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Interleukin-6 and insulin increase and nitric oxide and adiponectin decrease in blind dogs with pituitary-dependent hyperadrenocorticism

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ABSTRACT

In this study, two populations of dogs with pituitary dependent hypercortisolism (PDH) were compared over a 2-year period. One group had normal vision (Group A, $n = 27$) and one group was blind (Group B, $n = 20$). Group B was characterised by the rapid appearance of the clinical signs of PDH that precede blindness. We found increases in pre-adrenocorticotropic hormone cortisol ($P = 0.002$), IL-6 ($P = 0.0001$), insulin, and insulin sensitivity (detected with the Homeostatic Model Assessment, $P < 0.0001$) in Group B but not in Group A. The nitric oxide (NO) and the total adiponectin concentrations decreased ($P = 0.0001$ and $P = 0.02$, respectively) in Group B versus Group A. The IL-6 and insulin concentrations and the HOMA-A index were positively correlated with the cortisol concentration and were negatively correlated with the NO concentration. With the exception of adiponectin, the other variables were associated with blindness. We concluded that blindness in PDH is a haemodynamic event associated with metabolic changes, with the increase in the IL-6 concentration and the decrease in the NO concentration affecting the retinal vasculature and producing a high risk of vision loss.

1. Introduction

Pituitary-dependent hypercortisolism (PDH), also known as Cushing's disease, causes various metabolic changes (Ross and Linch, 1982; Arnaldi et al., 2003), including dyslipidemia, impaired fasting glucose, insulin resistance and hyperinsulinism, arterial hypertension, and obesity with a predominant deposit of abdominal fat (also referred to as visceral fat) (Andrews and Walker, 1999; Vegiopoulos and Herzig, 2007). Blindness of pituitary origin may also be present in Cushing's disease. Recently, we have observed (Cabrera Blatter et al., 2011) that the loss of vision is caused by compression of the optic chiasm in only 2.86% of blind dogs with PDH. In the rest, the loss of vision is associated with greater concentrations of triglycerides (Tg), glucose (G), and cortisol and changes in the vascular flow of the retina, similar to what has been described in non-diabetic humans and in rats (Irving et al., 2002; Wong et al., 2004; Yang and Zhang, 2004; Nguyen and Wong, 2006). In addition, these dogs are characterised by a rapid appearance of the typical clinical signs of PDH, especially an increase in weight with the development of abdominal obesity and the typical prominent or pendulum abdomen associated with PDH (Cabrera Blatter et al., 2011).

Visceral adipose tissue synthesises pro-inflammatory interleukins, such as interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) and a series of peptides called adipocytokines, which includes adiponectin (Adp) (Havel, 2002; German et al., 2010). Both the IL-6 and the TNF-α concentrations are elevated in abdominal obesity and are related to the development of insulin resistance and changes in the vascular endothelium (Duncan et al., 2000; Van Hecke et al., 2005; de Ferranti and Mozaffarian, 2008; Cartier et al., 2008).

Adp favours the sensitivity of the tissues that act on insulin (Brochu-Gaudreau et al., 2010). Three forms of Adp have been described: total (Adp), low molecular weight (LMW), and high molecular weight (HMW) (Yamauchi and Kadowaki, 2008). Adp concentrations decrease with obesity (Weyer et al., 2001; Ishioka et al., 2006; Kawano and Arora, 2009), affecting the action of insulin in the tissues. This action serves as one of the main proposed mechanisms that lead to insulin resistance and hyperinsulinism (Haluzic et al., 2004). Hyperinsulinism causes a thickening of the vascular endothelium and a resulting reduction of the vascular calibre, rigidness of the arteries or arterioles, and arterial hypertension (Maury and Brichard, 2010).

Nitric oxide (NO) produces a vasodilatory effect (Loscalzo, 1995; Luchi et al., 2003). Cortisol acts by inhibiting endothelial nitric oxide synthase (eNOS), thus decreasing the NO concentration (Yang and Zhang, 2004). Therefore, in individuals with hypercorti-

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solism due to different causes, inhibited eNOS is found, causing a difficulty with arterial vasodilation and a predisposition to arterial hypertension (Mitchell and Webb, 2002). In hypercortisolism, as with PDH, insulin resistance with hyperinsulinism is aggravated by the vascular problem (Hess et al., 2003).

Based on the above findings, along with the results we had obtained with a previous cohort (Cabrera Blatter et al., 2011), we analysed whether there are differences in the IL-6, TNF-α, insulin, Adp, and NO concentrations in dogs with PDH that either have preserved vision or are blind and whether PDH is related to the loss of vision among a new cohort.

2. Materials and methods

2.1. Study population

Out of a total of 186 dogs with a confirmed diagnosis of PDH over a 2-year period (2009–2010), 47 dogs ranging in age from 3 to 11 years (median of 8.5 years) were included in the study (27 were mixed breeds, and the rest belonged to various breeds; 15 males and 32 females; 4 spayed). These dogs were distributed into two groups according to whether they still had their vision. Dogs in Group A had PDH and preserved vision ($n = 27$; 9 males and 18 females; 3 spayed) and were examined during the first 4 months of the study. Group B was the group of blind PDH dogs ($n = 20$; 6 males and 14 females; 1 spayed) that had lost their vision shortly after displaying the clinical signs of this disease. These animals were included during the 2 years of the study as they presented themselves.

The dogs included in both groups displayed the characteristic clinical signs of PDH (polyuria, polyphagia, an increase in weight, a prominent abdomen, and dermatological changes). In addition to the other clinical signs that have been mentioned, blindness was a distinctive sign in the dogs of Group B and showed rapid onset (between 48 h and 1 month following the first evidence of the described clinical signs). Dogs that had PDH with blindness caused by a compression of the optic chiasm (evaluated by magnetic resonance imaging (MRI) with an axial cut) or that had systemic infectious or ophthalmological diseases, organ diseases such as cardiopathy or nephropathy, diabetes mellitus, or various malignancies were excluded from the study. None of the dogs was currently receiving or had received prior treatment for PDH.

At the time of the consultation, arterial tension (AT) was evaluated using high-definition oscillometric equipment (VetHDO monitor MD/90, Systeme & Beratung, Germany) to measure systolic pressure (Sys) and diastolic pressure (Dias). The measurements were taken after the animal was in a position of sternal recumbency; calm; and accustomed to the office, the bed, and the operator's touch. The dog's owner was present during the procedure. The cuff insufflator was positioned on the tail as close as possible to the rump. The lower third of the forearm (radial artery) was not used because we observed abrupt changes in the measurements if the dog moved the anterior limb. Based on our experience, the tail normally remains still when the animal is calm. The AT measurement was repeated four consecutive times, so the dog became used to the sensation of insufflation and the noise of the insufflator device. The last measurement was considered to be the reference measurement because the dog was more relaxed and accustomed to the procedure. Arterial hypertension was defined as Sys >160 mmHg and/or Dias >95 mmHg (Bodey and Michell, 1996; Brown et al., 2007).

2.2. Ophthalmological studies

A clinical ophthalmological study was performed on both groups using electroretinography (ERG) and an ocular Doppler echography, as previously described (Cabrera Blatter et al., 2011). These tests were performed 1 week before performing the biochemical studies.

2.3. Diagnosis of PDH (cortisol and plasma adrenocorticotropic hormone (pACTH) and the evaluation of Tg, total cholesterol (TCh) and G

PDH was diagnosed according to the protocol used in our institution (Gallelli et al., 2010; Cabrera Blatter et al., 2011). The diagnosis consisted of measuring the cortisol:creatinine ratio (CCR) in urine before and after inhibition with oral dexamethasone (Galac et al., 1997); analysing the ACTH stimulation test (tetracosactide 0.25 mg, Synacthen, Novartis); evaluating the baseline cortisol and measuring the cortisol concentration at 1-h post-intravenous ACTH delivery; measuring the plasma ACTH (pACTH) concentration; conducting an ultrasound of the adrenal glands; and performing MRI through the sagittal, coronal, and axial cuts of the sellar region (Gallelli et al., 2010).

The cortisol concentration was measured through a radioimmunoassay using a commercial kit (Cortisol DPC Corporation), with an intra-assay and inter-assay coefficient of variation of 5% and 8%, respectively.

The pACTH concentration was measured using an ELISA test (ACTH Alpco Immunoassays, Alpco Diagnostics, USA), as described elsewhere (Cabrera Blatter et al., 2011; Miceli et al., 2011). The intra-assay coefficient of variation was 2.30%, and the inter-assay coefficient was 6.9%. The sensitivity was 0.25 pmol/L.

The blood was taken (5 mL total) to measure the baseline cortisol concentration in serum. This blood was also used to measure the pACTH and Adp concentrations. For these analyses, 2 mL of blood was separated from the sample in a plastic refrigerated tube with the addition of an aprotinin anticoagulant [Trasylol, 0.1 mL = 10,000 KIU] and was immediately centrifuged and frozen at −80 °C. Tg, TCh, insulin, IL-6, TNF-α, and NO concentrations were also measured. For the latter, 2.5 mL of blood was placed in a glass tube with a subsequent separation of serum and was frozen at −30 °C. G was measured using 0.5 mL of blood placed in a plastic tube with a fluoride anticoagulant. Blood samples were collected with 12 h of fasting.

Tg, TCh, and G were measured using commercially available kits in an automatic analyser (Metrolab Autoanalyzer Merck, enzymatic colourimetric).

2.4. The evaluation of IL-6, TNF-α, insulin, HOMA-A, adiponectin and nitric oxide concentrations

To obtain reference values for the IL-6, TNF-α, Adp, and NO concentrations in our population, these variables were studied in the serum of 20 healthy dogs at normal weight (6 males and 14 females; ages between 3 and 10 years; 15 beagles and 5 mixed breeds). These dogs came from the vivarium of the College of Veterinary Sciences-UBA and made up the healthy control group for these variables.

The IL-6 (Quantikine Canine IL-6 Immunoassay, R&D System, USA) and the TNF-α (Quantikine Canine TNF-α/TNFSF1A Immunoassay, R&D System, USA) concentrations were analysed using an ELISA assay. The reference values for the IL-6 concentration obtained in the evaluated population were 5.3–31.2 pg/mL (median, 27.2 pg/mL). The intra-assay and inter-assay coefficients of variation were 2.4% and 4.5%, respectively, with a minimum sensitivity of 1 pg/mL.

The cut-off values obtained for the TNF-α concentration were 1.0–2.3 pg/mL (median, 1.5 pg/mL). The intra-assay and inter-assay coefficients of variation were 7.6% and 9.4%, respectively, with a minimum sensitivity of 0.5 pg/mL.

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Values below the detection limit for both cytokines were considered as "0" (zero) for graphing purposes.

The insulin concentration was measured through an enzyme-assay specific for canine-porcine species (ALPCO Insulin Porcine/Canine EIA, Alpco Immunoassays Diagnostic, USA), with inter- and intra-assay coefficients of variation (canine performance) of 4.2% and 4.3%, respectively, at a sensitivity of 0.05 pmol/L. The presence of insulin resistance was inferred through the Homeostatic Model Assessment (HOMA-A, insulin sensitivity) using the following formula: Insulin (μUl/mL) × Glycaemia (mmol)/22.5. Our cut-off HOMA-A value was <2.5 (Matthews et al., 1985; Miceli et al., 2011; Verkest et al., 2010).

Adp was evaluated using an ELISA assay (Canine Adiponectin ELISA, Millipore, USA), with intra- and inter-assay coefficients of variation of 4.2% and 9%, respectively, and a sensitivity of 0.048 ng/mL. The reference interval obtained in the normal weight population was 0.35–1.02 μg/mL (median, 0.71 μg/mL).

The NO concentration was analysed using an ELISA assay (Nitric Oxide Colorimetric Assay, BioVision, USA) and a calculation based on NO-producing nitrites and nitrates. The reading of absorbance (plot absorbance) was performed using a 540-nm filter to obtain the concentration of nitrates, following the manufacturer's instructions. The reference values obtained in the healthy population for the nitrate/nitrite ratio were 0.39 nmol/μL (range, 0.12–1.12 nmol/μL). The intra- and inter-assay coefficients of variation were 3.1% and 5.8%, respectively, with a sensitivity of 0.1 nmol nitrite/well.

2.5. Statistical analysis

To compare the IL-6, TNF-α, Adp, and NO concentrations in Group A and Group B versus the healthy controls, a non-parametric ANOVA was used (Kruskal–Wallis), followed by a Dunn's multiple comparison test. The comparison between Groups A and B for the remaining variables was performed with a non-parametric Mann–Whitney *U* test. Using the Spearman's test, we analysed whether there was a correlation between the biochemical variables studied and the conservation or loss of vision. Using a table of contingency with Fisher's test and calculating the odds ratio (OR), we determined whether these values were associated with blindness. With two-way ANOVA (multivariate analysis), the variables that interacted with vision were analysed. To perform these analyses, all of the data were normalised in their logarithmic form. The results are expressed as a median and a range, and a significance level of $P < 0.05$ was utilised.

2.6. Ethical approval

The study was approved by the Ethics Committee of the Faculty of Veterinary Sciences (CICUAL) and the Technical Evaluation Commission of the University of Buenos Aires (UBACyT V006) and complied with local and international laws for the use of animals in clinical research. The dogs' owners provided their signed consent to perform the studies.

3. Results

3.1. Evaluation of arterial hypertension and ophthalmological studies

The appearance of the clinical signs of PDH in Group B, as reported by the owners, was very abrupt, with blindness occurring between 48 h and 1.3 months after the dog first displayed other clinical signs of the disease, similar to what we had previously observed (Cabrera Blatter et al., 2011). Group B represented 10.7% (20/186) of the total of dogs with PDH who were attended during the 2-year period of study; in none of them did an adenoma compromise the optic chiasm. No significant differences were found in the AT, both for Sys (Group A: 141 [111–187] mmHg; Group B: 155 [140–180] mmHg) and Dias (Group A: 84 [70–141] mmHg; Group B: 84 [57–100] mmHg) between the two groups. Only 8/27 (29.6%) dogs from Group A displayed elevated Sys and Dias. In Group B, only Sys was elevated in 6/20 (30%) dogs.

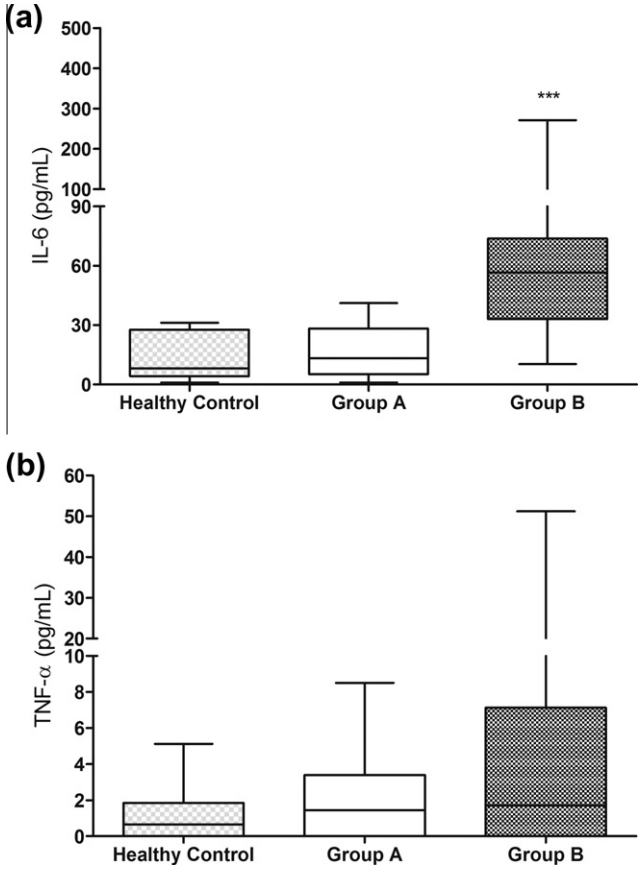

Fig. 1. The IL-6 (a) and TNF-α (b) concentrations in healthy control group dogs, dogs with PDH with preserved vision (Group A), and dogs with PDH that are blind (Group B). The IL-6 concentration in Group A is similar to the concentration in the control population. On the other hand, the elevation is notable in Group B, where it is elevated in almost all the dogs. The TNF-α did not exhibit changes among all three groups. ***$P = 0.0001$ Group B versus either Group A or the healthy controls.

The clinical ophthalmological evaluation and the ERG did not differ from what was observed in the previous study, with flat A and B waves present in Group B. The ophthalmological Eco-Doppler of the Group B revealed that venous flow was absent in 20/20 dogs and that arterial flow was absent in 4/20 dogs. Conversely, in all dogs of the Group A both arterial and venous flow were normal.

3.2. CCR, cortisol, pACTH, Tg, TCh and G concentrations

The CCR, pACTH and cortisol concentrations 1 h post-stimulation did not exhibit significant differences between the two groups (data not shown). The baseline cortisol concentrations were statistically higher ($P = 0.002$) in Group B (246 [57.9–463.5] mmol/L) versus Group A (107.4 [38.6–273] mmol/L). This observation was also true for Tg (Group B: 1.98 [1.2–3.4] mmol/L vs. Group A 1.22 [0.52–4.2], $P = 0.03$) and G (Group B: 5.2 [4.2–4.8] mmol/L vs. Group A: 4.4 [3.5–5.9] mmol/L; $P = 0.02$), with no differences found in the TCh concentration between the two groups, similar to what has already been stated.

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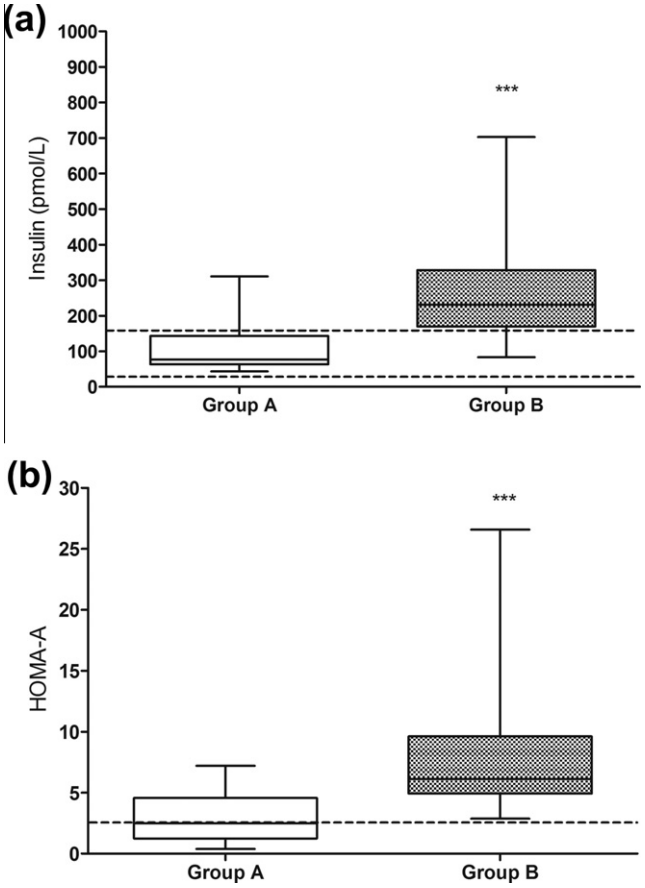

Fig. 2. The insulin concentration (a) and HOMA-A index (b) in dogs with preserved vision (Group A) and dogs that are blind (Group B). The insulin concentration in Group A is mostly within the reference values (dotted lines). In Group B, it is clearly elevated. The HOMA-A index increases slightly in Group A, indicating insulin resistance in 12/20 of these dogs. In Group B. this index is elevated for all the dogs. The dotted lines indicate the minimum (a) and maximum (a and b) reference ranges. $^{***}P < 0.0001$ Group B versus Group A, both for insulin and HOMA-A.

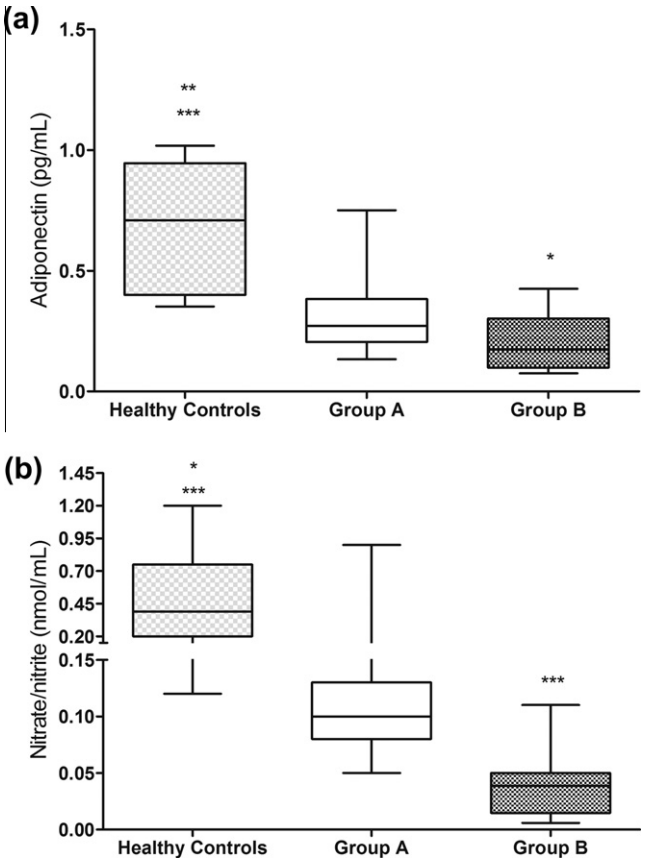

Fig. 3. The adiponectin (a) and NO (b) concentrations in healthy control dogs, dogs with PDH with preserved vision (Group A) and dogs with PDH that are blind (Group B). Note the decrease of both variables in the dogs with PDH with respect to the healthy control population. The greater decrease in the Adp and NO concentrations in the dogs of Group B with respect to those of Group A is striking. (a) $^{**}P = 0.01$ healthy control versus Group A; $^{***}P = 0.001$ healthy control versus Group B; $^{*}P = 0.02$ Group B versus Group A. (b) $^{*}P < 0.05$ healthy control versus Group A; $^{***}P < 0.001$ healthy control versus Group B; $^{***}P = 0.0001$ Group B versus Group A.

3.3. IL-6, TNF-α, insulin, Adp and NO concentrations and the HOMA-A index

The IL-6 (Fig. 1) concentration was significantly higher ($P = 0.0001$) for Group B compared to Group A. This cytokine was elevated in 5/27 (18.5%) of the Group A dogs and 16/20 (80%) of the Group B dogs. Comparing the IL-6 values of both groups of dogs with PDH versus the healthy control population, only the Group B dogs displayed significant differences in relation to the healthy control population ($P = 0.0001$). The TNF-α concentration did not display significant differences between groups ($P = 0.48$), and there were no differences between the PDH groups and the healthy control population (Fig. 1). The TNF-α concentration was elevated in only 4/27 of the Group A dogs and 4/20 of the Group B dogs.

The insulin concentration and the HOMA-A index (Fig. 2) were both significantly elevated ($P < 0.0001$) in the Group B dogs compared to the Group A dogs.

In the Group B dogs, the Adp ($P = 0.02$) and NO ($P < 0.0001$) concentrations were significantly lower than those found in the Group A dogs (Fig. 3). Comparing the values obtained in the healthy control population versus Groups A and B, both the Adp (healthy control versus Group A: $P = 0.01$; healthy control versus Group B: $P = 0.001$) and the NO (healthy control versus Group A: $P < 0.05$; healthy control versus Group B: $P < 0.001$) concentrations decreased significantly among the dogs with PDH, with a more marked decrease in the Group B dogs for both of the studied variables (Fig. 3).

The IL-6 ($r = 0.4$; $P = 0.016$), insulin ($r = 0.44$; $P = 0.003$), and NO ($r = -0.4$; $P = 0.018$) concentrations and the HOMA-A index ($r = 0.34$, $P = 0.03$) were correlated with the cortisol concentration (Fig. 4). No correlation was found between the cortisol and the Adp concentrations ($r = 0.25$; $P = 0.3$). The NO and the Adp concentrations were inversely correlated with the insulin concentration ($r = -0.41$, $P = 0.008$ and $r = -0.36$, $P = 0.03$, respectively). We did not find any correlations among the insulin, IL-6 and Adp concentrations.

Upon analysing whether there was a correlation between the loss of arterial and venous flow with any of the studied variables, we found that both flows were negatively correlated with the IL-6 ($r = -0.65$, $P = 0.002$), insulin ($r = -0.71$, $P < 0.001$), and cortisol ($r = -0.5$, $P = 0.03$) concentrations and positively correlated with the NO concentration ($r = 0.74$, $P = 0.003$).

Loss of vision was significantly associated with the IL-6 ($P < 0.0001$; OR: 17.6, 95% CI 4.07–76.12), insulin ($P = 0.002$; OR: 8.2, 95% CI 2.18–30.53), and NO ($P < 0.0001$; OR: 27.64, 95% CI 3.21–238) concentration and the HOMA-A index ($P = 0.003$; OR: 8.24, 95% CI 1.9–35.1). No association was found with the Adp concentration.

Using a two-way ANOVA, we found that the insulin, IL-6, NO and cortisol concentrations significantly ($P < 0.001$) interacted with vision, as increases or decreases (in the case of the NO) of the con-

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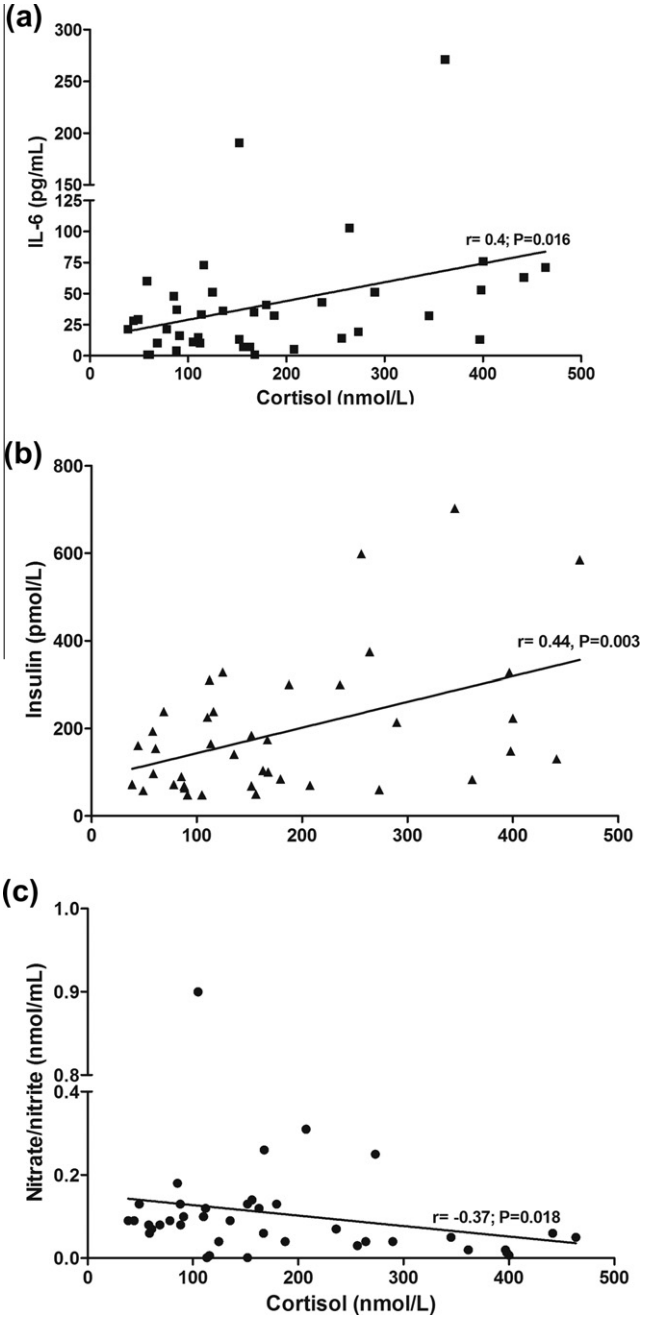

Fig. 4. The correlation between cortisol concentrations compared to the concentrations of IL-6 (a), insulin (b), and NO (c) in dogs with PDH with and without preserved vision. The increase in cortisol positively correlates with the IL-6 concentrations and the insulin concentrations (and therefore with the HOMA-A, not shown in the figure) and inversely correlates with the NO concentrations, indicating the inhibition of NO due to the effect of cortisol.

centrations of these variables affected vision ($P < 0.0001$, $F = 838.7$) and contributed to 93.61% of the variation.

4. Discussion

In this study, the number of cases of blind dogs with PDH was increased from our previous report. As previously reported (Cabrera Blatter et al., 2011), the clinical observation that differentiates the two groups is the rapid appearance of the clinical signs of PDH (almost simultaneously), in particular an increase in weight and an increase in abdomen size due to the increase in adipose tissue that characterises the Group B dogs and that precedes blindness. In the Group A dogs, the clinical signs appeared slowly, with ensuing polydipsia-polyuria and polyphagia.

Abdominal fat is the cause of metabolic changes (German et al., 2010), including an increase in Tg, G, hyperinsulinism and insulin resistance, and an increase in IL-6 and TNF-α production (Kershaw and Flier, 2004; Matsuzawa et al., 2011). These pro-inflammatory interleukins are involved in obesity and insulin resistance. IL-6 is more associated with visceral obesity when compared with TNF-α, which is related to subcutaneous obesity. Both cytokines are involved in the development of hyperinsulinemia (Park et al., 2005; Cartier et al., 2008). Strikingly, these variables have been found to affect the retinal vasculature and circulation in humans with obesity (both in type 2 obese diabetics and in obese non-diabetics), damaging the retina and potentially leading to blindness (Dodson et al., 1982; Irving et al., 2002; Wong et al., 2004). The abrupt increase in abdominal fat observed in the Group B dogs possibly caused rapid changes in the concentrations of these variables, among others. These changes then led to abrupt changes in the blood circulation of the retina, making it difficult for the dogs to adapt to these circulatory changes, and also affected nutrition, in contrast to what occurred with the dogs in Group A.

AT has been described as a common event with PDH (Nichols, 1990; Ortega et al., 1996; Lien et al., 2010). In addition to the increase of the AT it has been related to damage of the retinal tissue and blindness in humans (Wong and Mitchell, 2007; Bhargava et al., 2011). In our study, only 6/20 (30%) of the dogs in Group B (proportionally equal in the dogs in Group A) showed AT at the time of their consultation and did not display retinal tissue damage (e.g., tortuous vessels, haemorrhaging, and/or retinal detachment) during the ophthalmological examination. We cannot discard the idea that at the moment that the dog became blind, the AT rose rapidly and later normalised. This rapid onset may also have some effect on the blood circulation of the retina, and this possibility remains to be clarified.

As in the previous study, the venous flow was absent in all cases. This observation reinforces our impression that blood stasis, if found at the level of the capillaries, cannot be evaluated through an ophthalmological Eco-Doppler. It is necessary to perform in vitro studies and/or to use animal models to see what happens with the blood circulation in the retina at the moment the dog becomes blind. The dogs in the study were only evaluated after losing their vision, and the clinical data had to be collected from the owner accounts.

As previously mentioned, the adipose visceral tissue synthesises adipokines that affect various metabolic pathways and adversely influence different tissues. The increase in abdominal fat changes the expression of Type 1 11β-hydroxysteroid, a bidirectional enzyme that interconverts cortisol into cortisone, causing the enzyme level to increase and creating a prolonged persistence of cortisol circulation (Tomlinson et al., 2004; Iwasaki et al., 2008; Morton and Seckl, 2008; Wake and Walker, 2004), which is already increased due to PDH. Thus, the circadian regulation of cortisol is probably altered, as described in blind humans (Hollwich and Dieckhues, 1971; Orth et al., 1978; Sack et al., 1992); this alteration would explain the greater concentration of cortisol observed in Group B.

With respect to the IL-6 and TNF-α concentrations, the latter was not significantly elevated in the dogs of either group with respect to the healthy control population, except for four cases in each group. While the TNF-α concentration is thought to be elevated with obesity, which is associated with insulin resistance and may affect the vasculature and cause other alterations (Cartier et al., 2008; German et al., 2009), the TNF-α concentration was not relevant to the studied problem among the dogs with PDH in this study. On the contrary, the IL-6 concentration was elevated in 80%

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of the dogs of Group B, and in contrast to the other variables analysed, its alteration was almost exclusively limited to this group. In a study by Cartier et al. (2008) analysing obese men, the authors reported that the IL-6 concentration was more elevated in those obese individuals with a greater amount of visceral fat than in obese individuals with a lower amount of visceral adipose tissue, whose IL-6 concentrations did not differ from those of the normal weight population. Strikingly, the TNF-α concentration did not differ between the obese population and the thin population. These authors conclude that the IL-6 concentration is clearly associated with visceral obesity and states of hyperinsulinism. Our results are similar to those of Cartier et al. (2008) in that the rapid increase in weight due to visceral fat would explain the greater IL-6 concentrations in Group B and their adverse effects on this group. This hypothesis could be supported by evaluating whether the amount of visceral adipose tissue differs between the two groups of animals with PDH. Additionally, this cytokine has adverse effects on the homeostasis of G (Bastard et al., 2006; Cartier et al., 2008; German et al., 2009, 2010) and on the vascular endothelium, causing it to thicken (Vila and Salaices, 2005; Esteve et al., 2007). The latter action explains why the IL-6 concentration correlates with changes in retinal vascular flow. This cytokine is also referred to as a stimulant in the synthesis of both ACTH and cortisol due to a direct action on the adrenal gland (Jones, 1994; Tsagarakis et al., 1998; Zarković et al., 2008; Kageyama et al., 2010; Paoletta et al., 2011), thus explaining the correlation detected with the cortisol concentration. The IL-6 concentration could also be a factor that determines the greater concentrations of cortisol in the blind population. Therefore, its clear association with blindness in the Group B dogs and the fact that its increase represents a greater risk of vision loss (OR: 17.6) makes this cytokine one of the main factors that influence the loss of vision.

In addition to the effects of IL-6 on the vascular endothelium, insulin also exerts trophic actions on the endothelium and stimulates smooth arterial muscle cell proliferation (Meehan et al., 1993; Ridray, 1995; Steinberg and Baron, 2002). Among humans with obesity and insulin resistance (with or without type 2 diabetes), hyperinsulinism causes hypertrophy of the vascular endothelium and is one of the causes of vascular damage to the retina (Matthew and Davis, 1992). In our study, insulin was also associated with vision loss, in addition to being more elevated in Group B than in Group A, and this elevation was accompanied by greater insulin resistance, as estimated through the HOMA-A index, and was also correlated with the cortisol concentration. Hyperinsulinism and insulin resistance in the Group B dogs increased the risk of ending up blind due to affected vascular flow by up to eight times. Therefore, the greater IL-6 and insulin concentrations (along with the greater insulin resistance) in the Group B dogs play an important role in the progression toward blindness, as the respective OR values also indicate. The consequence is a decrease in the calibre of the retinal blood vessels (in particular arteries-arterioles or arteriolar capillaries), causing stasis or a decrease of blood circulation.

The previously described situation is complemented by the decreasing NO and Adp concentrations. The former has an important vasodilator function that is affected by the increased cortisol concentration upon inhibiting eNOS (Mitchell and Webb, 2002). This effect was clear in our study, where the NO values were lower among the dogs with PDH than in the healthy population (we have not found similar reports in this respect). It is also striking that in the Group B dogs, these concentrations were lower than in the Group A dogs, thus reinforcing the idea of vascular alteration and retinal circulation. In addition, it is important to note the inverse correlation between the NO concentration and the cortisol concentration. It is possible that in the Group A dogs, both the vascular calibre and the dilation were affected. However, the changes would not be so severe or abrupt as to cause damage to the retina, allowing it to adapt. On the other hand, in the Group B dogs, the rapid increase in weight with an increase in visceral adipose tissue would cause (in addition to the IL-6, insulin and cortisol changes already identified) the more pronounced decrease of the NO concentration and would prevent the retinal tissue from adapting to the reduced blood flow. This marked decrease of the NO concentration has turned out to be the greatest risk factor for blindness (OR: 27.64).

With regard to the Adp concentration, while we did not find that it was associated with vision loss or correlated with the cortisol concentration, it was inversely correlated with the insulin concentration, as expected. Because the Adp concentration is a fundamental factor for the action of insulin in the tissues, decreasing its concentrations through obesity will increase insulin resistance and increase the likeliness that the subject develops diabetes mellitus. Kato et al. (2008) determined that the HMW variant and the total Adp are elevated in humans with advanced stages of diabetic retinopathy, although the study was performed with normal body mass indices. We only measured the total Adp, and its decrease agrees with the findings of Ishioka et al. (2006), although this group only studied the Adp concentrations among dogs that were obese due to hypercaloric ingestion. To the best of our knowledge, the decrease of the Adp concentration among obese dogs with PDH has not been reported. Our results show that the Adp concentration was not associated with blindness or correlated with the loss of vascular flow or the IL-6 concentration; the cortisol concentration was inversely, though weakly, correlated with the insulin concentration but not with the HOMA-A index, as expected. In an interesting study by Verkest et al. (2011) performed on obese dogs, the authors postulate that there is no relationship among obesity, the decrease in the Adp concentration, and the sensitivity to insulin as determined by the HOMA-A index. Our results coincide with these findings. While IL-6 inhibits the synthesis of Adp (Fasshauer et al., 2003), we have not found a correlation between the two molecules. We cannot provide a satisfactory explanation for the greater decrease in the Adp concentration in the Group B dogs. The role of Adp in the development of blindness may be secondary, and Adp may be a predisposing factor but not a determinant of blindness itself. More studies are necessary to elucidate the role of this adipokine in dogs and the effect of cortisol over its synthesis, while its decrease must be taken into consideration because various authors report its effects on vascular protection, anti-inflammatory action, and antioxidant properties (Shimada et al., 2004; Ouchi and Walsh, 2007). We believe that it is useful to study the concentrations of the HMW variant to determine whether its concentrations are elevated among the blind population, as observed in humans with an advanced stage of diabetic retinopathy.

5. Conclusions

Blindness in dogs with PDH is a haemodynamic event caused by and associated with the metabolic changes inherent to this disease. A cascade or a series of interrelated events cause vision loss. The presence of elevated IL-6 concentrations and decreased NO concentrations in the Group B dogs are the main risk factors for vision loss, followed by the more marked hyperinsulinism and insulin resistance in this group. Therefore, these four factors in combination are determinants in this process, with the role of Adp yet to be determined.

The rapid increase in weight and visceral adipose tissue with the development of PDH due to the corticotroph adenoma could trigger the analysed changes, in addition to the increase in Tg and G observed in the Group B dogs. These changes affect the vas-

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cular endothelium, alter normal vasodilation, and decrease the vascular flow, thus affecting the nutrition and oxygenation of the retina and resulting in vision loss. It is important to analyse whether the changes found in the variables mentioned may also affect circulation in other organs and thus may be instituted as preventative therapy.

Conflict of interest statement

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

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