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Trace element status in canine endocrine diseases

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ABSTRACT

A balanced trace element status is essential for the optimal functioning of all organisms. However, their concentrations are often altered in diverse medical conditions. This study investigated the trace element profiles in plasma samples of dogs with endocrine diseases and used chemometric techniques to explore their associations with biochemical data. Thirteen elements (As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Se and Zn) were measured in 40 dogs with hyperadrenocorticism (HAC), 29 dogs with diabetes mellitus (DM), 11 dogs with hypothyroidism (HT) and 30 control dogs using inductively coupled plasma mass spectrometry (ICP-MS). Statistically significant differences were observed for As, Cu, Mo, Se and Zn. In comparison with the control group, the HT patients had higher As and lower Se levels, while the HAC group had higher concentrations of Mo. All three disease groups had higher Cu and Zn concentrations than the control group, with the DM group having higher Cu concentrations and the HAC group higher Zn concentrations than the other endocrinopathy groups. The chemometric analysis revealed distinctive association patterns for discriminating each pathology group and the control group. Moreover, the analysis revealed the following associations: Mo with glucose levels and Cu with fructosamine levels in the DM group, As with cortisol levels in the HAC group, and Se with TT4 levels and As with TSH levels in the HT group. The study findings provide valuable insights into the complex relationships between trace elements and endocrinopathies, elucidating the associations with biochemical markers in these diseases. Larger-scale studies are necessary to fully understand the observed relationships and explore the potential clinical applications.

1. Introduction

Trace elements are essential to all organisms as they perform structural, physiological, catalytic and regulatory functions (Suttle, 2022). They are involved in almost all biochemical reactions, acting as catalysts, cofactors or structural components of enzymes and hormones (Suttle, 2022). Trace elements thus play a vital role in metabolic, endocrine, immune and antioxidant systems, among other crucial functions. Although these elements are required by organisms in very small or trace concentrations, optimal intake is needed to maintain organismal homeostasis. However, trace element deficiencies remain widespread globally, posing a challenge to overall health (Angelo et al., 2015). These deficiencies or imbalances can lead to overt clinical disorders or, more frequently, manifest as subclinical processes, exacerbating susceptibility to other diseases. On the other hand, excessive concentrations of trace elements or exposure to toxic elements (e.g. As, Cd, Hg and Pb) can also have direct detrimental effects on the body and/or compete with essential elements.

Scientific research in human medicine has consistently highlighted the significance of trace elements in various medical conditions (Himoto

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and Masaki, 2020; Koekkoek and Van Zanten, 2016; Rodríguez-Tomàs et al., 2021; Shayganfard, 2022). While studies on small animal medicine are still relatively limited, emerging research is focusing on the exposure to trace elements or their alterations in conditions such as obesity, hypoxia and oncologic, neurologic or infectious diseases (Akkuş and Ekici, 2023; Bellocci et al., 2024; Cihan et al., 2023; Elsayed et al., 2020; Günay Uçmak et al., 2023; Harro et al., 2019; Rosendahl et al., 2023; Rosendahl et al., 2022; Tarhan and Dursun, 2022; Teodorowski et al., 2021). Chronically affected patients often experience an inflammatory state characterized by heightened oxidative stress, which contributes to organ damage and disease progression while compromising antioxidant defences. At the same time, trace elements that play a crucial role in antioxidant defence systems are frequently depleted in these patients. Conversely, the presence of excess concentrations of certain elements, such as Cu, has been found to be involved in generating reactive oxygen species, further intensifying oxidative stress and the progression of the pathologies (Lowe et al., 2017).

The relationship between trace elements and endocrinopathies has gained much attention in recent years (Qiu et al., 2017; Sanjeevi et al., 2018; Stojsavljević et al., 2020; Talebi et al., 2020). Similar to other medical conditions, imbalances in trace elements can predispose individuals to endocrine disorders because of the essential roles the elements play in chemical reactions or hormone structures. Elements such as Co, Cr, Fe, Se and Zn participate in glucose homeostasis while others such as Fe, Se and Zn are essential for thyroid homeostasis, hormone synthesis and metabolism (Błażewicz et al., 2021; Dubey et al., 2020; Köhrle, 2023). However, endocrinopathies can also induce or exacerbate existing alterations in trace element levels, owing to the endocrine system regulatory role in their metabolism and optimal utilization. Understanding the multifactorial aetiology of endocrine diseases is important for developing effective prevention and treatment strategies. In the field of human medicine, ongoing research is exploring potential interventions such as the use of Cu chelators in diabetes mellitus (Cooper, 2012; Lu et al., 2010; Tanaka et al., 2009) and Se supplementation in hypothyroidism (Filipowicz et al., 2021; Pirola et al., 2020; Wichman et al., 2016), in order to improve the treatment and outcome of these patients.

Endocrine system diseases are prevalent in the canine population, significantly impacting the overall well-being, quality of life and life expectancy of older dogs. While numerous studies have explored the clinical manifestations, aetiology and treatment options for these diseases, the trace element profile of dogs with endocrinopathies has yet to be investigated. In this retrospective study, we investigated the plasma trace element profiles of dogs with endocrine diseases and explored the potential associations between these elements and relevant biochemical data.

2. Material and methods

2.1. Sample collection, selection criteria and diagnosis

This retrospective study used plasma samples that had been previously stored in a biobank. The samples were taken between January 2019 and January 2021 from dogs admitted to the Endocrinology Service of the Hospital Veterinario de la Universidad de Zaragoza. The case records of 106 dogs with endocrinopathies were evaluated and all of the available data were recorded. A comprehensive record detailing the clinical characteristics of each case was available for consultation. Identical parameter information was not available for analysis, owing to the nature of routine procedures and the diverse range of cases encountered. Cases without clear and definitive diagnoses were excluded, resulting in the removal of 17 samples. Additionally, pathology groups consisting of fewer than 10 cases were also excluded, leading to the elimination of 9 samples (4 cases of pheochromocytomas and 5 cases of hypoadrenocorticism). The final endocrinopathy groups consisted of 40 dogs with hyperadrenocorticism (HAC), 29 with diabetes mellitus (DM) and 11 dogs with hypothyroidism (HT). Diagnosis were based on common clinical presentation (Behrend et al., 2018; Bugbee et al., 2023) and unequivocal laboratory results. Specifically, the diagnostic criteria for each condition were defined as follows: (i) HAC diagnosis based on the ACTH stimulation test or the low-dose dexamethasone suppression test; (ii) DM confirmed by persistent fasting hyperglycaemia and glucosuria; (iii) HT diagnosed by measurement of total thyroxine concentration (TT4) and/or free thyroxine concentration (fT4) and thyroid-stimulating hormone concentration (TSH).

A control group comprising 30 healthy dogs was also included in the study. The animals were considered eligible if they met the following criteria: (i) they were asymptomatic; (ii) attended the hospital for routine procedures (such as neutering or senior health evaluations); (iii) no alterations were found in the clinical examination and (iv) laboratory results (including blood cell counts and basic biochemistry panel) were available and fell within the reference range for the species provided by the laboratory devices. Among the eligible samples, preference was given to the oldest ones, considering that the endocrinopathy population was expected to be mostly geriatric. Blood cell counts were conducted with an automated blood cell counter (ProCyte Dx, IDEXX Laboratories, Westbrook, ME, USA) on whole blood collected in ethylenediaminetetraacetic acid tubes (EDTA K3E 1.3 mL, SARSTEDT, Sarstedtstraße, Germany), and biochemistry panels were analyzed in a Catalyst One analyser (IDEXX Laboratories, Inc., Westbrook, ME, USA) with plasma collected in heparin tubes (Lithium heparin LH 1.3 mL, SARSTEDT, Sarstedtstraße, Germany). For cortisol, fructosamine, TSH, TT4 and fT4 measurements, analyses were carried out by an external clinical laboratory testing service (Albeitar Laboratories, Zaragoza, Spain) using serum collected in serum separator tubes (Aquisel tube with gel serum separator, Centaruro, Barcelona, Spain). Sample collection adhered to the clinical standards and ethical guidelines of the Hospital Veterinario de la Universidad de Zaragoza, which is regularly monitored by the Ethical Committee for Animal Experimentation of the Universidad de Zaragoza. Data collection followed Directive 2010/63/EU on the protection of animals used for scientific purposes (European Parliament, 2010), and the trial complied with the Spanish legislation on animal care (Real Decreto 53/2013). In this retrospective study, all samples were remainders of plasma samples collected in heparin tubes during routine clinical procedures and subsequently stored in a biobank at -20 °C. No additional sample collection procedures were performed specifically for the purpose of this study.

2.2. Sample preparation and ICP-MS analysis for trace elements

For trace element determination, the plasma samples were subjected to acid digestion before analysis by inductively coupled plasma mass spectrometry (ICP-MS), as previously described (Luna et al., 2019). Specifically, 0.2 mL of plasma was mixed with 1 mL concentrated HNO3 and 0.5 mL H2O2 in propylene tubes. The mixture was maintained at 60 °C for 2 h to allow digestion of the samples. The resulting digest was diluted by adding 2.5 mL of ultrapure water. The digest was centrifuged at 2000 rpm for 5 min, and the supernatant was collected for subsequent analysis of trace and toxic elements by ICP-MS. The concentrations of the following 13 elements were determined: arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), selenium (Se) and zinc (Zn).

ICP-MS determinations were conducted employing an Agilent 7900 ICP-MS system (Agilent Technologies, Tokyo, Japan). The sample introduction system was comprised of an autosampler, a double-pass spray chamber with Peltier system (Agilent Technologies, Tokyo, Japan), a glass concentric nebulizer (MicroMist low-flow nebulizer, Glass Expansion, West Melbourne, Australia), and a quartz torch (Agilent Technologies, Tokyo, Japan). Quantification of elemental concentrations was achieved using Agilent ICP-MS MassHunter 5.1 (Version D.01.01, Agilent Technologies, Tokyo, Japan). The operational

parameters were set as follows: plasma flow rate at 15 L/min, nebulizer flow rate at 1.1 L/min, sample depth at 8, sample flow rate at 0.1 rpm, plasma radiofrequency power at 1550 W, and spray chamber temperature maintained at 2 °C. Additionally, helium (He) (4 L/min) or hydrogen (H_2) (4.2 L/min) gases were used to correct interferences.

To ensure the accuracy of the procedure, daily calibration curves (ranging from 0.2 to 10,000 μg/L) were prepared using fresh standard solutions before analysis of the plasma samples. The correlation coefficients of the detection responses of the ICP-MS instrument were higher than 0.999 and the relative standard deviations were lower than 5%. An analytical quality control programme was used to verify the results, and the main results are summarized in Table 1. Analytical blanks were included during processing of all batches, and the limit of detection (LOD) was calculated as 3 times the standard deviation of the blanks. The LOD values were low enough to enable determination of all elements, except Cd. The accuracy of the method was checked by using certified reference material (CRM) of animal serum NIST-1598a (National Institute of Standards and Technology, Gaithersburg, MA, USA) and also dog serum samples spiked in our laboratory with appropriate concentrations of the elements (up to 2–10 times higher than the normal levels in the samples). Overall, good recoveries were achieved for both the CRM and the spiked serum samples (Table 1).

2.3. Data analysis

All statistical analyses were carried out with Statgraphics Centurion XVIII, ver. 18.1.12 (Statistical Graphics, Rockville, MD, USA) and SPSS Statistics, ver. 29.0.1.0 (IBM, International Business Machines Corporation, Armonk, NY, USA). The data distribution was checked with the Kolmogorov–Smirnov (K—S) test. The data were first examined to determine if there were significant differences in sex and reproductive status among groups using a Chi-square test, and in age using a one-way ANOVA. Only differences in age were found to be statistically significant. Subsequently, differences in trace element levels among the groups were assessed using a general linear model, with the type of pathology included as the main factor and age included as a covariate. When differences were observed, group means were compared with Tukey test. All differences were considered significant at $p < 0.05$.

The relationships between trace elements and the three endocrinopathies were comprehensively examined by using chemometric procedures. The primary aim was to reveal any latent patterns and correlations as well as potential associations between samples, variables and disorders. For this purpose, two display chemometric techniques were used: Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). All variables were autoscaled by subtraction of the variable mean and dividing the value obtained by the variable standard deviation, to avoid the potential influence of the different size of the variables, for both PCA and HCA. New variables with zero mean and unity standard deviation were thus obtained.

Principal Component Analysis is a chemometric method that simplifies data by transforming it into a new coordinate system, thus highlighting the most significant patterns and reducing the dimensionality. This method identifies and represents the principal components, which are orthogonal axes capturing the maximum variance in the data (Deming et al., 1988). In the present study, PCA analysis was initially applied to the autoscaled matrix X_{110x13}, where the rows correspond to the 110 dogs studied (80 with an endocrinopathy and 30 healthy controls) and the rows include the concentrations of the 13 elements analyzed. However, according to preliminary assays and considering the most useful information contained in the data set (avoiding the inclusion of non-informative variables in the chemometric analysis), only those trace elements for which statistically significant differences between groups were observed were included (As, Cu, Mo, Se and Zn).

Hierarchical Cluster Analysis (HCA) is a chemometric technique that organizes data into a tree-like structure based on similarities. It starts with individual data points and gradually merges them into clusters, forming a hierarchy. The process continues until all data points are included in a single cluster, revealing the inherent structure and relationships within the data (Deming et al., 1988). This technique was used to reveal, separately for each studied pathology, the relationships between groups for statistically significant variables (As, Cu, Mo, Se and Zn) and the key variables for the diagnosis of each pathology (fructosamine and glucose for DM, basal cortisol and p-ACTH cortisol for HAC and TT4 and TSH for HT). Different data matrices were constructed for each pathology by including the five trace elements that differed significantly between groups and the two key diagnostic biochemical variables in each case as variables. The numbers of cases were 26 patients for DM, 22 patients for HAC and 10 patients for HT. Therefore, the data arrays used for each group were X_{26x7} for DM, X_{22x7} for HAC and X_{10x7} for HT. In all cases, the variables were autoscaled. The similarity between variables was calculated from the squared Euclidean distance, and the clusters were obtained by Ward agglomerative method (Massart and Kaufman, 1983). The resulting clusters were visualized in the form of dendrograms, i.e. tree diagrams illustrating the arrangement of clusters generated by HCA, for each endocrinopathy group. Moreover, after examination of the tree clusters, the observed relationships between biochemistry parameters and trace elements were tested by Pearson's correlation coefficient, considering a significance level of $p < 0.05$.

Table 1
Results of the analytical quality program applied for the ICP-MS determination of the essential trace and toxic elements in plasma of dogs in the present study.

Element	Limit of detection (μg/L)	Animal Serum NIST 1598a		Spiked samples
		Certified value (mean ± SD; μg/L)	Recovery (mean ± SD; %)	Recovery (mean ± SD; %)
As	0.001	(0.3)	90.1 ± 6.0	108 ± 5
Cd	0.014	0.048 ± 0.004	91.0 ± 5.1	99.6 ± 5.7
Co	0.002	1.24 ± 0.07	92.3 ± 4.6	104 ± 7
Cr	0.005	0.33 ± 0.08	96.2 ± 4.1	108 ± 7
Cu	0.007	1580 ± 90	93.5 ± 3.5	102 ± 3
Fe	0.045	1680 ± 60	105 ± 8.0	102 ± 4
Hg	0.005	0.32 ± 0.19	95.1 ± 5.7	102 ± 6
Mn	0.030	1.78 ± 0.33	107 ± 11	107 ± 6
Mo	0.005	5.5 ± 1.0	97.3 ± 4.7	102 ± 3
Ni	0.008	0.94 ± 0.18	94.4 ± 4.4	101 ± 5
Pb	0.003	–		109 ± 7
Se	0.003	134.4 ± 5.8	98.8 ± 2.1	98.1 ± 5.6
Zn	0.096	880 ± 24	94.8 ± 4.1	105 ± 5

In brackets only indicative values.

3. Results

3.1. Dog characteristics

The information gathered from both the endocrine and control groups is summarized in Table 2. All dogs were fed a non-prescription commercial diet and received no additional vitamin or mineral supplementation.

Table 2
Characteristics of the diabetes mellitus (DM), hyperadrenocorticism (HAC), hypothyroidism (HT) and control groups. Different letters indicate statistically significant differences.

Group	Sex (%)		Reproductive status (%)		Age (years)
	Male	Female	Intact	Neutered	Mean ± SD
DM	72.4	27.6	55.2	44.8	10.2 ± 1.95 [a]
HAC	42.5	57.5	45.0	55.0	10.4 ± 2.23 [a]
HT	63.6	36.4	36.4	63.6	6.82 ± 3.22 [b]
Control	50.0	50.0	60.0	40.0	8.30 ± 2.67 [b]

Sex and reproductive status showed no significant differences between dogs with endocrinopathies and control group ($p = 0.082$ and $p = 0.441$, respectively). A statistically significant difference was observed in age distribution between the HT and control group compared to the DM and HAC groups ($p < 0.05$). These age differences were anticipated, given the known variation in age of presentation among these endocrinopathies (Nelson and Couto, 2019).

3.2. Trace and toxic element concentrations in canine endocrinopathies

The concentrations of trace and toxic elements are shown, along with statistically significant differences ($p < 0.05$) between groups, as box-whisker plots for each trace element in Fig. 1. Descriptive statistics are also detailed in supplementary material (S1). Overall, the trace element concentrations of the control group closely aligned with our laboratory's

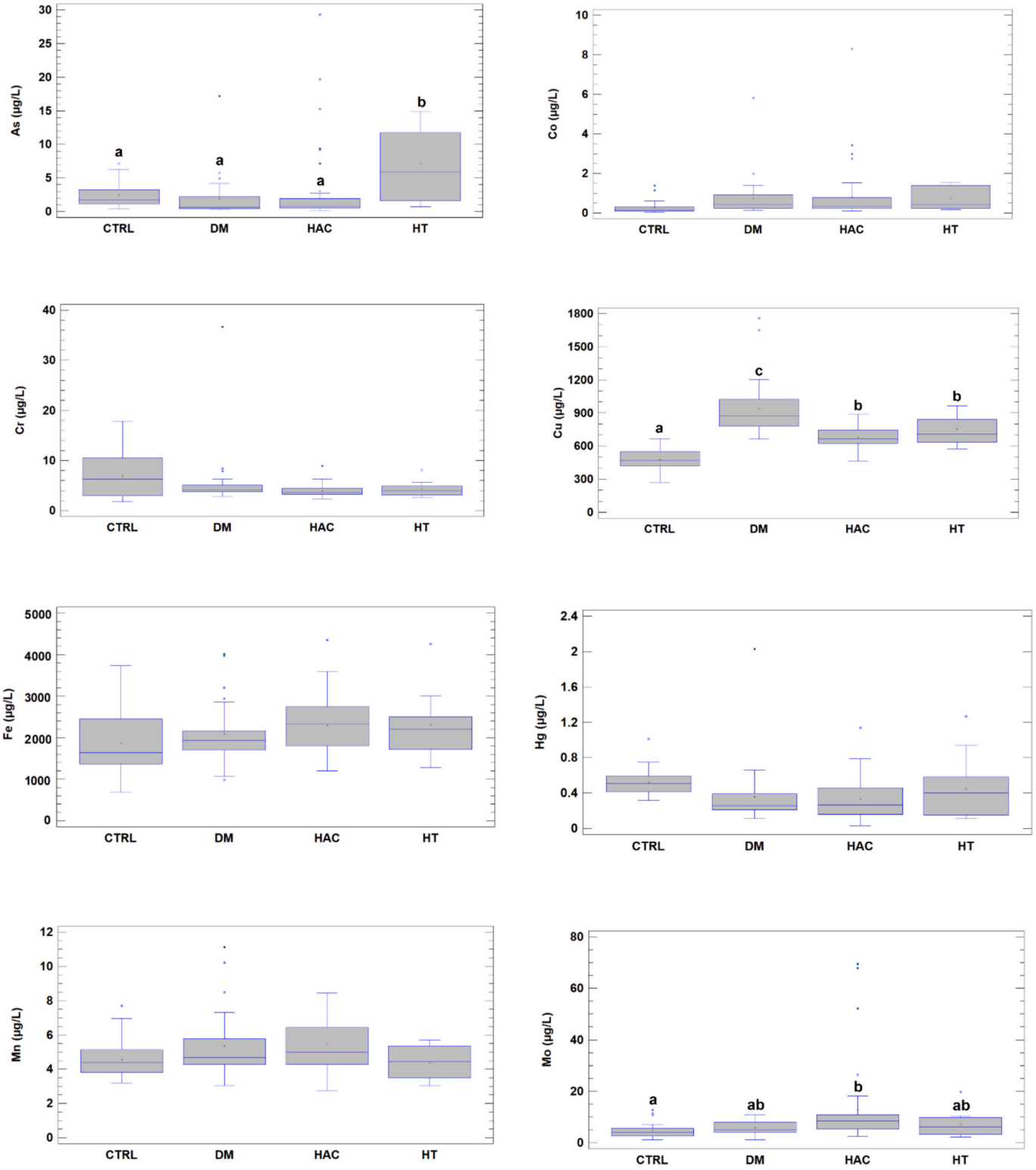

Fig. 1. Box-and-whisker plot showing the plasma concentrations of trace elements in healthy dogs (CTRL) and dogs with diabetes mellitus (DM), hyperadrenocorticism (HAC) and hypothyroidism (HT). All results are in µg/L. Different letters indicate statistically significant differences between groups ($p < 0.05$).

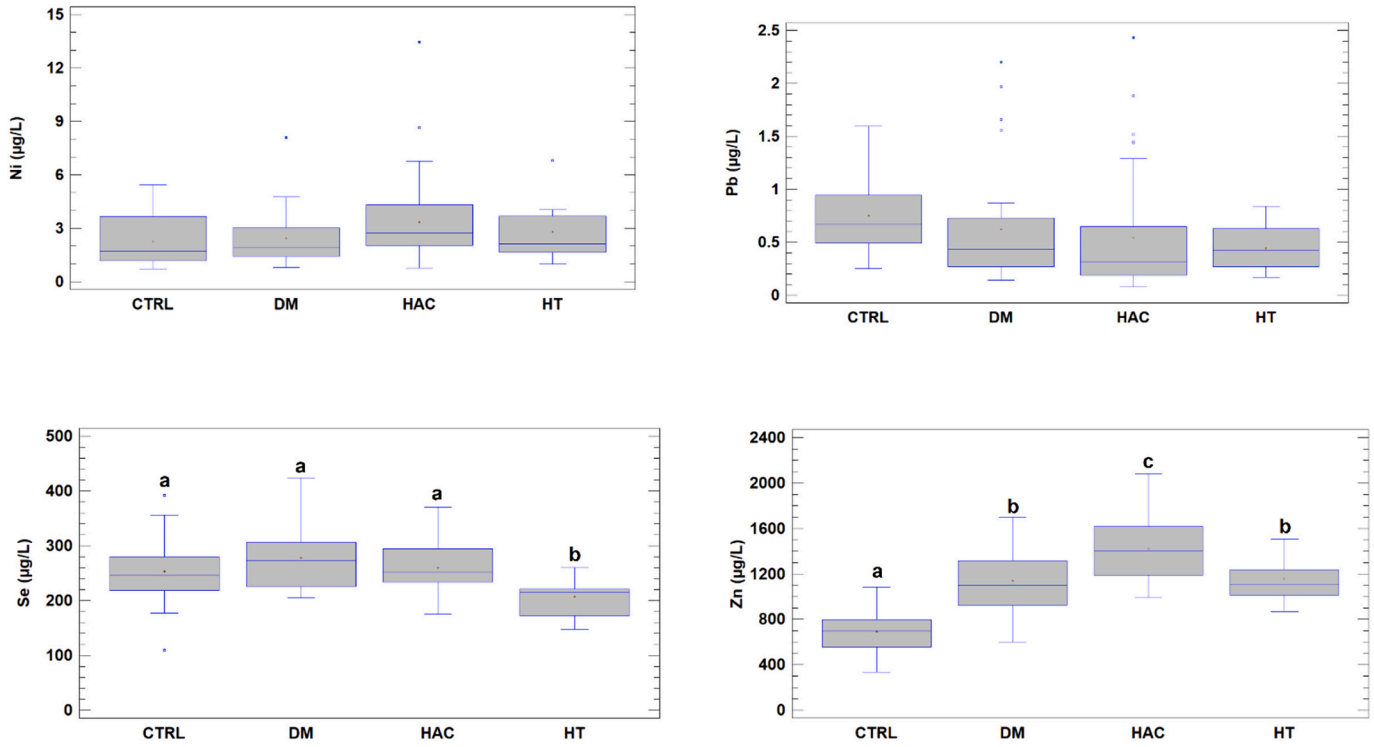

Fig. 1. (*continued*).

reference ranges for the species in serum (Cedeño et al., 2020a).

The results of the general linear model showed statistically significant differences in the concentrations of five trace elements (As, Cu, Mo, Se and Zn) across the groups. Notably, age was not identified as a significant factor in the analysis. The concentrations of As ($p = 0.012$) were higher and those of Se ($p = 0.002$) were lower in the HT group than in the control group, while the concentrations of Mo were higher in the HAC group than in the control group ($p = 0.003$). The concentrations of Cu and Zn were higher in all three disease groups than in the control group ($p < 0.001$). Specifically, within the endocrinopathy-affected groups, the Cu levels were highest in the DM group and the Zn levels were highest in the HAC group.

When comparing the significant trace elements (As, Cu, Mo, Se and Zn) to the reference range of our laboratory (Cedeño et al., 2020a), some differences were observed for the diseased groups. The levels of Cu exceeded the reference values in 82.8%, 40.0%, and 45.5% of patients in the DM, HAC, and HT groups, respectively. The concentrations of Se fell below the reference range for 9.1% of patients in the HT group. The levels of Zn surpassed the reference range in 93.1% of patients with DM and in all cases from the HAC and HT groups. The concentrations of Mo were above the reference interval in the 24.1% of the patients with HAC.

3.3. Chemometric analysis showing the relationship between trace and toxic elements and biochemical parameters

PCA was used, as outlined in Section 2.3, to reveal the relationships between the samples from different pathologies in the five-dimensional space of the significant trace element variables (As, Cu, Mo, Se, Zn). The loading plots of the samples from different pathologies and controls in the space of two (Fig. 2a) and three (Fig. 2b) dimensions for the first principal components preserved respectively 57.1 and 76.2% of the total data variance of the original variables.

In both cases, control samples (depicted in blue) formed a distinct cluster separate from the pathological samples. Additionally, samples representing the different pathologies formed separate groups in different positions within the feature space, justifying the value of the selected variables for differentiating between pathologies. While most samples within each group formed obvious clusters, there was slight overlapping between DM, HAC and HT. This result can be attributed to the clinical and pathological/analytical similarities between the diseases, which are consistent with the Cu and Zn concentrations (see Fig. 1).

As a second step in the chemometric analysis, HCA was applied to the

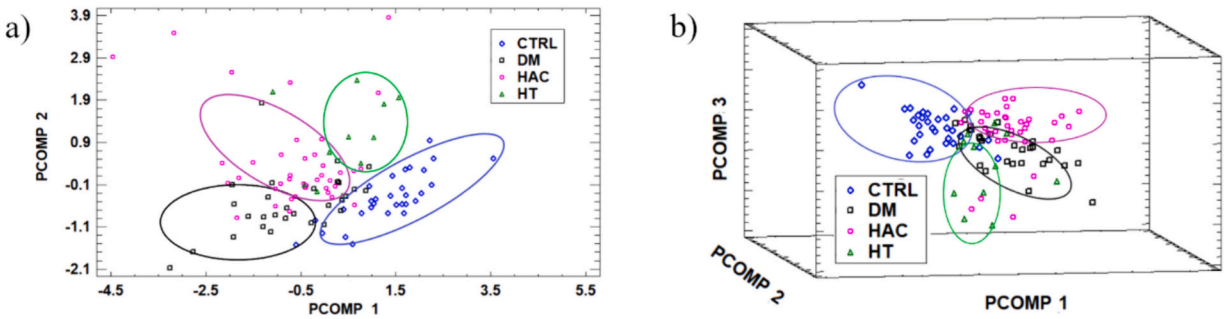

Fig. 2. Loading plot of the control (CTRL) and pathological (diabetes mellitus, DM, hyperadrenocorticism, HAC, and hypothyroidism, HT) samples in the space of the first two (a) and three (b) principal components obtained by PCA.

data matrices for DM (X_{26x7}), HAC (X_{22x7}) and HT (X_{10x7}) to establish the relationships between the trace elements and the key diagnostic biochemical parameters, as specified in Section 2.3. While the relationships between trace elements have been well described in the literature, the use of HCA in this context revealed some interesting associations between biochemical parameters and trace elements. Thus, associations between Mo and glucose levels and between Cu and fructosamine concentrations were observed in the DM group (Fig. 3a). A relationship between As and cortisol levels, encompassing both basal cortisol and post-ACTH cortisol levels, was detected in the HAC group (Fig. 3b). Finally, different relationships were observed in the HT group, particularly between Se and TT4 levels and between As and TSH concentrations (Fig. 3c).

The observed relationships were explored using Pearson correlation. In the DM group, positive correlations between Cu and fructosamine ($R = 0.397$; $p = 0.045$) and between Mo and glucose ($R = 0.513$; $p = 0.006$) were observed. However, no significant correlations between the elements and biochemical parameters were observed in the HAC or HT groups. Assessment of the correlation between TT4 and As involved only six TT4 values as in five cases the hormone levels were below the detection limit of the apparatus (6.44 pmol/L).

4. Discussion

The findings of the study reveal significant alterations in trace elements in the most prevalent canine endocrinopathies. Some of these changes are consistent with previous findings in human medicine, providing evidence for the role of these elements in the pathophysiology of these conditions. Imbalances in trace element status can contribute to the development of these pathologies, while abnormal concentrations may also arise as a consequence of the pathologies themselves.

Differences in the concentrations of Se and As, as well as a relationship between Se and TT4 and As and TSH were observed in the dogs with HT. Canine HT can result from lymphocytic thyroiditis or idiopathic atrophy of the thyroid gland, with the latter potentially representing a final stage of the former (Nelson and Couto, 2019). In humans, Hashimoto's disease is the most frequent cause of HT (Błażewicz et al., 2021), sharing similarities with canine lymphocytic thyroiditis as an immune-mediated condition. Selenium concentrations were significantly lower in the dogs with HT. Similar findings have been observed in a recent review and in a meta-analysis of human medical studies (Błażewicz et al., 2021; Talebi et al., 2020), as well as in rats with induced HT (Baltaci et al., 2013; Chanoine et al., 1992). Different mechanisms can explain the relationship between Se, TT4 and HT. Selenium deficiency could lead to thyroid dysfunction as Se forms part of the active site of the selenoenzymes glutathione peroxidase, thioredoxin reductase and iodothyronine deiodinase (Błażewicz et al., 2021). The former two enzymes are associated with protection against oxidative damage, while the latter is responsible for the activation and inactivation of thyroid hormones. Additionally, because Se plays a crucial role in the immune system, low Se levels may predispose to immune dysregulation and the development of immune-mediated thyroiditis, as well as other autoimmune diseases (Talebi et al., 2020). Conversely, the immune dysregulation and increased oxidative stress that is sometimes observed in HT patients (Lassoued et al., 2010) may also lead or contribute to the reduction in Se levels. Considering the consistent observation of Se deficiency in human studies, different clinical trials have been undertaken to explore the potential benefits of Se supplementation. These trials have demonstrated normalization of TSH in subclinical HT (Pirola et al., 2020) and a reduction in thyroid autoantibody levels (Wichman et al., 2016), indicating the involvement of Se in HT.

Regarding As, increased levels of this element were also observed in HT patients, and the corresponding dendrogram revealed a relationship between As and TSH (Fig. 3). An association between As and HT has been reported in various species, including humans (Mohammed Abdul et al., 2015; Stojsavljević et al., 2020), rats (Ahangarpour et al., 2018), guinea pigs (Mohanta et al., 2014) and amphibians (Davey et al., 2008). This toxic metalloid is known to be an endocrine disruptor and the thyroid gland has a high capacity to accumulate toxic substances (Stojsavljević et al., 2020). Arsenic is considered an antagonist of Se and a negative correlation between these elements has been observed in both serum and thyroid tissue in humans with HT (Stojsavljević et al., 2020).

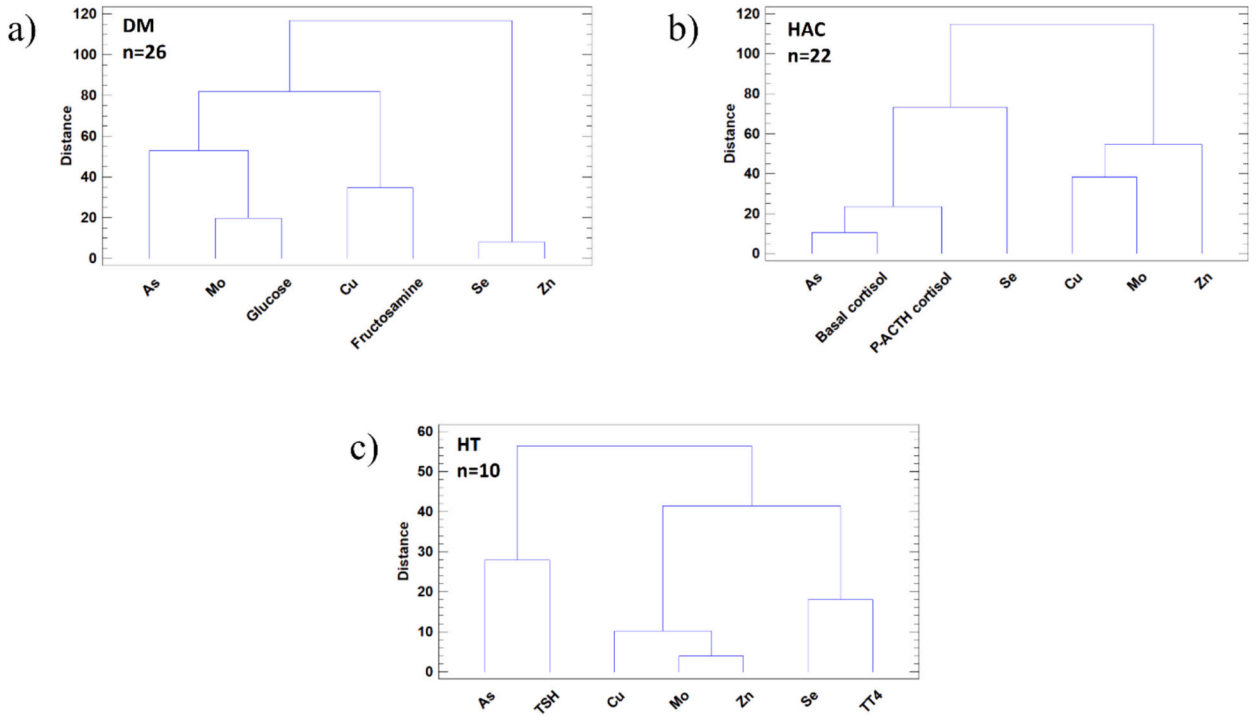

Fig. 3. Dendrograms obtained by the squared Euclidean distance and the Ward agglomerative method for the diabetes mellitus (a) (DM), hyperadrenocorticism (b) (HAC) and hypothyroidism (c) (HT) groups.

In the present study, we observed a negative correlation between the two elements ($R = -0.481$), but it did not reach statistical significance ($p = 0.134$), probably due to the small sample size ($n = 11$). While As thyrotoxicity is well established, the precise mechanisms of disturbance remain to be elucidated (Sun et al., 2016). Studies suggest that As can interfere with normal thyroid metabolism, alter thyroid hormone nuclear receptors and even replace Se in thyroid tissue (Ahangarpour et al., 2018; Stojsavljević et al., 2020; Sun et al., 2016), contributing to thyroid dysfunction, as previously mentioned. Finally, elevated As concentrations have been linked to an increased prevalence of autoimmune diseases, which can probably be attributed to immune system disorders (Mohammed Abdul et al., 2015). Such disorders could also play a role in the development of lymphocytic thyroiditis.

The DM patients had significantly higher Cu concentrations than the other groups, which is consistent with the findings of two meta-analyses conducted in diabetic humans (Qiu et al., 2017; Sanjeevi et al., 2018). There are several possible mechanisms accounting for the elevated Cu levels observed in DM. Hilário-Souza et al. (2016) found that ATP7B, the ATPase responsible for Cu biliary excretion, is stimulated by insulin and inhibited by glucagon. Therefore, the hormonal abnormalities characteristic of DM may contribute to the accumulation of Cu. The significance of this ATPase in Cu homeostasis is evident in Wilson disease and also in Labrador retrievers and Bedlington terriers with Cu toxicosis, all of which present some form of ATP7B dysfunction (Wu et al., 2016). Ceruloplasmin is another factor contributing to elevated plasma Cu levels. This cuproprotein is a moderate positive acute phase protein in dogs (Cray, 2012) and can bind up to six atoms of Cu, accounting for 40–70% of total plasma Cu (Linder, 2016). Ceruloplasmin levels are elevated in canines (Ismail et al., 2021) and humans (Memişoğullari and Bakan, 2004) with DM, probably due to its association with increased inflammation and oxidative stress. Finally, studies conducted in both human (Nangliya et al., 2015) and canine (Cedeño et al., 2020a) patients with hepatopathies have shown higher serum Cu levels than in controls. Although Cu levels were higher in the DM group, the concentration of Cu was higher than in the control group in all three pathologies and above the published reference ranges (Cedeño et al., 2020a). All of these endocrinopathies are characterized by liver involvement to some extent. This is represented by elevated activity of liver enzymes, particularly alkaline phosphatase (ALP), which is primarily associated with hepatobiliary function (Nelson and Couto, 2019). Since Cu is primarily eliminated through bile, hepatic dysfunction could lead to functional cholestasis, hindering Cu excretion and contributing to the elevated Cu levels observed in patients with hepatopathies. In contrast to our results, no alterations were found in the serum Cu levels of dogs with methimazole-induced HT (Dodurka et al., 2005). However, the hepatic function of these dogs was not studied.

The development, progression and complications of DM have been found to be associated with elevated Cu levels in human diabetic patients (Bjørklund et al., 2019; Lowe et al., 2017). However, the mechanisms underlying these associations are still not fully understood. Elevated levels of Cu enhance the production of reactive oxygen species and glycation end products (Lowe et al., 2017), thereby exacerbating oxidative stress, which plays a central role in the progression of DM. Moreover, poor glycemic control has been associated with elevated levels of Cu (Qiu et al., 2017; Viktorínová et al., 2009), and treatment of DM tends to decrease Cu serum concentrations (Naka et al., 2013). These findings support the observed positive correlation between Cu and fructosamine, a marker of glycaemic control. The same correlation has been reported in diverse human studies (Król et al., 2019; Skalnaya et al., 2017; Viktorínová et al., 2009; Xu et al., 2013). The consistent association between Cu and DM suggests that Cu chelation may be a promising strategy in DM. Although the number of studies exploring this hypothesis in human medicine is limited, the initial findings provide valuable information indicating potential benefits in improving glycaemia control and reducing DM-related complications (Cooper, 2012; Lu et al., 2010; Tanaka et al., 2009). Further research is needed to understand the observed positive correlation between Mo and glucose. Knowledge of Mo metabolism in canines is currently limited. Molybdenum is mainly excreted in the urine (Suttle, 2022), and a relationship between Mo and creatinine and blood urea nitrogen levels has been demonstrated, even when the renal parameters were within the physiological range (Cedeño et al., 2020b). This suggests that Mo levels could potentially provide valuable insights into incipient kidney injury. However, renal disease is not commonly observed in diabetic dogs, in which glucosuria is mainly produced when glycaemia exceeds the renal threshold. We speculate that altered renal function in diabetic dogs may potentially interfere with Mo metabolism. Some studies conducted in human diabetic patients have also reported an association between Mo and DM (Flores et al., 2011; Tadayon et al., 2012), DM related complications (Flores et al., 2011) and an association between urinary Mo and fructosamine (Yang et al., 2022). In an in vitro study of pancreatic β-cells, Yang et al. (2016) concluded that Mo may have cytotoxic effects on pancreatic β-cells. However, despite these findings, the exact involvement of Mo in DM remains unclear.

In the present study, patients with HAC had higher levels of Mo and Zn than all other groups. In some cases (27.6%), Mo was also higher than the published reference intervals (Cedeño et al., 2020a). As far as we are aware, this study represents the first evaluation of trace element levels in patients with HAC, as analogous investigations are absent in human medicine. As previously mentioned, Mo is primarily excreted through the kidneys, and dogs with HAC often experience hypertension and impaired glomerular and tubular function (Nelson and Couto, 2019; Smets et al., 2012). Renal abnormalities could potentially account for the observed increase in Mo levels in these patients. Regarding Zn, the concentrations in all three disease groups were found to be statistically different from those in the control group and were particularly high in HAC patients. Moreover, almost all samples surpassed the published reference values (Cedeño et al., 2020a). Conversely, human patients with DM or HT typically demonstrate lower levels of Zn, although conflicting findings have also been reported in various studies (Błażewicz et al., 2021; Dubey et al., 2020; Hanif et al., 2018; Sanjeevi et al., 2018; Stojsavljević et al., 2018; Talebi et al., 2020). In dogs with induced HT, Dodurka et al. (2005) found no significant alterations in Zn serum levels. Zinc is a constituent of the active site of the metalloenzyme ALP (Pereira et al., 2021), which is frequently elevated in the three endocrinopathies studied. This association may explain the positive correlation between Zn and ALP ($R = 0.425$, $p < 0.001$; data not shown). Notably, increased ALP is typically more pronounced and prevalent in HAC, with approximately 85% of dogs exhibiting increased serum activity (Nelson and Couto, 2019). This pattern was also evident in the present study, as mean ALP activities were 49%, 105% and 932% higher in dogs with HAC than in the DM, HT and control groups, respectively. Additionally, considering that Zn excretion has a significant biliary component (Pereira et al., 2021) it is possible that secondary hepatopathy may also contribute to Zn accumulation, following the same reasoning for Cu. It should also be considered that, in addition to HAC being an endocrine disease, spontaneous HAC is also caused by pituitary or adrenal neoplasia. Indeed, Cedeño et al. (2020a) reported elevated Zn levels in over 25% of oncologic canine patients under study. Despite these observations, high plasma Zn concentrations are rarely observed in pathologies, and the role of Zn in canine conditions is not clear, highlighting the need for further investigation into the role of Zn in canine diseases. A relationship between As and cortisol (both basal and post-ACTH) was observed in the dendrograms. Arsenic is widely considered to be a carcinogen (Mohammed Abdul et al., 2015) and may contribute to the development of adrenal or hypophyseal neoplasia. This metalloid can also potentially disrupt the hypothalamic-pituitary-adrenal axis, although the specific mechanisms involved are not yet fully understood (Mohammed Abdul et al., 2015).

There were several limitations to this study. First, it was a retrospective study conducted in a heterogeneous population and with small sample sizes, as a preliminary investigation of the selected pathologies.

Owing to its retrospective nature, the mineral content of each diet was not assessed, and tubes specifically designed for trace element measurement were not employed. Moreover, quantification of the iodine (I) levels in the samples would have been valuable, especially in the group with HT, considering the association between I, the thyroid gland and Se metabolism. However, measurement of I requires a different extraction technique from that of the other elements considered in this study. Given the small volume of the remnant specimens analyzed, performing two different digestions was not feasible. Despite the limitations inherent in the study, the results revealed consistent changes in some trace elements, which are consistent with previous findings in the field of human medicine. These findings emphasize the need for further investigation into the trace element status of canine endocrinopathies, ideally in a prospective study with a larger sample size. Additionally, studying the variations in trace elements during the treatment of these diseases could provide insights into whether the observed alterations are of a causal or consequential nature.

5. Conclusion

This study provides valuable insights into the complex relationship between trace elements and endocrine system diseases in dogs. Significant differences in As, Cu, Mo, Se and Zn profiles were observed, similar to findings in human endocrinopathies. Some elements may act as risk factors, i.e. As and Se for HT, and Cu for DM. However, the diseases may also influence metabolism of the elements, as possibly in the case of Zn and Mo in HAC. It remains unclear whether the observed alterations precede the onset or are a result of the pathology, as causal effects cannot be detected in this type of study. Larger-scale prospective studies are essential to clarify the observed relationships. Such studies could pave the way to addressing trace element imbalances and offering simple complementary therapeutic interventions, mirroring progress in human medical studies and of particular importance in the management of chronic pathologies.

CRediT authorship contribution statement

Belén Larrán: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Araceli Loste:** Writing – original draft, Supervision, Resources, Project administration, Methodology. **Marta Miranda:** Resources, Methodology, Data curation. **Marta López-Alonso:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Carlos Herrero-Latorre:** Software, Resources, Formal analysis, Data curation. **M. Carmen Marca:** Resources, Methodology. **Inmaculada Orjales:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105309.

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