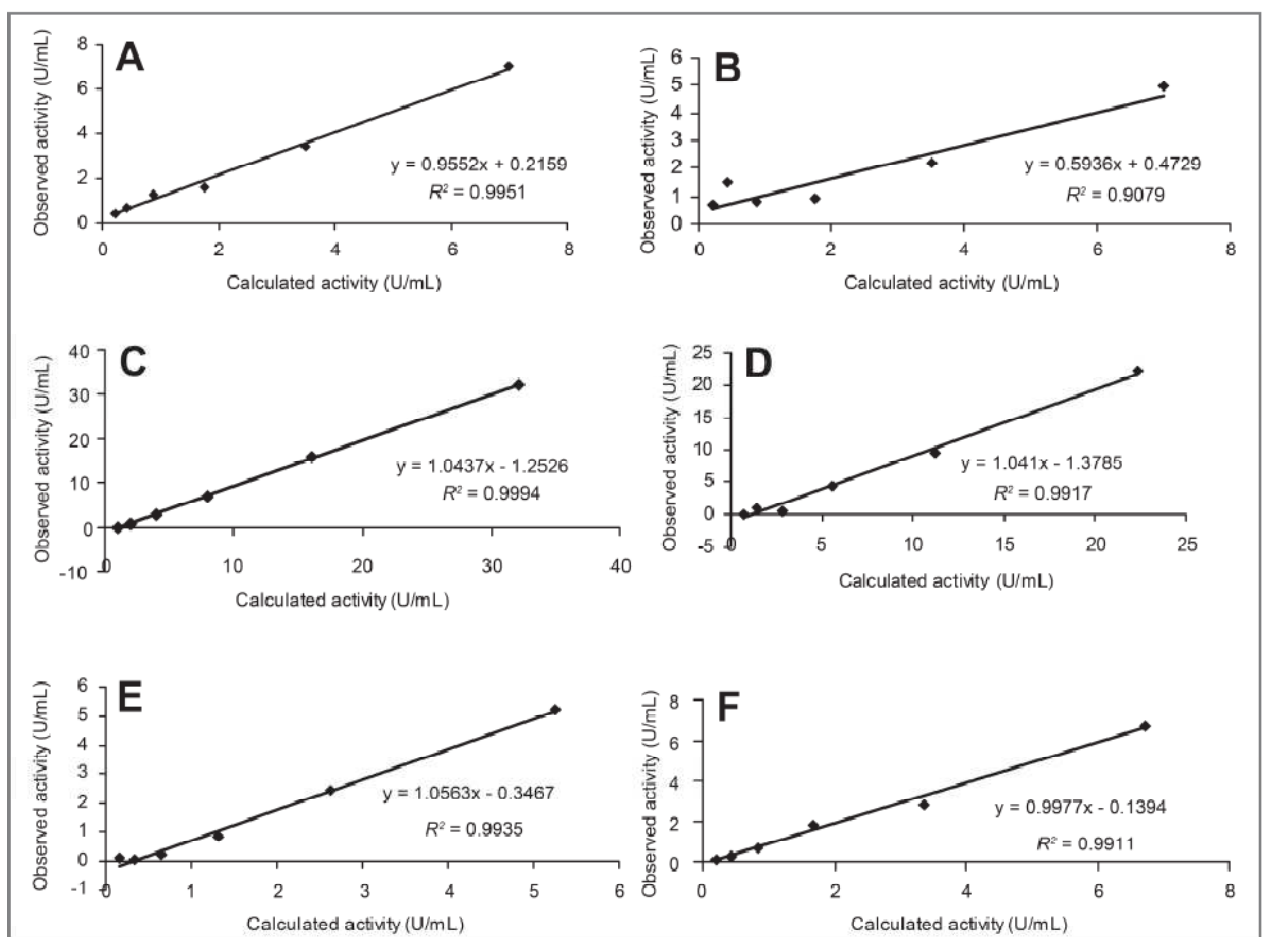


Figure 1—Regression lines showing the linearity of PON1 activity in each of 2 canine serum samples at various dilutions by use of 3 assays: lactonase activity via measurement of TBBL hydrolysis (A and B), arylesterase activity via measurement of phenyl acetate hydrolysis (C and D), and arylesterase activity via measurement of p-nitrophenyl acetate hydrolysis (E and F). Regression equations and coefficients of determination (R^2) are shown.

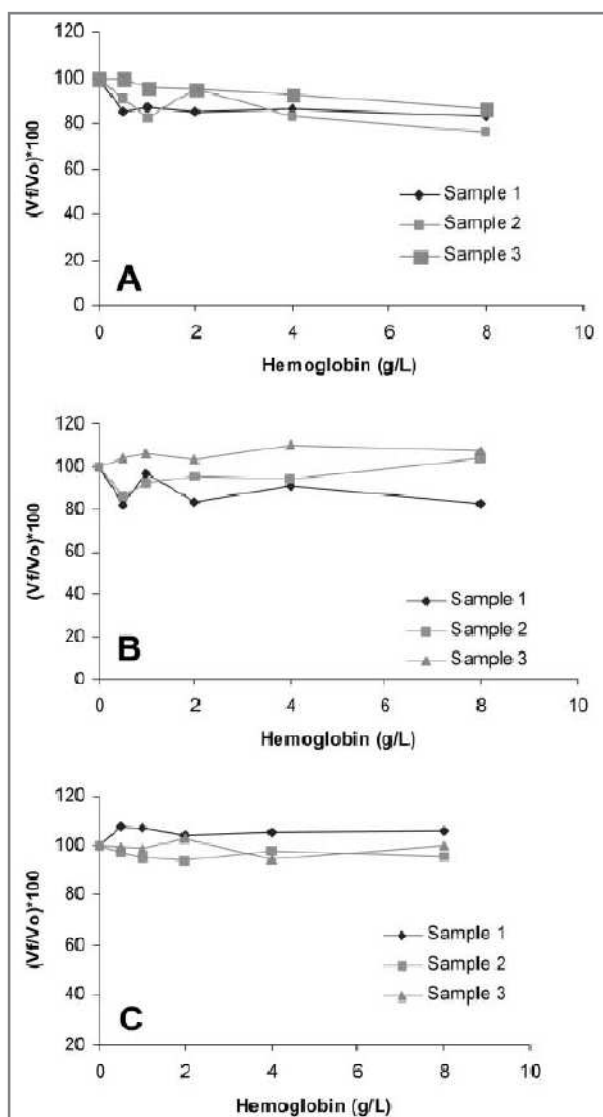


Figure 2—Interferograms corresponding to the effect of hemoglobin concentration on PON1 activity as measured by use of 3 assays: lactonase activity via measurement of TBBL hydrolysis (A), arylesterase activity via measurement of phenyl acetate hydrolysis (B), and arylesterase activity via measurement of p-nitrophenyl acetate hydrolysis (C). Values for each given concentration (V_f) are reported as a percentage of the original value (V_o).

the 3 methods (Table 3). The Spearman correlation test revealed a positive, significant correlation between TACs and results of assays A ($\rho = 0.64$; $P = 0.002$), B ($\rho = 0.49$; $P = 0.027$), and C ($\rho = 0.48$; $P = 0.033$).

Discussion

In the study reported here, 3 spectrophotometric methods for measurement of PON1 activity were evaluated and validated. A different substrate was used in each assay: TBBL for lactonase activity and p-nitrophenyl acetate and phenyl acetate for arylesterase activity. Paraoxon is a highly toxic and unstable compound that is unsuitable for routine high-throughput use,² so measurements of paraoxonase activity of PON1 were not included in the present study. In humans, arylesterase

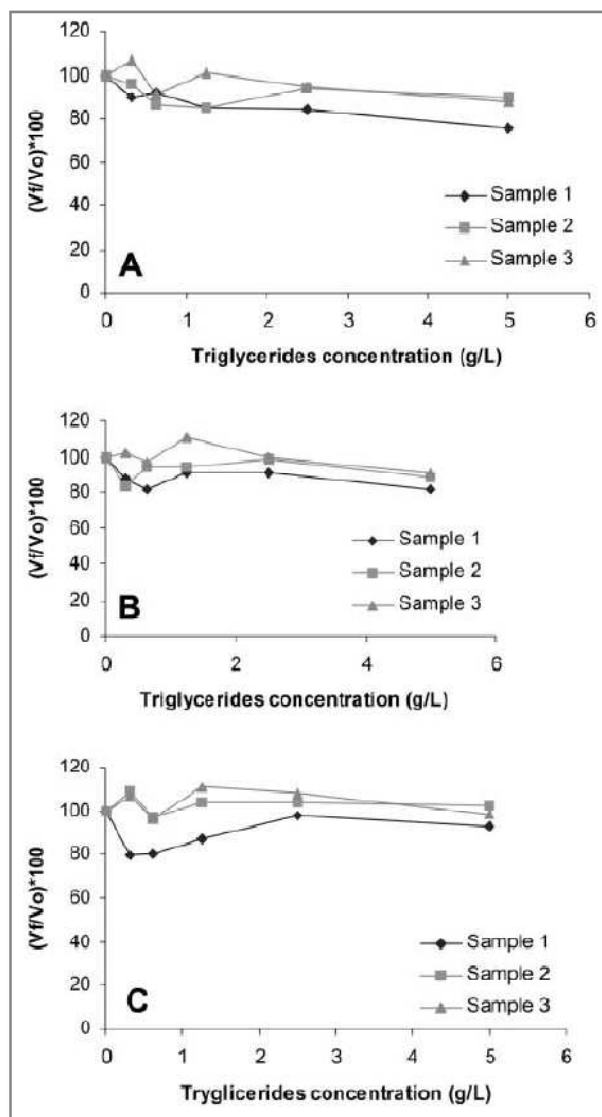


Figure 3—Interferograms corresponding to the effect of triglycerides concentration on PON1 activity as measured by use of the assays in Figure 2. See Figure 2 for remainder of key.

or lactonase activities are less variable between subjects than is paraoxonase activity, which is influenced by genetic variation.^{2,6,10}

Although PON1 was long considered to be an arylesterase and paraoxonase and its activity was measured accordingly, it recently became apparent that PON1 can catalyze the hydrolysis and formation of various lactones. Lactonase activity is the only activity shared by all other members of the PON family, some of which have no paraoxonase or arylesterase activity.¹⁸ Some investigators have consequently postulated that use of phenyl acetate or paraoxon would not have physiologic relevance and have suggested that assays of PON1 activity should mainly address the lactonase activity.¹² However, because the substrate for lactonase activity measurements is not widely available and a correlation between arylesterase and lactonase activities has been reported for humans,⁷ we aimed to validate assays not only for lactonase activity but also for arylesterase activity.

Table 2—Activities of PON1 in serum and EDTA-treated plasma sample aliquots as measured by use of 3 assays.

Assay	Sample	Activity in serum (U/mL)	Activity in plasma with EDTA (U/mL)
A	1	4.59	1.03
	2	4.36	0.46
	3	4.25	1.26
	4	3.67	0.34
	5	4.71	1.95
	6	4.36	0.69
	Mean $\pm$ SD	4.32 $\pm$ 0.36	0.96 $\pm$ 0.60*
B	1	21.28	9.10
	2	21.28	9.10
	3	18.78	9.10
	4	18.78	9.10
	5	20.03	11.27
	6	15.02	9.10
	Mean $\pm$ SD	19.20 $\pm$ 2.33	9.46 $\pm$ 0.89*
C	1	6.51	1.87
	2	4.83	1.09
	3	5.33	4.22
	4	4.92	1.05
	5	5.63	2.38
	6	4.69	1.17
	1	5.32 $\pm$ 0.68	1.96 $\pm$ 1.22*

*Value differs significantly ($P < 0.001$) from value for serum.

Table 3—Mean $\pm$ SD PON1 activity determined by use of 3 assays and TAC in 10 dogs with eccentricity and 22 healthy dogs.

Analyte	Dogs with eccentricity	Healthy dogs	P value
PON1 activity (U/mL)			
Assay A	4.10 $\pm$ 1.22	4.82 $\pm$ 0.50	0.048
Assay B	14.97 $\pm$ 6.13	20.00 $\pm$ 2.37	0.023
Assay C	4.82 $\pm$ 1.44	6.31 $\pm$ 0.68	0.010
TAC (mmol)	0.35 $\pm$ 0.29	0.66 $\pm$ 0.18	0.011

A value of $P < 0.05$ was considered significant.

Initially, we intended to fully automate all 3 methods to improve the assay precision and decrease the time needed to analyze PON1 activity in each sample. However, the assays that involved phenylacetate and lactonase were validated in a 96-well ELISA plate format because the absorbance of phenylacetate at < 340 nm and instability of lactonase in aqueous solutions precluded its adaptation to automated analyzers. Although these assays were not fully automated, analysis involving a 96-well ELISA plate is fast and enables simultaneous processing of large batches of serum samples. In addition, spectrophotometric readers for 96-well ELISA plates are widely available in biochemistry laboratories, so this technology could be suitable for use in veterinary diagnostic laboratories.¹⁹

The validation results showed that the 3 methods were practical, rapid, and allowed evaluation of multiple samples at the same time; they also required a low volume of sample. These characteristics are also important if the measurement is to be included in a battery of several tests or in situations in which sample volume might be limited. Both arylesterase assays had CVs $< 10\%$ in all assessed situations, which is similar to assay validation findings in humans.²⁰ The low CVs suggest a good assay reproducibility and support their suitability for use in other studies. However, the lactonase method

had an interassay CV of 17.7%, which is similar to values reported when the method is used in humans.² Ideally, when lactonase activity is the analyte of interest, serum samples should be analyzed in the same batch to avoid these interassay variations. Perhaps because its suitability for automation diminishes analytic errors, p-nitrophenyl acetate is reportedly more sensitive than other substrates for detecting variations in PON1 activity.²¹ Moreover, p-nitrophenyl acetate is a nontoxic ester that is less volatile than lactone and thus would be more suitable for routine measurement of PON1 activity.²¹

Hemolysis did not result in substantial interferences in the arylesterase assays, and only a significant decrease in serum PON1 activity was evident with the lactonase method at a high hemoglobin concentration (8 g/L). This finding is similar to findings in humans regarding the lactonase assay; in humans, results are not affected by hemolysis when hemoglobin values of < 6 g/L are present,² indicating hemolysis has no influence on PON1 assay results unless it is severe. In lipemic serum samples, significant decreases in PON1 activities were observed with increasing triglycerides concentration with all 3 methods. A negative correlation between serum PON1 activity and triglycerides has been reported for humans.²² In addition, lipemic samples can interfere with many clinical chemistry tests through various mechanisms, the most common of which is the scattering of light rays by the lipids (mainly chylomicrons and very low-density lipoproteins).²³

Results of all the methods used in the present study were highly correlated. In humans, similarly significant correlations exist between results of arylesterase and lactonase assays.⁷ However, results of PON1 activity testing in humans with certain diseases can vary depending of the substrate used, so the simultaneous use of at least 2 substrates to measure PON1 activity is recommended to improve the reliability of the results.²⁴

To test the ability of the methods to detect and differentiate between PON1 activities, we performed in vitro assays to assess the influence of EDTA and oxidative dog illness (eccentricity) on PON1 activity. Reportedly, PON1 has an absolute requirement for Ca^{2+} for activity and stability, so use of EDTA can inactivate PON1.²⁵ Our results are in agreement with previous findings²⁶ that a significant decrease of PON1 activity occurs when EDTA-treated plasma is used instead of serum.

Eccentricity are erythrocytes in which hemoglobin is concentrated on 1 side of the cell, leaving a pale or clear eccentric space. These cells are associated with oxidative injury of erythrocytes in humans and animals.^{27–29} Increased amounts of endogenous oxidants are generated in various disorders, such as inflammation, neoplasia, and diabetes, or when reducing pathways in erythrocytes are defective, as occurs with glucose-6-phosphate dehydrogenase and flavin adenine dinucleotide deficiencies.^{30,31} In the present study, dogs with eccentricity had lower TAC values than healthy dogs, indicating oxidative damage.^{32,33} The decrease of PON1 in dogs with eccentricity in the present study indicates that the validated methods were able to detect lower PON1 values associated with oxidative stress. As

has been reported for humans,³⁴ a significant correlation was detected between PON1 activities and TACs. These data indicate that the PON1 methods described here can be used to evaluate oxidative status in dogs.

The 3 assays described here will allow a wider use of PON1 activity as a biomarker of oxidative stress in dogs because the assays were robust, precise (with the exception of the interassay values for lactonase method), and easy to set up in laboratories. As in human medicine, these assays could allow additional research into PON1 behavior in situations that can alter oxidative status, such as sepsis and renal or chronic liver disease in dogs; eventually, such studies could lead to applications already described for humans, such as the use of PON1 as a prognostic marker of disease progress and recovery in patients with chronic liver disease^{2,35} or sepsis.^{18,36}

- a. TapVal, Aquisel, Barcelona, Spain.
- b. Advia 120 hematology system, Siemens, Italy.
- c. AU 400 automated biochemical analyzer, Olympus, Minneapolis, Minn.
- d. 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt, Sigma-Aldrich Co, St Louis, Mo.
- e. Cobas Mira Plus multiparametric autoanalyser, ABX Diagnostics Inc, Montpellier, France.
- f. (R)-(+)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich Co, St Louis, Mo.
- g. Tris(hydroxymethyl)aminomethane, Sigma-Aldrich Co, St Louis, Mo.
- h. Calcium chloride dihydrate, Sigma-Aldrich Co, St Louis, Mo.
- i. 5,5'-Dithiobis(2-nitrobenzoic acid), Sigma-Aldrich Co, St Louis, Mo.
- j. Dimethyl sulfoxide, Sigma-Aldrich Co, St Louis, Mo.
- k. Acetonitrile, Multisolvant HPLC grade ACS, Sharlau Chemie SA, Sentmenat, Spain.
- l. Microtiter plates, polystyrene 96-wells, Sigma-Aldrich Co, St Louis, Mo.
- m. Provided by Dr. Khersonsky, Weizmann Institute of Science, Israel.
- n. PowerWave XS, Bio-Tek Instruments Inc, Winooski, Vt.
- o. Phenyl acetate, Sigma-Aldrich Co, St Louis, Mo.
- p. 4-nitrophenyl acetate, Sigma-Aldrich Co, St Louis, Mo.
- q. Methanol, Sigma-Aldrich Co, St Louis, Mo.
- r. Coulter CPA, Kemia Cientifica SA, Madrid, Spain.
- s. Lipofundina 20%, Braun Medical SA, Barcelona, Spain.
- t. BD Vacutainer, BD, Madrid, Spain.
- u. GraphPad Prism, version 5 for Windows, Graph Pad Software Inc, San Diego, Calif.

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3.5.1.2. Article 19



Serum butyrylcholinesterase and paraoxonase 1 in a canine model of endotoxemia: Effects of choline administration

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ABSTRACT

Butyrylcholinesterase (BChE) and paraoxonase 1 (PON1) are two serum enzymes synthesized by the liver that are related with inflammation. The main objectives of this study were to determine changes in serum BChE and PON1 by using a canine model of endotoxemia, and to evaluate whether choline alters BChE and PON1 activities during inflammation. For this purpose, a total of 20 mongrel dogs were divided into four groups: control, choline (C), lipopolysaccharide (LPS), and LPS + C. Dogs in the control group were injected with 0.9% NaCl (0.2 ml/kg, i.v.). Dogs in C and LPS + C groups received choline chloride (20 mg/kg, i.v., three times with 4 h intervals). Endotoxin was injected (0.02 mg/kg, i.v., once) to the dogs of LPS and LPS + C groups. Statistically significant decreases in BChE and PON1 activities in LPS group were detected 24 and 48 h post injection, respectively. No statistically significant changes in BChE and PON1 activities at different times were detected in control, C, or LPS + C groups. In conclusion, the data obtained in present study revealed a decrease in serum BChE and PON1 activities in dogs during experimentally induced endotoxemia and that choline administration attenuates these changes.

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1. Introduction

Sepsis, the systemic inflammatory response to infection, is characterized by dysregulated production of cytokines, a pathologic state that causes tissue injury, which leads to organ dysfunction and death. In its early stages, unrestrained production of pro-inflammatory cytokines, such as TNF and IL-1b triggers a systemic inflammatory cascade mediated by chemokines, vasoactive amines, the complement and coagulation systems, reactive oxygen species, and acute phase proteins, among others. Despite intensive research and attempts to improve treatment strategies for sepsis and septic shock, infection remains a major cause of mortality in intensive care units (Balk, 2000).

Butyrylcholinesterase (BChE) and paraoxonase 1 (PON1) are two serum enzymes synthesized by the liver that are related with inflammation (Das, 2007). Both enzymes seem to be functionally related since BChE is inherently protected from oxidative stress by PON1 (Ofek et al., 2007). The physiological function of BChE is unknown in humans and animals; although it has been related with lipid metabolism, obesity and endocrine disorders, such as diabetes mellitus (Iwasaki et al., 2007; Rao et al., 2007), and with

detoxification processes of drugs and organophosphate and carbamate insecticides (Munro et al., 1991; Sakaguchi et al., 1997; Tecles et al., 2002). Increased activity of BChE leads to reduced levels of acetylcholine (ACh) in this way reducing anti-inflammatory actions exerted by this molecule. That, in turn, enhances local and systemic inflammation (Das, 2007). Serum PON1 is an HDL-associated enzyme that protects LDL and HDL particles from oxidative stress by promoting degradation of lipid peroxides contained in cholesterol esters and phospholipids (Mackness et al., 1991) and in this way limiting production of proinflammatory mediators (Watson et al., 1995). A drop in activity of the two enzymes (BChE and PON1) has been observed in naturally occurring sepsis and following LPS application in humans and experimental animals, such as hamsters and rats (Novak et al., 2010; Feingold et al., 1998; Ofek et al., 2007; Draganov et al., 2010).

Choline is an agonist of acetylcholine receptors and a precursor of neurotransmitter acetylcholine (Ulus et al., 1988; Zeisel, 2006). Choline treatment stimulates acetylcholine synthesis and release, increases cholinergic neurotransmission, and activates both the sympathoadrenal and parasympathetic systems (Ilcol et al., 2002, 2003, 2007, 2008; Cansev et al., 2007, 2008); these two branches of the autonomic nervous system are involved in the body's integrated response to endotoxin (Nance and Sanders, 2007; Tracey, 2007; Gallowitsch-Puerto and Pavlov, 2007). Choline stimulates

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phospholipids synthesis, and treatment with choline-containing phospholipids reduces organ injury/dysfunction and improves survival in experimental endotoxemia and/or sepsis (Gordon et al., 2005; Ilcol et al., 2005, 2009).

To the authors knowledge there are no reports about the behavior of BChE and PON1 in sepsis in dogs. In addition, there are no published studies about possible influence of choline administration on these enzymes. So the principal objectives of this study were (1) to determine changes in serum BChE and PON1 by using a canine model of endotoxemia, and (2) to evaluate whether choline alters BChE and PON1 activities during inflammation. Moreover, since the acute phase response is characterized by profound alterations in the hepatic synthesis both of lipids and a number of plasma proteins during inflammation, the relationship between BChE, PON1, acute phase proteins (C-reactive protein – CRP, haptoglobin – Hp and ceruloplasmin – Cp) and lipids was also investigated in this experimental model.

2. Material and methods

2.1. Animal and general procedures

A total of 20 adult healthy mongrel dogs (12 male and eight female) were used in this study. The dogs weighed 14.2–31.3 kg (mean $\pm$ SD, 24.4 $\pm$ 4.8 kg) and ranged from 2 to 5 years (mean $\pm$ SD, 3.6 $\pm$ 1.9 years). The animals have a body condition score (BCS) of three in all cases. BCS was evaluated in accordance with a 5-point scale (McGreevy et al., 2005).

The dogs were housed in individual cages in a controlled room (18–24 °C and 12/12 h light/dark cycle) for 3 days before the experiment at Veterinary Teaching Hospital, Uludağ University, Bursa, Turkey. All dogs were kept in similar conditions, provided ad libitum water, and were fed twice daily with extruded diet (IAMS-Propet, Istanbul, Turkey). The dogs were clinically healthy based in normal clinical examinations and the results of complete blood cell count and biochemistry including C-reactive protein were within reference limit.

The experimental protocol was approved by the Animal Care and Use Committee of the University of Uludağ (protocol No: 2009-01/08).

2.2. Experiments

The dogs were divided into four groups of five dogs (two female and three male) in each: (1) control group, (2) choline (C) group, (3) lipopolysaccharide (LPS) group, and (4) LPS + C group.

Dogs in the control group were injected intravenously (i.v.) via the cephalic veins with 0.9% NaCl (0.2 ml/kg). Dogs in group C received 20 mg/kg of choline chloride in 0.2 ml/kg 0.9% NaCl, i.v., three times with 4 h intervals. Dogs in LPS group received, saline containing endotoxin (LPS) solution (0.2 ml/kg, 0.9% NaCl) i.v. slowly at the dose of 0.02 mg/kg for only once. In LPS + C group, the first choline chloride injection was performed 5 min after LPS infusion, and the second and third choline chloride doses were injected at the same dose, with 4 h intervals (Yilmaz et al., 2006).

The experiment was initiated at 9:00 am, after 14 h of fasting in all groups of dogs. All dogs were monitored clinically for 48 h after the treatment. During this period, dogs were fed four times (at 12, 24, 36 and 48 h just after blood sampling).

2.3. Sample collection and measurements

Clinically body temperature, heart and respiratory rates, and non-invasive mean arterial blood pressure (MAP, BM3-Vet; Bionet Co. Ltd.) were recorded for this study. Venous blood samples were

collected into tubes with and without EDTA (Vacutest EDTA K₃, BD, 3 ml, and Vacutest, BD, 10 ml, Hema & Tube Tic. Ltd. Sti., Ankara-Turkey) before (baseline) and at 0.5, 1, 4, 24 and 48 h post-treatment.

BChE activity was measured by previously reported method by Tecles et al. (2000) and adapted for an automated analyser (Olympus AU2700, Olympus Diagnostica GmbH).

Serum PON1 activity was determined measuring arylesterase activity following a previously described method (Haagen and Brock, 1992) with some modifications (Browne et al., 2007). This method was previously validated in our laboratory for use in dogs (Tvarijonavičiute et al., in press).

CRP concentration was measured using a human immunoturbidimetric assay (CRP OSR 6147 Olympus Life and Material Science Europe GmbH, Lismeehan, O'Callaghan's Mills, Co. Clare, Ireland) that showed a correlation of 0.98 with a specific canine ELISA assay (Tridelta Phase range canine CRP kit, Tridelta Development Ltd., Brey, Ireland) which has been validated for use in dogs (Martinez-Subiela and Cerón, 2005).

Hp concentration was measured by a commercially available colorimetric method (Tridelta Phase range haptoglobin kit, Tridelta Development Ltd.) that was validated previously for use in dogs (Martinez-Subiela and Cerón, 2005).

Serum concentration of ceruloplasmin was measured by use of a spectrophotometric method based on the in vitro oxidase activity of Cp with *p*-phenylenediamine based validated for use in canine samples (Ceron and Martinez-Subiela, 2004).

Total cholesterol, HDL-C, and LDL-C were performed on the automated clinical chemistry analyser (Olympus AU2700, Olympus Diagnostica GmbH) following the instructions of the manufacturer.

2.4. Chemicals

Endotoxin (lipopolysaccharide, *Escherichia coli* serotype 055:B5, purity >97%) and choline chloride were purchased from Sigma Chemical (St. Louis, MO), and dissolved in sterile saline solution (0.9% NaCl) immediately before the experiment. The volume of endotoxin or choline chloride containing solution injected intravenously was 0.2 ml/kg.

2.5. Statistical analysis

Summarized data are shown as mean $\pm$ SD. The results for each parameter were evaluated for approximate normality of distribution by using the Kolmogorov–Smirnov statistics. Because the data were not distributed normally, data were log transformed to assess normality. Repeated measures ANOVA with Newman-keuls Multiple Comparison post test were used to evaluate changes of proteins in dogs' sera in different groups. Spearman correlation test was preceded in order to verify the possible correlations between BChE, PON1, APPs, and lipids. Values of $p < 0.05$ were considered significant.

3. Results

There were no statistically significant alterations of clinical parameters monitored throughout the study in control (saline-treated) dogs (Fig. 1), whereas choline caused increase in respiratory rate (Fig. 1b) and decrease in heart rate (Fig. 1c). Also, choline produced some physiological symptoms of a cholinergic nature such as respiratory stress, hypersalivation, lacrimation and increase in gastrointestinal motility (vomiting and defecation) within 15 min after its intravenous administration. These symptoms disappeared within 1 h following the treatment, and did not recur throughout the study, whereas respiratory rate in response to cho-

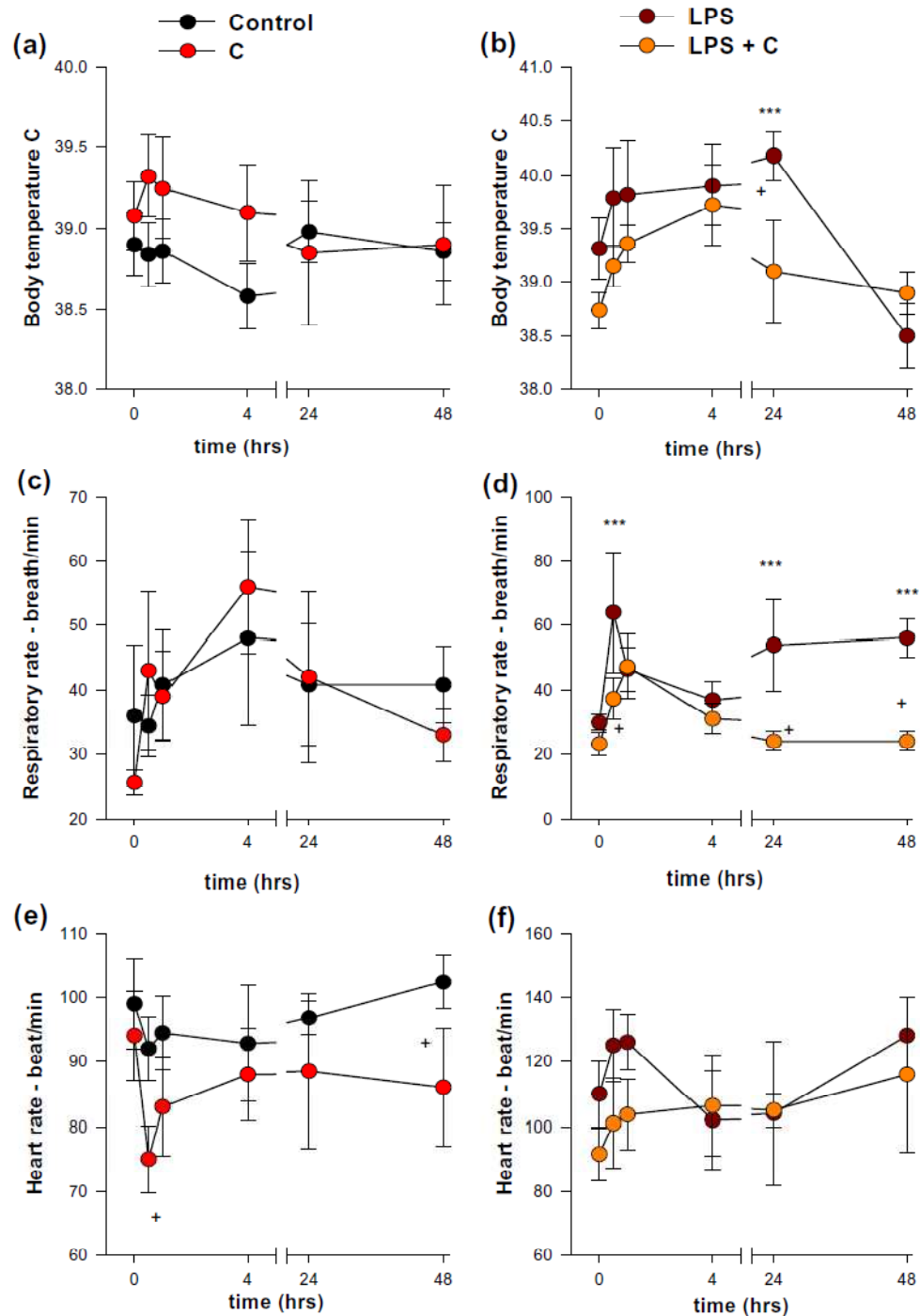


Fig. 1. Changes in clinical parameters (body temperature, and respiratory and heart rates) within and between groups. (a), (c), and (e): Dogs receiving normal saline (control group) or choline chloride (group C); (b), (d), and (f): dogs receiving LPS alone (LPS group) or in combination with choline chloride (LPS + C group). *** $p < 0.001$ When compared with baseline value (0 h), * $p < 0.01$; when compared with respective value from control group or LPS group.

line was stable high to 4 h, and then returned to baseline (Fig. 1c). MAP was increased slightly, but not significantly, from baseline by about 8% at 0.5 h and 6% at 1 h after choline administration (Fig. 2a).

Endotoxin caused a rapid increase in body temperature (Fig. 1b), heart and respiratory rates (Fig. 1d and e), and decrease in MAP by about 25% at 0.5 h ($p < 0.001$) and 22% at 1 h ($p < 0.001$) from its baseline (Fig. 2b). Prolonged capillary refill

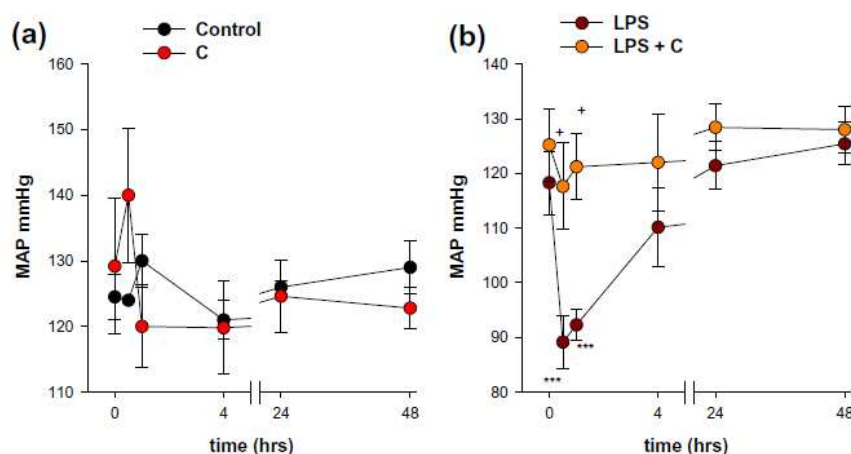


Fig. 2. Changes in mean arterial blood pressure (MAP) measured by non-invasive blood pressure monitor (BM3-Vet; Bionet Co. Ltd.) within and between groups. (a): Dogs receiving normal saline (control group) or choline chloride (group C); (b): dogs receiving LPS alone (LPS group) or in combination with choline chloride (LPS + C group). *** $p < 0.001$ When compared with baseline value (0 h), * $p < 0.001$; when compared with respective value from LPS group.

time, weak peripheral pulse quality, depression, anorexia, vomiting, and diarrhea were also observed within 0.5 h after LPS injection. The severity of clinical and haematological findings such as marked leucopenia (data not shown) increased during 1 h after LPS administration. Heart and respiratory rates as well as body temperature returned to baseline limits within 48 h. Two dogs of LPS group were still depressive and had mild diarrhea at 48 h.

Choline administration protected or attenuated the clinical abnormalities in response to endotoxin in LPS + C group (Fig. 1b, d, and f; Fig. 2b). There were significant differences on body temperature at 24 h ($p < 0.001$, Fig. 1b), respiratory rate at 0.5–48 h ($p < 0.05$ – $p < 0.001$, Fig. 1d), heart rate at 0.5–1 h ($p < 0.05$, Fig. 1f), and MAP at 0.5–1 h ($p < 0.001$, Fig. 2b) between groups; LPS vs LPS + C.

Changes of the analytes measured in the study in the four groups appear in Fig. 3. A statistically significant decrease in BChE activity in LPS group was recorded 24 h post injection (2.34 ± 1.2 UI/L) when compared with BChE activity recorded at time 0 (3.36 ± 1.1 UI/L, $p < 0.05$). No statistically significant changes in BChE activity at different times were detected in control, C, or LPS + C groups.

A statistically significant decrease in PON1 activity was recorded in LPS group 48 h post injection (2.36 ± 0.6 UI/L) when compared with PON1 activity recorded at time 0, 0.5, 1, 4, and 24 h post injection (2.70 ± 0.6 UI/L, $p < 0.05$; 2.75 ± 0.4 UI/L, $p < 0.05$; 2.90 ± 0.6 UI/L, $p < 0.05$; 3.20 ± 0.8 UI/L, $p < 0.01$; 2.96 ± 1.0 UI/L, $p < 0.05$; respectively). No statistically significant changes in PON1 activity were detected in control, C, or LPS + C groups.

No variations in CRP, Hp or Cp were detected in the control or choline groups. In LPS and LPS + C groups a significant increase in CRP started to appear at 4 h and reached a peak at 24 h, with a 27 and 14.6-fold increase, respectively. In LPS and LPS + C groups, Hp started to increase significantly at 24 h and reached a peak at 48 h with a 1.8 and 2.4-fold increase, respectively. Statistically significant increase in Cp levels was detected 48 h after injection with a 1.9 and 1.4-fold increase in LPS and LPS + C groups of dogs, respectively.

Table 1 shows Spearman correlation coefficients and significance between the two enzymes (BChE, PON1) and other analytes (APPs, TC, HDL-C, and LDL-C). Positive correlation was observed between BChE and PON1 ($r = 0.494$, $p < 0.0001$). BChE activity was negatively correlated with CRP and Cp, and positively with TC, HDL-C, and LDL-C. Weak negative correlation was detected

between PON1 activity and Cp, and positive with TC, HDL-C, and LDL-C.

4. Discussion

Sepsis is the leading cause of death in critically ill patients (Balk, 2000). The pathophysiological mechanisms implicated in the development of sepsis and organ failure are complex and involve activation of systemic inflammatory response syndrome (SIRS) and coagulation together with endothelial dysfunction. Endotoxemia experimental models have been extensively used for induce an inflammatory response in the dog, since is useful in replicating the signs and laboratory findings observed in human sepsis/endotoxemia which is a common an serious condition in intensive care unit patients worldwide (Yilmaz et al., 2006; Ilcol et al., 2005). LPS, a heat-stable endotoxin, initiates consecutive intracellular events in immune, endothelial and neuroendocrine cells in sepsis. While pro-inflammatory mediators (TNF- α , etc.) eradicate invading microorganisms by initiating acute phase reaction, anti-inflammatory mediators (IL-4, etc.) adapt the immune system and leukocyte responses to this new condition in order to take these reactions under control (Van Amersfoort et al., 2003). Using LPS induced endotoxemia as an experimental model, we aimed to increase the knowledge about the behavior of BChE and PON1 in inflammation in dogs that has been an unexplored field until now.

In the present study, endotoxemia was characterized by increased body temperature and respiratory rate, and decreased MAP and circulating white blood cell count, 0.5 h after LPS administration. These findings are in accordance with previous studies (Yilmaz et al., 2010; Ilcol et al., 2005, 2009), and indicate the presence of SIRS in LPS-treated dogs. Choline administration, on the other hand, reversed the enhancement in body temperature and respiratory rate, and the reduction in MAP observed in dogs receiving LPS. Although choline alone reduced heart rate in normal dogs, it did not affect the heart rate significantly when combined with LPS administration, possibly because the effects of the two agents negate each other to produce a relatively normal hearth rate. These observations suggest that choline treatment, at least in the dose range used in our study, does not cause a cardiovascular collapse in LPS-induced experimental endotoxemia.

The increase of CRP after LPS injection that reached significance at 4 h and peaked at 24 h with a more than 10-fold increment and

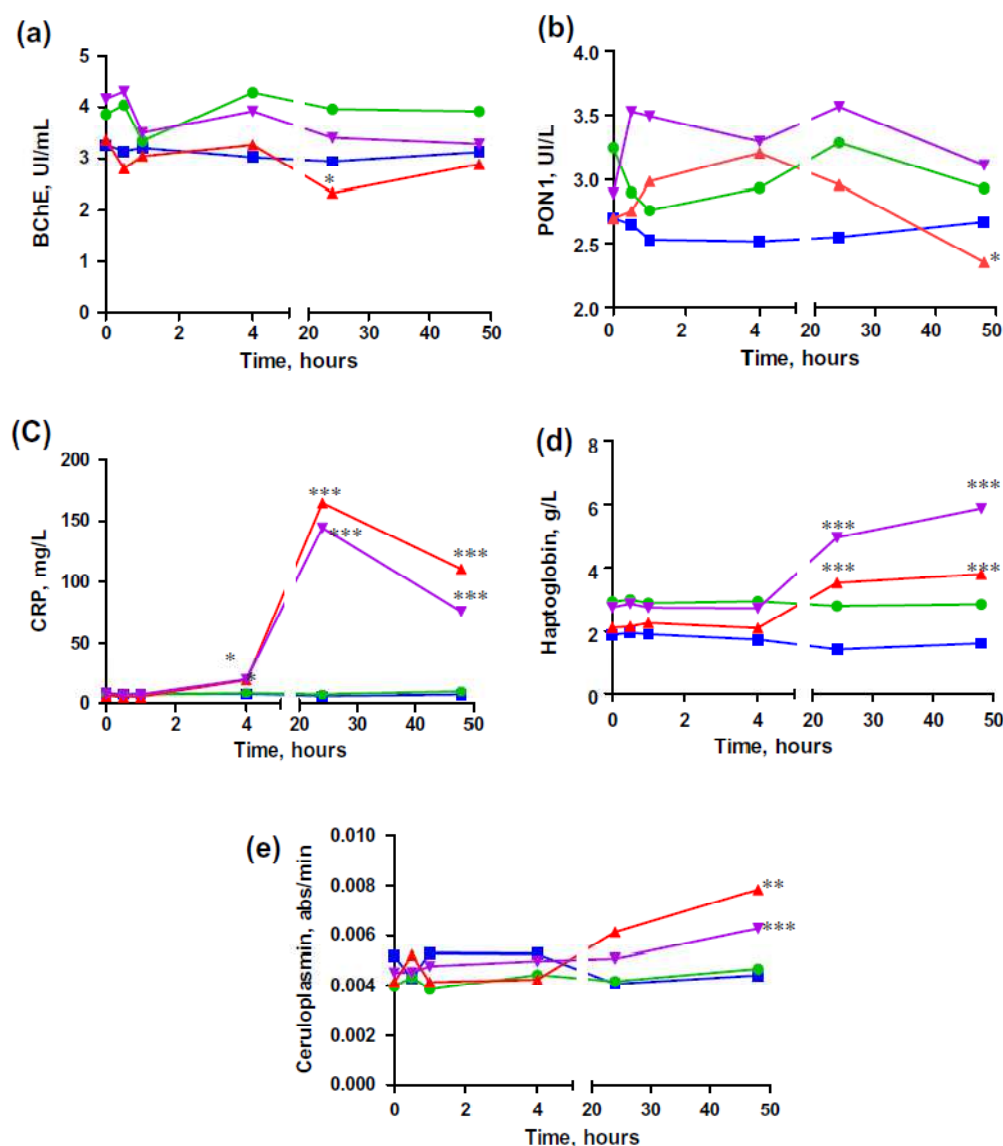


Fig. 3. Variations of BChE (butyrylcholinesterase) (a), PON1 (paraoxonase) (b), CRP (C reactive protein) (c), Haptoglobin (d), and Ceruloplasmin (e) levels during experimental period in dogs treated with 0.9% NaCl sol. (Control group, ■), in dogs that received choline (C group, ●), in dogs injected with toxin (LPS group, ▲), and in dogs that received choline and toxin injection (LPS + C group, ▼). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs time 0.

Table 1
Spearman's correlation coefficients and significances within the whole group.

Analyte	Number	BChE		PON1	
		R	P	R	P
CRP	120	-0.316	0.0007	-0.099	0.3
Hp	120	0.1558	0.1	0.14	0.14
Cp	120	-0.401	<0.0001	-0.226	0.017
TC	120	0.311	0.004	0.449	<0.0001
HDL-C	120	0.352	0.001	0.494	<0.0001
LDL-C	120	0.281	0.0096	0.448	<0.0001
BChE	120			0.494	<0.0001

CRP – C reactive protein; BChE – butyrylcholinesterase; PON-1 – paraoxonase-1; Hp – haptoglobin; Cp – ceruloplasmin; TC – total cholesterol; HDL-C – high density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol.

the increases in Hp and Cp that peaked later, at 48 h, with a lower than 2-fold increment (Fig. 3), indicated that an inflammatory acute phase response was produced in our experimental model, as evidenced by the behavior of these proteins which are major (CRP) and moderate (Hp) positive acute phase proteins (Ceron et al., 2005).

In our work, a significant decrease in BChE in endotoxemia in dogs appeared at 24 h. Moreover, a negative significant correlation between BChE and CRP and Cp was detected. These findings agree with the decreases in BChE described in previous reports with sepsis or infection in humans. A significant decrease in the level of cholinesterase has been found in human patients with systemic sepsis syndrome (septic shock) in an intensive care unit (Al-Kassab and Vijayakumar, 1995). Similarly a decrease in BChE activity was observed in patients with postoperative infections (Blanceil and Delaroche, 1996). The inflammatory reaction associated to a path-

ogenic stimulus is followed by a fast and crucial anti-inflammatory response, defined as compensatory anti-inflammatory response syndrome – CARS (Adib-Conquy and Cavaillon, 2009). This limits the inflammatory processes below a certain threshold, ascertaining survival and avoiding autoimmune diseases or spreading of the inflammatory components into the bloodstream, which may lead to septic shock (Tracey, 2002). Neither the origin(s) of individual variability in response to inflammation, nor the underlying mechanism(s) are fully understood. However, cholinergic signaling is involved in anti-inflammatory reactions, mediated by acetylcholine an inhibitor of the activation of macrophages and release of pro-inflammatory cytokines (Tracey, 2002). The decrease of BChE observed at 24 h after the stimulus in our study could be an organic reaction with the purpose of increasing the acetylcholine levels and limiting the inflammatory response (Rosas-Ballina and Tracey, 2009).

Oxidative stress is a major promoter and mediator of the systemic inflammatory response. Serum PON1 has been demonstrated in multiple clinical and animal studies to protect against oxidative stress, but also to undergo inactivation upon that condition (Draganov et al., 2010). In present study we found a decrease in PON1 activity in LPS treated dogs at 48 h. It was suggested that PON1 is predominantly expressed in the liver and is carried in plasma bound to HDL, which provides the optimal acceptor complex in terms of both stimulating PON1 secretion and/or stabilizing the secreted PON1 protein. Changes in lipid and protein composition of HDL in response to inflammation influence PON1 activity and function (James and Deakin, 2004). During the acute phase response, HDL is losing apolipoprotein A1, esterified cholesterol, and most of the HDL-associated enzymes (including PON1). PON1 is replaced mainly by serum amyloid A with the concomitant loss of HDL anti-oxidative properties (Van Lenten et al., 1995). A dose-dependent decrease in serum PON1 activity and liver PON1 expression (36–62%) during sepsis has been demonstrated in the serum of Syrian hamsters, a model of Gram-negative bacterial infection, within 24 h following LPS treatment (Feingold et al., 1998). Moreover, administration of tumor necrosis factor- α and interleukin-1 cytokines without LPS treatment moderately decreased serum PON1 activity and PON1 mRNA levels in the liver, indicating a partial direct negative effect of these cytokines on PON1 expression (Feingold et al., 1998). Additionally, Novak et al. (2010) describe PON1 as a negative acute phase protein as observed decreased PON1 activity during inflammation and detected negative correlation between PON1 activity and CRP levels. In the present study, PON1 activity did decrease during inflammation, but statistically significant negative correlation was detected only between PON1 and ceruloplasmin.

BChE as well as PON1 positively correlated with total cholesterol, HDL-C, and LDL-C. These results are in accordance with data reported in human medicine (Novak et al., 2010; Iwasaki et al., 2007). It has been proposed that BChE may be involved in lipid metabolism and flux of free fatty acids between adipose tissue and liver, thus regulate adiposity (Iwasaki et al., 2007). Moreover, significant positive correlation between the two enzymes studied in present study has been observed, confirming a functional relationship between BChE and PON1 during inflammation in dogs.

In the present study no statistically significant changes in BChE and PON1 activities were detected in dogs that received endotoxin and choline in comparison with the decrease found in both enzymes in LPS group. Even a trend of BChE and PON1 activities to increase was observed when choline was administered. The mechanism(s) that are responsible of the observed effect of choline on BChE and PON1 activities are not clear. The choline protective effect over liver in the dog by restore endotoxin-induced hypotension and improve tissue perfusion during endotoxemia could be one of the reasons by these enzymes do not decrease after LPS (Il-

col et al., 2005). Also, it was described that cholinesterase decreases during inflammation in order to increase acetylcholine, an inhibitor of pro-inflammatory cytokines, and in this way limits the inflammatory response (Rosas-Ballina and Tracey, 2009). It could be postulated that lack of decrease in BChE activity found after choline administration in sepsis is due to the attenuation in the inflammatory response that is produced by choline (Ilcol et al., 2005). One sign of this response could be the lower magnitude of CRP and Cp increments in LPS + C group in comparison with LPS group indicating a reduced inflammatory reaction. Additionally, choline administration could attenuate the decrease of PON1 activity during inflammation due to its inhibitory effect of the release of tumor necrosis factor- α (TNF- α) from the Kupffer cells (Rivera et al., 1998; Ilcol et al., 2005); cytokine that has been demonstrated to have a direct negative effect on PON1 mRNA levels in the liver (Feingold et al., 1998).

It can be observed that choline prevented LPS-mediated decreases in BChE and PON1 activity, but comparatively seemed to have little ability to mitigate CRP, ceruloplasmin and haptoglobin increases. Although choline has been shown to inhibit TNF- α and IL-6 (Ilcol et al., 2005; Murch et al., 2008) it would be interesting in the future to perform a complete panel of cytokines in these situations in order to evaluate if selective inhibition of these mediators could be involved in the variability of the response.

In conclusion, the data obtained in present study revealed a decrease in serum BChE and PON1 activities in dogs during experimentally induced endotoxemia and that choline administration attenuates these changes. These findings could be related with the improvements of the endotoxin-induced symptoms in dogs when choline is administered.

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3.5.1.3. Article 20



Relationship between serum butyrylcholinesterase and obesity in dogs: A preliminary report

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ABSTRACT

The aim of this study was to analyse serum butyrylcholinesterase (BChE) values in experimentally developed obesity in Beagle dogs. A short-term fattening protocol was applied to 11 dogs to obtain a wide range of bodyweight (BW) gains and body condition scores (BCS) of 4 and 5; four other dogs with BCS scores of 3 were used as controls. A significant increase in serum BChE activity in overweight dogs was observed when compared with the group of optimal weight dogs. Significant correlation was detected between BChE and BCS ($r = 0.911$), BW ($r = 0.538$) and morphological parameters (waist and thorax circumference, $r = 0.563$ and $r = 0.552$, respectively). Serum BChE concentration had a negative correlation with adiponectin concentration ($r = 0.719$) and a positive correlation with serum lipid profile (cholesterol ($r = 0.781$), HDL-cholesterol ($r = 0.763$), LDL-cholesterol ($r = 0.878$)). It was concluded that serum BChE activity is increased in experimentally overweight dogs and is correlated with other physical and biochemical markers of obesity.

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Introduction

Two classes of cholinesterase (ChE) enzyme that are capable of hydrolysing acetylcholine exist in the blood of most mammals in various proportions. True cholinesterase or acetylcholinesterase (AChE, EC 3.1.1.7) is a specific ChE, which hydrolyses acetylcholine and propionylcholine (but not butyrylcholine) and is present at high levels in brain, nerve and red blood cells. The other type, called butyrylcholinesterase (BChE, EC 3.1.1.8), is a non-specific ChE (also called pseudocholinesterase) that hydrolyses butyrylcholine at higher rate than acetylcholine and propionylcholine.

BChE is produced and secreted by the liver and is present in the serum, pancreas, and central nervous system (Kaplay, 1976; Jope et al., 1985; Dass et al., 1994). Both AChE and BChE are inhibited by organophosphate and carbamate insecticides, although they can have different affinity for specific inhibitors: e.g., tetraisopropylpyrophosphoramidate or phenothiazine derivatives are selective BChE inhibitors (Augustinsson et al., 1978).

In veterinary medicine, ChE has attracted attention as a bioscavenger of drugs and organophosphate and carbamate insecticides (Munro et al., 1991; Atkinson et al., 1994; Sakaguchi et al., 1997). However, studies in humans have reported significant associations between serum BChE activity and parameters of adiposity, serum lipid profile and glucose metabolism, as well as a potential involvement of BChE activity in lipid metabolism in adipose tissue

(Magarian and Dietz, 1987; Alcatara et al., 2003; Randell et al., 2005; Calderon-Margalit et al., 2006; Iwasaki et al., 2007).

The objective of the present study was to evaluate whether there is a relationship between serum BChE and obesity in dogs. For this purpose, serum BChE activity was correlated with morphological (bodyweight [BW], body condition score [BCS], thorax and waist circumference) and biochemical biomarkers of adiposity, such as adiponectin and serum lipid profile (including total cholesterol, high density lipoprotein cholesterol [HDL-C], low density lipoprotein cholesterol [LDL-C], triglycerides [TG]) in Beagle dogs with different weights and body conditions obtained after an experimental short-term induced overweight trial.

Materials and methods

Animals and experimental set-up

A total of 15 adult intact, university-owned Beagle dogs were used in this study (7 females, 8 males; age range 5.1–7.4 years; BW range 12.4–18.6 kg). Dogs were maintained in their usual kennel (1 or 2 dogs per kennel) under controlled temperature ($23 \pm 2^\circ\text{C}$) and light (lights on at 08:00–20:00 h). The size of each kennel was 3×4 m.

To obtain a wide range of weights and body conditions, the protocol described by Sagawa et al. (2002) was used with some modifications. Dogs were separated into two groups and maintained for 10 weeks as follows: group A (4 dogs; 2 males, 2 females) were fed a standard dry food (Premium Croc Adult, Affinity Petcare), containing 24.0% crude protein, 16.1% crude fat, 2.5% crude fibre, 6% crude ash, 8% moisture; group B (11 dogs; 6 males, 5 females) were fed a high-energy diet (Advance Performance, Affinity Petcare) containing 31.0% crude protein, 21.0% crude fat, 2.0% crude fibre, 6.5% crude ash, 8% moisture. For all dogs food and water were available *ad libitum*.

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The assessment of the nutritional condition was based on a 5-scale BCS: 1, thin; 2, lean; 3, optimal; 4, obese; 5, gross (Laflamme, 1997). At the beginning of the study all dogs in group A had optimal weight (BCS 3); in group B, seven dogs (4 males and 3 females) had optimal weight (BCS 3) and four dogs (2 males and 2 females) were obese (BCS 4).

All dogs passed general health examinations weekly throughout and complete blood count and biochemical profiles were performed at the beginning of the study confirmed that there were no apparent abnormalities (except obesity). All animals had a negative serological titre for *Leishmania infantum* and *Ehrlichia canis*. Animal care and procedures were in accordance with the guidelines of the University of Murcia.

Blood samples for BChE and the other biochemical assays were collected from all dogs at the end of the experimental period. Collections were made in the morning (after an overnight fast of at least 12 h) by venipuncture of the cephalic vein into tubes containing clotting accelerator (TapVal, Aquisel). Samples were centrifuged at 2000 g for 10 min at room temperature to obtain serum, which was stored in plastic vials at -20°C until analysis. On the day of analysis, samples were brought to room temperature and thoroughly vortexed prior to measurements.

Morphological parameters (thorax and waist circumference) were measured on the morning after blood sample collection. The circumference of the thorax was measured just behind the front legs at the widest point. Waist circumference was measured just in front of the back legs (Burkholder and Toll, 2000).

Biochemical parameters

Serum BChE activity was measured using a previously reported method by Tecles et al. (2000) using butyrylthiocholine iodide as substrate and adapted for an automated analyser (Olympus AU2700, Olympus Diagnostica). The activity of BChE, which represents the total amount of ChE in dog serum, could be quantified (Tecles et al., 2000). Plasma total cholesterol, HDL-C, LDL-C, TG and glucose were also measured using the analyser and following the instructions of the manufacturer. Plasma adiponectin concentration was determined by human high sensitivity adiponectin ELISA (Human Adiponectin ELISA, High Sensitivity Kit, BioVendor-Laboratori medicina), previously validated in our laboratory.

Statistical analysis

Summarised data are shown as means $\pm$ SD. Data were log transformed to assess normality. Unpaired *t* tests were used to compare levels of ChE activity, morphological parameters and biochemical biomarkers of adiposity in different groups. A paired *t* test was used to compare changes of BW at the beginning and the end of the study in the same groups. Correlations between variables were estimated using Pearson's correlation coefficient. $P < 0.05$ was considered significant.

Results

The feeding protocol produced a wide range of weight gains and BCS. The differences allowed us to divide the dogs into three groups based on different BCS: group A had four dogs (2 males, 2 females) with BCS 3, group B had five dogs (3 males, 2 females) with BCS 4, and group C had six dogs (3 males, 3 females) with BCS 5. Results for weight gain and BCS are shown in Table 1. In

Table 1

Mean bodyweight and mean body condition score of dogs in groups A, B and C. Dogs in group A were fed a standard dry food, whereas in groups B and C they were fed high-energy food. BCS was expressed on 5-point scale: 1, thin; 2, lean; 3, optimal; 4, obese; 5, gross.

Dogs	Week 0	Week 10	P
Group A			
M/F	2/2		
BW (kg)	13.8 $\pm$ 1.7	14.5 $\pm$ 1.7	NS
BCS	3	3	
Group B			
M/F	3/2		
BW (kg)	13.9 $\pm$ 1.3	15.7 $\pm$ 1.1	0.019
BCS	3	4	
Group C			
M/F	3/3		
BW (kg)	14.4 $\pm$ 2.4	19.15 $\pm$ 3.5	0.002
BCS	3	5	

M/F, male/female; BW, bodyweight; BCS, body condition score; NS, not significant.

group A there were no significant differences in the BW between week 0 and week 10. In groups B and C a significant increase in BW was observed ($P = 0.019$ and $P = 0.002$, respectively). Dogs in group C showed higher food consumption than those in group B.

Table 2 shows the values of the morphological measurements and biochemical analytes of the different groups of dogs. A significant increase in plasma BChE activity in both groups of overweight dogs was observed, when compared with the group of optimal weight dogs. Plasma BChE activity in the optimal weight dogs (BCS 3) was 3.1 ± 0.5 kU/L, in obese dogs (BCS 4) 3.8 ± 0.5 kU/L, and in gross dogs (BCS 5) 5.4 ± 0.5 kU/L. Plasma adiponectin concentrations were lower, whilst concentrations of lipid (total cholesterol, HDL-C, LDL-C and TG), as well as values of morphological measurements (BW, thorax and waist circumference) were higher in groups B and C compared with group A, but the differences were only significant between groups A and C (Table 2).

The relationship between plasma BChE and other variables determined at the end of the feeding period are listed in Table 3. A significant correlation was detected between ChE and BCS ($r = 0.911$; $P < 0.001$), BW ($r = 0.538$; $P < 0.047$) and morphological parameters (waist and thorax circumference; $r = 0.563$; $P < 0.05$ and $r = 0.552$; $P < 0.05$, respectively). Plasma BChE concentration had a significant negative correlation with adiponectin ($r = -0.719$; $P < 0.005$), and positive correlation with serum cholesterol profile (cholesterol ($r = 0.781$; $P < 0.001$), HDL-C ($r = 0.763$; $P < 0.005$), LDL-C ($r = 0.878$; $P < 0.001$), but not with plasma concentrations of TG and glucose (Table 3).

Table 2

Values of the morphological measurements and biochemical analytes of the groups A, B and C.

Dogs	Week 10	P
Group A		
BW (kg)	14.5 $\pm$ 1.7	
Thorax circumference (cm)	59.0 $\pm$ 3.3	
Waist circumference (cm)	50.3 $\pm$ 4.0	
ChE (kU/L)	3.1 $\pm$ 0.3	
Adiponectin (ng/mL)	14.3 $\pm$ 1.2	
Cholesterol (mg/dL)	147.2 $\pm$ 22.3	
HDL-C (mg/dL)	118.8 $\pm$ 15.6	
LDL-C (mg/dL)	31.3 $\pm$ 4.1	
TG (mg/dL)	38.7 $\pm$ 9.6	
Glucose (mg/dL)	106.3 $\pm$ 7.6	
Group B		
BW (kg)	15.7 $\pm$ 1.1	NS
Thorax circumference (cm)	59.7 $\pm$ 2.2	NS
Waist circumference (cm)	52.4 $\pm$ 4.4	NS
ChE (kU/L)	3.82 $\pm$ 0.5	<0.05
Adiponectin (ng/mL)	13.2 $\pm$ 1.6	NS
Cholesterol (mg/dL)	168.3 $\pm$ 23.2	NS
HDL-C (mg/dL)	132.8 $\pm$ 18.2	NS
LDL-C (mg/dL)	44.4 $\pm$ 8.5	NS
TG (mg/dL)	40.7 $\pm$ 6.4	NS
Glucose (mg/dL)	97.0 $\pm$ 12.2	NS
Group C		
BW (kg)	19.15 $\pm$ 3.5	<0.05
Thorax circumference (cm)	65.6 $\pm$ 4.3	<0.05
Waist circumference (cm)	59.3 $\pm$ 4.5	0.05
ChE (kU/L)	5.4 $\pm$ 0.5	<0.0005
Adiponectin (ng/mL)	8.1 $\pm$ 2.3	<0.001
Cholesterol (mg/dL)	235.2 $\pm$ 21.5	<0.001
HDL-C (mg/dL)	169.6 $\pm$ 11.1	<0.001
LDL-C (mg/dL)	87.6 $\pm$ 16.7	<0.0005
TG (mg/dL)	59.4 $\pm$ 19.1	NS
Glucose (mg/dL)	100.5 $\pm$ 6.8	NS

BW, bodyweight; BCS, body condition score; ChE, cholinesterase; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides. Significance compared to group A ($P < 0.05$); NS, not significant.

Table 3Correlation coefficients (*r*) between plasma BChE and other analytical variables at week 10.

Variable	<i>n</i>	<i>r</i>	<i>P</i>
BW (kg)	15	0.538	0.047
BCS	15	0.911	<0.001
Thorax circumference (cm)	15	0.552	0.041
Waist circumference (cm)	15	0.563	0.036
Plasma adiponectin	15	−0.719	0.004
Plasma total cholesterol	15	0.781	0.001
Plasma HDL-C	15	0.763	0.002
Plasma LDL-C	15	0.878	<0.001
Plasma TG	15	0.519	0.057
Plasma glucose	15	−0.081	0.782

BW, bodyweight; BCS, body condition score; ChE, cholinesterase; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides.

Discussion

The primary objective of this study was to analyse the possible relationship between serum BChE and obesity in dogs. Increased serum BChE activity of 19.7% and 42.9% in dogs with BCSs 4 and 5 was observed when compared with optimal weight dogs (BCS 3), but did not exceed the reported serum BChE reference range of 3.41–6.56 kU/L previously established for healthy dogs (Abdelkader and Hauge, 1986; Furlanello et al., 2006). Similarly, increases in BChE have been reported in obese humans compared with those of optimal bodyweight (9.0 kU/L vs. 8.1 kU/L) (Calderon-Margalit et al., 2006), although all values were inside the reference range established in humans for BChE (4.6–14 kU/L) (Vaisi-Raygani et al., 2007).

The significant correlation between serum BChE and BW, BCS, waist circumference, and thorax circumference could suggest a direct relationship between BChE activity and obesity in dogs. Similar results were published in human medicine, where an association of BChE with waist circumference and body mass index has been reported (Alcatara et al., 2003; Randell et al., 2005). Morphological parameters (waist and thorax circumference) are relatively homogeneous in humans in contrast to dogs of different breeds (German, 2006). For this reason, only dogs from one breed were included in present study.

Serum BChE activity was significantly positively correlated with total cholesterol, HDL-C and LDL-C, and a positive trend was found in the correlations between TG concentration and serum BChE activity – findings that are consistent with those described in humans (Randell et al., 2005). As a possible explanation for the relationship between BChE and lipids, it has been proposed that an incremental flux of free fatty acids from adipose tissue to the liver might stimulate the hepatic synthesis of plasma BChE (Cuauianu et al., 2002). In present study, there was no correlation between serum BChE and glucose. This result is also in agreement with reports evaluating humans, in which no association between BChE and glucose was found in healthy people, and only correlation between both analytes have been detected in case of diabetic patients (Calderon-Margalit et al., 2006).

A significant negative correlation between serum BChE and adiponectin concentrations was found in the present study. Adiponectin has been investigated as a good index of adiposity in dogs, as serum adiponectin concentrations decrease with weight gain (Ishioka et al., 2006; Brunson et al., 2007). The correlation between BChE and adiponectin is further evidence of the association between obesity and BChE activity in dogs.

The main limitations of this study are the relatively low number of animals used and the use of only one breed. However, the number of dogs is in line with other experimental studies of obesity in dogs (Ishioka et al., 2002; Jeusette et al., 2006; Briand et al., 2008).

In addition, this was a preliminary study and we wanted to limit the possible confounding effect of breed variations, especially in morphological measurements. Further studies with a higher number of animals and a variety of breeds will be necessary to evaluate if serum BChE keeps a similar correlation with obesity markers and if it could be an useful biomarker of obesity in dogs in routine practice.

Conclusions

Serum BChE activity was increased in overweight Beagle dogs fed a high-energy diet and correlated with other obesity markers, such as changes of morphological parameters, increment of serum lipids (total cholesterol, HDL-C, LDL-C) and decrease of adiponectin concentrations.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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3.5.1.4. Article 21

Brief Communication

Acetylcholinesterase and butyrylcholinesterase activities in beagle dogs before and after weight loss.

Running head: AChE and BChE in dogs before and after weight loss

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Keywords: Acetylcholinesterase, Butyrylcholinesterase, Dog, Obesity, Weight loss.

Abstract

Background: Obesity is the most common nutritional disorder in today's dog population and the major risk factor for a number of diseases. However, the pathogenesis of this relationship is still not clear. Butyrylcholinesterase (BChE) in humans has been associated with the lipoprotein metabolism and pathogenesis of inflammation; factors that could be involved in the development of obesity related diseases.

Objectives: To study the possible effects of experimentally induced weight loss on BChE and acetylcholinesterase (AChE) in dogs in order to increase the knowledge about the possible relationship between these enzymes and obesity.

Methods: Six, female, obese, non-castrated, beagle dogs remained in a weight loss programme for 3 months. BChE was measured in serum samples using butyrylcholine as substrate, while AChE was measured in whole blood samples after BChE has been inhibited with ethopropazine, using acetylcholine as a substrate.

Results: Serum BChE showed a statistically significant decrease ($p < 0.05$), whereas, a highly significant increase ($p < 0.01$) was detected in AChE in dogs after rapid weight loss. A significant positive correlation was detected between serum BChE activity and levels of total cholesterol ($r = 0.745$), LDL-C ($r = 0.752$), HDL-C ($r = 0.744$), and TG ($r = 0.602$). A significant negative correlation was detected between serum BChE and AChE activities ($r = -0.826$), and between AChE activity and serum levels of total cholesterol ($r = -0.634$), LDL-C ($r = -0.687$), and HDL-C ($r = -0.560$).

Conclusions: Short-term weight loss in the experimental conditions of this study did produce different effects in the two cholinesterase isoenzymes, with significant decreases in BChE and increases in AChE activities.

Approximately 25 to 41% of dogs receiving veterinary care are overweight or obese.^{1,2} Obesity in dogs is associated with increased risk for other diseases, such as diabetes, pancreatitis, dyslipidemia, osteoarthritis, hypertension, altered kidney function, and respiratory distress.³

Mammalian blood contains at least two types of cholinesterase (ChE): erythrocyte cholinesterase (EC 3.1.1.7), also known as acetylcholinesterase (AChE), which is characterized by high activity in brain, nerve and red blood cells, and plasma cholinesterase (PsChE; EC 3.1.1.8), also known as butyrylcholinesterase (BChE), which is mainly synthesized in the liver with posterior distribution to several parts of the organism, being more abundant in plasma.⁴ AChE catalyzes hydrolysis of acetylcholine within cholinergic synapses of the brain and autonomic nervous system. Although BChE shares some of these functions, its biological role has not been clearly established.⁵ It has been reported that BChE may be involved in lipoprotein metabolism and pathogenesis of inflammation.^{6,7}

Recent studies in humans have described significant associations of serum BChE activity with the body weight, body mass index, and different serum analytes related with adiposity, such as triglycerides or cholesterol.⁸⁻¹⁰ In addition, the increase of serum BChE activity in experimentally induced obesity in dogs has been reported.¹¹ However, to the authors knowledge no studies about possible effects of an induced weight loss programme on BChE activity in dogs have been published. Additionally, there are no reports that evaluate if there is also a relation between the other type of cholinesterase, AChE, and obesity in this species.

We hypothesized that weight loss could produce changes in BChE and AChE activities in dogs. So the aim of this study was to evaluate the possible variations in serum BChE and whole blood AChE activities after an experimentally induced rapid weight loss in dogs.

A total of six adult female obese, non-castrated beagle dogs (age range, 5.2 – 6.5 years), were used in this study. These dogs had participated in a study aiming investigate

effects of weight loss on inflammatory biomarkers.¹² Briefly, all dogs began the study with >30% obesity (body condition score [BCS] 5/5) and remained on the weight loss study until they reached a BCS 3/5 compatible with their ideal body weight.¹³ Animals received restricted amount of hypo-energetic commercial diet (Obesity Management, Royal Canin) once a day. The amount of food was progressively adjusted to induce a rapid weight loss (2-3% of body weight [BW]/week) and to cover the minimal requirements in proteins.¹⁴ Drinking water for all dogs was available *ad libitum*. BCS and BW were determined weekly. Body fat mass (FM) was obtained at the obese stage and after weight loss, using dilution of a single dose of deuterium oxide (275 mg/kg).¹⁵

At the beginning and the end of experimental weight loss period a total of five mL of blood from each dog were obtained by venipuncture of the cephalic vein on the morning before feeding. Four mL were placed in tubes with clot accelerator (TapVal, Aquisel) and 1 mL in tubes with EDTA (TapVal, Aquisel). EDTA tubes were strictly filled till the limit recommended by the manufacturer in order to avoid differences in final EDTA concentrations. After complete clotting, samples of tubes with clot accelerator were rimmed and centrifuged at 2000g for 10 min at room temperature. Then the serum was separated and immediately stored in plastic vials at -20°C until analysis. Whole blood samples with EDTA were diluted 1:50 with distilled water and frozen at -20°C until analysis.¹⁶ All samples were analysed in one batch less than 6 months after collection. ChE has been shown to be stable at -20°C for at least 6 months.¹⁷

Animal care and procedures were in accordance with the guidelines of the University of Murcia that follows European legislation and were approved by the Local Ethical Committee.

Serum BChE activity was measured using butyrylthiocholine as a substrate following previously reported procedures¹⁶ and adapted for an automated analyser (Olympus AU2700, Olympus Diagnostica GmbH). Whole blood AChE (AChE) activity was measured¹⁸ after

inhibition of BChE with ethopropazine using acetylthiocholine as a substrate. The procedure described by Worek et al.¹⁹ was followed with some modifications (the ethopropazine concentration was increased to 0.3 mM) in order to adapt its use in dogs and assure a total inhibition of BChE. This method is considered as a gold standard for measurement of AChE.²⁰ The intra- and inter-assay coefficients of variation were below 10%.

Cholesterol, HDL- cholesterol, LDL- cholesterol, and triglycerides were analyzed in the automated clinical chemistry analyser (Olympus AU2700, Olympus Diagnostica GmbH).

Data is presented as median (range) unless otherwise stated. The results for each parameter were evaluated for approximate normality of distribution by using the Kolmogorov–Smirnov normality test statistics. Data giving a non parametric distribution were *log* transformed for subsequent statistical analysis. Paired t test of log transformed data was used to compare changes in BChE and AChE at the beginning and the end of the weight loss study in dogs. Correlations between variables were estimated using Pearson’s correlation coefficient of log transformed data. Values of $p < 0.05$ for two-sided analyses were considered significant.

Serum BChE showed a statistically significant decrease in dogs after weight loss (6.05 (5.70-8.20) vs. 5.20 (3.10-5.60) $\mu\text{mol/mL/min}$; $p < 0.05$) and a significant increase was detected in AChE (0.65 (0.49-0.79) vs. 0.80 (0.80-0.99) $\mu\text{mol/mL/min}$; $p < 0.01$).

Positive correlation was detected between serum BChE activity and levels of total cholesterol ($r = 0.745$, $p < 0.001$), LDL-C ($r = 0.752$, $p < 0.001$), HDL-C ($r = 0.744$, $p < 0.001$), and TG ($r = 0.602$, $p < 0.05$). Negative correlation was detected between serum BChE and AChE activities ($r = -0.826$, $p < 0.0001$). Negative correlation was detected between AChE activity and serum levels of total cholesterol ($r = -0.634$, $p < 0.01$), LDL-C ($r = -0.687$, $p < 0.01$) and HDL-C ($r = -0.560$, $p < 0.05$).

The primary objective of this paper was to analyse the evolution of BChE and AChE during weight loss in dogs. To the authors' knowledge, this is the first report in literature that evaluates simultaneously the behaviour of both isoenzymes, BChE and AChE, in relation with weight loss in this species.

The BW reduction in animals of our study was accompanied by the decrease in cholesterol, HDL-C, and LDL-C and the improvement in insulin resistance during experimentally induced weight loss.¹² These results indicate that the protocol used in present study lead to rapid reduction in weight loss accompanied by changes in serum biochemical analytes that have been shown in previous studies to be related with obesity, such as lipidic profile.¹³

We determined BChE in serum and AChE in whole blood after BChE inhibition, as recommended by different authors.^{19,20} Use of serum and butyrylthiocholine as substrate to measure BChE in dogs is indicated since no AChE activity is detected with this procedure.²¹ AChE was measured in whole blood, since the use of whole blood has been recommended for international standardization of AChE measurements.²² When whole blood AChE is measured, use of selective inhibitor of BChE is recommended, as it was demonstrated that BChE is able to hydrolyse acetylcholine substrate.²¹ Naik et al.²⁰ have reported that ethopropazine is much more specific inhibitor of BChE compared with Iso-OMPA. For this reason in present study ethopropazine as a specific inhibitor of BChE was used for AChE measurements.

Serum butyrylcholinesterase activity decreased in average 23.4% (range, 8.2 – 48.3%) in dogs in response to weight loss during a 12 weeks period. We have previously reported that serum BChE increased in dogs within a range of 19.7-42.9% after short-term induced weight gain.¹¹ In the present study, a correlation between BChE and cholesterol profile and triglycerides was found. In human medicine an association between BChE and obesity markers, like lipid profile, BW and waist circumference, has been reported.⁸ It has been proposed that an incremental flux of free fatty acids from adipose tissue to the liver might stimulate the

hepatic synthesis of plasma BChE as a possible explanation for the relationship between ChE and lipids.²³ On the other hand, it has been described that serum BChE produces hydrolysis of acetylcholine and it is implied in the maintenance of cholinergic function.⁷ Increased activity of BChE leads to reduced levels of acetylcholine, in this way reducing the anti-inflammatory actions exerted by this molecule. That, in turn, enhances local and systemic inflammation.⁷ Thus, the decrease of BChE activity observed in dogs after weight loss could indicate decreased predisposition to inflammation of obese dogs.

In the present study, mean increase of 31% (range, 11.9 – 61.9%) in AChE activity was detected after weight loss in beagle dogs. In human medicine, a significantly lower AChE activity in overweight/obese persons in comparison with normal weight ones have been reported (1.18 ± 0.13 vs. 1.23 ± 0.12 nmol hydrolysate/mg protein/min, respectively).²⁴ It was suggested that the decrease in AChE could be related with impairment of the erythrocyte membrane physical-chemical properties in overweight and obese people as a consequence of oxidative injury that could be part of a pathogenic mechanism responsible for obesity related pathologies.²⁴

Overall it can be concluded that a short-term weight loss in the experimental conditions of this study produced significant decrease in BChE and increase in AChE activities. Further studies should be undertaken to clarify the physiopathological mechanisms involved in these changes and the possible implication that these variations in cholinesterase isoenzymes activities can have in the development and complications associated with obesity.

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07-May-2012

Dear Dr. Tecles:

I am pleased to inform you that your manuscript entitled "Acetylcholinesterase and butyrylcholinesterase activities in beagle dogs before and after weight loss" is accepted for publication in Veterinary Clinical Pathology pending editorial review. You may consider your manuscript as "in press". Your manuscript will be edited prior to publication and any additional reviewer comments can be addressed at that time.

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Sincerely,

Dr. Karen Young
Editor-in-Chief
Veterinary Clinical Pathology

3.5.1.5. Article 22



Serum butyrylcholinesterase activity in dogs with diabetes mellitus

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ABSTRACT

Increased serum butyrylcholinesterase (BChE) activity is a feature of diabetes mellitus (DM) in humans and rats. The objective of this study was to evaluate serum BChE activity in diabetic dogs. The activity of the enzyme was assessed in three cohorts of animals: (1) dogs with naturally occurring DM ($n = 74$); (2) clinically normal dogs ($n = 74$); and (3) dogs with various other diseases ($n = 74$). A statistically significant increase in BChE activity was found in the diabetic dogs (7.59 ± 2.9 kU/L) compared with the clinically normal animals (6.12 ± 1.94 kU/L; $P < 0.05$), and with the dogs with other diseases (5.55 ± 2.06 kU/L; $P < 0.01$). Such increased activity could be the result of the altered glucose and lipid metabolism that occurs in DM.

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Introduction

Cholinesterase is a mammalian enzyme found in two forms, namely, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8). AChE is present in the central nervous system and in platelet and erythrocyte membranes, while BChE is more abundant in the serum and is synthesised by the liver.

Despite research spanning a number of decades, the physiological function of BChE remains unknown. In humans, a direct association has been demonstrated between serum BChE activity and obesity, the serum lipid profile and insulin resistance (Iwasaki et al., 2007), and it has been suggested that BChE is involved in the pathophysiology of metabolic syndrome, which is characterised by visceral obesity, dyslipidaemia, hypertension and glucose intolerance. Although the exact role of this enzyme remains unclear, its activity is associated with insulin deficiency and/or resistance and with incremental trafficking of free fatty acids from adipose tissue to the liver (Cucianu et al., 2002). The inhibition of the activity of BChE has been mooted as a treatment for obesity where there is concurrent dyslipidaemia and insulin resistance (Iwasaki et al., 2007).

There is evidence linking BChE and diabetes mellitus (DM), where increased serum BChE activity is found in both type 1 and 2 forms of the disease in humans (Abbott et al., 1993; Cucianu et al., 2002). There is also an association between serum BChE activity and high fasting insulin levels where insulin resistance is demonstrated (Randell et al., 2005). Furthermore, induction of type 1 DM in rats following treatment with streptozocin results in ele-

vated BChE activity (Annapurna et al., 1991). To the authors' knowledge, there are no reports of the activity of serum BChE in dogs with DM.

DM is one of the most commonly diagnosed endocrine diseases in the dog and there is evidence that it is increasing in incidence (Guptill et al., 2003) as in humans (Magori et al., 2005). The objective of the present study was to determine whether dogs with naturally occurring DM have increased serum BChE activity similar to that in humans and rats. We also investigated the inter-relationship between the activity of this enzyme and the serum concentrations of cholesterol and triglyceride.

Materials and methods

Study design

The San Marco Veterinary Hospital database was interrogated to identify all dogs with DM admitted between January 2007 and 2009. Clinically normal dogs and dogs with diseases other than DM of the same breed, age, weight, and sex, were also identified. A total of 222 dogs was then divided into three groups. Group 1 consisted of 74 animals with DM of which 43 were female and 31 were male, with an average age of 113.81 ± 33.6 months and an average weight of 16.59 ± 12.7 kg. Twenty-nine of the group were of mixed breed, and the remainder consisted of seven Labrador Retrievers, seven Toy Poodles, six Yorkshire Terriers, four English Setters, three Siberian Huskies, two of each of the Samoyed, Fox Terrier, and Papillon Spaniel breeds, and one of each of the following breeds: American Pit-bull Terrier, Teckel, Bolognese, Kurzhaar, Chihuahua, Chow-Chow, Dalmatian, Doberman Pinscher, Rottweiler, Shih-Tzu, and Zwergspinscher.

The diagnosis of DM was based on clinical signs and laboratory findings (Duracher et al., 2008) including polyuria, polydipsia, polyphagia (excluding dogs with ketosis), glucosuria and hyperglycaemia (glucose >200 mg/dL). Only dogs not previously treated with insulin were eligible for inclusion in the study. Twenty-eight of this group presented with diabetic ketoacidosis, and the following disorders were diagnosed in 46 of the animals: pancreatitis ($n = 16$); inflammatory bowel disease ($n = 11$); hyperadrenocorticism ($n = 8$); hypothyroidism ($n = 7$); renal failure ($n = 4$); sepsis ($n = 3$); neoplasia ($n = 3$); pyometra ($n = 2$); single animals had poly-

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neuropathy, a portosystemic shunt, cardiac tamponade, and uveitis, respectively. Eleven of this sub-group of 46 dogs exhibited two of these disorders contemporaneously.

Group 2 consisted of 74 clinically normal animals that had been presented for routine health checks, elective surgery or that were used as blood donors. Of this group 43 were female and 31 were male, with an average age of 112.59 ± 34.0 months and an average weight of 16.06 ± 12.0 kg. Although healthy, a proportion of this group were considered obese as determined by a five-point scale body condition score (BCS) (McGreevy et al., 2005). Dogs with a BCS of 4–5 were considered obese. Group 3 consisted of 74 dogs that presented with diseases other than DM. Of this group 43 were female and 31 were male, with an average age of 113.37 ± 33.7 months and an average weight of 15.85 ± 12.3 kg. Thirty-six of this group presented with inflammatory disease, 24 with neoplasia, five with trauma or infection, respectively, and four with cardiac or renal failure. These three groups were further divided into non-obese (a) and obese (b) sub-groups based on the criteria outlined above.

Sample collection and biochemical analysis

Blood samples were collected by cephalic/jugular venepuncture into Vacuette tubes containing clotting accelerator (Greiner, Bio-One). Samples were centrifuged at 2000 g for 10 min at room temperature and the separated serum analysed within 30 min. Owner consent was obtained prior to sample collection.

Serum BChE activity was measured by spectrophotometry (Furlanello et al., 2006) in an automated Olympus AU2700 analyser (Olympus Diagnostica). Glucose, cholesterol, and triglyceride concentrations were measured in the same analyser, according to the manufacturer's instructions.

Statistical analysis

The D'Agostino and Pearson omnibus normality test was used to assess the normality of the data with parametric distribution. An ANOVA followed by the Bonferroni's multiple comparison test was used to compare the BChE values between the different groups and sub-groups. Summarised data are shown as mean $\pm$ SD. Values of $P < 0.05$ were considered significant.

Results

No significant differences in age or weight were found between the three groups. Mean serum glucose, total cholesterol and triglyceride levels are presented in Table 1. These analytes were significantly higher in group 1 dogs when compared with animals in groups 2 and 3. No significant differences in these three parameters were detected between groups 2 and 3.

Significantly increased BChE activity was found in the dogs of group 1 (7.59 ± 2.9 kUI/L) compared to those in groups 2 (6.12 ± 1.94 kUI/L, $P < 0.05$) and 3 (5.55 ± 2.06 kUI/L, $P < 0.01$) (Fig. 1). When divided into non-obese (a) and obese (b) sub-groups, significantly increased BChE activity was detected in sub-groups 2b and 3b relative to their respective 'a' group counterparts ($P < 0.01$ in both cases) (Fig. 2). No significant difference in serum BChE activity was found between the group 1 sub-groups. Group 1b dogs did not have significantly different BChE activity than those in sub-groups 2b and 3b. However, animals in sub-group 1a, had significantly higher BChE compared with those in sub-groups 2a and 3a ($P < 0.01$ in both cases).

When dogs in group 1 were subdivided based on whether or not they had uncomplicated DM (UDM), diabetic ketoacidosis (DKA), or DM with concurrent disease (DMC), no significant differences were found (Fig. 3). A Spearman correlation test revealed that serum BChE and cholesterol correlated in groups 1 ($R = 0.25$; $P < 0.05$),

Table 1

Mean serum glucose, total cholesterol (Chol), and triglyceride (TG) concentrations ($\pm$ SD) in: dogs with naturally occurring DM (group 1); clinically normal dogs (group 2); and dogs with various other diseases (group 3).

Group	Glucose (mg/dL)	Chol (mg/dL)	TG (mg/dL)
1	527.3 ± 305.1	354.1 ± 164.9	288.1 ± 386.5
2	$102.1 \pm 11.4^*$	$238.8 \pm 58.7^*$	$76.7 \pm 40.1^*$
3	$109.8 \pm 38.1^*$	$265.1 \pm 125.4^*$	$133.7 \pm 211.6^*$

* $P < 0.01$ vs. group 1.

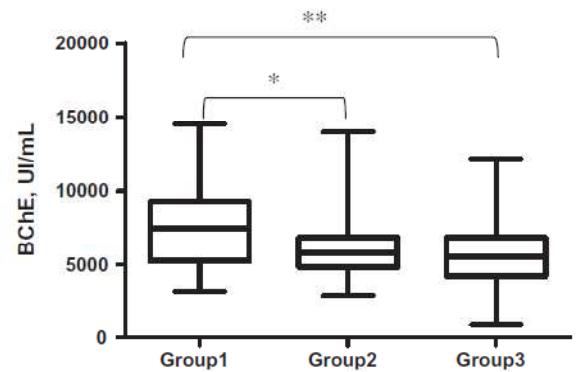


Fig. 1. Butyrylcholinesterase (BChE) activity in: dogs with DM (group 1); clinically normal dogs (group 2); and dogs with various other diseases (group 3). * $P < 0.05$, ** $P < 0.01$.

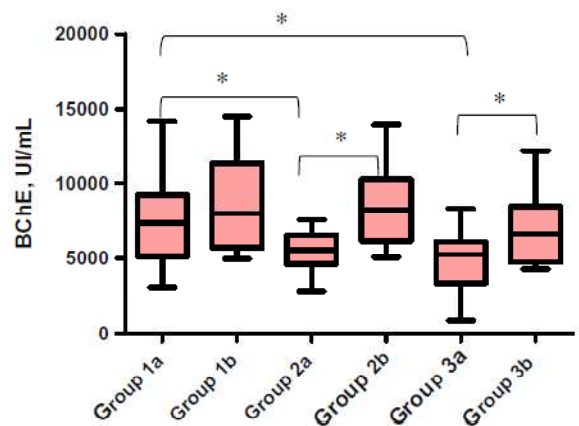


Fig. 2. Butyrylcholinesterase (BChE) activity in non-obese ('a' sub-groups) and obese ('b' sub-groups): dogs with DM (group 1); clinically normal dogs (group 2); and dogs with various other diseases (group 3). * $P < 0.01$.

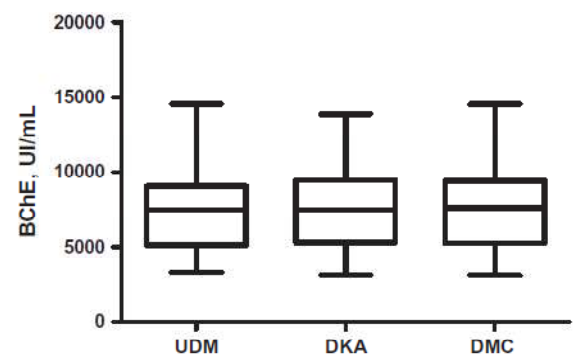


Fig. 3. Butyrylcholinesterase (BChE) activity in dogs with: uncomplicated diabetes mellitus (UDM); DM and ketoacidosis (DKA); and DM with concurrent disease (DMC).

and 3 ($R = 0.45$; $P < 0.0001$) and the BChE concentration correlated with triglyceride in group 3 ($R = 0.39$; $P < 0.001$). There was no correlation between the serum BChE, cholesterol and triglyceride concentrations in group 2.

Discussion

Previously, BChE has been used as a biomarker of organophosphate and carbamate intoxication in dogs (Munro et al., 1991; Sakaguchi et al., 1997; Tecles et al., 2002). More recently however, there has been increased interest in the role of this enzyme in lipid metabolism, obesity and in endocrine disorders such as DM in humans (Iwasaki et al., 2007; Rao et al., 2007). This is the first study investigating the potential role of BChE in canine DM.

We found a significant 1.5-fold increase in serum BChE concentrations in dogs with DM compared to non-obese controls. Increases of a similar magnitude have been reported in diabetic humans (Abbott et al., 1993). Despite the significant increase in BChE concentration in the dogs with DM, there was a considerable overlap in the levels of this enzyme between healthy and diabetic animals, similar to the situation in humans (Abbott et al., 1993), suggesting that the measurement of BChE would not facilitate the diagnosis of DM.

The cause of increased BChE concentration in DM remains unclear. Elevated glucose concentrations can stimulate BChE activity in vitro (Lunkes et al., 2006), and a link between cholinesterase activity and lipid metabolism has been described in human diabetics (Alcântara et al., 2002), where serum BChE activity positively correlates with cholesterol and triglyceride concentrations (Abbott et al., 1993; Randell et al., 2005). However, in another study, no such correlation was found (Sridhar et al., 2005). The metabolic mechanisms underpinning a relationship between BChE, cholesterol and triglyceride concentrations and lipid biosynthesis remains unclear (Rustemeijer et al., 2001). However, it has been postulated that BChE has a role in altered lipoprotein metabolism and in the hypertriglyceridaemia that occurs in DM (Iwasaki et al., 2007). Administration of a specific BChE inhibitor after the induction of type I DM in rats and mice reverses the hypertriglyceridaemia (Annapurna et al., 1991), and BChE has been used as a biomarker in the evaluation of drugs used to treat hypertriglyceridaemia in human diabetics (Rustemeijer et al., 2001).

Insulin may have a direct effect on BChE that could account for the increases in BChE in type II DM, as it may stimulate the production of BChE by a CaCo-2 intestinal cell line (Randell et al., 2001), and increase the synthesis of BChE by the liver in cases of insulin resistance (Randell et al., 2005). In human patients, serum BChE activity has a significant positive correlation with serum levels of insulin and with insulin resistance in patients with type II DM and metabolic syndrome (Abbott et al., 1993; Cucuianu et al., 2002; Randell et al., 2005).

Given that both canine and human type I DM (Feldman and Nelson, 2004), are characterised by low insulin concentrations, the causes of the increased BChE activity in our study could relate to the increased serum glucose and lipid levels. In our group 1 animals with DM, although cholesterol, triglyceride and BChE concentrations were increased, a significant positive correlation was only detected between the BChE and cholesterol levels, a finding in line those in humans (Sridhar et al., 2005).

When the dogs were subdivided into obese and non-obese categories, obesity was found to be associated with increased BChE activity in both the clinically normal dogs and in the dogs with non-diabetic disease. Tvarijonavičiūtė et al. (2010) reported similar finding in healthy beagles. It has been proposed that incremental trafficking of free fatty acids from adipose tissue to the liver might stimulate the hepatic synthesis of BChE (Cucuianu et al., 2002), and result in the release of BChE by adipocytes (Randell et al., 2005). However BCS did not influence BChE concentrations in dogs with DM, as reported in humans (Abbott et al., 1993; Randell et al., 2005).

A limitation of this study was that 62% of the diabetic dogs had concurrent disease. However, this high frequency of contempora-

neous disease typically reflects the clinical situation in diabetic dogs (Fincham et al., 2004). The group 3 dogs with other non-diabetic diseases were included in the study in order to evaluate the effect of such diseases on the BChE concentration in breed-, age-, weight-, and sex-matched animals. The fact that this group of dogs had lower BChE values than the diabetic animals, taken together with the lack of differences found between dogs with and without the complications of DM, indicates that concurrent disease may not have a significant effect on serum BChE concentrations in dogs with DM.

Conclusions

Our study found that dogs with DM presented with elevated BChE concentrations as described in human patients. Such increased activity could be linked to the altered glucose and lipid metabolism that occurs in DM and further studies will be required to elucidate the causes and consequences of this finding for diabetic animals.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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3.5.2. Proteomic

3.5.2.1. Article 23



A proteomic analysis of serum from dogs before and after a controlled weight-loss program

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Abstract

The objective of this study was to investigate how weight-loss program would alter the proteome of the serum of Beagle dogs. For this purpose, serum samples from 5 Beagle dogs, before and after weight loss, were analyzed using 2-dimensional electrophoresis. Protein profiles of all samples were obtained, divided into 2 classes (obese and lean), and compared using specific 2-dimensional software, giving a total of 144 spot matches. Statistical analysis revealed 3 spot matches whose expressions were modulated in response to weight loss: 2 protein spots were upregulated and 1 protein spot was downregulated in the obese state in comparison with the lean state of the dogs. Mass spectrometric identification of differentially regulated spots revealed that these protein spots corresponded to retinol-binding protein 4, clusterin precursor, and α -1 antitrypsin, respectively, which could be considered potential markers of obesity and obesity-related disease processes in dogs.

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Keywords: Dog; Obesity; Proteomics; Serum; Weight loss

1. Introduction

Obesity is the most common nutritional disorder in today's dog population and the major risk factor for a number of diseases such as diabetes mellitus, hypothyroidism, pancreatitis, and respiratory distress [1,2]. It is a growing concern in companion animals and its increasing incidence appears to be mirroring the trend

observed in humans [3]. Obesity is considered an extremely complex disorder and, despite its importance, its mechanisms and possible links with related diseases are still incompletely understood [4]. Thus, there is a need to investigate the molecular mechanisms leading to obesity understanding and to promote the implementation of control measures at different levels and the design of new therapies.

Global protein expression analysis, known as proteomics, has currently emerged as a novel scientific technology successfully applied to several fields of medicine [4]. The separation, identification, and characterization of proteins and an understanding of their interactions with other proteins are the essential aims of

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proteomic analysis. Two-dimensional electrophoresis (2-DE) coupled with mass spectrometry (MS) is considered a powerful tool in the separation and identification of hundreds of plasma proteins [5]. This approach enables comparison of control and diseased samples revealing differentially expressed proteins.

Changes in plasma proteins caused by obesity-related factors are connected with the development of metabolic disorders [4]. Proteome analysis has recently been performed in studies of obesity in humans and experimental animal models such as mice and rats to clarify the physiopathology of obesity and its associated diseases [6–8]. In dogs, proteomic approaches have been used for the identification of cancer biomarkers such as in lymphoma [9] or transitional cell carcinoma [10], for the study of induced hepatic hypertrophy [11], and more recently for a screening of proteins differentially expressed in dogs infected with leishmania vs noninfected dogs [12]. However, to the best of the authors' knowledge, proteomic studies have not been performed in obesity under experimental conditions in dogs.

Thus, the objective of this study was to perform a proteomic analysis on the serum of Beagle dogs before and after an experimental weight-loss program to identify new potential serum biomarkers involved in or linked with obesity that could contribute to the disclosure of its physiopathological mechanisms.

2. Materials and methods

2.1. Animals and experimental setup

The experimental study was performed at the Veterinary Medicine School, University of Murcia, Spain. Animal care and procedures were in accordance with the guidelines of the University of Murcia and approved by the local ethical committee.

A total of 5 adult, female, obese, noncastrated Beagle dogs (age range, 5.2 to 6.5 yr) were used in this study. The dogs had developed nutritional obesity 3 mo before the beginning of the study. Briefly, the dogs were fed ad libitum a hyperenergetic commercial diet (Advance Performance, Affinity Petcare SA, Barcelona, Spain) that was formulated to contain 31.0% crude protein, 21.0% crude fat, 2.0% crude fiber, 6.5% crude ash, 8% moisture, and 2,840 kcal metabolizable energy/kg. One dog per kennel was maintained during the whole study period with controlled temperature ($23 \pm 2^\circ\text{C}$) and light (lights on from 6:00 AM to 6:00 PM). The size of the kennel was 3×4 m. These dogs had participated in a study aiming to investigate the possible effects of weight loss on inflammatory biomarkers

[13]. All dogs began the study $>30\%$ overweight (body condition score [BCS] 5/5) and remained on the weight-loss study for 3 mo until they reached a BCS 3/5 compatible with their ideal body weight (BW) [14]. All bitches were in anestrus during the weight-loss period. Animals received a restricted amount of hypoenergetic commercial diet (Obesity Management, Royal Canin, Aimargues, France) once a day. The hypoenergetic diet was formulated to contain 34.0% crude protein, 10% crude fat, 8.2% crude fiber, 7.9% ash, 9% moisture, and 3,275 kcal metabolizable energy/kg. The amount of food was progressively adjusted to induce a rapid weight loss (2% to 3%/wk) and to cover the minimal requirements in proteins [15]. Drinking water for all dogs was available ad libitum.

Blood samples were collected on the morning after an overnight fasting by venipuncture in the cephalic vein from all dogs at the beginning (obese state, BCS 5/5, T1) and at the end (lean state, BCS 3/5, T2) of the weight-loss study. Serum samples were obtained after centrifugation of blood samples at 2,000g for 10 min and were stored at -20°C until analysis.

All dogs were subjected to general health examinations weekly. They showed no abnormalities on physical examination (except obesity), and routine hematological and biochemical analyses were within our laboratory reference ranges during the whole study. All animals had a negative serologic titer for *Leishmania infantum* and *Ehrlichia canis* as confirmed using commercial tests (Canine SNAP tests, IDEXX Laboratories, Westbrook, ME, USA).

Body condition score and BW were also determined weekly. Body fat mass (BFM) was obtained at the obese stage and after weight loss, using dilution of a single dose of deuterium oxide (275 mg/kg) [16].

During the weight-loss period, the BCS of all dogs decreased from 5/5 to 3/5. In parallel, a significant reduction of BW, BFM (%), total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, insulin, insulin:glucose ratio, fructosamine, and insulin-like growth factor 1 and a significant increase in circulating adiponectin levels were observed [13].

2.2. Two-dimensional polyacrylamide gel electrophoresis

Serum samples of T1 and T2 of all 5 dogs were analyzed in duplicate for 2-DE. The total protein content of the serum sample was quantified using a commercial kit (RC-DC Bio-Rad, Hercules, CA, USA). The first dimensional isoelectric focusing was performed on

homemade immobilized pH gradient strips (11 cm) with a nonlinear pH range of 4 to 10. Strips were previously rehydrated for 8 h in a commercial rehydration tray (Bio-Rad) with 360 μ L of standard rehydration solution [17]. A total of 50 μ g/strip of serum proteins was applied to the strips in a total volume of 20 μ L of standard sample buffer with sample application pieces. Proteins were focused overnight by slowly raising the voltage to 2,000 V until 15 kVh in a horizontal electrophoresis unit (Multiphor II electrophoresis unit, GE Healthcare Europe GmbH, Freiburg, Germany). Sample application pieces were removed 3 h before the end of the electrophoresis.

For the second dimension, strips were equilibrated for 10 min in 2% dithiothreitol in equilibration solution (6 M urea, 30% glycerol, 2% sodium dodecyl sulfate, 0.05 M Tris-HCl, pH 6.8) followed by 5 min with 2.5% iodoacetamide in equilibration solution, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on homemade gradient 10%–15% polyacrylamide gels (140 $\times$ 140 $\times$ 1.5 mm) in a vertical electrophoresis chamber (SE 600 Ruby electrophoresis chamber, GE Healthcare Europe GmbH) during 5 h at 15°C and 25 mA/gel. After electrophoresis, the gel was stained with silver according to standard protocols as reported previously [18].

2.3. Two-dimensional electrophoresis gel image analysis

Stained 2D gels were scanned in an ImageScanner II (GE Healthcare Europe GmbH) and evaluated using specific software (Image Master 2D Platinum 7.0, GE Healthcare Europe GmbH). Images of all animal samples were digitalized and aligned. Afterward, the specific software mentioned above allowed the automatic detection of all specific spots in the gels and quantified them as the percentage volume. The relative percentage volume of a spot is a normalized value that remains relatively independent of variations resulting from protein loading and staining by considering the total volume over all spots in the image and is calculated as follows according to the manufacturer's instructions:

$$\%Vol = Vol^n \sum_{s=1}^n VolS \times 100,$$

where VolS is the volume of spot S in a gel containing n spots.

Once spots of all gels were detected, an automatic match set was performed. The image-matching algorithm compares gel images to find matches between related spots, that is, spots representing the same protein in the gels. Then, matched gel images were divided

into 2 different classes according to the experimental design corresponding to class 1 for obese dogs at T1 and class 2 for lean dogs at T2.

A class statistical analysis was carried out to evaluate possible differentially expressed proteins between states T1 and T2 using statistical software (GraphPad Prism 5 demo, GraphPad Software Inc, San Diego, CA, USA) by performing a paired *t* test ($P < 0.05$).

2.4. Mass spectrometry identification

Those spots that appeared to be differentially expressed between the obese and lean states of all animals were subjected to MS identification. For MS identification, additional 2-DE gels were prepared as previously described with several modifications. In brief, the total amount of protein applied was 60 μ g/strip and aldehydes were omitted in some of the steps during silver staining [19].

Protein identification was performed on spots of interest. Selected spots were excised and in-gel digestion with trypsin was performed. After a desalting step using C18 Zip-Tip, the entire peptides were analyzed by matrix-assisted laser desorption/ionization time of flight/time of flight MS (Ultraflex II, Bruker Daltonics, Bremen, Germany).

2.5. Mass spectrometry data analysis

Processed spectra were searched using Mascot (<http://www.matrixscience.com>) in Swiss-Prot database (release 56.5) or the NCBI nr database (20090314). The following search parameters were set: taxonomy mammalia, global modifications carbamidomethylation on cysteine, variable modifications oxidation on methionine, MS tolerance 100–150 ppm, MS/MS tolerance 1 Da, 1 missed cleavage allowed. The MS and MS/MS identifications were considered significant ($\alpha < 5\%$).

3. Results

The mean $\pm$ SD percentage weight loss and percentage change in BFM were $31 \pm 3\%$ and $45 \pm 17\%$, respectively (Table 1).

The results of protein mapping of canine serum using 2-DE revealed more than 170 individual spots with mo-

Table 1
Mean ($\pm$ SD) body weight (BW) and body fat mass (BFM) before (T1, obese state) and after (T2, lean state) the BW loss period of 5 Beagle dogs.

	T1	T2
BW (kg)	20.6 ± 2.5	14.1 ± 1.3
BFM (%)	46.7 ± 6.3	25.1 ± 6.3

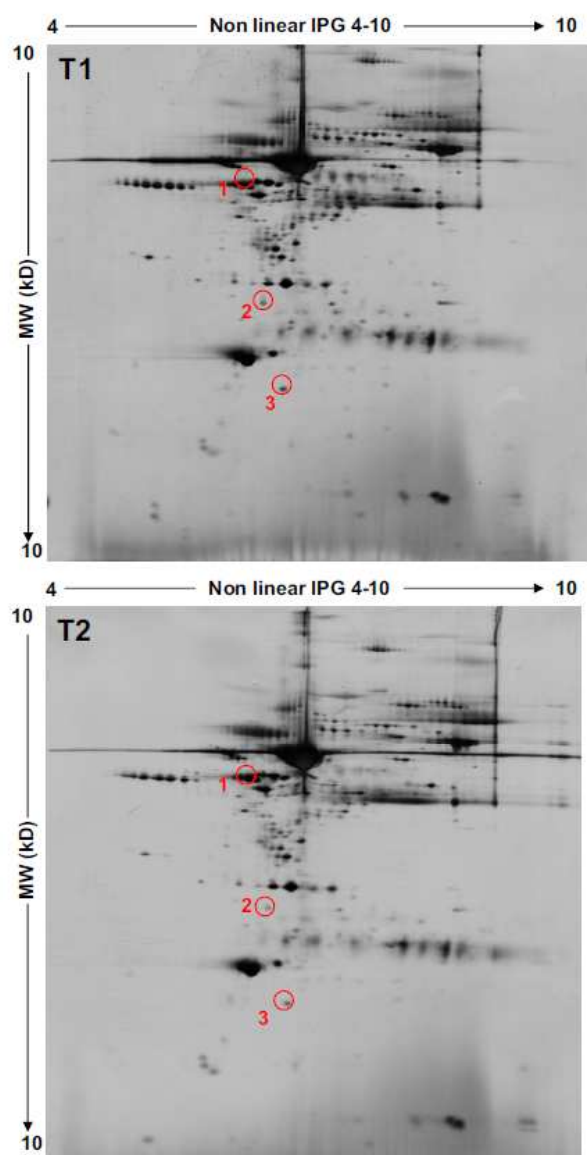


Fig. 1. Representative 2-dimensional electrophoresis gel images of silver-stained proteins of canine serum obtained before (T1, obese state) and after (T2, lean state) body weight loss corresponding to dog 1. Differentially expressed protein spots between the obese and lean states of the dogs are circled and numbered: (1) retinol-binding protein 4, (2) clusterin precursor, and (3) serpin peptidase inhibitor, clade A (α -1 antiproteinase, antitrypsin), member 1.

lecular weights between 10 and 100 kDa over a pH range of 4 to 10 in each analyzed silver-stained 2-DE gel from the serum samples of both obese and lean dogs (Fig. 1). Protein profiles obtained for each class (dogs in an obese and a lean state) were homogeneous because no differences were detected between replicates and between images from samples of the same class.

Image analysis was used for automatic spot detection and

for comparison of the spot volumes obtained in the 2 image classes: images from obese and lean serum samples. A total of 144 spot matches, corresponding to the detection and quantification of the same spot in the different classes, were obtained. A statistical class analysis allowed the detection of 3 spot matches that were modulated in response to weight loss (Fig. 2). Among the 3 spots differentially expressed in the obese vs the lean state, 2 were upregulated and 1 was downregulated in the obese state in comparison with the lean state of the dogs (Table 2).

Matrix-assisted laser desorption/ionization time of flight/time of flight MS allowed the identification of the 3 protein spots with differential expression levels as belonging to retinol-binding protein 4 (RBP4), clusterin precursor (CLU), and α -1 antitrypsin (α -AT; Table 3).

4. Discussion

In this study we used an experimental model of Beagle dogs that were subjected to a weight-loss program that produced an improvement of the metabolic status of the animals with a decrease in lipids, insulin, insulin:glucose ratio, and insulin-like growth factor 1 and an increase in adiponectin [13], similar to previous reports by different authors [14,15,20,21]. A limitation of this study could be that the experimental model used does not reflect a real clinical situation of canine obesity, especially in chronic obesity cases, because this experimental protocol aims to avoid different variations that can occur when studies are performed in a clinical situation such as the use of different diets and environmental conditions, different lengths in the onset of obesity, medications, and possible secondary diseases. On the other hand, it could be postulated that the use of 5 animals is limited and the study should be considered preliminary, although it has been indicated that 3 biological samples are sufficient to detect induced biological variations in the proteome of animal experiments [17].

There are limited data of serum proteomics in dogs. To the authors' knowledge, only 2 studies have been reported to date, including the 2-DE protein profile in canine serum from healthy dogs [22,23]. However, 1 study focused on a specific malignancy [23], hemangiosarcoma, whereas the second study was performed on the follicular fluid of dogs [22]. In the present study, serum proteome analysis was performed in Beagle dogs before and after a controlled weight-loss program. Statistical comparison of spot volumes revealed 3 spots differentially expressed between the obese and lean states of the animals. The detection of only 3 proteins significantly affected by obesity would be at odds with expectations because, for example, a range of adipo-

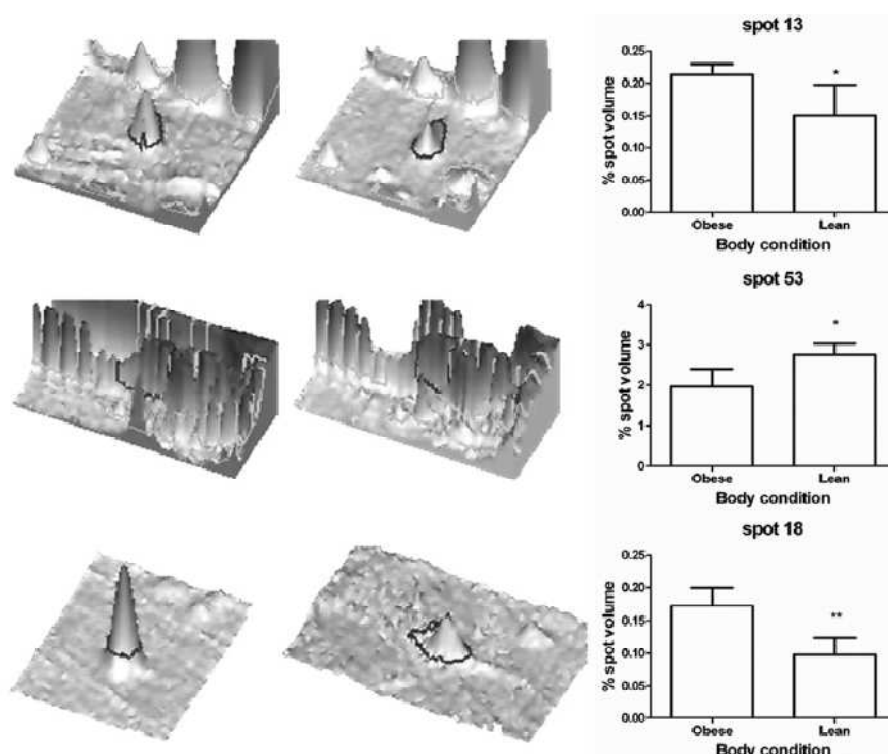


Fig. 2. Example of the 3-dimensional images of differentially regulated spot proteins 1, 2, and 3 from dog 1 under the obese state (left) and the lean state (right). Graphics represent the statistical analysis of the spot volumes indicating the mean (box) and standard deviation (vertical bars).

kines are altered in canine obesity [2]. This finding could be explained by the insensitivity of the technique, because when 2-DE gel methods are used for proteome analysis, the detection of some biomarkers can be very challenging due to the presence of high-abundance proteins (present in the serum at milligram-per-milliliter levels) [24]. Hence, the depletion of major proteins has been suggested to be a potential strategy for enhancing detection sensitivity in serum [24]. However, this study could serve as the basis for further proteomic investigations in canine obesity.

Mass spectrometric analysis revealed that serum CLU,

RBP4, and α -AT were the proteins involved in the spot changes obtained in the 2-DE analysis. CLU and RBP4 have been previously identified in the serum and follicular fluid of healthy dogs with similar pI and MW characteristics [22]. Our results showed that CLU and RBP4 spots were expressed significantly more before vs after weight loss, whereas the α -AT spot volume was lower in obesity and significantly increased after weight loss in dogs.

In serum, CLU acts as an apolipoprotein, which at least partly associates with HDL [25]. Several functions have been proposed for CLU, including lipoprotein transport, and a positive correlation between changes of CLU with body fat mass has been described in humans independent of age, gender, HbA1c, and fasting plasma insulin concentrations [26]. This is the first report in which a relation between serum CLU and canine obesity is postulated.

RBP4 is structurally related to a number of extracellular proteins involved in the transport of small hydrophobic compounds, the lipocalins [27,28]. RBP4 has gained substantial attention since the initial notion that it is elevated in the serum of insulin-resistant humans and mice [29]. Moreover, increased RBP4 serum concentrations are associated with many components of

Table 2
Differentially expressed spot matches in obese vs lean dogs.

Spot ^a	Obese vs lean	Obese state (n = 5)		Lean state (n = 5)		P value
		X	SD	X	SD	
1	↑	0.2091	0.0155	0.1534	0.0409	0.0316
2	↑	0.1639	0.0303	0.0964	0.0234	0.0072
3	↓	0.1998	0.3169	2.726	0.3001	0.0310

X, media of normalized volumes; SD, standard deviation.

^a Spot label number from annotated gel image (see Figs. 1 and 2).

Table 3

Mass spectrometry identification of proteins from canine serum samples.

Spot label ^a	Accession No. ^b	Protein identification	Theoretical MW (kDa)	Theoretical pI	Number of unique peptides	Sequence coverage ^c (%)	Score
1	gil73998292	Retinol-binding protein 4 (<i>Canis lupus familiaris</i>)	29.8	9.4	3	19.5	390.2
2	gil50979240	Clusterin precursor (<i>Canis lupus familiaris</i>)	51.8	5.6	3	9.7	81.3
3	gil121583756	Serpin peptidase inhibitor, clade A (α -1 antiproteinase, antitrypsin), member 1 (<i>Canis lupus familiaris</i>)	46.3	5.5	2	6.5	239.2

MW, molecular weight; pI, isoelectric point.

^a Spot label number from annotated gel image (see Fig. 1).^b NCBI (gi) protein database accession numbers.^c Sequence coverage: percentage of identified sequence to the complete sequence of the known protein.

the metabolic syndrome, such as increased body mass index, waist-to-hip ratio, serum triglycerides, systolic blood pressure, insulin resistance, low-grade chronic inflammation, or type 2 diabetes [30]. Circulating RBP4 levels also correlate with ectopic fat accumulation in the liver, visceral fat, and skeletal muscle [31]. Furthermore, a strong association between RBP4 and reduced body fat has been found after 6 mo of human weight loss [32,33], and it has been suggested that the mechanism of decrease of RBP4 levels after weight loss could be a reduction in these ectopic fat depots [26]. In the present study, increased levels of an RBP4 spot have been observed in the obese state of the dogs, which decreased after weight loss in accordance with the data reported in humans. Moreover, RBP4 has been identified as a marker of obesity in rats being increased in obese vs lean animals [7].

α -AT is the most abundant serine proteinase inhibitor in human plasma. Circulating levels of human α -AT increase in response to inflammation or infection [34,35], obesity [36,37], insulin resistance [38], and atherogenesis [39]. Thus, α -AT in humans is considered a positive acute-phase protein [40], whereas in dogs little variation was observed in α -AT levels, even following surgical trauma, suggesting a low relationship between inflammation and canine α -AT [41]. In contrast, an interaction between α -AT and LDL, the predominant lipoprotein, has been reported in humans [39]. However, in dogs the predominant lipoprotein is HDL [42]. Furthermore, in obesity HDL is increased in dogs in contrast to humans [43,44]. These differences between human and dog lipid metabolism could in part be responsible for the results obtained in the present study, where lower values of an α -AT spot have been observed in the obese in comparison with the lean state of the dogs. Further studies would be necessary to elucidate the metabolism of α -AT and its possible role in obesity and related diseases in dogs.

5. Conclusion

In this study, 3 differentially expressed protein spots were noted in obese in comparison with lean dogs: CLU, RBP4, and α -AT. These could be considered potential markers of obesity and obesity-related disease processes in dogs. In addition, serum levels of CLU, RBP4, and α -AT may be useful quantitative markers of positive lifestyle changes or therapeutic effectiveness of obesity reduction that would likely increase the average lifespan of dogs. In future studies, CLU, RBP4, and α -AT should be quantified using species-specific assays in the serum of obese and normal-weight dogs to verify the results obtained in this report.

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4. Conclusions

1. Commercially available assays for adiponectin and IGF-1 showed adequate results in our validation studies when using with dogs and cats samples. Commercially available leptin and ghrelin ELISA kits tested in our study showed to have low sensitivity and/or accuracy; and development of new kits to measure these adipokines would be recommended (Articles 2-6).
2. Serum adiponectin and IGF-1 are not influenced by feeding/fasting cycles or by a single dose corticoid administration. Also circulating adiponectin levels are not affected by orquidectomy. However, caution should be taken when interpreting IGF-1 values of castrated dogs, or adiponectin values obtained from bitches in oestrus, since lower and higher, respectively, concentrations were observed in these situations. Also inflammatory status of the dog should always be evaluated when analyzing both adiponectin and IGF-1, since inflammation decreases concentrations of these proteins in significant way (Articles 7-10).
3. Canine and feline obesity is associated with decreased circulating adiponectin concentrations, which increase after weight loss. But it seems to not produce an evident inflammation, at least that can be detected with APPs (Articles 11-14, 16).
4. Up to one third of obese dogs suffer from obesity-related metabolic dysfunction, characterized by hypoadiponectinemia and hyperinsulinemia, similarly as occur in the human metabolic syndrome (Article 14).
5. Weight loss in dogs is associated with the improvement of serum markers of renal dysfunction, such as clusterin, homocystein, cystatin C (Article 15).
6. Therapy of dogs with hypothyroidism results in decreased adiponectin concentrations and increased BChE activity (Article 17).

7. Serum BChE showed to be a good marker of adiposity in dogs and is increased in canine diabetes mellitus. No changes were observed in PON1 activity in obese dogs, and BChE and PON1 in obese cats. Although in cats pre-weight loss PON1 could be used as a predictor of successfulness of weight loss program. BChE activity is increased in dogs with diabetes mellitus (Articles 18-22).
8. Proteomic analysis revealed three proteins (clusterin, retinol-binding protein 4, and alpha-1 antitrypsin) that could be considered as potential markers of obesity and obesity-related disease processes in dogs (Article 23).

5. Summary

1. INTRODUCTION

Obesity is the most common nutritional disorder in dogs and cats (Roudebush et al., 2008; Lusby et al., 2009) being a growing concern nowadays. Its increasing incidence appears to be mirroring the trend observed in humans (Rennie and Jebb, 2005). In humans almost two-thirds of adults in the United States are overweight or obese (Flegal et al., 2002). Similarly, studies in companion animals from various parts of the world have estimated the incidence of obesity in the dog and cat population to be between 22 and 40% (Burkholder et al., 2000; McGreevy et al., 2005).

The objectives of this PhD Thesis were:

1. To validate commercially available assays for adipokines and IGF-1 for use in dogs and cats.
2. To analyze possible causes of the variations due to other but obesity factors, (such as diurnal variation, effect of food intake, castration, renal failure, and sepsis). For this purpose the analytes that gave adequate validation results in point 1 and were more economic, easy and fast to perform would be selected.
3. To clarify the possible effect of obesity on adiponectin and acute phase proteins in dogs and cats. Also to explore if obese dogs suffer from metabolic syndrome, evaluate if obesity could be related with changes in renal biomarkers, and to study how adipokines and acute phase proteins are affected by an obesity-related disease, such as hypothyroidism.
4. To study and identify new potential serum biomarkers involved/linked to obesity.

2. VALIDATION STUDIES

2.1. Material and methods

Serum samples were used to evaluate the ability of different commercially available assays to measure adiponectin, leptin, ghrelin, and IGF-1 in dogs and adiponectin, ghrelin, and IGF-1 in cats. Evaluated assays appear in table 2.1. Leptin in cats was not studied due to the lack of reactivity of this adipokine with human or mouse/rat ELISAs and due to lack of commercially available species-specific assays at the time in which the analysis were performed.

For analytical validation of the methods previously published protocols were followed (Jensen and Kjelgaard-Hansen, 2010). Therefore, three basic parameters were studied: precision, accuracy, and limit of detection. Additionally, overlap performance was carried out for all methods using samples with high and low concentrations corresponding to normal weight and obese animals or vice versa. With the exception of ghrelin, in which the samples for overlap performance were obtained at different times after glucose administration to dogs, as glucose is the most potent inhibitor of systemic ghrelin levels in humans and rodents (McCowen et al., 2002).

2.2. Results

2.2.1. Analytical validation

Analytical validation results of different methods for analytes determination in canine and feline serum, are presented in tables 2.1 and 2.2, respectively.

2.2.2. Overlap performance

2.2.2.1. Canine serum samples

Adiponectin. Samples collected from obese dogs analysed with canine-specific and human adiponectin ELISA assays showed significantly lower adiponectin concentrations than samples collected from normal weight dogs ($p < 0.005$ and 0.0005 , respectively) (Table 2.3).

Leptin. When assay A was used, samples collected from overweight-obese dogs had higher leptin concentrations than those collected from thin-ideal weight dogs ($p < 0.001$). No significant difference ($p = 0.89$) was observed in leptin concentration when serum samples from overweight-obese and thin-ideal weight dogs were analyzed with assay B (Table 2.3).

Ghrelin. Changes in circulating levels of ghrelin after glucose administration determined with canine and human assays are presented in figure 2.1. In comparison to the basal values, a mean decrease of 17% and a mean increase of 19% in ghrelin concentrations were observed 5 and 120 minutes after glucose administration, respectively, when analyzed with the canine assay. However, no statistically significant changes were recorded. When a human assay was used, a decrease of >30 % in plasma ghrelin levels was detected 10 min after glucose administration ($p<0.05$), followed by 50% increase in plasma ghrelin levels 120 minutes after glucose injection ($p<0.05$) when compared to the basal levels.

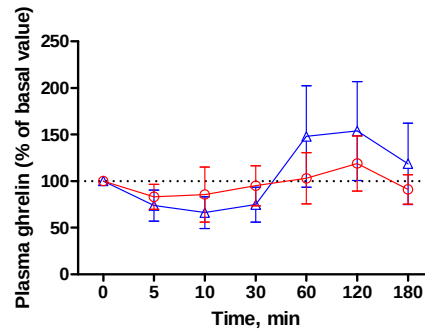


Figure 2.1. Plasma ghrelin response to IV glucose (0.5/kg of body weight) injection in four Beagle dogs measured with canine (○) and human (Δ) assays.

IGF-1. Serum IGF-1 values in lean dogs were higher ($p<0.05$) in comparison with obese dogs (Table 2.3).

2.2.2.2. Feline serum samples

Adiponectin, ghrelin, and IGF-1. Serum adiponectin ($p=0.01$) and ghrelin ($p=0.03$) concentrations were lower in obese cats ($n=10$) compared with normal weight cats ($n=10$), but there was no difference in IGF-1 concentrations between groups ($p=0.12$) (Table 2.4).

Table 2.1. Analytical validation data of different assays to measure adiponectin, leptin, ghrelin, and IGF-1 in canine serum samples.

Analyte	Assay	Units	Precision		Accuracy			Detection limit ^d
			CV, %		Linearity		Recovery ^c %	
			Intra	Inter	Equation	R ²		
Adiponectin ^a	Canine ELISA Kit, Millipore, USA	µg/mL	<7	<14	y=0.982x+0.65; y=0.999x+0.66	0.9996; 0.997	NE	0.098
	Human ELISA, High Sensitivity Kit, BioVendor-Laboratorni medicina, Czech Republic	µg/mL	<8	<11	y=0.991x+0.11; y=0.976x+0.37	0.996; 0.999	NE	0.74
Leptin ^b	Canine ELISA kit, Millipore, USA [assay A]	µg/L	<6	<8	y=1.037x-0.06; y=0.987x-0.79	0.985; 0.986	77 - 101	-
	Canine leptin, BPB Biomedicals Inc, USA [assay B]	µg/L	<14	<14	y=0.928x+0.38; y=0.907x+3.75	0.955; 0.970	67 - 125	9.08
Ghrelin	Canine ELISA, Phoenix Pharmaceuticals Inc, USA	ng/mL	<10	>20%	y=1.059x – 0.71; y=1.006x – 1.04	0.991; 0.924	101-121	0.6
	Human unacylated ELISA, BioVendor-Laboratorni medicina, Czech Republic	pg/mL	<7	<20	y=0.977x+15.46; y=1.006x+5.06	0.999; 0.998	83-96	-
IGF-1	Immulite 1000 assay, Diagnostic Products, USA	ng/mL	<8	<10	y=0.993x+4.11; y=0.992x+0.18	0.999; 0.999	NE	0.01

CV, coefficient of variation.

^a a canine serum sample with an adiponectin concentration determined by the canine ELISA was used as a standard for curve calibration of the human ELISA.

^b 3 more kits (Human EASIA, BioSource Europe S.A., Belgium; Human ELISA, BioVendor-Laboratorni medicina a.s., Czech Republic; Mouse/rat enzyme immunoassay kit, SPIbio, France) were evaluated for leptin measurements in canine serum, but no reactivity was observed.

^c recovery was performed only with those kits where purified canine protein was available; NE, not evaluated.

^d detection limits for leptin assay A and human ghrelin assay were not possible to be calculated as the blanks did not give any value.

Table 2.2. Analytical validation data of different assays to measure adiponectin, ghrelin, and IGF-1 in feline serum samples.

Analyte	Assay	Units	Precision		Accuracy			Detection limit
			CV, %		Linearity		Recovery %	
			Intra	Inter	Equation	R ²		
Adiponectin	Human ELISA, High Sensitivity Kit, BioVendor-Labaratorni medicina, Czech Republic	ng/mL	<7	<16	y=0.894x+997.37; y=0.882x+605.43	0.974; 0.987	100-112	2.0
Ghrelin	Human unacylated ELISA, BioVendor-Labaratorni medicina, Czech Republic	pg/mL	<15	<15	y=0.984x-11.90; y=0.963x+5.88	0.962; 0.998	91-112	9.6
IGF-1	Immulite 1000 assay, Diagnostic Products, USA	ng/mL	<3	<6	y=1.022x+6.85; y=1.022x+0.83	0.987; 0.994	100-112	0.01

CV, coefficient of variation.

Table 2.3. Serum levels of adiponectin, leptin, and IGF-1 lean and obese dogs.

Analyte	Assay	Lean			Obese			P
		n	median	10th-90th percentile	n	median	10th-90th percentile	
Adiponectin, µg/mL	canine	8	16.10	9.82-28.56	22	5.05	0.45-16.87	<0.01
	human	8	16.32	10.93-26.69	22	5.54	3.55-14.94	<0.001
Leptin, ng/mL	assay A	8	< 3.6	3.6-5.4	37	7.2	4.2-25.0	<0.001
	assay B	8	27.6	20.8-81.0	37	28.1	13.7-92.3	>0.05
IGF-1, ng/mL		8	102.0	90.0-180.0	8	151.0	95.0-200.0	<0.05

Table 2.4. Serum adiponectin, ghrelin, and IGF-1 in obese (n=10) and normal weight (n=10) cats.

	Obese cats		Lean cats		P
	Median	10th-90th percentile	Median	10th-90th percentile	
Adiponectin, pg/mL	735.8	(204.3-2675.0)	3117.0	(387.5-6457.1)	<0.01
Ghrelin, pg/mL	216.2	(130.21-306.6)	312.1	(196.0-332.9)	<0.05
IGF-1, ng/mL	434.0	(296.8-556.2)	453.0	(291.0-998.0)	>0.05

2.3. Discussion

Validation studies are needed to ensure that analytical methods can successfully measure the analyte and provide reproducible and accurate results that are useful to discriminate between healthy and diseased animals (Jensen and Kjelgaard-Hansen, 2010). Four observations about our validation studies are worth to be pointed out:

1. The use of a canine serum sample with a known adiponectin concentration would be recommended to calibrate the human adiponectin ELISA. Since it contributed to a better differentiation between low and high adiponectin concentrations. This is in line with the recommendation of use of species-specific standards to achieve similar affinity of antiserum against standards and samples (Eckersall, 1995). Also the use of serum samples as standard is preferred in many cases to purified protein, since it is more economic, yields more suitable physiological matrix and can ensure a consistent, long-lasting supply of standard material (Eckersall, 1995).

2. The two methods used for leptin measurements in canine serum samples presented some disadvantages. The assay A, showed good precision and accuracy results, although gave no result for 7 out of 8 serum samples collected from normal weight dogs, indicating a lack of sensitivity. While using assay B, leptin values were highly under- or over-estimated (reaching up to 33% of the expected value) when compared with expected values in the recovery study. This low performance in the recovery studies probably was the reason of the limited ability of this ELISA to discriminate differences in leptin concentration between obese and lean dogs. Thus, the development and/or evaluation of new commercially available assays for leptin measurements in dogs would be recommended.

3. Interestingly, the human ELISA evaluated for ghrelin measurement in canine serum samples gave better results than species-specific ELISA. Firstly, while human assay showed intra and inter-assay CVs below 20%, the canine assay showed high inter-assay CVs (up to 54%), which was probably influenced by the high inter-kit variability. Secondly, the greater relative changes in ghrelin values obtained in the overlap performance test with human assay in comparison with canine assay, suggest the reduced ability of the canine assay to discriminate between high and low ghrelin values. Although the human ELISA assay gave better validation results, the high inter-assay CV (19.5%) could be considered as a limitation of the kit. Therefore, similarly as happened with leptin, new commercially available assays for ghrelin measurement with better analytical performance should be developed.

4. Overall, adiponectin and IGF-1 assays showed the best results in the validation studies with adequate precision, accuracy, and ability to differentiate between low and high values of the analytes. Also these assays were economic and easy to perform, so were selected to be used in the following experiences of this PhD work.

3. VARIATIONS IN ADIPONECTIN AND IGF-1 DUE TO OTHER BUT OBESITY FACTORS

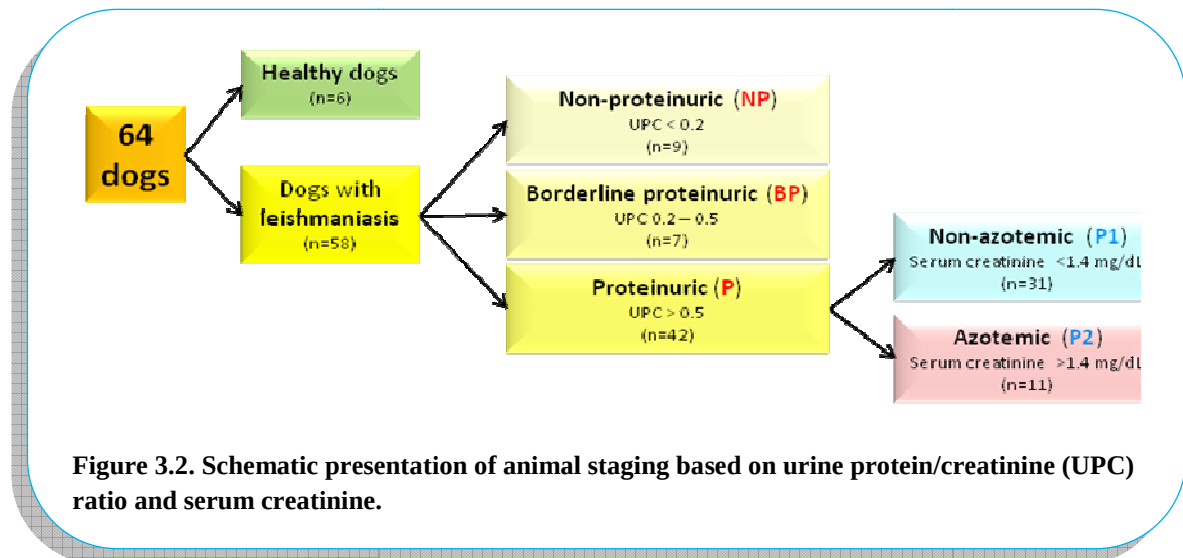
3.1. Material and methods

-Diurnal variation. This study was performed based on a previously described protocol (Ishioka et al., 2005). Eight beagle dogs were assigned into two groups of four dogs (2 males and 2 females in each). One group, defined as group Fed+, was fed at 9:00 as in the previous days. Other group, defined as group Fed-, was fasted on the day of experiment. Blood samples were taken at 8:00, 10:00, 12:00, 16:00, 20:00, 24:00, and 4:00 h.

-Effect of corticosteroids. The design of this study was based on previously described protocols (Martinez-Subiela et al., 2004; Yilmaz et al., 2007). Fourteen beagles were included in this experiment. None of these dogs had a history of exogenous glucocorticoid exposure. The dogs were assigned to control (n=4) and test groups (n=10). Dogs in the control group (2 males and 2 females) were injected SC 0.9% NaCl (0.1ml/kg). The dogs in the test groups were distributed to two subgroups: group 1 (n=5, 3 males and 2 females) and group 2 (n=5, 3 males and 2 females). Methylprednisolone (Aventis Pharma S.A., Spain) was injected once SC 1mg/kg and 5mg/kg in groups 1 and 2 respectively. The experiment was started at 8:00 am after 12 h fasting in all groups. Blood samples were collected prior to treatment at 8:00, 10:00, 12:00, 16:00, and at 20:00 h.

-Effect of castration. A total of five male adult, university-owned beagle dogs were used in this study. All dogs were castrated using open pre-escrotal method as described by Fossum (2007). Blood samples were collected for biochemical assays from all dogs before castration (D1), during six consecutive days (D2-D7) and on weeks 2, 4, 8 and 12 (W2 – W12) after castration.

-Effect of renal failure. Sixty-four dogs, 39 males and 25 females were included in this study. Six dogs were healthy dogs and 58 were dogs previously diagnosed to have leishmaniasis between 2008 and 2010. Schematic presentation of staging of dogs with leishmaniasis based on urine protein/creatinine (UPC) ratio and serum creatinine is presented in figure 3.2.



In this study, unfortunately, IGF-1 could not be evaluated because of the low volume of the samples. Since the used samples in this retrospective study came from the serum left after routine biochemical profiles and serology protocols of Veterinary Clinical Pathology Service of Murcia of Murcia.

-Effect of sepsis. The experimental procedures with animals were performed in Uludag University (Turkey) by a collaborative project with this institution. A total of 10 adult healthy mongrel dogs (6 Male and 4 female) were used. The dogs were assigned to the control (n=5; 2 male and 3 female) and *lipopolysaccharide* (LPS) group (n=5; 2 male and 3 female). Dogs in the control group received an intravenous vehicle (0.9% NaCl, 0.2 mL/kg), whereas endotoxin was injected intravenously once at 0.02 mg/kg to the dogs of LPS group. The experiment was initiated at 9:00 am, after 14 hrs of fasting in both groups of dogs. Venous blood samples were collected before (baseline) and at 0.5, 1, 4, 24 and 48 h post-treatment.

Statistical analysis

The statistical procedures were performed using a standard software (Microsoft Office Ecsel 2007, Microsoft Corporation, Redmond, WA, USA, and GraphPad Prism, GraphPad Software, San Diego, CA, USA). The results for each parameter were

evaluated for approximate normality of distribution by using the D'Agostino & Pearson omnibus or Kolmogorov-Smirnov normality test statistics. To compare changes of BW and biochemical analytes in the same animals at different time points paired *t test* was used for data that showed normal distribution, and *Wilcoxon* signed rank test was used for data with non-normal distribution. To compare changes of BW and biochemical analytes in different groups of dogs *ANOVA* test was used for data that showed normal distribution and *Kruskal-Wallis* test was used for data with non-normal distribution. Correlations between variables were estimated using Spearman correlation coefficient. Values of $p < 0.05$ for two-sided analyses were considered significant.

3.2. Results

-Diurnal variation. Figure 3.3 shows mean $\pm$ sd serum glucose, adiponectin and IGF-1 levels over a 20 h period in fed and fasted dogs. There were significant diurnal rhythms of serum glucose in fed and fasted dogs. However, no consistent diurnal patterns in total adiponectin or IGF-1 levels in both groups of dogs were detected.

-Effect of corticosteroids. Methylprednisolone 1 mg/kg and 5 mg/kg subcutaneously produced a significant increase in serum cortisol levels (180.5% and 1318.9%, respectively) 2h after injection. But did not produce alterations in adiponectin and IGF-1 concentrations, and no significant differences in these analytes were found between control group and groups that received glucocorticoids at different time-points. (Figure 3.4).

-Effect of castration. Serum adiponectin levels significantly decreased on days 3 and 4 after orchidectomy (Figure 3.5.a). After this initial change, adiponectin returned to the initial levels, and did not show any significant alterations during the 3 months of the experimental period.

Serum IGF-1 concentrations were significantly decreased at days 2–5 after castration (Fig. 3.5.b), then began to increase and showed similar values as recorded before castration on weeks 2 and 4 after surgery. However, on weeks 8 and 12 serum IGF-1 concentrations were significantly lower in comparison with the values recorded before castration.

-Effect of renal failure. No significant differences in adiponectin were found between healthy dogs and dogs with different degrees of proteinuria. However, when the group

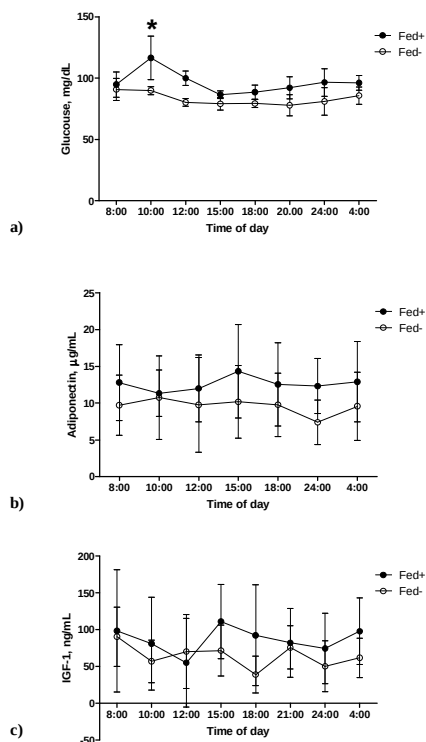


Figure 3.3. Post-prandial variation of serum glucose (a), adiponectin (b), and IGF-1 (c) concentrations. Four beagle dogs were fed (Fed+) at 9:00h and 4 beagle dogs were fasted (Fed-) during all day long. *, $p < 0.05$.

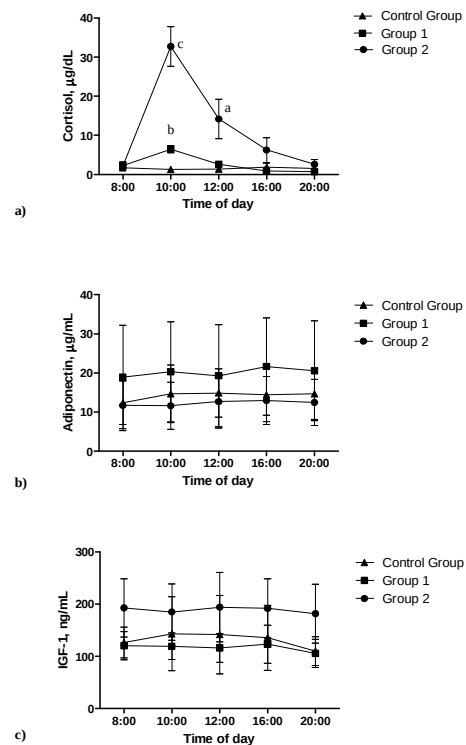


Figure 3.4 Mean ± SD plasma cortisol (a), adiponectin (b), and IGF-1 (c) response to s.c. bolus of methylprednisolone: 1mg/kg (group 1; 5 dogs) and 5mg/kg (Group 2; 5 dogs). Four dogs in the control group were injected SC 0.9% NaCl (0.1ml/kg). *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0005$.

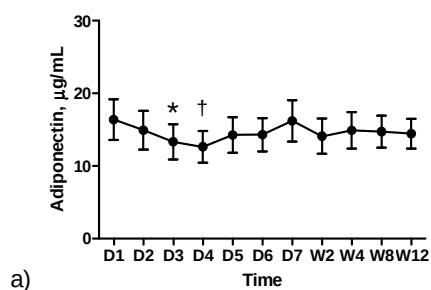
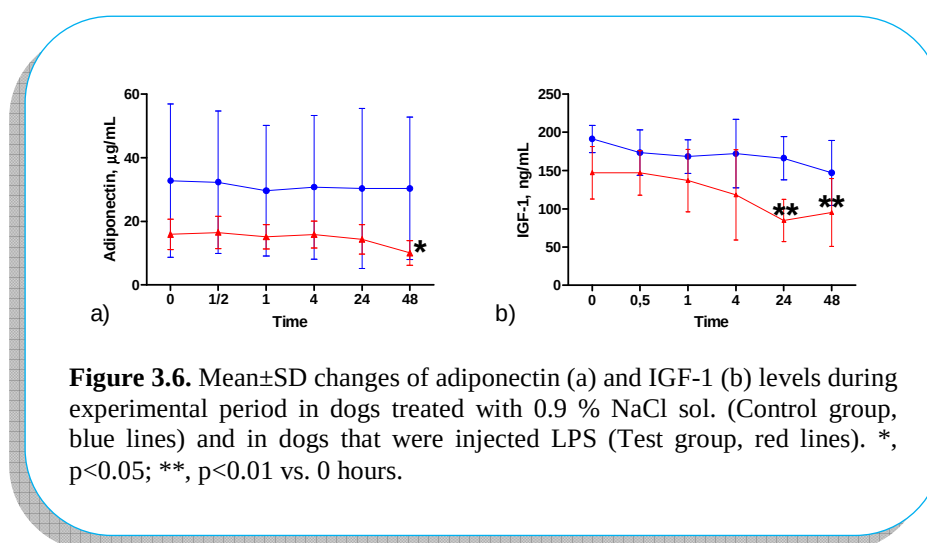


Figure 3.5. Evolution of mean ± SD values of serum adiponectin (a) and IGF-1 (b) during study period. *, $p < 0.05$; †, $p < 0.01$; ‡, $p < 0.001$. D1-D7, days 1-7; W2-12, weeks 2-12.

of dogs with proteinuria was subdivided into 2 subgroups according presence or not of azotemia, serum adiponectin concentrations were higher in azotemic dogs (median [25th-75th percentil], 13.7 µg/mL [10.6-18.7]) in comparison with non-azotemic dogs (median [25th-75th percentil], 6.4 µg/mL [3.7-12.8]).

-Effect of sepsis. Serum adiponectin and IGF-1 did not have significant changes in the control dogs in the different samples times during the experimental period. However in dogs injected with LPS a significant decrease was observed in adiponectin levels at 48 h and IGF-1 levels at 24 and 48 h after the LPS administration when compared with values recorded at time 0 (Figure 3.6.).



3.3. Discussion

Diurnal variation. Our results indicated that regular feeding or one day fasting does not affect serum adiponectin and IGF-1 levels in dogs and that these analytes did not present diurnal variations. These data agree with studies conducted in humans and other animal species that found no diurnal variation or post-prandial alterations in adiponectin (Shand et al., 2006) or IGF-1 (Juul, 2003) concentrations. This fact represents an advantage of adiponectin and IGF-1 over leptin which is greatly influenced by feeding-fasting cycles, as up to 5 fold increase in leptin levels after food intake has been reported in dogs (Ishioka et al., 2005).

Effect of corticosteroids. Methylprednisolone up to 5 mg/kg did not have a short-term effect on adiponectin and IGF-1. There are contradictory results in the literature regarding glucocorticoid effects on adiponectin and IGF-1, as one group of studies

suggested a direct inhibitory effect of glucocorticoids on adiponectin (Fallo et al., 2004); others did not observe the difference in adiponectin or IGF-1 levels (Wolthers et al., 1994; Lewandowski et al., 2006), while a third group of authors described increased serum adiponectin and IGF-1 levels after glucocorticoid administration (Raff and Bruder, 2006; Jang et al., 2008). Use of different corticoid, dose, way, and time of administration could be responsible of contradictory results obtained by distinct authors, thus further studies are needed in order to clarify this topic. However, our data points out another advantage of adiponectin and IGF-1 over leptin, which is affected by methylprednisolone administration (can increase or decrease up to 2 fold depending of the dose) (Yilmaz et al., 2007).

Castration. Orchidectomy induced a short-term inflammatory process due to the surgical procedure that could be associated with the decrease in serum levels of adiponectin and IGF-1. However, orchidectomy did not result in long-term changes of circulating adiponectin concentrations, while a decrease in IGF-1 levels was observed on weeks 8 and 12 post-castration in dogs. The observed changes in IGF-1 levels could be attributed to the direct effect of the decrease in androgens, since expression of IGF-1 is through gonadotropins through cAMP-signal transduction induced (Sirotkin, 2005), or to the food restriction applied in the study in order to maintain stable body weight, as IGF-1 levels had been described to be susceptible to feed restriction (Prewitt et al., 1982; Jull, 2003).

Sepsis. Both adiponectin and IGF-1 decreased a mean of 37% and 42%, respectively, in response to endotoxins in our dogs, similarly as it has been described in human medicine (Colson et al., 2003; Tsuchihashi et al., 2006). The decrease in IGF-1 after an inflammatory-infectious stimulus could be attributed to the inhibitory effect of proinflammatory cytokines on the IGF-1 expression in the liver (Colson et al., 2003). While the decrease in adiponectin levels could be related with low IGF-1 concentrations, as IGF-1 has been demonstrated to induce adiponectin synthesis in adipose tissue (Meier and Gressner, 2004). This data indicate that inflammatory status of the dogs should always be taken in account when the serum adiponectin and IGF-1 are analyzed.

4. VARIATIONS DUE TO OBESITY AND OBESITY-RELATED DISEASES

4.1. Material and methods

In order to try to clarify the two controversies and the three aspects not studied regarding this point, the following trials were performed:

4.1.1. Experimental studies in dogs

-Weight gain. A total of fourteen adult intact, university-owned beagle dogs were used to obtain a wide range of weights and body condition scores (BCS), following a previously described protocol (Sagawa et al., 2002). Schematic presentation of used protocol is presented in Figure 4.1. Blood samples were collected from all dogs at the end of the experimental period.

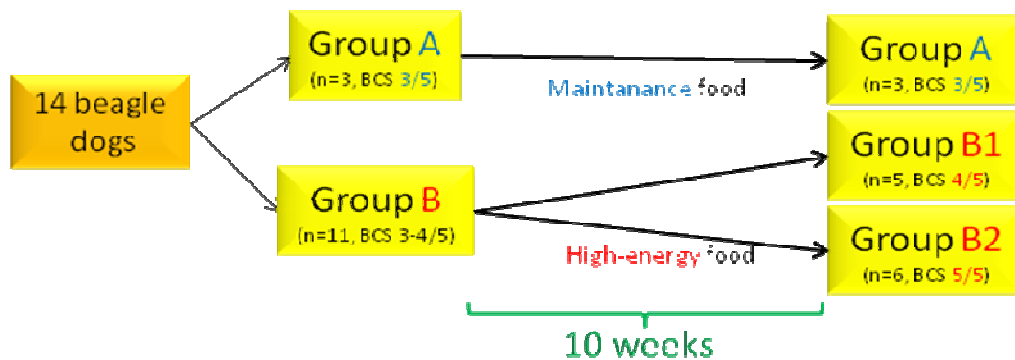


Figure 4.1. Schematic presentation of experimental protocol used in order to obtain dogs with a wide range of body weights and body condition scores. BCS 3/5, lean dogs; BCS 4/5, overweight; BCS 5/5, obese dogs.

-Weight loss. A total of six female non-castrated beagle dogs were used. Schematic presentation of protocol used to induce weight loss in dogs is presented in Figure 4.2. Blood samples were collected from all dogs at the beginning (obese state, T0) and every month (T1-T3) during experimental weight loss period.

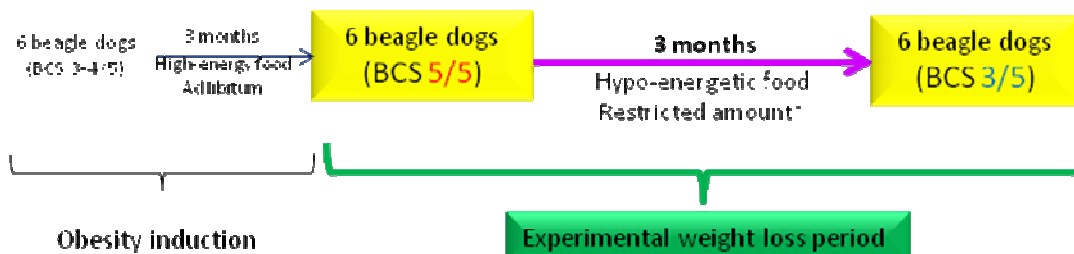


Figure 4.2. Schematic presentation of experimental protocol used to induce rapid weight loss in dogs. *, the amount of food was progressively adjusted to induce a rapid weight loss (2-3%/week) and to cover the minimal requirements in proteins (Blanchard et al., 2004).

Adiponectin was determined in all samples in order to study if this adipokine was affected by changes in BW in dogs. In addition, in order to evaluate the possible presence of inflammation in canine obesity, APPs (high sensitivity C-reactive protein (hs-CRP), haptoglobin (Hp), ceruloplasmin (Cp), and serum amyloid A (SAA)) were assessed in both weight gain and weight loss studies. Also in the weight loss study, immunoglobulins (Igs) were measured. For performing Igs, automated methods originally designed for human Igs determinations were validated for use in dogs giving adequate precision, accuracy, and overlap performance results.

4.1.2. Clinical studies in dogs

-*Weight loss.* The study was performed in collaboration with University of Liverpool (United Kingdom). Plasma and urine from 35 client-owned obese dogs were assessed before and after a weight loss programme in order to evaluate:

- 1) effect of weight loss on circulating adiponectin.
- 2) the possible presence of inflammation in canine obesity by measuring hs-CRP.
- 3) presence of canine obesity-related metabolic dysfunction (ORMD, using a definition modified from that used for human metabolic syndrome). For this purpose, guidelines of the International Diabetes Federation (International diabetes federation) were modified in order to produce an accessible system for dogs. Therefore, the final definition of ORMD was as follows:

a) BCS 7-9/9

b) AND any **two** of the following:

1. Triglycerides >200 mg/dL, (2.3 mmol/L).
2. Total cholesterol > 300 mg/dL (7.8 mmol/L).
3. Systolic blood pressure >160 mmHg.
4. Fasting plasma glucose >100 mg/dL (5.6 mmol/L).

Insulin and adiponectin were measured in all dogs to evaluate if these analytes could change in ORMD.

- 4) effect of weight loss on plasma renal markers. For this purpose classic (serum urea, serum creatinine, urine specific gravity [USG], UPCr, and urine albumin corrected by creatinine [UAC]) and new (homocysteine, cystatin C, clusterin) biomarkers of renal function were evaluated.

4.1.3. Clinical studies in cats

-*Weight loss.* The study was performed in collaboration with University of Liverpool (United Kingdom). Blood samples collected prior to and after the weight loss period from 37 overweight neutered cats were studied in order to evaluate:

- 1) effect of weight loss on plasma adiponectin.
- 2) possible presence of inflammation in feline obesity by measuring Hp and SAA.

4.1.4. Hypothyroidism study in dogs

The study was performed in collaboration with Nantes Atlantic College of Veterinary Medicine (France). 39 hypothyroid client-owned dogs were included in the study. Primary hypothyroidism was diagnosed based on clinical and laboratorial signs of primary hypothyroidism i.e. lethargy, bodyweight (BW, kg) gain, dermatologic signs, high cholesterol (>6.5 mmol/L), high c-TSH (>0.5 ng/mL) and low fT4 values (≤ 12 pmol/L) (Tyler, 2007). All dogs were subjected to thyroxin therapy (mean $\pm$ sd, 14.4 ± 5.7 μ g/kg/day) for 3 months. Good therapeutic response was considered, if the dogs exhibited a significant clinical improvement (including weight loss, increase activity, improvement of the skin) and at least a 30% decrease in c-TSH value.

Blood samples were collected when hypothyroidism was diagnosed (T1), and after treatment with thyroxin during 3 months (T2), and APPs (CRP, SAA, and Hp) and adiponectin were assessed. In this study IGF 1 was not evaluated, since a recently published study by Jaillardon et al. (2011) dealt with this topic.

4.2. Results

4.2.1. Experimental studies in dogs

-*Weight gain.* Table 4.1 shows the values of the body weight, adiponectin, and APPs of the different groups of dogs at the end of the trial. SAA concentrations were below detection limit of the assay in six animals, so the statistical study could not be performed for this acute phase protein. Dogs with BCS 5/5 (group B2) showed significantly lower serum adiponectin and Hp concentrations in comparison with normal weight dogs (group A, BCS 3/5), while no statistically significant differences were detected in serum concentration of hs-CRP, CRP and Cp between the groups of dogs.

-*Weight loss.* Evolution of the concentrations of adiponectin, APPs, and Igs at different time points is presented in Figure 4.3. Serum adiponectin showed a statistically

significant increase at two (T2) and three (T3) months after beginning the weight loss when compared with pre-weight loss adiponectin concentrations (T0). No statistically significant changes were detected in CRP, Hp, Cp, IgG, and IgM concentrations at one, two and three months after beginning the weight loss period (T1, T2, and T3, respectively) when compared with their levels before weight loss (T0). Serum IgA levels were significantly decreased at T1, T2, and T3 when compared with T0.

4.2.2. Clinical studies in dogs

-Weight loss.

(1) Adiponectin. Plasma total adiponectin significantly increased in dogs after the weight loss program.

(2) Inflammation. In obese state of dogs hsCRP concentrations were within the range for normal healthy dogs and did not change with weight loss.

(3) Canine obesity-related metabolic dysfunction (ORMD). Prior to weight loss, 10 dogs were defined as having ORMD. Plasma adiponectin concentration was less ($P=0.004$), and plasma insulin concentration was greater ($P=0.01$) in dogs with ORMD.

(4) Renal markers. Urea and USG significantly increased after weight loss, whilst UPCR, UAC, serum creatinine, homocysteine, cystatin C, and clusterin all decreased.

4.2.3. Clinical studies in cats

-Weight loss. The results for all plasma biomarkers are shown in Table 4.2. A significant increase in plasma adiponectin was observed after weight loss. APPs in obese cats before weight loss were within the range for normal healthy cats, and no significant changes were found in APPs after weight loss.

Table 4.1. Values of the body weight (BW) and biochemical analytes in dogs with BCS 3/5 (group A), BCS 4/5 (group B1), and BCS 5/5 (group B2) after experimentally induced weight gain trial.

	Group A	Group B1	Group B2
BW, kg	14.53±1.7	15.66±1.1	19,15±3,46*
Adiponectin, µg/mL	14.3±1.2	13.2±1.6	8.1±2.3
hs-CRP, mg/L	1.58±1.91	1.89±1.29	2.76±2.65
Hp, g/L	1.20±0.16	1.17±0.17	0.71±0.24**
Cp*10⁻³, Δabs/min	6.60±0.75	6.64±2.45	7.02±2.96

*, p<0.05; **, p<0.01 vs group A.

Table 4.2. Plasma biomarkers in cats undergoing a weight loss program.

Analyte	Pre-weight loss	Post-weight-loss
<i>Adiponectin (pg/mL)</i>	2661 (978 to 6039)	3174 (668 to 7820) ^a
<i>Haptoglobin (g/L)</i>	1.7 (0.2 to 3.1)	1.8 (0.2 to 2.9)
<i>SAA (mg/L)</i>	0.38 (0.38 to 0.38)	0.38 (0.38 to 0.38)

All data are expressed as median (range).

^a, p<0.05 versus successful cats pre-weight loss.

Table 4.3. Median (range) data of analytes of successfully (Group A) and unsuccessfully treated hypothyroid dogs (Group B).

	Group A			Group B		
	T1	T2	P	T1	T2	P
TSH	1.55 (0.60-8.30)	0.40 (0.03-4.80)	<0,001	1.00 (0.60-4.30)	0.90 (0.50-4.20)	NS
FT4	10.00 (0.0-16.00)	16.50 (10.00-29.00)	<0,001	10.00 (7.00-11.00)	14.00 (12.00-17.00)	<0,001
BW	35.50 (6.00-69.00)	33.45 (15.00-70.00)	0,023	39.00 (26.00-54.00)	38.50 (26.00-51.00)	NS
Adiponectin, µg/mL	13.30 (5.23-23.78)	9.48 (0.43-27.92)	0,001	16.97 (8.02-29.87)	14.53 (5.92-23.64)	0,038
CRP, mg/L	5 (5.00-53.80)	5 (5.00-46.20)	NS	5 (5.00-18.70)	5 (5.00-5.00)	NS
Hp, g/L	2.15 (0,09-7.4)	2.4 (0.28-9.20)	0,039	2.11 (0.05-4.03)	2.17 (0.20-3.72)	NS
SAA, µg/mL	5.04 (0.68-116.00)	1.83 (0.20-24.88)	NS	2.21 (0.20-90.60)	3.57 (0.84-33.95)	NS
Cp, ΔA/min	0.024 (0.001-0.042)	0.022 (0.005-0.055)	NS	0.027 (0.003-0.052)	0.020 (0.004-0.033)	NS

T1, before therapy; T2, after 3 month therapy; NS, not significant.

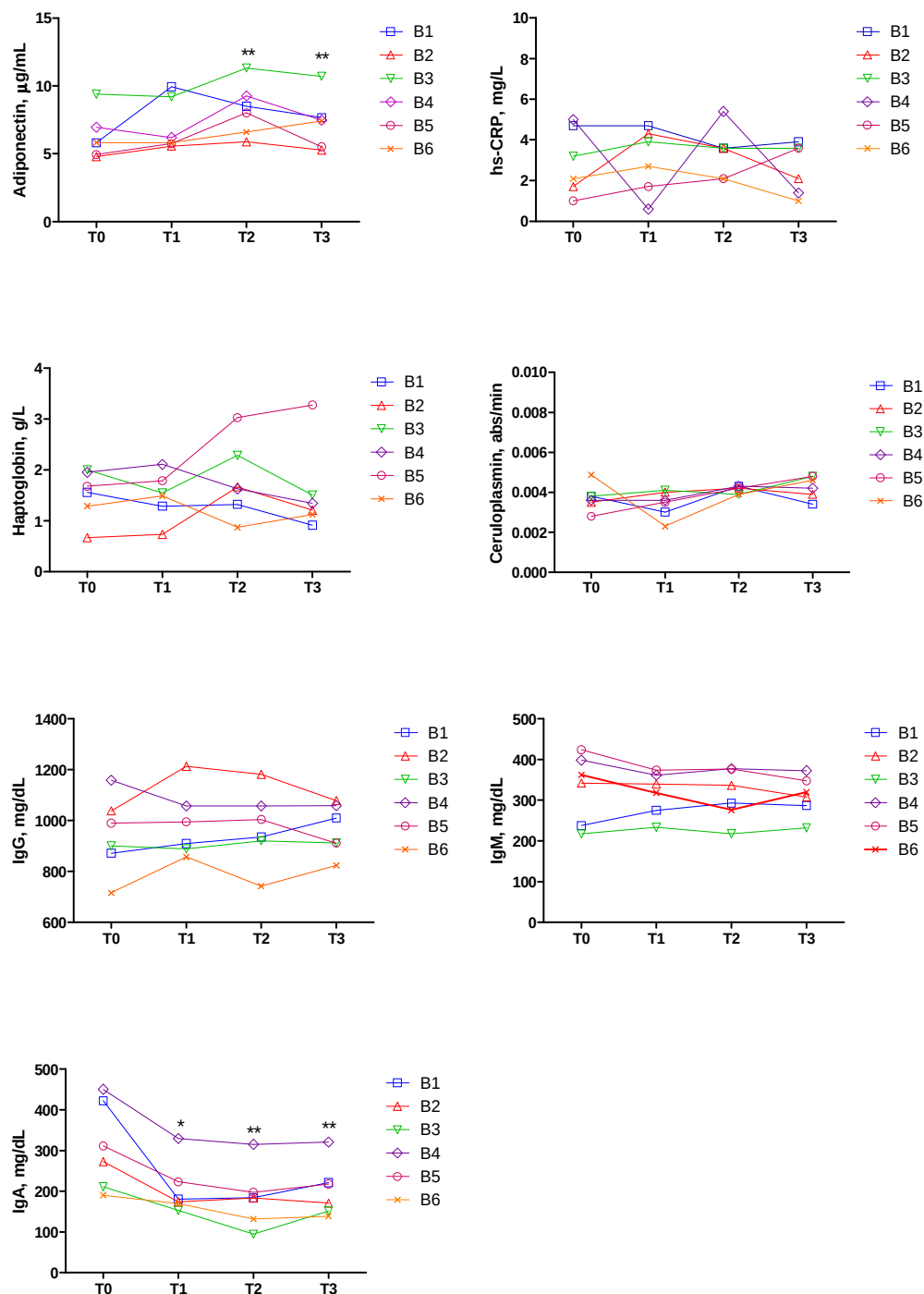


Figure 4.3. Evolution of the levels of serum biomarkers associated with inflammation in six Beagle dogs (B1-B6) before (T0) and during weight loss program (T1-3, 1-3 months after beginning weight loss program, respectively). CRP, C-reactive protein; IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A. *, $P<0.05$; **, $P<0.01$ vs. T0.

4.2.4. Obesity-related diseases in dogs

-Hypothyroidism. Twenty eight of 39 dogs exhibited a good therapeutic response (group A), whereas the rest of the dogs were considered insufficiently-treated (group B). Median (range) results of evaluated analytes in both group of dogs is presented in table 4.3. After treatment, group A dogs demonstrated a significant decrease in c-TSH and increase in fT4 concentration, respectively, accompanied by significant decrease in BW and adiponectin, and an increase in Hp. Group B dogs showed no statistically significant changes in c-TSH, but had a significant increase in fT4, accompanied by a significant decrease in adiponectin, of lower magnitude than group A.

4.3. Discussion

a) Relation obesity-adiponectin. Our longitudinal experimental as well as clinical studies indicated that serum adiponectin is decreased in obese dogs and significantly increase after weight loss. Also our clinical studies demonstrated that in cats adiponectin has a similar behaviour as in dogs. These results are in accordance with those reported by some authors in obesity studies in dogs (Ishioka et al., 2006) as well as in human medicine (Arita et al., 1999; Chandran et al., 2003). Although contradict those reported by Verkest et al. (2011), who found no correlation between adiponectin and canine obesity. It is worth pointing out that the lack of differences in adiponectin levels between obese and lean dogs found in some previous studies could be attributed to high inter-individual variability of adiponectin, not taking in account the inflammatory status or oestrus cycle phase, and/or due to the use of methods that were not validated for adiponectin measurement in canine samples.

b) Relation obesity-inflammation. Our results would indicate that there is no existence of evident inflammation in canine and feline obesity. Previous papers published showed contradictory results about values of CRP in canine obesity. Some authors found increases and others decreases, although CRP levels in these studies were within the range that indicates a non-inflammatory condition (Veiga et al., 2008; German et al., 2009). In obese humans, CRP concentrations are between 3 mg/L (concentrations <3 mg/L are considered in human as normal values) and 10 mg/L (>10mg/L values are compatible with inflammation). It has been postulated that these intermediate values are not directly related with inflammation and could reflect genetic

factors, demographic variables, or behavioural and dietary patterns (Kushner et al., 2006).

c) *Canine obesity-related metabolic dysfunction*. Definition of “obesity related metabolic dysfunction (ORMD)” and not “metabolic syndrome” was chosen since syndrome suggests a disease process related to obesity which has not yet been identified in dogs.

When a modified diagnostic criteria of the International Diabetes Federation was used, up to third of obese dogs showed to be suffering from obesity-related metabolic dysfunction (ORMD) characterized by hypoadiponectinaemia and hyperinsulinaemia, similarly as it occurs in humans (Leroith, 2012) and horses (Johnson, 2002). This study could represent the basis of further investigations to determine pathogenetic mechanisms and the health significance of ORMD for dogs, in terms of disease associations and outcomes of weight loss.

d) *Weight loss effect on renal biomarkers*. Our data demonstrated a decrease in a variety of renal biomarkers (such as clusterin, homocystein, cystatin C) in obese dogs undergoing weight loss. These results might suggest possible subclinical alterations in renal function in canine obesity, and could establish a link between the kidney problems that have been described to be associated with obesity (German et al., 2010).

e) *Hypothyroidism*. Our data indicates that decrease in adiponectin after the treatment may be directly attributed to the influence of thyroid hormones, as it has been suggested in humans (Iglesias et al., 2003), as no correlation between this adipokine and BW was observed.

No significant difference were found in mean circulating levels of APPs before and after treatment; with the exception of a significant increase in Hp concentrations in successfully treated dogs, although these values were within the range observed in healthy animals (Mylonakis et al., 2011). Our CRP results are in agreement with many previous reports, indicating, that disturbances in thyroid status do not produce evident increases in CRP in dogs (Nakamura et al., 2008) as well as humans (Pérez et al., 2004; Caixás et al., 2009). However, SAA higher than the limit of our laboratory reference range (<5 mg/L) was found in 14 dogs before the treatment. And all of them, with the

exception of 2 dogs, showed a decrease in these SAA after the therapy, indicating the need of further studies to explore the role of SAA in canine hypothyroidism.

5. STUDY AND IDENTIFICATION OF NEW POTENTIAL SERUM BIOMARKERS INVOLVED/LINKED TO OBESITY

5.1. PON1 and BChE

5.1.1. *Material and methods.*

5.1.1.1. Validation of PON1 assays in dogs and cats. Three different spectrophotometric assays for measurements of serum PON1 activity in canine serum samples were evaluated. Two methods were adapted to 96-well microplate format using phenyl acetate and 5-thiobutyl butyrolactonase (TBBL) and one was adapted to an automated analyser using p-nitrophenyl acetate as substrate. The analytical validation was performed as described in Section 2, precision, accuracy, and limit of detection were calculated.

The methods for BChE measurements in dogs and cats were not evaluated in this PhD work, because they were previously validated (Tecles et al., 2000).

5.1.1.2. Variations due to inflammation

-Effect of sepsis. For material and methods description of experimental sepsis in dogs see Section 3.1.

5.1.1.3. Variations due to obesity and obesity-related diseases

5.1.1.3.1. Experimental studies in dogs

For material and methods description of experimental induction of obesity and weight loss in dogs see Section 4.1.1.

5.1.1.3.2. Clinical studies in dogs

For material and methods description of experimental induction of obesity and weight loss in dogs see Section 4.1.2.

5.1.1.3.3. Clinical studies in cats

For material and methods description of weight loss study in cats see Section 4.1.3.

5.1.1.3.4. Obesity-related diseases in dogs

-Hypothyroidism.

For material and methods description see section 4.1.4.

-*Diabetes mellitus (DM)*. A Total of 222 dogs were included in present study. All dogs were divided into 3 groups:

Group 1 was formed by 74 dogs with diabetes mellitus. The diagnosis of diabetes mellitus was made on the basis of previously described criteria (Durocher et al., 2008). Only those dogs that were not treated with insulin previously were eligible for inclusion in the study.

Group 2 included 74 dogs that were healthy dogs with no clinical evidences of acute or chronic disease, except in some cases obesity.

Group 3 was formed by 74 dogs that presented different diseases except diabetes mellitus.

5.1.2. Results.

5.1.2.1. *Validation PON1 in dogs and cats*. Analytical validation data of the three methods in dog and one in cat serum are presented in table 5.1.1.

Table 5.1.1. Analytical validation data of different methods to measure PON1 activity (UI/mL) in canine and feline serum samples.

Species	Assay	Precision		Accuracy		Detection limit
		CV, %		Linearity		
		Intra	Inter	Equation	R ²	
Dog	Serum lactonase activity by measuring the hydrolysis of 5-thiobutyl butyrolactonase (TBBL) [assay A]	<7	<18	y=0.955x+0.22; y=0.594x+0.47	0.995; 0.908	1.0
	Serum arylesterase activity by measuring the hydrolysis of phenyl acetate [assay B]	<5	<10	y=1.041x-1.38; y=1.044x-1.25	0.999; 0.992	9.1
	Serum arylesterase activity by measuring the hydrolysis of p-nitrophenyl acetate [assay C]	<6	<9	y=0.998x-0.14; y=1.056x-0.35	0.994; 0.991	0.6
Cat ^a	Serum arylesterase activity by measuring the hydrolysis of p-nitrophenyl acetate [assay A]	<5	<7	y=0.897x+0.18; y=0.960x+0.17	0.999; 0.999	0.3

CV, coefficient of variation.

5.1.2.2. Variations due to inflammation

-*Effect of sepsis*. Serum BChE and PON1 did not have significant changes in the control dogs in the different samples times during the experimental period. However in dogs injected with LPS a significant decrease was observed in BChE activity at 24 h and PON1 activity at 48 h after the LPS administration when compared with values recorded at time 0 (Figure 5.1.1.).

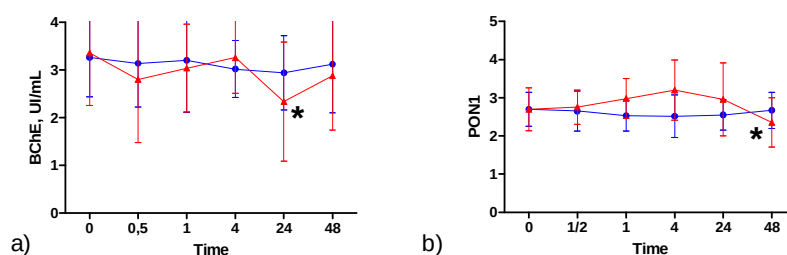


Figure 5.1.1. Mean±SD changes of BChE (a) and PON1 (b) concentrations during experimental period in dogs treated with 0.9 % NaCl sol. (Control group, blue lines) and in dogs that were injected LPS (Test group, red lines). *, $p < 0.05$ vs. 0 hours.

5.1.2.3. Variations due to obesity and obesity-related diseases

5.1.2.3.1. Experimental studies in dogs

-Weight gain. Serum BChE activity was lower in dogs with BCS 3/5 (group A, 3.1 kU/L [2.8-3.3]) when compared with dogs with BCS 4/5 (group B1, 3.8 kU/L, [3.2-4.4] $P < 0.05$) and dogs with BCS 5/5 (group B2, 5.4 kU/L [4.6-6.1], $P < 0.001$). While no significant differences were observed in PON1 activity between the different groups of dogs (group A, 5.7 kU/L [5.1-6.3]; group B1, 5.1 kU/L [4.9-6.2], and group B2, 5.4 kU/L [5.0-6.3]).

-Weight loss. Serum BChE showed a statistically significant decrease in dogs after weight loss (6.05 [5.70-8.20] vs. 5.20 [3.10-5.60] kU/L; $p < 0.05$), while no statistically significant differences were observed in PON1 activity in dogs before (6.0 kU/L [4.9-7.2]) vs. after (5.83 kU/L [5.4-6.7]) weight loss

5.1.2.3.2. Clinical studies in dogs

BChE activity significantly decreased in dogs after weight loss, while no statistically significant changes were observed in PON1 activity in dogs before and after weight loss (Table 5.1.3).

Table 5.1.3. BChE and PON1 in dogs before and after weight loss.

Criterion	Pre-weight loss	Post-weight-loss	<i>p</i>
BChE (kU/L)	7.4 (2.4 to 14.8)	5.9 (2.5 to 14.0)	<0.05
PON-1 (kU/L)	2.5 (0.5 to 3.8)	2.23 (0.5 to 3.4)	>0.05

5.1.2.3.3. Clinical studies in cats

-Weight loss. There were no differences in BChE ($P=0.737$) or (PON-1) activity after weight loss in obese cats (Table 5.1.4). However, pre-weight-loss PON-1 activity ($P=0.004$) was lower in cats that failed to complete weight loss than those who were successful, no differences were noted for BChE, $P=0.777$.

Table 5.1.4. BChE and PON1 in cats that completed and not completed weight loss program.

Criterion	Cats not completing	Cats completing	
		Pre-weight loss	Post-weight-loss
BChE (kU/L)	2.8 (1.8 to 3.9)	2.9 (1.7 to 5.1)	2.8 (1.7 to 4.3)
PON-1 (kU/L)	4.6 (3.1 to 6.9) ^a	5.5 (3.8 to 7.3)	5.6 (3.3 to 7.4)

All data are expressed as median (range).

BChE, butyrylcholinesterase; PON-1, paraoxonase type 1.

^a, $p<0.01$ versus successful cats pre-weight loss.

5.1.2.3.4. Obesity-related diseases in dogs

-Hypothyroidism. Dogs that demonstrated a good therapeutic response showed significant increase in BChE activity from 5.20 kU/L (2.90-9.50) to 5.65 kU/L (2.70-10.70). No statistically significant changes were observed in PON1 activity in both groups of dogs.

-Diabetes mellitus. A significantly higher BChE activity was recorded in dogs with diabetes mellitus (group 1) when compared with healthy dogs or dogs with diseases different than diabetes mellitus (groups 2 and 3) (Figure 5.1.2).

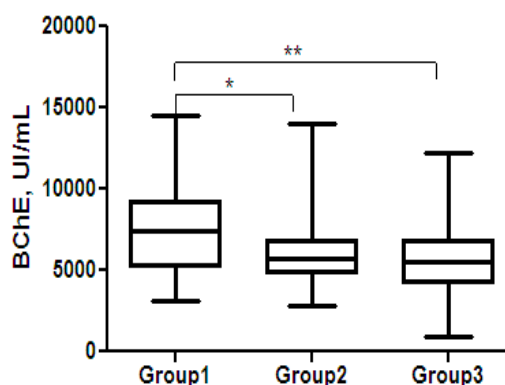


Figure 5.1.2. Butyrylcholinesterase (BChE) activity in dogs with diabetes mellitus (Group 1), healthy dogs (Group 2), and in dogs with different diseases except diabetes mellitus (Group 3). *, $P<0.05$; **, $P<0.01$.

5.1.3. Discussion

a) *PON1* assays. Three methods were validated for measurement of PON1 activity in dog and one in cat serum. A different substrate was used in each assay:

TBBL for lactonase activity and p-nitrophenyl acetate and phenyl acetate for arylesterase activity. All the assays were robust, precise (with the exception of the interassay values for lactonase method for canine samples), and easy to set up in laboratories. These assays will allow additional research into PON1 behavior in obesity and obesity-related diseases that are known to alter oxidative status in dogs and cats.

b) Inflammation. Our data indicate that severe inflammation decrease both BChE and PON1 activity in dogs (approximately 30% and 20%, respectively), similarly as occur in humans (Al-Kassab and Vijayakumar, 1995; Blanceil and Delaroche, 1996). The decrease of BChE could be an organic reaction with the purpose of increasing the acetylcholine levels and limiting the inflammatory response (Rossas-Ballina and Tracey, 2009). While observed decrease in PON1 activity could be attributed mainly to two mechanisms: (1) direct negative effect of cytokines (TNF- α , IL-1) on PON1 expression (Feingold et al., 1998); and (2) changes in lipid and protein composition of HDL in response to inflammation, as during the acute phase response, HDL is losing most of the HDL-associated enzymes (including PON1) (Van Lenten et al., 1995; James and Deakin, 2004).

c) Obesity. Serum BChE showed to be a good marker of adiposity in dogs, similarly as occur in humans (Randell et al., 2005), while no changes were observed in obesity in PON1 activity in dogs, and BChE and PON1 in cats. Although in cats pre-weight loss PON1 could be used as a predictor of successfulness of weight loss program, suggesting that pre-weight loss PON1 could be used as a predictor of the success of a weight loss regime in cats. However, since these findings are preliminary, further studies should be undertaken to clarify the relation between BChE and PON1, and obesity in the cat.

d) Hypothyroidism. The successful treatment of hypothyroid dogs was also accompanied by the increase in serum BChE activity, similarly as it has been described in humans (Popović et al., 1998). The increase of BChE activity could be involved in the hydrolysis of triglycerides (Popović et al., 1998) and would contribute to the improvement of lipidic profile described in hypothyroid dogs after thyroxin therapy (Dixon et al., 2002).

e) *Diabetes mellitus*. The evaluation of BChE in canine DM was performed because of two main reasons. Firstly, although increase in BChE was described in human DM possibly due to altered lipid metabolism, there were no reports about this topic in dogs. Secondly, BChE is included in the general biochemical profile in “San Marco” veterinary hospital, what allowed us to perform a retrospective study with more than 200 dogs in order to evaluate possible changes in BChE activity due to presence of DM. This is a preliminary study, and further studies evaluating PON1 and other biomarkers used in this PhD (such as adiponectin, IGF-1 and APPs) should be performed in canine diabetes mellitus in order to clarify its physiopathological mechanisms.

Dogs with diabetes mellitus showed higher BChE activity than healthy controls, similarly as described in humans (Abbott et al., 1993). However body condition score did not affect BChE values in dogs with diabetes mellitus, as reported in humans (Abbott et al., 1993) Suggesting that in diabetes mellitus insulin insensitivity or deficiency could have a major influence on BChE activity than body weight.

5.2. Proteomics

5.2.1. Material and methods

-Animals and experimental set-up

For animal and experimental set-up description see section 4.1.1.

-Two dimensional polyacrylamide gel electrophoresis (2DE)

Serum samples before (T1) and after (T2) weight loss of five dogs were analyzed in duplicate for 2DE. The first dimensional isoelectric focusing was performed on homemade immobilized pH gradient strips (11cm) with a non linear pH range of 4 to 10. For the second dimension was performed on homemade gradient 10-15% polyacrylamide gels 140 x 140 x 1.5mm. After electrophoresis, the gel was stained with silver according to standard protocols as reported before (Miller and Gemeiner, 1992).

-2DE gel image analysis

Stained 2D gels were scanned in an ImageScanner II (GE Healthcare Life Sciences) and evaluated by using specific software (Image Master 2D Platinum 7.0, GE Healthcare Life Sciences).

-MS identification and data analysis

Those spots that appeared significantly differentially expressed between obese and lean state of all animals were subjected to MS identification. Selected spots were excised and in-gel digestion with trypsin was performed. After desalting step by using C18 Zip-Tip the entire peptides were analyzed by MALDI-TOF/TOF mass spectrometry (Ultraflex II, Bruker Daltonics).

Processed spectra were searched using Mascot (www.matrixscience.com) in Swiss-Prot database (release 56.5) or the NCBI nr database (20090314). Following search parameters were set: taxonomy mammalia, global modifications carbamidomethylation on cysteine, variable modifications oxidation on methionine, MS tolerance 100-150 ppm, MS/MS tolerance 1 Da, one missed cleavage allowed. MS and MS/MS-identifications were considered as statistically significant ($\alpha < 5\%$).

5.2.2. Results

Results of protein mapping of canine serum using 2DE revealed more than 170 individual spots with molecular weights between 10 and 100 kDa over a pH range of 4 to 10 in each analyzed silver stained 2DE gels from both, obese and lean dog's serum samples (Figure 5.2.1). Protein profiles obtained of each class (dogs in an obese and lean state) were homogeneous since no differences were detected between replicates and between images from samples of the same class.

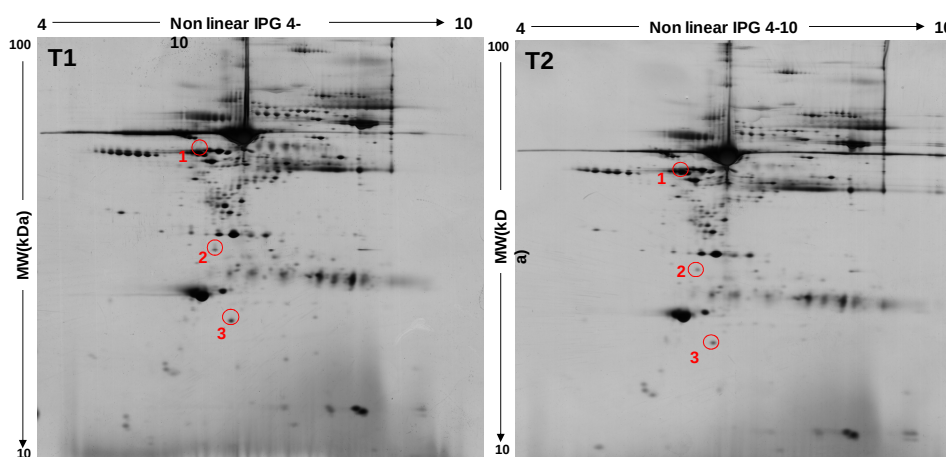


Figure 5.2.1. Representative 2DE gel images of silver-stained proteins of canine serum obtained before (T1, obese state) and after (T2, lean state) weight loss correspond to dog number 1. Differentially expressed protein spots between the obese and lean states of the dogs are encircled and numbered: (1) retinol-binding protein 4; (2) Clusterin precursor; (3) Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1.

Image analysis was used for automatic spot detection and for the comparison of the spot volumes obtained in the two image classes: images from obese and lean serum samples. A total of 144 spot matches, corresponding to the detection and quantification of the same spot in the different classes, were obtained. A statistical class analysis allowed the detection of 3 spot matches that were modulated in response to weight loss. Among the three spots differentially expressed in obese versus lean state, two were up-regulated and one was down-regulated in obese state in comparison with lean state of the dogs (table 5.2.1).

Table 5.2.1. Differentially expressed spot matches in obese versus lean state of dogs. X: media of normalized volumes. SD: standard deviation.

Normalized volumes: SD: standard deviation.						
Spot ^a	Obese vs Lean	<i>Obese state</i>		<i>Lean state</i>		P value
		<i>(n = 5)</i>		<i>(n = 5)</i>		
		X	SD	X	SD	
1	↑	0.2091	0.0155	0.1534	0.0409	0.0316
2	↑	0.1639	0.0303	0.0964	0.0234	0.0072
3	↓	0.1998	0.3169	2.726	0.3001	0.0310

^aSpot label number from annotated gel image (see figure 1 & 2).

MALDI-TOF/TOF MS allowed the identification of the three protein spots with differential expression levels as belonging to retinol-binding protein 4 (RBP4), clusterin precursor (CLU), and alpha-1 antitrypsin (AAT) (Table 5.2.2).

Table 5.2.2. MS identification of proteins from canine serum samples. MW: molecular weight. pI: isoelectric point.

Spot label ^a	Accession number ^b	Protein identification	Theoretical MW [kDa]	Theoretical pI	Number of unique peptides	Sequence coverage ^c [%]	Score
1	gi 73998292	Retinol-binding protein 4 [Canis lupus familiaris]	29.8	9.4	3	19.5	390.2
2	gi 50979240	Clusterin precursor [Canis lupus familiaris]	51.8	5.6	3	9.7	81.3
3	gi 121583756	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 [Canis lupus familiaris]	46.3	5.5	2	6.5	239.2

^aSpot label number from annotated gel image (see Figure 1).

^bNCBI (gi) protein database accession numbers.

^cSequence coverage: percent of identified sequence to the complete sequence of the known protein.

5.2.3. Discussion

In the present study, serum proteome analysis was performed in beagle dogs before and after controlled weight loss program. Statistical comparison of spot volumes

revealed three spots differentially expressed between obese and lean state of the animals. The detection of only 3 proteins significantly affected by obesity would be in odds with what would be expected, as for example, a range of adipokines are known to be altered in canine obesity (German et al., 2010). This could be explained by the insensitivity of the technique, as when 2DE gel methods are used for proteome analysis the detection of some biomarkers can be very challenging due to the presence of high – abundance proteins (presented in serum at mg/mL levels) (Selvaraju and Rassi, 2012).

Mass spectrometric analysis revealed that serum CLU, RBP4, and AAT were the proteins involved in the spot changes obtained in the 2DE analysis. These proteins could be considered as potential markers of obesity and obesity-related diseases processes in dogs. The data from our clinical study of weight loss in dogs support this finding, since serum CLU was significantly lower in dogs after weight loss (section 4.1.2). Future studies, evaluating RBP4 and AAT by using species-specific assays in serum of obese and normal weight dogs, would be recommended in order to verify the results obtained in this report.

6. CONCLUSIONS

1. Commercially available assays for adiponectin and IGF-1 showed adequate results in our validation studies when using with dogs and cats samples. Commercially available leptin and ghrelin ELISA kits tested in our study showed to have low sensitivity and/or accuracy; and development of new kits to measure these adipokines would be recommended.
2. Serum adiponectin and IGF-1 are not influenced by feeding/fasting cycles or by a single dose corticoid administration. Also circulating adiponectin levels are not affected by orquidectomy. However, caution should be taken when interpreting IGF-1 values of castrated dogs, since lower concentrations were observed in these animals. Also inflammatory status of the dog should always be evaluated when analyzing both adiponectin and IGF-1, since inflammation decreases concentrations of these proteins in significant way.

3. Canine and feline obesity is associated with decreased circulating adiponectin concentrations, which increase after weight loss. But it seems to not produce an evident inflammation, at least that can be detected with APPs.
4. Up to one third of obese dogs suffer from obesity-related metabolic dysfunction, characterized by hypoadiponectinemia and hyperinsulinemia, similarly as occur in the human metabolic syndrome.
5. Weight loss in dogs is associated with the improvement of serum markers of renal dysfunction, such as clusterin, homocystein, cystatin C.
6. Therapy of dogs with hypothyroidism results in decreased adiponectin concentrations and increased BChE activity.
7. Serum BChE showed to be a good marker of adiposity in dogs and is increased in canine diabetes mellitus. No changes were observed in PON1 activity in obese dogs, and BChE and PON1 in obese cats. Although in cats pre-weight loss PON1 could be used as a predictor of successfulness of weight loss program. BChE activity is increased in dogs with diabetes mellitus.
8. Proteomic analysis revealed three proteins (clusterin, retinol-binding protein 4, and alpha-1 antitrypsin) that could be considered as potential markers of obesity and obesity-related disease processes in dogs.

6. Resumen

1. Introducción

La obesidad es el trastorno nutricional más común en perros y gatos (Roudebush et al., 2008; Lusby et al., 2009). Su creciente incidencia parece reflejar la tendencia observada en los humanos (Rennie and Jebb, 2005) donde casi dos tercios de los adultos en los Estados Unidos tienen sobrepeso o son obesos (Flegal et al., 2002). Del mismo modo, los estudios en animales de compañía procedentes de diversas partes del mundo han estimado que la incidencia de la obesidad en la población de perros y gatos oscila entre el 22 y el 40 por ciento (Burkholder et al., 2000; McGreevy et al., 2005).

Los objetivos de esta tesis doctoral fueron:

1. Validar los ensayos disponibles comercialmente de adipoquinas (adiponectina, leptina y grelina) e IGF-1 (factor de crecimiento insulínico-1) para su uso en perros y gatos.
2. Estudiar los efectos de las variaciones debidas a factores no relacionados con obesidad (como variaciones diurnas, el efecto de la ingesta de alimentos, la castración y sepsis). Para este propósito, se seleccionaron los analitos que dieron resultados adecuados en la validación realizada en el punto 1 y fueron más económicos, fáciles y rápidos de realizar.
3. Estudiar el efecto de la obesidad sobre la adiponectina y las principales proteínas de fase aguda en perros y gatos y explorar si los perros obesos sufren de síndrome metabólico. También evaluar si la obesidad podría estar relacionada con cambios en biomarcadores renales, y estudiar cómo las adipoquinas y proteínas de fase aguda se ven afectados por enfermedades relacionadas con la obesidad, como el hipotiroidismo.
4. Estudiar e identificar nuevos biomarcadores séricos relacionados con la obesidad canina. Para esto se evaluará el PON1 (paraoxonasa 1) y la colinesterasa y se realizarán estudios de proteómica.

2. Estudios de validación

2.1. Material y métodos

Se evaluó la capacidad de los diferentes métodos disponibles comercialmente en el momento de la realización de la Tesis, para medir la adiponectina, la grelina, leptina, e IGF-1 en perros y adiponectina, la grelina, y el IGF-1 en los gatos. Los métodos evaluados aparecen en tabla 2.1. La leptina en los gatos no se estudió debido a la falta de reactividad de esta adipoquina con anticuerpos de humana o de ratón/rata utilizados en ELISAs y a la no existencia de ELISAs específicos de gato en el momento en que se realizaron las pruebas.

Para la validación de los métodos analíticos se ha seguido protocolos publicados previamente (Jensen and Kjelgaard-Hansen, 2010) estudiándose tres parámetros básicos: precisión, exactitud y límite de detección. Además, se realizó una validación de la capacidad de los métodos para diferenciar muestras con concentraciones altas y bajas. Para esto se usaron muestras de animales delgados y obesos, o viceversa, salvo para la grelina, en la que las muestras fueron obtenidas a diferentes tiempos después de administración de glucosa intravenosa en los perros, ya que la glucosa es un potente inhibidor de la grelina (McCowen et al., 2002).

2.2. Resultados

2.2.1. Validación analítica

Los resultados de la validación analítica de diferentes métodos para perro y gato aparecen en las tablas 2.1 and 2.2, respectivamente.

2.2.2. Capacidad para diferenciar muestras de diferentes concentraciones

2.2.2.1. Perros

Adiponectina. Las muestras obtenidas de perros obesos y analizadas con los ELISAs específico-canino y humano tuvieron concentraciones de adiponectina mas bajo que las muestras recolectadas de los perros delgados ($p < 0.005$ and 0.0005 , respectivamente) (Tabla 2.3).

Leptina. Con el ensayo A, las muestras de perros obesos tuvieron una concentración mas alta de leptina que las de perros de peso normal ($p < 0.001$). No se observaron

diferencias significativas ($p=0.89$) en las concentraciones de leptina cuando las muestras de los perros obesos y de peso normal se analizaron con el ensayo B (Tabla 2.3).

Grelina. Los cambios en las concentraciones de grelina en suero canino después de inyectar glucosa, medidos con ensayos canino y humano, aparecen en la figura 2.1. En comparación con los valores basales, hay una disminución media del 17% y un incremento medio de 19% en las concentraciones de ghrelin tras 5 y 120 minutos después de la administración de glucosa, respectivamente, cuando se analizaron con el ensayo canino. Sin embargo, no se detectaron cambios estadísticamente significativos. Cuando se utilizó el ensayo humano, se detectó una disminución del 30% en los niveles de grelina en plasma 10 min después de la administración de glucosa ($p < 0,05$), seguido por un aumento de 50% a los 120 minutos ($p < 0,05$), con respecto a los niveles basales.

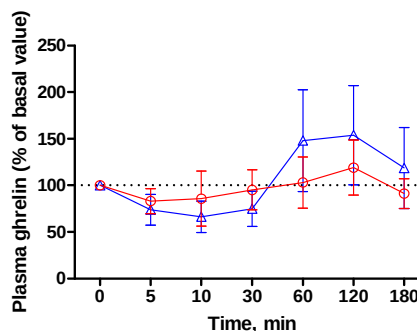


Figura 2.1. Respuesta de grelina plasmática a la glucosa IV (0.5/kg de peso corporal) inyectada a cuatro perros de raza beagle y medida con ensayos canino (○) y humano (Δ).

IGF-1. Las concentraciones de IGF-1 sérico en los perros delgados fueron mas altas ($p < 0,05$) que las de los perros obesos (Tabla 2.3).

2.2.2.2. Gatos

Adiponectina, grelina, y IGF-1. Las concentraciones de adiponectina ($p=0.01$) y grelina ($p=0.03$) fueron mas bajas en gatos obesos ($n=10$) en comparación con gatos de peso normal ($n=10$). Pero no se observaron diferencias significativas en los niveles de IGF-1 entre los dos grupos de gatos ($p=0.12$) (Tabla 2.4).

Tabla 2.1. Datos de la validación analítica de diferentes ensayos para medir la adiponectina, leptina, grelina, e IGF-1 en muestras de suero canino.

Camino:	Analito	Ensayo	Unidades	Precisión		Exactitud			Límite de detección
				CV, %		Linealidad		Recuperación ^c %	
				Intra	Inter	Ecuación	R ²		
Adiponectina ^a	Canine ELISA Kit, Millipore, USA	µg/mL	<7	<14	y=0.982x+0.65; y=0.999x+0.66	0.9996; 0.997	NE	0.098	
	Human ELISA, High Sensitivity Kit, BioVendor-Labartorni medicina, Czech Republic	µg/mL	<8	<11	y=0.991x+0.11; y=0.976x+0.37	0.996; 0.999	NE	0.74	
Leptina ^b	Canine ELISA kit, Millipore, USA [assay A]	µg/L	<6	<8	y=1.037x-0.06; y=0.987x-0.79	0.985; 0.986	77 - 101	-	
	Canine leptin, BPB Biomedicals Inc, USA [assay B]	µg/L	<14	<14	y=0.928x+0.38; y=0.907x+3.75	0.955; 0.970	67 - 125	9.08	
Grelina	Canine ELISA, Phoenix Pharmaceuticals Inc, USA	ng/mL	<10	>20%	y=1.059x – 0.71; y=1.006x – 1.04	0.991; 0.924	101-121	0.6	
	Human unacylated ELISA, BioVendor-Labartorni medicina, Czech Republic	pg/mL	<7	<20	y=0.977x+15.46; y=1.006x+5.06	0.999; 0.998	83-96	-	
IGF-1	Immulite 1000 assay, Diagnostic Products, USA	ng/mL	<8	<10	y=0.993x+4.11; y=0.992x+0.18	0.999; 0.999	NE	0.01	

CV, coeficiente de variación.

^a se usó una muestra de suero canino, con una concentración de adiponectina determinada con ELISA canino, como estándar para la curva de calibración de la ELISA humana.

^b se evaluaron 3 ensayos mas (Human EASIA, BioSource Europe S.A., Belgium; Human ELISA, BioVendor-Laboratorni medicina a.s., Czech Republic; Mouse/rat enzyme immunoassay kit, SPIbio, France) para las medir leptina en suero canino, pero no hubo reactividad.

^c los ensayos de recuperación se realizaron sólo con los kits donde se disponía de la proteína canino purificada; NE, no evaluado.

^d los límites de detección no se han podido calcular para el ensayo A de la leptina y ensayo humano de grelina, ya que blancos no dieron ningún valor.

Tabla 2.2. Datos de la validación analítica de diferentes ensayos para medir adiponectina, grelina, y IGF-1 en suero felino.

Tabla 2.2. Datos de la validación analítica de diferentes ensayos para medir adiponectina, grelina, y IGF-1 en suero humano.							
Analito	Ensayo	Unidades	Precisión		Exactitud		Límite de detección
			CV, %		Linealidad	Recuperación	
			Intra	Inter	Ecuación	R ²	
Adiponectina	Human ELISA, High Sensitivity Kit, BioVendor-Labaratorni medicina, Czech Republic	ng/mL	<7	<16	y=0.894x+997.37; 0.974; y=0.882x+605.43 0.987	100-112	2.0
Grelina	Human unacylated ELISA, BioVendor-Labaratorni medicina, Czech Republic	pg/mL	<15	<15	y=0.984x-11.90; 0.962; y=0.963x+5.88 0.998	91-112	9.6
IGF-1	Immulite 1000 assay, Diagnostic Products, USA	ng/mL	<3	<6	y=1.022x+6.85; 0.987; y=1.022x+0.83 0.994	100-112	0.01

CV, coeficiente de variación.

Tabla 2.3. Valores de adiponectina, leptina, y IGF-1 en suero de perros delgados y obesos.

Analito	Ensayo	Delgados			Obesos			P
		n	mediana	10-90 percentil	n	mediana	10-90 percentil	
Adiponectina, µg/mL	perro	8	16.10	9.82-28.56	22	5.05	0.45-16.87	<0.01
	Humana	8	16.32	10.93-26.69	22	5.54	3.55-14.94	<0.001
Leptina, ng/mL	A	8	< 3.6	3.6-5.4	37	7.2	4.2-25.0	<0.001
	B	8	27.6	20.8-81.0	37	28.1	13.7-92.3	>0.05
IGF-1, ng/mL		8	102.0	90.0-180.0	8	151.0	95.0-200.0	<0.05

Tabla 2.4. Valores de adiponectina, grelina, y IGF-1 en suero de gatos obesos (n=10) y delgados (n=10).

	Obesos		Delgados		P
	Mediana	10-90 percentil	Mediana	10-90 percentil	
Adiponectina, pg/mL	735.8	(204.3-2675.0)	3117.0	(387.5-6457.1)	<0.01
Grelina, pg/mL	216.2	(130.21-306.6)	312.1	(196.0-332.9)	<0.05
IGF-1, ng/mL	434.0	(296.8-556.2)	453.0	(291.0-998.0)	>0.05

2.3. Discusión

Los estudios de validación son necesarios para garantizar que los métodos analíticos funcionan adecuadamente, proporcionando resultados reproducibles y precisos y siendo útiles para discriminar entre animales sanos y enfermos (Jensen and Kjelgaard-Hansen, 2010). Se pueden destacar cuatro puntos con respecto a los estudios de validación realizados en esta Tesis:

1. Se recomienda usar una muestra de suero canino con una concentración conocida para calibrar el ELISA adiponectina humana. Ya que produce una mejor diferenciación entre las concentraciones altas y bajas de adiponectina. Este dato está en línea con la recomendación del uso de estándares específicos de especie para lograr una afinidad similar de antisuero frente a estándares y muestras (Eckersall, 1995). También el uso de muestras de suero como estándar se prefiere en muchos casos a la proteína purificada, ya que es más económico, produce una matriz fisiológica más adecuada y puede asegurar un suministro constante y de larga duración de material estándar (Eckersall, 1995).

2. Los dos métodos usados para las mediciones de leptina en las muestras de suero canino presenta algunas desventajas. El ensayo A, mostró una buena precisión y exactitud de los resultados, aunque no dió resultado para 7 de 8 muestras de perros de peso normal, lo que indica una falta de sensibilidad del ensayo. Mientras, que en el ensayo B, los valores de leptina fueron significativamente sub o sobre-estimados en comparación con los valores esperados en el estudio de la recuperación. Este bajo rendimiento de los estudios de recuperación probablemente fue la causa de la baja capacidad de este ELISA para detectar diferencias significativas entre las concentraciones de leptina de perros obesos y delgados. Así sería recomendable el desarrollo de nuevos ensayos para determinar la leptina canina de una forma fiable .

3. Curiosamente, el ELISA humano evaluado para la medición de la grelina en perro dio mejores resultados que el ELISA canino. Ya que, mientras el ensayo humano mostró unos CV intra e inter-ensayo inferiores al 20%, el ensayo canino tuvo unos CVs inter-ensayo muy alto (hasta 54%), probablemente influenciado por la alta variabilidad inter-kit. Además, el ensayo humano mostró unos cambios superiores en los valores de grelina obtenidos durante la prueba de administración de glucosa; indicando una reducida capacidad del ensayo canino discriminar entre los valores altos y bajos de grelina. Aunque el ensayo humano proporcionó unos mejores resultados en la validación, el alto CV de inter-ensayo (19,5%) se puede considerar como una limitación

del kit. Por lo tanto, al igual como ocurre con los ensayos de leptina, se deberían desarrollar nuevos ensayos para la medición de la grelina en suero canino.

4. En general, los ensayos de adiponectina y de IGF-1 mostraron los mejores resultados en los estudios de validación, con una adecuada precisión y exactitud, y una buena capacidad de diferenciar entre los valores altos y bajos de los analitos. También estos ensayos fueron económicos y fáciles de realizar; por ello fueron seleccionados para ser utilizados en las experiencias siguientes de este trabajo de doctorado.

3. VARIACIONES DE LAS ADIPOQUINAS POR FACTORES NO RELACIONADOS CON LA OBESIDAD

3.1. Material y métodos

-Variaciones diurnas. Este estudio se realizó basándose en un protocolo previamente descrito (Ishioka et al., 2005). Ocho perros de raza beagle se distribuyeron en dos grupos de cuatro perros (2 machos y 2 hembras en cada uno). Un grupo, Fed+, se alimentó a las 9:00 como se realiza habitualmente. Mientras que los perros del otro grupo, Fed-, se mantuvieron en ayunas durante el día de experimento. Se sacaron muestras de sangre a las 8:00, 10:00, 12:00, 16:00, 20:00, 24:00, y 4:00 h.

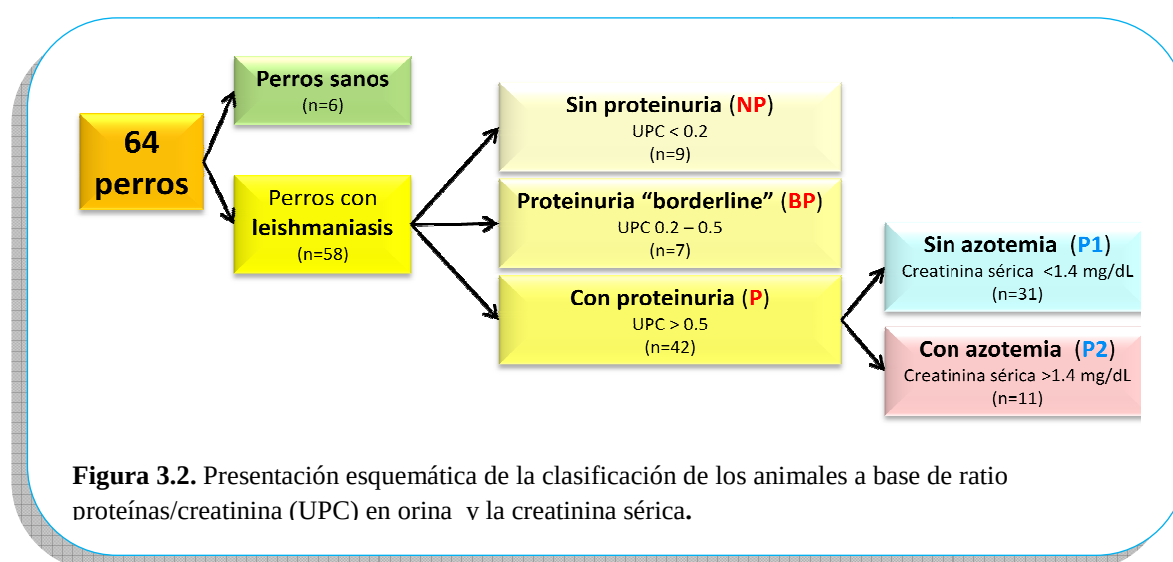
-Efecto de los corticosteroides. El diseño de este estudio se realizó según protocolos previamente descritos (Martinez-Subiela et al., 2004; Yilmaz et al., 2007). Se emplearon 14 perros de raza beagle sin historia de exposición a glucocorticoides exógenos, que se distribuyeron en un grupo control (n=4) y un grupo prueba (n=10). A los perros del grupo control (2 machos y 2 hembras) se les inyectó SC 0.9% NaCl (0.1ml/kg). Los perros del grupo de prueba se dividieron en dos subgrupos: grupo 1 (n=5, 3 machos y 2 hembras) y grupo 2 (n=5, 3 machos y 2 hembras). A los grupos 1 y 2 se les inyectó metilprednisolona (Aventis Pharma S.A., Spain) en una sola dosis SC a dosis de 1mg/kg y 5mg/kg respectivamente. El experimento comenzó a las 8:00 am después de 12 h de ayuno y se tomaron muestras de sangre en todos los grupos a las 8:00, 10:00, 12:00, 16:00 y a las 20:00 h.

-Efecto de castración. Se usaron 5 perros Beagle que se castraron usando la técnica abierta pre-escrotal descrita por Fossum (2007). Se tomaron muestras de sangre antes de

la castración (D1), durante seis días consecutivos (D2-D7) y 2, 4, 8 y 12 (W2 – W12) semanas después de castración.

-Efecto de daño renal. Se incluyeron 64 perros, 39 machos y 25 hembras, en el estudio. Seis eran perros sanos y 58 eran perros con leishmaniasis. Estos perros se dividieron en grupos según su ratio proteína/creatinina en orina (UPC) y creatinina en suero como se representa en la figura 3.2.

En este estudio, el IGF-1 no pudo ser evaluado debido al reducido volumen de las muestras. Ya que las muestras utilizadas en este estudio procedían del suero que había sobrado después de la realización de los perfiles bioquímicos y protocolos de serología de Servicio de Patología Clínica de Universidad de Murcia.



-Efecto de sepsis. Los procedimientos experimentales con animales se realizaron en Uludag University (Turquía) dentro de un proyecto de colaboración con esta institución. Se incluyeron un total de 10 perros adultos mestizos (6 machos y 4 hembras) que fueron asignados a un grupo control (n=5; 2 machos y 3 hembras) y grupo de lipopolysaccharido (LPS) (n=5; 2 machos and 3 hembras). A los perros del grupo control se les inyectó IV 0.9% NaCl (0.2 mL/kg), mientras que a los perros del grupo LPS se les inyectó endotoxina IV en una sola dosis (0.02 mg/kg). El experimento se inició a las 9:00 am, después de 14 horas de ayuno en los dos grupos de perros. Se tomaron muestras de sangre venosa antes (basales) y a 0,5, 1, 4, 24 y 48 h post-tratamiento.

Análisis estadístico

Los análisis estadísticos se realizaron usando un software estándar (Microsoft Office Ecel 2007, Microsoft Corporation, Redmond, WA, USA, and GraphPad Prism, GraphPad Software, San Diego, CA, USA). Los resultados de cada parámetro se evaluaron para la normalidad de la distribución mediante el uso de la D'Agostino y Pearson omnibus o Kolmogorov-Smirnov. Para comparar los cambios de peso corporal y analitos bioquímicos en los mismos animales a diferentes tiempos de una prueba se utilizó prueba t pareada para los datos que mostraron una distribución normal, y el test de Wilcoxon para datos con distribución no normal. Para comparar los cambios de peso corporal y analitos bioquímicos en diferentes grupos de perros se utilizó el test ANOVA para los datos que mostraron una distribución normal y el Kruskal-Wallis para datos con distribución no normal. Las correlaciones entre variables se estimaron mediante el coeficiente de correlación de Spearman. El límite para el nivel de significación se estableció en $p > 0.05$.

3.2. Resultados

-Variaciones diurnas. La Figura 3.3 muestra la media $\pm$ sd de glucosa sérica, adiponectina y IGF-1 durante un período de 20 h en el grupo de perros alimentados y el grupo en ayunas. Se encontraron ritmos diurnos significativos de glucosa sérica en los dos grupos. Sin embargo, no se detectó ningún patrón cíclico consistente en las variaciones de adiponectina total o IGF-1 en ambos grupos de perros.

-Efecto de corticoesteroides. La metilprednisolona a 1 mg/kg y 5 mg/kg por vía subcutánea produjo un incremento significativo en los niveles de cortisol en suero (180,5% y 1318,9%, respectivamente) 2h después de la inyección. Sin embargo, no produjo alteraciones en las concentraciones de adiponectina e IGF-1, y no se observaron diferencias significativas en estos analitos a diferentes tiempos entre el grupo control y los grupos que recibieron glucocorticoides (Figura 3.4).

-Efecto de castración. Los niveles séricos de adiponectina disminuyeron significativamente en los días 3 y 4 después de la orquidectomía (Figura 3.5.a). Después de este cambio inicial, la adiponectina volvió a los niveles iniciales, y no mostró alteraciones significativas durante los 3 meses del período experimental

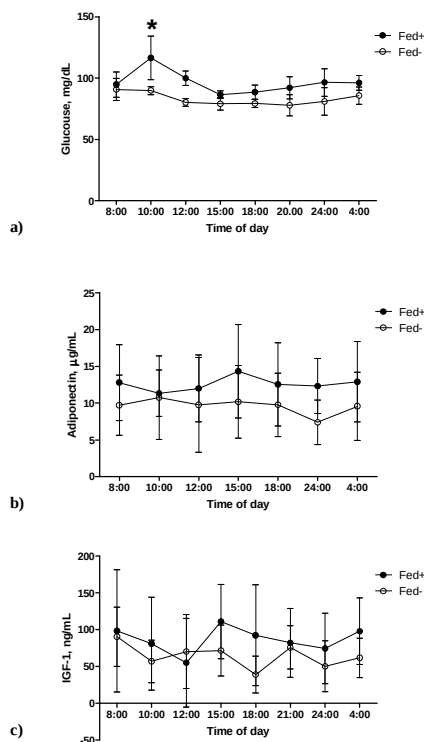


Figura 3.3. Variación post-prandial de concentraciones séricas de glucosa (a), adiponectina (b), y IGF-1 (c). Cuatro perros de raza beagle han sido alimentados (Fed+) a las 9:00 h y 4 perros de raza beagle estaban en ayuno (Fed-) durante todo el día. *, $p < 0.05$.

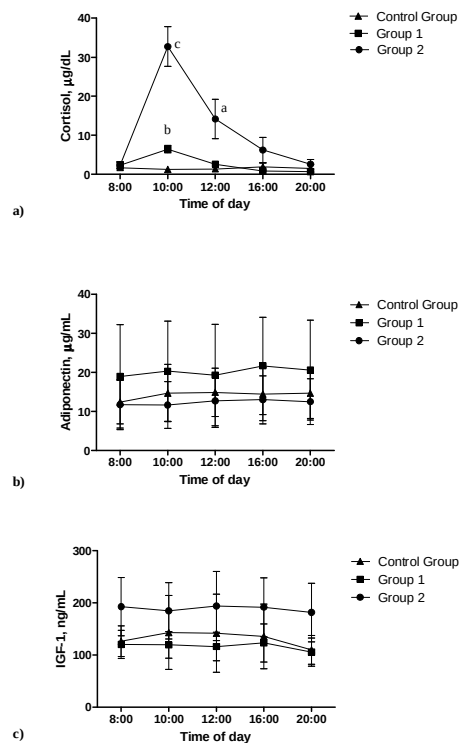


Figura 3.4 Respuesta de valores medios $\pm$ SD de cortisol (a), adiponectina (b), y IGF-1 (c) a una inyección s.c. de metilprednisolona: 1mg/kg (grupo 1; 5 perros) y 5mg/kg (Grupo 2; 5 perros). Cuatro perros en grupo control se inyectaron s.c. con 0.9% NaCl (0.1ml/kg). *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0005$.

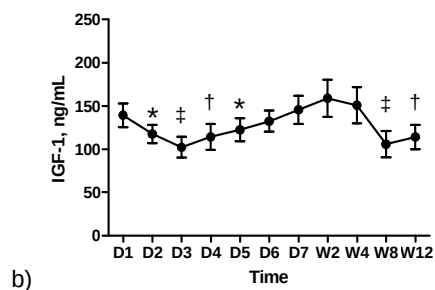
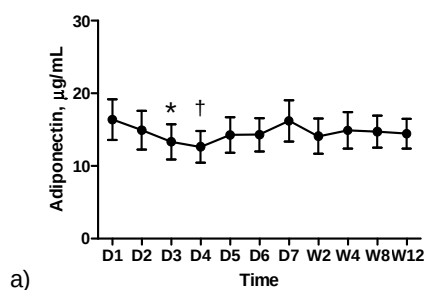
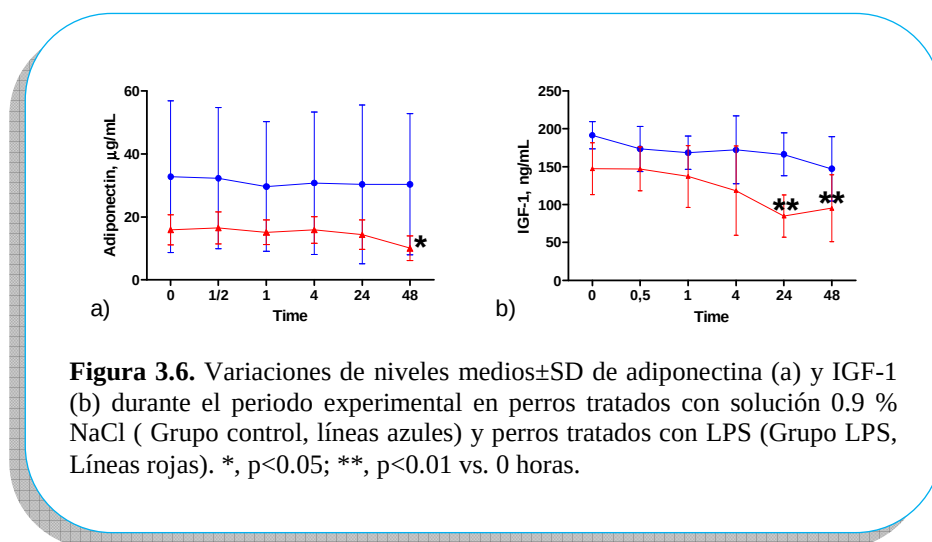


Figura 3.5. Evolución de valores medios $\pm$ SD de adiponectina (a) y IGF-1 (b) séricas durante el periodo de estudio. *, $p < 0.05$; †, $p < 0.01$; ‡, $p < 0.001$. D1-D7, días 1-7; W2-12, semanas 2-12.

Las concentración de IGF-1 sérico se redujo significativamente 2-5 días después de la castración (Fig. 3.5.b), y luego comenzó a aumentar, mostrando valores similares a los registrados antes de la castración en las semanas 2 y 4 después de la cirugía. Sin embargo, en la semanas 8 y 12 las concentraciones de IGF-1 fueron significativamente más bajas en comparación con los valores registrados antes de la castración.

-Efecto de daño renal. No se observaron diferencias significativas en la adiponectina entre perros sanos y perros con distintos grados de proteinuria. Sin embargo, cuando el grupo de perros con proteinuria fue subdividido en 2 subgrupos en función de la presencia o no de azotemia, las concentraciones séricas de adiponectina fueron más altas en los perros con azotemia (mediana [25-75 percentil], 13,7 mg/ml [10.6-18.7]) en comparación con perros sin azotemia (mediana [25-75 percentil], 6,4 mg/ml [3.7-12.8]).

-Efecto de sepsis. La adiponectina e IGF 1 no mostraron cambios significativos en los perros en grupo control en las distintas tomas efectuadas durante todo período experimental. Sin embargo, en los perros inyectados con LPS, se observó una disminución significativa en los niveles de adiponectina a las 48 h y en los niveles de IGF-1 a las 24 y 48 h después de la administración de LPS en comparación con los valores registrados a tiempo 0 (Figura 3.6).



3.3. Discusión

Variaciones diurnas. Nuestros resultados indican que la alimentación regular o un día de ayuno no afecta la adiponectina o IGF-1 en los perros y que estos analitos no presentan variaciones diurnas. Estos datos concuerdan con estudios realizados en

humanos y otras especies, donde no se encontraron variaciones diurnas o alteraciones post-prandiales en las concentraciones de adiponectina (Shand et al., 2006) o IGF-1 (Juul, 2003). Este hecho representa una ventaja de la adiponectina y la IGF-1 sobre la leptina que está fuertemente influenciada por ciclos de la alimentación y ayunas; ya que se han descrito en el perro incrementos de hasta 5 veces en los niveles de leptina después de la ingesta de alimentos (Ishioka et al., 2005).

Efecto de corticosteroides. La methylprednisolona hasta 5 mg/kg no tuvo un efecto a corto plazo sobre las concentraciones séricas de adiponectina e IGF-1. Existen resultados contradictorios en la literatura sobre los efectos de los glucocorticoides en la adiponectina y el IGF-1. Ya que se ha indicado un efecto inhibitor directo de los glucocorticoides sobre la adiponectina (Fallo et al., 2004); otros autores no observan diferencias en los niveles de adiponectina o IGF-1 (Wolthers et al., 1994; Lewandowski et al., 2006); mientras que en otros estudios se ha descrito un aumento de la adiponectina sérica y los niveles de IGF-1 después de la administración de glucocorticoides (Raff and Bruder, 2006; Jang et al., 2008). El uso de diferentes tipos de corticoides, dosis, modo y tiempo de administración podría ser los factores responsables de los resultados contradictorios obtenidos por distintos autores, y se necesitarían más estudios para aclarar este tema. Sin embargo, nuestros resultados ponen de manifiesto otra ventaja de la adiponectina y la IGF-1 sobre la leptina en perros; ya que la leptina que se afecta por la administración de metilprednisolona (puede aumentar o disminuir hasta 2 veces dependiendo de la dosis de corticoide administrado) (Yilmaz et al., 2007).

Castración. La orquidectomía indujo un proceso inflamatorio a corto plazo debido a la intervención quirúrgica, que podría estar asociada con la disminución de los niveles séricos de adiponectina y IGF-1. Sin embargo, la orquidectomía no produjo cambios a largo plazo en las concentraciones de adiponectina, mientras que se observó una disminución en los niveles de IGF-1 en las semanas 8 y 12 después de la castración en perros. Los cambios observados en los niveles de IGF-1 podrían atribuirse a la disminución de los andrógenos, ya que la expresión de IGF-1 es inducida por gonadotropinas a través de la transducción de señal del AMP cíclico (Sirotkin, 2005). O también a la restricción de alimentos que se realizó en los animales del estudio con el fin de mantener el peso corporal estable, ya que se descrito que los niveles de IGF-1 son susceptibles a la restricción de alimento (Prewitt et al., 1982; Jull, 2003).

Sepsis. Tanto la adiponectina como la IGF-1 disminuyeron una media de 37% y 42%, respectivamente, como respuesta a la endotoxina en nuestro estudio, de manera similar como se ha descrito en humana (Colson et al., 2003; Tsuchihashi et al., 2006). La disminución de IGF-1 podría ser atribuida al efecto inhibitor de las citoquinas proinflamatorias en la expresión de IGF-1 en el hígado (Colson et al., 2003). Mientras que la disminución en los niveles de adiponectina podría estar relacionada con concentraciones bajas de IGF-1, ya que la IGF-1 induce la síntesis de adiponectina en el tejido adiposo (Meier and Gressner, 2004). Estos datos indican que siempre que se analice la adiponectina y/o la IGF-1 se debe tener en cuenta el estado inflamatorio de los perros.

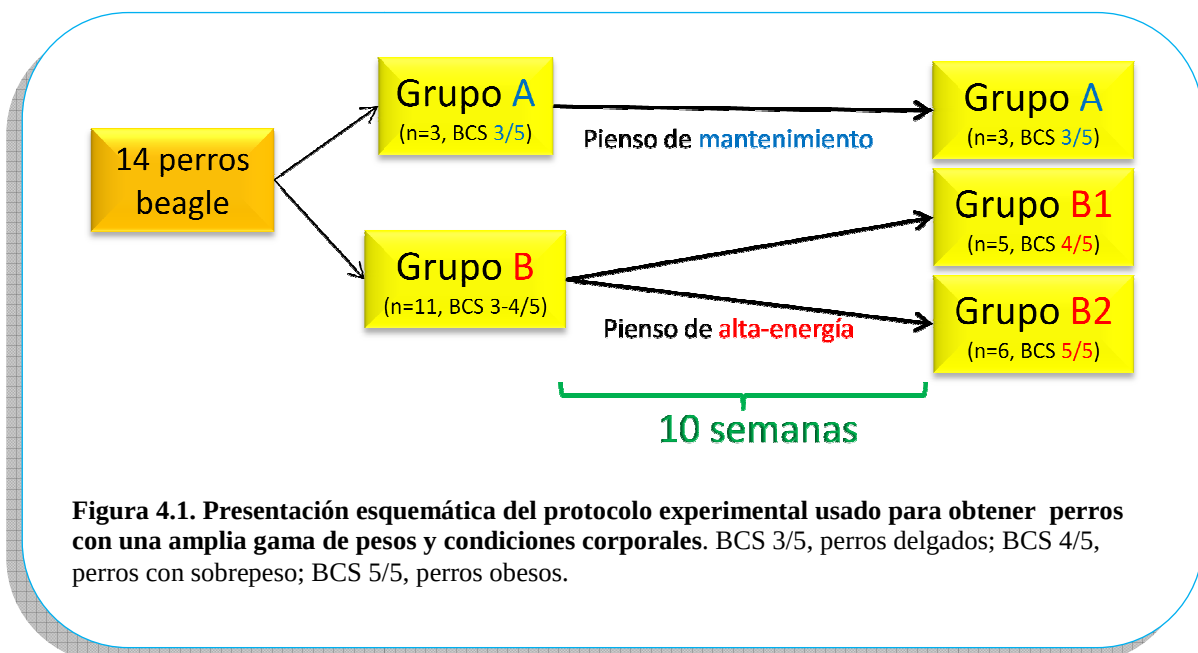
4. VARIACIONES DEBIDAS A LA OBESIDAD Y ENFERMEDADES RELACIONADAS

4.1. Material y métodos

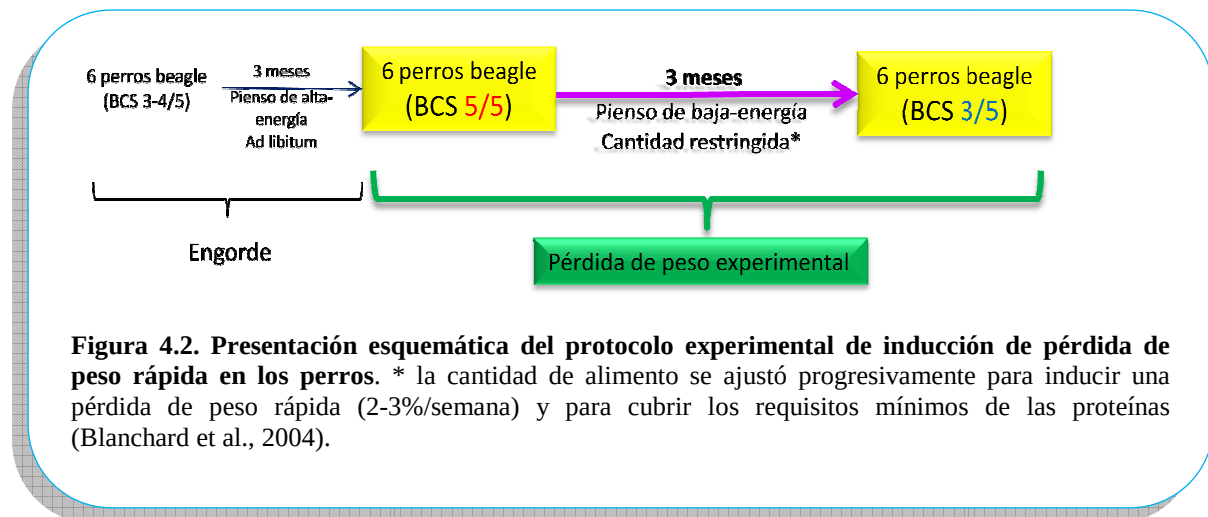
Se realizaron los siguientes ensayos:

4.1.1. Estudios experimentales en perros

-Engorde. Se usaron catorce perros adultos intactos de raza beagle, para obtener una amplia gama de pesos y condición corporal (BCS), siguiendo un protocolo descrito previamente (Sagawa et al., 2002). El esquema del protocolo utilizado aparece en la Figura 4.1. Las muestras de sangre se recogieron de todos los perros en el final del período experimental.



-Pérdida de peso. Se emplearon un total de seis perras de raza beagle, no castradas. El esquema del protocolo utilizado para inducir la pérdida de peso en los perros se presenta en la Figura 4.2. Las muestras de sangre se recogieron de todos los perros en el comienzo (estado obeso, T0) y cada mes (T1-T3) durante el periodo experimental de pérdida de peso.



En todas las muestras se midió la adiponectina con el fin de estudiar si esta adipoquina se afecta por los cambios en el peso corporal de perros. Además, con el fin de evaluar la posible presencia de inflamación en la obesidad canina, en los estudios de ganancia y pérdida de peso se evaluaron las principales proteínas de fase aguda (PFAs): la proteína C reactiva de alta sensibilidad (hs-CRP), haptoglobina (Hp), ceruloplasmina (Cp) y el amiloide A sérico (SAA). También en el estudio de la pérdida de peso, se cuantificaron las inmunoglobulinas (Igs). Para ello, se validaron métodos automatizados originalmente diseñados para determinaciones de Igs in humana para su uso en perros; mostrando una satisfactoria precisión y linealidad.

4.1.2. Estudios clínicos en perros

- Pérdida de peso. El estudio se realizó en colaboración con la Universidad de Liverpool (Reino Unido). Se evaluaron plasma y orina de 35 perros obesos antes y después de un programa de pérdida de peso con el fin de estudiar:

- 1) el efecto de la pérdida de peso en la adiponectina.
- 2) la posible presencia de inflamación en la obesidad canina mediante la medición de hs-CRP.

3) la presencia de disfunción metabólica canina relacionada con la obesidad (ORMD siglas en ingles de obesity related metabolic disfunction) utilizando una definición modificada a partir de la usada para el síndrome metabólico humano. De esta forma se empleó como criterio para considerar a un perro con ORMD::

- a) Una condición corporal de 7-9/9
- b) Y tener dos de las siguientes:
 - 1. Triglicéridos >200 mg/dL, (2.3 mmol/L).
 - 2. Colesterol total > 300 mg/dL (7.8 mmol/L).
 - 3. Presión arterial sistólica >160 mmHg.
 - 4. Glucosa en ayunas >100 mg/dL (5.6 mmol/L).

La insulina y adiponectina se midieron en todos los perros para evaluar si estos analitos podrían cambiar en ORMD.

4) efecto de la pérdida de peso en marcadores renales. Para esto se evaluaron biomarcadores de la función renal clásicos (urea sérica, creatinina sérica, la densidad de orina, UPCR y albúmina en la orina corregida por creatinina [UAC]) y nuevos (homocisteína, cistatina C y clusterina).

4.1.3. Estudios clínicos en gatos

- *Pérdida de peso.* Este estudio se realizó en colaboración con la Universidad de Liverpool (Reino Unido). Se recogieron las muestras de sangre antes y después de pérdida de peso de 37 gatos castrados con sobrepeso para evaluar:

- 1) efecto de la pérdida de peso en la adiponectina plasmática.
- 2) posible presencia de inflamación en la obesidad felina midiendo Hp y SAA.

4.1.4. Estudio sobre hipotiroidismo en perros

El estudio se realizó en colaboración con el Atlantic College de Medicina Veterinaria de Nantes (Francia). Se incluyeron en el estudio 39 perros hipotiroideos. El hipotiroidismo primario se diagnosticó en base a signos clínicos y de laboratorio, como letargo, ganancia de peso corporal, alteraciones dermatológicas, colesterol alto (> 6,5 mmol / L), c-TSH alta (> 0,5 ng / ml) y bajos niveles de fT4 ($\leq$ 12 pmol / L) (Tyler, 2007). Todos los perros fueron sometidos a una terapia con tiroxina (media $\pm$ sd, 14,4 $\pm$ 5,7 mg/kg/día) durante 3 meses. Se consideró como una buena respuesta terapéutica, si los perros mostraron una significativa mejoría clínica (incluyendo pérdida

de peso, aumento de la actividad, la mejora de la piel) y al menos una disminución del 30% en los niveles de c-TSH.

Las muestras de sangre fueron obtenidas en el momento del diagnóstico del hipotiroidismo (T1) y después del tratamiento con tiroxina durante 3 meses (T2), y se midieron en ellas las principales proteínas de fase aguda (CRP, SAA, y Hp) y la adiponectina. En este estudio no se evaluó la IGF 1, ya que un trabajo recientemente publicado por Jaillardon et al. (2011) trató este tema.

4.2. Resultados

4.2.1. Estudios experimentales en perros

-Ingorde. La Tabla 4.1 muestra los valores del peso corporal, la adiponectina y PFAs de los diferentes grupos de perros al final del ensayo. Las concentraciones de SAA estaban por debajo del límite de detección del ensayo en los seis animales. Los perros con BCS 5/5 (grupo B2) mostraron unas concentraciones de adiponectina y Hp significativamente más bajas en comparación con los perros con peso normal (grupo A, BCS 3/5). Mientras que no se observaron diferencias estadísticamente significativas en las concentración séricas de hs-CRP y Cp entre los grupos de perros.

-Pérdida de peso. La evolución de las concentraciones de adiponectina, PFAs e Igs se presenta en la Figura 4.3. La adiponectina sérica mostró un aumento estadísticamente significativo a los dos (T2) y tres (T3) meses después del inicio de la pérdida de peso, en comparación con las concentraciones de antes de perder peso (T0). No se observaron cambios estadísticamente significativos en los niveles de CRP, Hp, Cp, IgG, y IgM en los 3 (T1, T2 y T3) meses posteriores tras comenzar el período de pérdida de peso en comparación con los niveles previos a la pérdida de peso (T0). Los niveles séricos de IgA se redujeron significativamente en T1, T2, y T3 en comparación con T0.

4.2.2. Estudios clínicos en perros

-Pérdida de peso.

(1) Adiponectina. La adiponectina total aumentó significativamente en los perros después del programa de pérdida de peso.

(2) Inflamación. En los perros obesos, las concentraciones de hsCRP estaban dentro del rango de valores de perros normales. Y la hsCRP no cambió con la pérdida de peso.

(3) Disfunción metabólica canina relacionada con la obesidad (ORMD). Antes de la pérdida de peso, 10 perros fueron identificados con ORMD. En estos perros la concentración plasmática de adiponectina fue menor ($P = 0,004$), y la concentración plasmática de insulina fue mayor ($P = 0,01$) que en el resto de perros obesos.

(4) Marcadores renales. La urea y la densidad aumentaron significativamente después de la pérdida de peso, mientras que UPCR, UAC, la creatinina sérica, la homocisteína, la cistatina C y la clusterina disminuyeron.

4.2.3. Estudios clínicos en gatos

-*Pérdida de peso.* Los resultados de todos los biomarcadores se muestran en la Tabla 4.2. La adiponectina aumentó de forma significativa después de la pérdida de peso. Las PFAs en los gatos obesos estaban dentro del rango de referencia para gatos normales sanos, y no cambiaron significativamente después de la pérdida de peso.

4.2.4. Enfermedades relacionados con obesidad

-*Hipotiroidismo.* Veintiocho de los 39 perros mostraron una buena respuesta terapéutica (grupo A), mientras que el resto de los perros fueron considerados como que no habían tenido una respuesta adecuada al tratamiento (grupo B). La mediana (rango) de los resultados de los analitos evaluados en ambos grupos de perros se presenta en la tabla 4.3. Después del tratamiento, los perros del grupo A mostraron una disminución significativa en c-TSH y un aumento de la concentración de T4 libre, respectivamente, acompañado de un descenso significativo en el peso corporal y la adiponectina, y un aumento de Hp. Los perros de grupo B no mostraron cambios estadísticamente significativos en c-TSH, pero tenían un aumento significativo en fT4, acompañado de una disminución significativa de la adiponectina, aunque inferior que el grupo A.

Tabla 4.1. Valores del peso corporal y analitos bioquímicos en los perros con BCS 3/5 (grupo A), BCS 4/5 (grupo B1), y BCS 5/5 (grupo B2) después del engorde.

	Grupo A	Grupo B1	Grupo B2
Peso, kg	14.53±1.7	15.66±1.1	19,15±3,46*
Adiponectina, µg/mL	14.3±1.2	13.2±1.6	8.1±2.3
hs-CRP, mg/L	1.58±1.91	1.89±1.29	2.76±2.65
Hp, g/L	1.20±0.16	1.17±0.17	0.71±0.24**
Cp*10⁻³, Δabs/min	6.60±0.75	6.64±2.45	7.02±2.96

*, p<0.05; **, p<0.01 vs grupo A.

Tabla 4.2. Biomarcadores plasmáticos antes y después de pérdida de peso en gatos.

<i>Analito</i>	<i>Antes</i>	<i>Después</i>
<i>Adiponectina (pg/mL)</i>	2661 (978 to 6039)	3174 (668 to 7820) ^a
<i>Hp (g/L)</i>	1.7 (0.2 to 3.1)	1.8 (0.2 to 2.9)
<i>SAA (mg/L)</i>	0.38 (0.38 to 0.38)	0.38 (0.38 to 0.38)

Todos los datos se expresan como mediana (rango).

^a, p<0.05 vs. antes de pérdida de peso.

Tabla 4.3. Mediana (rango) de los analitos medidos en los perros hipotiroideos que mostraron buena (Grupo A) o no suficiente (Grupo B) respuesta al tratamiento.

	Grupo A			Grupo B		
	T1	T2	P	T1	T2	P
TSH	1.55 (0.60-8.30)	0.40 (0.03-4.80)	<0,001	1.00 (0.60-4.30)	0.90 (0.50-4.20)	NS
FT4	10.00 (0.0-16.00)	16.50 (10.00-29.00)	<0,001	10.00 (7.00-11.00)	14.00 (12.00-17.00)	<0,001
Peso	35.50 (6.00-69.00)	33.45 (15.00-70.00)	0,023	39.00 (26.00-54.00)	38.50 (26.00-51.00)	NS
Adiponectina, µg/mL	13.30 (5.23-23.78)	9.48 (0.43-27.92)	0,001	16.97 (8.02-29.87)	14.53 (5.92-23.64)	0,038
CRP, mg/L	5 (5.00-53.80)	5 (5.00-46.20)	NS	5 (5.00-18.70)	5 (5.00-5.00)	NS
Hp, g/L	2.15 (0,09-7.4)	2.4 (0.28-9.20)	0,039	2.11 (0.05-4.03)	2.17 (0.20-3.72)	NS
SAA, µg/mL	5.04 (0.68-116.00)	1.83 (0.20-24.88)	NS	2.21 (0.20-90.60)	3.57 (0.84-33.95)	NS
Cp, ΔA/min	0.024 (0.001-0.042)	0.022 (0.005-0.055)	NS	0.027 (0.003-0.052)	0.020 (0.004-0.033)	NS

T1, antes de la terapia; T2, 3 meses después de la terapia; NS, no significativo.

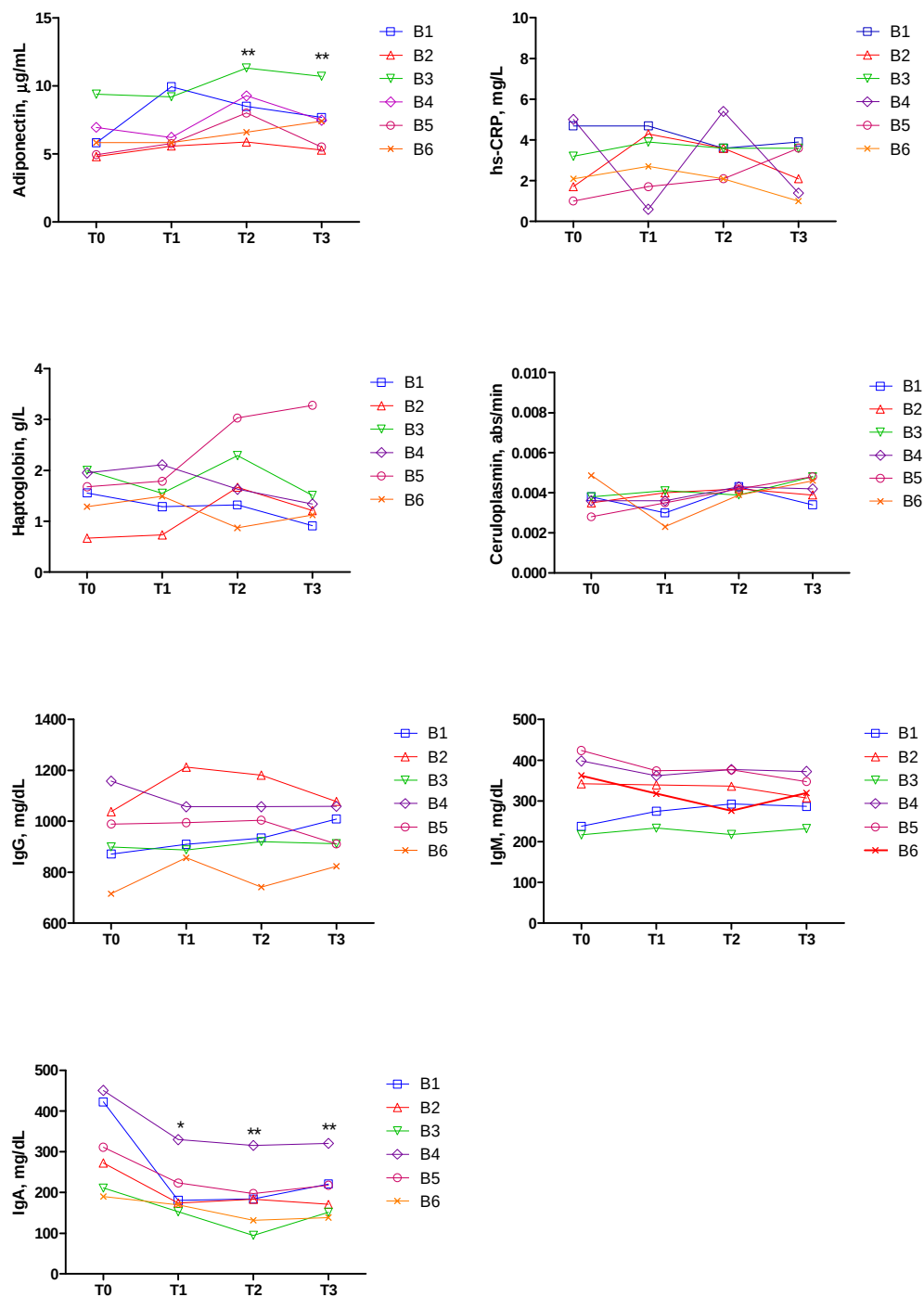


Figura 4.3. Evolución de los niveles de biomarcadores séricos asociados con la inflamación en seis perros Beagle (B1-B6) antes (T0) y durante el programa de pérdida de peso (T1-3, 1-3 meses después de comenzar el programa de pérdida de peso, respectivamente). CRP, protein C-reactiva; IgG, inmunoglobulina G; IgM, inmunoglobulina M; IgA, inmunoglobulina A. *, $P < 0.05$; **, $P < 0.01$ vs. T0.

4.3. Discusión

a) *Relación obesidad-adiponectin.* Nuestro estudio experimental, así como los estudios clínicos indicaron que la adiponectina sérica está disminuida en los perros obesos y aumenta significativamente después de la pérdida de peso. También nuestros estudios clínicos demostraron que la adiponectina en los gatos tiene un comportamiento similar al de los perros. Estos resultados están de acuerdo con lo descritos por algunos autores en los estudios de la obesidad en perros (Ishioka et al., 2006) así como en medicina humana (Arita et al., 1999; Chandran et al., 2003). Aunque contradicen los obtenidos por Verkest et al. (2011), quien no encontró correlación entre la obesidad y la adiponectina caninos. La ausencia de diferencias en los niveles de adiponectina entre perros obesos y delgados que se encontraron en estos estudios previos se podría atribuir a la alta variabilidad interindividual de la adiponectina, el no tener en cuenta el estado inflamatorio, y/o debido a la utilización de métodos que no han sido validados para la medición de la adiponectina en el perro.

b) *Relación obesidad-inflamación.* Nuestros resultados indican que no hay signos que indiquen la existencia de una inflamación evidente en la obesidad canina y felina. Los artículos publicados anteriormente muestran resultados contradictorios acerca de los valores de proteína C reactiva en la obesidad canina. Unos autores encontraron aumentos, mientras que otros encontraron disminuciones, aunque los niveles de CRP en todos estos estudios estaban dentro del rango que indica una condición no inflamatoria (Veiga et al., 2008; German et al., 2009). En humanos obesos, los valores de CRP están entre 3 mg/L (las concentracones <3 mg/L se consideran en humanos como valores normales) y 10 mg / L (valores > 10 mg / L son compatibles con la inflamación). Se ha postulado que estos valores intermedios no están directamente relacionadas con la inflamación y podría reflejar factores genéticos, variables demográficas, o patrones de comportamiento y de dieta (Kushner et al., 2006).

c) *Disfunción metabólica canina relacionada con la obesidad.* En nombre de "disfunción metabólica canina relacionada con la obesidad (ORMD)" y no "síndrome metabólico" se eligió por que el síndrome sugiere un proceso de enfermedad relacionada con la obesidad que aún no ha sido identificado en los perros.

En nuestro estudio se encontró que un 30% de perros obesos mostraron presencia de ORMD, caracterizada por hipoadiponectinemia e hiperinsulinemia, de manera

similar como ocurre en seres humanos (Leroith, 2012) y caballos (Johnson, 2002). Este estudio podría ser la base de nuevas investigaciones para determinar los mecanismos patogénicos y la importancia de la salud de ORMD para perros, en términos de asociaciones de enfermedades y los resultados de la pérdida de peso.

d) Efecto de pérdida de peso sobre los biomarcadores renales. Nuestros datos demuestran una disminución de varios biomarcadores renales como la clusterina, homocisteína y cistatina C en perros obesos sometidos a la pérdida de peso. Estos resultados sugerirían que en la obesidad canina se pueden producir posibles alteraciones subclínicas de la función renal, y explicarían los problemas renales que se han descrito asociados a la obesidad (German et al., 2010).

e) Hipotiroidismo. Nuestros datos indican que la disminución de la adiponectina después del tratamiento puede ser atribuida directamente a la influencia de las hormonas tiroideas, como se ha sugerido en los seres humanos (Iglesias et al., 2003), ya que no se observó correlación entre esta adipocitina y el peso corporal.

No hemos encontrado diferencias significativas en la media de los niveles circulantes de PFAs antes y después del tratamiento, con la excepción de un incremento significativo en las concentraciones de haptoglobina en los perros tratados con éxito, aunque estos valores estaban dentro del rango para animales sanos (Mylonakis et al., 2011). Los resultados de CRP concuerdan con anteriores publicaciones, indicando que las alteraciones en el estado de la tiroides no producen aumentos evidentes de CRP en perros (Nakamura et al., 2008) así como en los humanos (Pérez et al., 2004; Caixás et al., 2009). Sin embargo, se encontraron valores de SAA mayores que el límite del rango de referencia de laboratorio (<5 mg / L) en 14 perros antes del tratamiento. Y todos ellos, con la excepción de 2 perros, mostraron una disminución en valores de SAA después de la terapia, lo que indica la necesidad de estudios adicionales para investigar el papel de SAA en el hipotiroidismo canino.

5. ESTUDIO E IDENTIFICACIÓN DE NUEVOS BIOMARCADORES RELACIONADOS CON LA OBESIDAD

5.1. PON1 y BChE

5.1.1. Material y métodos.

5.1.1.1. Validación de PON1 en perros y gatos. Se evaluaron tres ensayos espectrofotométricos diferentes para la medición de la actividad PON1 en muestras de suero canino. Dos métodos se realizaron en formato de microplaca de 96 pocillos usando acetato de fenilo y 5-thiobutyl butyrolactonase (TBBL) y uno fue adaptado a un analizador automatizado usando p-nitrofenil acetato como sustrato. Por otra parte se evaluó un método automatizado basado en el p-nitrofenil acetato en gatos. La validación analítica se realizó tal como se describe en la Sección 2, calculándose la precisión, exactitud, y el límite de detección. No se validaron métodos para determinación de BChE en perros y gatos en este trabajo de doctorado, ya que han sido previamente validados (Tecles et al., 2000).

5.1.1.2. Variaciones por inflamación

-Efecto de sepsis. La descripción de materiales y métodos de la prueba inducción experimental de sepsis en perros aparece en la Sección 3.1.

5.1.1.3. Variaciones debidas a la obesidad y enfermedades relacionadas

5.1.1.3.1. Estudios experimentales en perro

La descripción de materiales y métodos de las pruebas de inducción experimental de la obesidad y pérdida de peso en perros aparece en la Sección 4.1.1.

5.1.1.3.2. Estudios clínicos en perros

La descripción de materiales y métodos de las pruebas de inducción de la pérdida de peso en perros aparece en la Sección 4.1.2.

5.1.1.3.3. Estudios clínicos en gatos

La descripción de materiales y métodos de la prueba de pérdida de peso en gatos aparece en la Sección 4.1.3.

5.1.1.3.4. Enfermedades relacionados con la obesidad en perros

-Hipotiroidismo.

Para la descripción de material y métodos ver sección 4.1.4.

-Diabetes mellitus (DM). Se incluyeron en este estudio un total de 222 perros. Todos los perros se dividieron en 3 grupos:

Grupo 1: formado por 74 perros con diabetes mellitus. El diagnóstico de la diabetes mellitus se basó en los criterios descritos por Durocher et al (2008). Se incluyeron en el estudio sólo perros que no habían sido tratados previamente con insulina.

Grupo 2: formado por 74 perros sanos sin evidencia clínica de enfermedad aguda o crónica, excepto obesidad en algunos casos.

Grupo 3: formado por 74 perros que presentaban diferentes enfermedades, excepto diabetes mellitus.

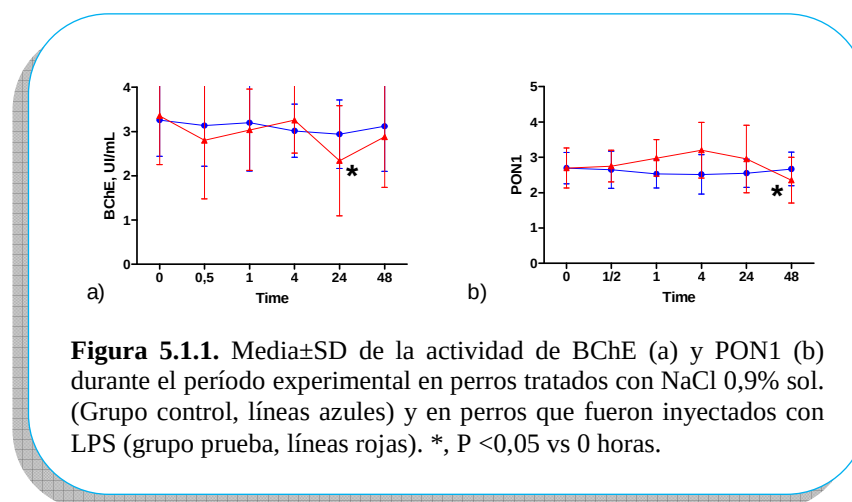
Tabla 5.1.1. Datos de validación analítica de los diferentes métodos evaluados para medir la actividad de PON1 (UI/mL) en muestras de suero canino y felino.						
Especie	Ensayo	Precisión		Exactitud		Límite de detección
		CV, %		Linealidad		
		Intra	Inter	Ecuación	R ²	
Perro	Actividad de lactonasa en suero mediante la medición la hidrólisis de la 5-thiobutyl butyrolactonase (TBBL) [ensayo A]	<7	<18	y=0.955x+0.22; y=0.594x+0.47	0.995; 0.908	1.0
	Actividad de arilesterasa en suero mediante la medición de la hidrólisis de acetato de fenilo [Ensayo B]	<5	<10	y=1.041x-1.38; y=1.044x-1.25	0.999; 0.992	9.1
	Actividad de arilesterasa en suero mediante la medición de la hidrólisis de p-nitrofenil acetato [ensayo C]	<6	<9	y=0.998x-0.14; y=1.056x-0.35	0.994; 0.991	0.6
Gato	Actividad de arilesterasa en suero mediante la medición de la hidrólisis de p-nitrofenil acetato	<5	<7	y=0.897x+0.18; y=0.960x+0.17	0.999; 0.999	0.3
CV, coeficiente de variación.						

5.1.2. Resultados.

5.1.2.1. Validación de PON1 en perros y gatos. Los datos analíticos de la validación de los tres métodos en perros y el de gatos se presentan en la tabla 5.1.1.

5.1.2.2. Variaciones por inflamación

-Efecto de sepsis. La BChE y PON1 en suero no mostraron cambios significativos en los perros de grupo control durante el período experimental. Sin embargo en los perros inyectados con LPS se observó una disminución significativa en la actividad BChE a las 24 h y en la actividad PON1 a las 48 h en comparación con los valores obtenidos en el tiempo 0 (Figure 5.1.1.).



5.1.2.3. Variaciones debidas a la obesidad y las enfermedades relacionadas.

5.1.2.3.1. Estudios experimentales en perros

-*Engorde.* La actividad de BChE fue menor en los perros con BCS 3/5 (grupo A, 3.1 kU/L [2.8-3.3]) que en los de BCS 4/5 (grupo B1, 3.8 kU/L, [3.2-4.4] $P<0.05$) y BCS 5/5 (grupo B2, 5.4 kU/L [4.6-6.1], $P<0.001$). No se observaron diferencias significativas en la actividad PON1 entre los diferentes grupos de perros (grupo A, 5.7 kU/L [5.1-6.3]; grupo B1, 5.1 kU/L [4.9-6.2], and grupo B2, 5.4 kU/L [5.0-6.3]).

-*Pérdida de peso.* La BChE sérica disminuyó de forma significativa en los perros después de la pérdida de peso (6.05 [5.70-8.20] vs. 5.20 [3.10-5.60] kU/L; $p<0.05$), mientras que no se observaron diferencias estadísticamente significativas en la actividad PON1 en los perros antes (6.0 kU/L [4.9-7.2]) vs. después (5.83 kU/L [5.4-6.7]) de la pérdida de peso.

5.1.2.3.2. Estudios clínicos en perros

La actividad de la BChE sérica disminuyó significativamente en los perros después de la pérdida de peso, mientras que no se detectaron cambios estadísticamente significativos en la actividad sérica de PON1 (Tabla 5.1.3).

Tabla 5.1.3. BChE y PON1 en perros antes y después de pérdida de peso.

	Antes de pérdida de peso	Después de pérdida de peso	<i>P</i>
<i>BChE (kU/L)</i>	7.4 (2.4-14.8)	5.9 (2.5-14.0)	<0.05
<i>PON-1 (kU/L)</i>	2.5 (0.5-3.8)	2.23 (0.5-3.4)	>0.05

Tabla 5.1.4. BChE y PON1 en los gatos del programa de pérdida de peso.

	Gatos que no lo completaron	Gatos que lo completaron	
		Antes de pérdida de peso	Después de pérdida de peso
BChE (kU/L)	2.8 (1.8-3.9)	2.9 (1.7 -5.1)	2.8 (1.7-4.3)
PON-1 (kU/L)	4.6 (3.1- 6.9) ^a	5.5 (3.8 -7.3)	5.6 (3.3 -7.4)

Todos los datos se expresan como mediana (rango).

BChE, butirilcolinesterasa; PON-1, paraoxonasa tipo 1.

^a, p<0.01 comparado con gatos que completaron el programa successful cats pre-weight loss.

5.1.2.3.3. Estudios clínicos en gatos

-Pérdida de peso. No hubieron diferencias significativas en las actividades de BChE ($P = 0,737$) o (PON-1) entre antes y después de la pérdida de peso en gatos (Tabla 5.1.4). Sin embargo, actividad de PON-1 antes de la pérdida de peso fue menor ($P = 0,004$) en los gatos que no completaron el programa de pérdida de peso que en aquellos que tuvieron éxito.

5.1.2.3.4. Enfermedades relacionadas con la obesidad en perros

-Hipotiroidismo. Los perros que tuvieron una buena respuesta terapéutica mostraron un aumento significativo en la actividad de BChE de 5.20 kU/L (2.90-9.50) a 5.65 kU/L (2.70-10.70). No se observaron cambios estadísticamente significativos después de tratamiento en la actividad de PON1 en los dos grupos de perros.

-Diabetes mellitus. Se observó que la actividad de BChE era significativamente más alta en perros con diabetes mellitus (grupo 1) en comparación con los perros sanos o perros con enfermedades diferentes a la diabetes mellitus (grupos 2 y 3) (Figura 5.1.2).

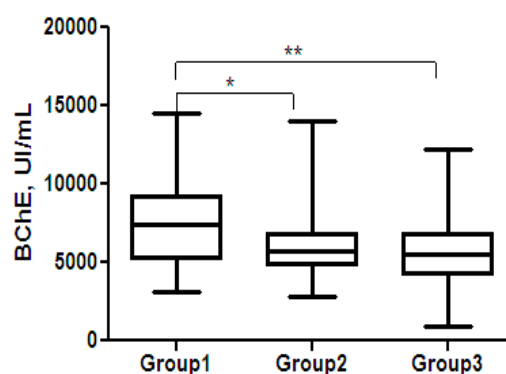


Figura 5.1.2. Actividad de butyrylcholinesterasa (BChE) en perros con diabetes mellitus (Grupo 1), perros sanos (Grupo 2), y perros con diferentes enfermedades, excepto diabetes mellitus (Grupo 3). *, $P<0.05$; **, $P<0.01$.

5.1.3. Discusión

a) *Ensayos para el PON-1.* Todos los ensayos evaluados fueron precisos (con la excepción de los valores de interensayo de método para medir lactonasa en muestras caninas), exactos y fáciles de instalar en el laboratorio. Estos ensayos facilitarán la implantación del PON-1 en los laboratorios de veterinaria y la investigación del comportamiento de esta enzima en la obesidad y las enfermedades relacionadas ella en perros y gatos.

b) *Inflamación.* Nuestros datos indican que la inflamación severa disminuye tanto la actividad de BChE como la de PON1 en perros (aproximadamente 30% y 20%, respectivamente), de manera similar como ocurre en humanos (Al-Kassab and Vijayakumar, 1995; Blanceil and Delaroche, 1996). La disminución de BChE podría ser una respuesta del organismo con el fin de aumentar los niveles de acetilcolina y así limitar la respuesta inflamatoria (Rossas-Ballina and Tracey, 2009). Mientras que la disminución observada en la actividad PON1 podría atribuirse principalmente a dos mecanismos: (1) efecto negativo directo de citoquinas (TNF- α , IL-1) sobre la expresión de PON1 (Feingold et al., 1998); y (2) cambios en los lípidos y la composición de proteínas de HDL como respuesta a la inflamación, ya que durante la respuesta de fase aguda el HDL pierde la mayor parte de las enzimas asociadas (incluyendo PON1) (Van Lenten et al., 1995; James and Deakin, 2004).

c) *Obesidad.* La BChE sérica demostró ser un buen marcador de adiposidad en los perros, de forma similar a lo descrito en humana (Randell et al., 2005). No se observaron cambios en la actividad PON1 en la obesidad en perros, y BChE y PON1 en gatos. Aunque en los gatos los valores de PON1 podrían ser utilizado como un factor predictivo del éxito de programa de pérdida de peso. Sin embargo, estos resultados deben considerarse preliminares, y se deberían realizar más estudios para aclarar la relación entre BChE y PON1 y la obesidad en el gato.

d) *Hipotiroidismo.* Cuando los perros hipotiroideos respondieron adecuadamente al tratamiento, se produjo un aumento de la actividad BChE en suero, de manera similar como se ha descrito en humana (Popović et al., 1998). El aumento de la actividad de BChE podría estar implicado en la hidrólisis de los triglicéridos (Popović et al., 1998), contribuyendo a la mejora del perfil lipídico que se ha descrito en perros hipotiroideos después de la terapia de tiroxina (Dixon et al., 2002).

e) *Diabetes mellitus*. La evaluación de la BChE en la diabetes mellitus canina se realizó debido a dos razones principales. En primer lugar no hay publicaciones acerca de este tema en perros. Y además la BChE está incluida en el perfil bioquímico general del hospital veterinario "San Marco", lo que nos permitió realizar un estudio retrospectivo con más de 200 perros con el fin de evaluar los posibles cambios en la actividad BChE debido a la presencia de DM. Se trata de un estudio preliminar, y se deberían realizar estudios adicionales que evalúen el PON1 y otros marcadores utilizados en este estudio de doctorado (como adiponectina, IGF-1 y PFAs) en la diabetes mellitus canina con el fin de aclarar sus mecanismos fisiopatológicos.

Los perros con diabetes mellitus mostraron mayor actividad BChE que los perros sanos, de forma similar a como se describe en humana (Abbott et al., 1993). Sin embargo, la condición corporal no afectó los valores de BChE en perros con diabetes mellitus, como también se ha descrito en humana (Abbott et al., 1993). Esto indica que la insensibilidad o deficiencia de insulina en diabetes mellitus podría tener una mayor influencia sobre la actividad BChE que el peso corporal.

5.2. Proteómica

5.2.1. Material y métodos

-Animales y desarrollo experimental

La descripción de los animales y desarrollo experimental aparece en la Sección 4.1.1.

- Electroforesis de dos dimensiones en gel de poliacrilamida (2DE)

Las muestras de suero antes (T1) y después (T2) de la pérdida de peso se analizaron por duplicado en 2DE. El isoelectroenfoco de primera dimensión se realizó en tiras (11 cm) caseras en gradiente inmovilizado de pH con intervalo de pH no lineal comprendido entre 4 y 10. La segunda dimensión se realizó en geles de poliacrilamida caseras 140 x 140 x 1.5mm de gradiente 10-15%. Después de la electroforesis, se tiñeron los geles con plata de acuerdo con protocolos estándar anteriormente descritos (Miller and Gemeiner, 1992).

- *Análisis de imágenes de geles 2DE*

Los geles 2D teñidos fueron escaneados con ImageScanner II (GE Healthcare Life Sciences) y evaluados mediante el uso de un software específico (imagen principal 2D Platinum 7.0, GE Healthcare Life Sciences).

- *Identificación con espectrometría de masas (MS) y análisis de los datos*

Las manchas (o “spots” en inglés) que aparecieron expresados de forma diferente en los animales obeso en comparación con los delgados fueron sometidos a una identificación por espectrometría de masas MALDI-TOF/TOF (Ultraflex II, Bruker Daltonics).

Los datos fueron analizados utilizando Mascot (www.matrixscience.com) en las bases de datos Swiss-Prot (versión 56.5) o NCBIInr (20090314).

5.2.2. Resultados

Los resultados del “mapa” de proteínas de suero canino utilizando 2DE reveló más de 170 “spots” individuales con pesos moleculares entre 10 y 100 kDa en un intervalo de pH de 4 a 10 (Figura 5.2.1). Los perfiles proteicos obtenidos en los estados de obesidad y delgadez fueron homogéneos, ya que no se detectaron diferencias entre repeticiones y entre las imágenes de las muestras en los mismos estados.

Mediante análisis por imagen se detectaron 3 “spots” que fueron expresados de forma diferencial en la situación de pérdida de peso en comparación con el estado de obesidad. De estos tres puntos, dos habían incrementado y uno se había reducido (tabla 5.2.1).

El MALDI-TOF/TOF MS permitió la identificación de las 3 “spots” de proteínas, correspondiendo a la proteína retinol-binding 4 (RBP4), precursor de clusterina (CLU), y antitripsina alfa-1 (AAT) (Tabla 5.2.2).

Tabla 5.2.1. Manchas expresadas diferencialmente en estado de obesidad versus delgadez en perros. X: media de los volúmenes normalizados. SD: desviación estándar.

Spots ^a	Obesidad vs Delgadez	Obesidad (n = 5)		Delgadez (n = 5)		P
		X	SD	X	SD	
1	↑	0.2091	0.0155	0.1534	0.0409	0.0316
2	↑	0.1639	0.0303	0.0964	0.0234	0.0072
3	↓	0.1998	0.3169	2.726	0.3001	0.0310

^a Numeros de “spots” anotados en la imagen de gel (ver figuras 1 & 2).

Tabla 5.2.2. Identificación MS de proteínas de las muestras obtenidas de suero canino. MW: peso molecular. PI: punto isoeléctrico.

Spot ^a	Número de acceso ^b	Identificación de proteínas	Teórico MW [kDa]	Teórico pI	Número de péptidos únicos	Cobertura de secuencia ^c [%]	Puntuación
1	gi 73998292	Retinol-binding protein 4 [Canis lupus familiaris]	29.8	9.4	3	19.5	390.2
2	gi 50979240	Clusterin precursor [Canis lupus familiaris]	51.8	5.6	3	9.7	81.3
3	gi 121583756	Serpin peptidase inhibitor, clade A (alpha-1 antitrypsin), member 1 [Canis lupus familiaris]	46.3	5.5	2	6.5	239.2

^a Numero de “spot” anotado en los imagenes de geles (ver Figura 1).

^b Número de acceso de base de datos NCBI (gi).

^c Cobertura de secuencia: por ciento de secuencia identificado a la secuencia completa de la proteína conocida.

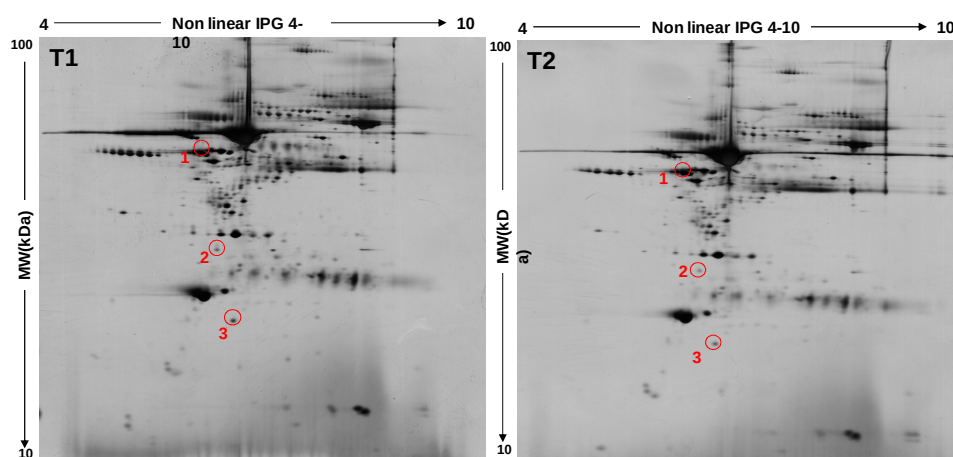


Figura 5.2.1. Imágenes representativas de geles 2DE teñidos con plata de suero canino obtenido antes (T1, estado obeso) y después de la pérdida de peso (T2, estado delgado), que corresponden al perro número 1. “Spots” de proteínas expresados diferencialmente entre los estados de obesidad y delgadez de los perros están rodeados y numerados: (1) proteína retinol-binding 4; (2) precursor de clusterina; (3) inhibidor de peptidasa de Serpin. clade A (alfa-1 antiproteínasa. antitripsina). miembro 1.

5.2.3. Discusión

Este estudio consistió en el análisis de proteoma del suero en perros beagle antes y después de un programa controlado de pérdida de peso. La comparación estadística de los volúmenes de las “spots” reveló tres que mostraban diferencias en la expresión entre el estado de obesidad y delgadez de los animales. No se detectó ninguna de las citoquinas o adipocinas que se saben que están alteradas en situaciones de obesidad (German et al., 2010). Esto podría explicarse por la falta de sensibilidad de la técnica, ya que cuando los métodos 2DE de gel se utilizan para el análisis del proteoma, la detección de algunos biomarcadores puede ser muy difícil debido a la presencia de proteínas de alta abundancia que enmascaran otras proteínas (Selvaraju and Rassi, 2012).

El análisis de espectrometría de masas reveló que CLU, RBP4, y AAT séricos fueron las proteínas correspondientes a las “spots” que mostraron cambios en el análisis 2DE. Estas proteínas podrían ser consideradas como posibles marcadores de obesidad y de las enfermedades relacionadas con la obesidad en perros. Los datos de nuestro estudio clínico de pérdida de peso en perros apoyan este hallazgo, ya que CLU sérico fue significativamente menor en perros después de la pérdida de peso (Sección 4.1.2). Sería recomendable realizar estudios futuros, evaluando RBP4 y AAT en el suero de perros de peso normal y obesos mediante el uso de ensayos especies específicos, con el fin de verificar los resultados obtenidos en proteómica.

6. CONCLUSIONES

1. Los ensayos disponibles comercialmente en la actualidad para la adiponectina y la IGF-1 mostraron unos resultados adecuados de validación en el perro y en el gato. Sin embargo, los ensayos para leptina y grelina, probados en nuestro estudio, mostraron una sensibilidad y/o exactitud baja, y por ello se recomendaría el desarrollo de nuevos kits para medir estas adipocinas en perros y gatos.
2. La adiponectina e IGF-1 séricas no están influenciados por los ciclos de alimentación/ayuno o por la administración de una dosis única de corticoides. También los niveles circulantes de adiponectina no se ven afectados por una

orquidectomía. Sin embargo, hay que tener precaución al interpretar los valores de IGF-1 de los perros castrados o valores de adiponectina obtenidos de perras en celo, ya que pueden estar descendidos o aumentados respectivamente en estas situaciones. También se debe evaluar el estado inflamatorio del animal siempre que se analice tanto la adiponectina como la IGF-1, ya que la inflamación disminuye las concentraciones de estas proteínas en forma significativa.

3. La obesidad canina y felina está asociada con una disminución de la concentración de adiponectina, aumentando después de la pérdida de peso. Sin embargo, la obesidad no parece producir una inflamación evidente, al menos que se puede detectar con las proteínas de fase aguda.
4. Hasta un tercio de los perros obesos sufren del síndrome de disfunción metabólica relacionado con la obesidad. Este síndrome está caracterizado por hipoadiponectinemia y la hiperinsulinemia, de manera similar como ocurre en el síndrome metabólico en humana.
5. La pérdida de peso en perros está asociada con un descenso de los marcadores séricos de disfunción renal, como clusterina, homocisteína, cistatina C.
6. La terapia adecuada de los perros con hipotiroidismo disminuye las concentraciones de adiponectina y aumenta la actividad de BChE.
7. La BChE sérica demostró ser un buen marcador de la adiposidad en los perros y está aumentada en la diabetes mellitus canina. No se observaron cambios en la actividad de PON1 en perros obesos, ni BChE y PON1 en gatos obesos. Aunque en los gatos, el PON1 podría ser utilizado para predecir el éxito del programa pérdida de peso. La actividad BChE está incrementada en perros con diabetes mellitus.
8. El análisis proteómico reveló tres proteínas (clusterina, proteína retinol-binding 4 y alfa-1 antitripsina) que podrían ser considerados como posibles marcadores de obesidad y enfermedades relacionadas en el perro.

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8. Apendix

8.1. Journal impact factor

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☑	5	RES VET SCI	0034-5288	3938	1.649	1.760	0.237	186	10.0	0.00682	0.474
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