Title: Mineral status in canine medial coronoid process disease: A cohort study using analysis of hair by mass-spectrometry

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Abstract

Background: In several species developmental skeletal diseases involving abnormal endochondral ossification have been associated with imbalanced mineral intake. Hair analysis reflects long-term mineral status.

Aim: To determine the mineral content of hair from dogs with or without medial coronoid process disease (MCPD). Hypothesis: dogs with MCPD have a different profile of minerals known to influence metalloenzymes involved in endochondral ossification.

Methods: After cleansing, chelation and acid-digestion of hair samples (n=79 in total: Control dogs, n=70 vs. MCPD, n=9) mineral profile (7 major and 25 trace elements) was determined by inductively coupled plasma-mass spectrometry (ICP-MS). Dogs were of similar age (Control, 4.05 [1.85 – 7.70] vs. MCPD, 4.30 [3.25 – 6.53] median (IQR) years; P=0.78) and gender (Control, n=43/27 vs. MCPD, n=4/5 males/females). 28/70 (40%) of control and 8/9 (88%) of MCPD dogs were neutered, respectively.

Results: Hair from dogs with MCPD contained significantly lower amounts (µg/g DM^{-1}) of copper, sulphur and zinc (all at P<0.001). Age, sex and neutered status had no effect on hair mineral status.

Conclusions: Based on hair analysis, a role for mineral imbalance including copper, sulphur and zinc in the aetiopathogenesis of canine MCPD is suggested. Hair mineral analysis may prove useful as a biomarker for susceptible puppies.

Introduction

In young mammals, linear skeletal growth occurs at the cartilaginous growth plate or physis with subsequent endochondral ossification (Mackie and others 2008) and structural remodelling in response to biomechanical forces under 'Wolffs Law' (Frost 1994). Canine developmental skeletal disease (DSD) is an over-arching term used to describe several conditions including hip dysplasia; premature closure of growth plates; ununited anconeal process (UAP); fragmentation of the coronoid process (medial coronoid process disease - MCPD) and osteochondrosis (OCD). DSD in the elbow is called 'elbow dysplasia' (Lavrijsen and others 2014; Scott 1998).

Excessive forces applied to insertions on bone usually result in an avulsion fracture. In MCPD, the coronoid process onto which the collateral ligament and brachialis muscle tendon insert develops fissures and disintegrates into multiple fragments (Fitzpatrick and others 2009); (Lau and others 2013b) endochondral ossification is delayed (Lau and others 2013a) and affected processes are significantly less mineral dense than processes from unaffected dogs (Burton and others 2010). Occurrence of MCPD, together with other DSDs (Meyer-Lindenberg and others 2006), suggests either a common aetiopathogenesis or a degree of linked heritability.

Observational studies across several species have associated DSDs with dietary mineral imbalance. This may involve high or low intake of a specific trace mineral or competitive interactions amongst divalent transition metals in the gastrointestinal tract, which reduces mineral bioavailability. For example, high phosphorus (Savage and others 1993a, b) or zinc intake (Gunson and others 1982) have been implicated in the occurrence of OCD in foals. Zinc directly inhibits osteoclast activity (Moonga and Dempster 1995) and high intake reduces the bioavailability of copper resulting in frank

deficiency, as has been reported in pigs (Hill and others 1983). Dietary supplementation with copper (Knight and others 1990) or magnesium (Counotte and others 2014) has been shown to reduce the occurrence rate of OCD in foals, highlighting the essentiality of good mineral balance during growth for development of the skeletal system. High calcium intake has been associated with DSDs in dogs (Schoenmakers and others 2000).

Precisely how imbalanced mineral nutrition causes DSD has not been determined, although it has been suggested that altered activity of endochondral-located metalloenzymes (e.g. zinc-dependent, matrix metalloproteinases (MMPs)) are involved (Page-McCaw and others 2007; Takaishi and others 2008). MMPs are important for normal bone development and bone remodelling after repair and maintenance; experimental deletion in mice, particularly of MMP-9 & MMP-13, leads to disrupted bone development (Page-McCaw and others 2007; Takaishi and others 2008). In dogs, MMPs have been proposed to underpin the development of OCD (Kuroki and others 2005). Hence, endochondral metalloenzyme activity may be impaired by inappropriate mineral status thereby increasing the likelihood of DSD.

Mineral status may be assessed in a number of ways. Blood and urine samples provide only a short-term assessment of mineral status (i.e. hours to days) which would not cover the growth period. Tissue biopsy can be definitive for identifying micronutrient deficiencies (e.g. in liver), but is invasive and traumatic and therefore unacceptable as a routine diagnostic or screening tool. We consider that mineral composition of hair is a better indicator of long-term mineral nutrition status. Hair concentrations of some minerals are 10-15 times the levels in blood or urine (Ahmad and others 2013), sampling is non-invasive (e.g. by grooming or clipping) and reflects mineral accumulation over a long-period (months to years). Many factors are involved in mineral incorporation into the

structure of hair: at the time of synthesis of each hair in the follicle, through absorption from sebaceous or other secretions, and through environmental contamination. Studies using radioactive isotopes have shown that mineral uptake into hair can occur within hours of dietary intake, but that uptake rates vary from one mineral to another; for example, zinc incorporation into hair takes longer than other minerals (Strain and others 1971). Similar to other keratinous structures, like horse hooves or human nails, hair does not accurately reflect diet, but dietary fluctuations can influence hair composition. In cattle, variation in dietary magnesium (low *vs.* high intake; (Anke 1966; Fisher and others 1985)), or dietary zinc and selenium (Perry and others 1976) were reflected as high or low content in hair. Whilst accurate data are not available for all dog breeds and variable rates of growth or periods between shedding may affect hair content, it is generally accepted that dog hairs remain in the inactive, 'resting' telogen phase for a long period (e.g. months to years) and for some breeds such as Nordic dogs, hair may only be shed after many years.

Based on an accumulation of evidence, we posit that imbalanced mineral nutrition or impaired bioavailability, is an underlying cause of canine MCPD. We hypothesise that the mineral composition of hair from dogs with MCPD will be significantly different, especially in zinc and copper content, to the mineral composition of hair from dogs that are free from MCPD.

This study was approved by the Ethics Committee of the School of Veterinary Medicine and Science, University of Nottingham

Materials and methods

Collection and preparation of hair for elemental analysis: A total of 79 samples (10-200g) of hair were collected from 32 different breeds of dogs without DSD during routine grooming from a UK Veterinary Hospital and from 9 dogs with confirmed elbow dysplasia and fragmentation of the coronoid process (MCPD) attending several UK practices during September and October 2015. Breeds with MCPD included Labradors (n=6 of 9; 66%), two crossbred dogs (22%) and one Staffordshire bull terrier. Hair was placed into sealed plastic bags and labelled with the dog's name, age, breed, sex and neutered status. Of the 79 hair samples, n=70 (males, n=43; females, n=27) were healthy dogs free from any clinical signs of developmental skeletal disease based on their clinical records (Control). The remaining n=9 dogs (males, n=4; females, n=5), were confirmed to have MCPD by experienced radiologists assessing CT scans. 28 of the healthy dogs and one of the dogs with MCPD were not neutered. The age range of each population of dogs was similar: Control, 4.05 [1.85 – 7.70] vs. MCPD, 4.30 [3.25 – 6.53] median (IQR) years; P=0.78).

During preparation of hair, gloves were worn at all times to avoid trace ion contamination of the samples. In order to remove external contaminants (dust, dirt, skin cells, cosmetic and cleaning treatments) a sample of hair (10-50g) was washed in a series of steps, as described previously (Forte and others 2005). In brief, the sample was immersed in a mixture of 3:1 (v/v) di-ethyl ether-acetone (Sigma, UK) and stirred at room temperature for 10 min to remove any sebaceous film covering the hair. At this time, samples were re-immersed and stirred for 1 h in 5% sodium ethylenediamine tetracetic acid (EDTA) (Sigma, UK) to chelate free chemical elements present on the surface of the hair. Finally, samples were repeatedly rinsed for 10 mins (x3) in ultrapure milli-Q water (18.2 MΩ cm) (Fisher Scientific UK Ltd, Loughborough, UK). After cleansing, a known quantity of hair was

placed into a 250ml solvent-resistant container (Sarstedt, UK) and contents freeze dried. After drying, moisture content was calculated by difference (<5% for all samples) and a known weight of dry hair (100-200 mg) acid-digested using standard techniques for inductively-coupled plasma mass-spectrometry (ICP-MS). Briefly, each sample was microwave-digested in 3.0 mL of 70% Trace Analysis Grade (TAG) HNO_3, 2.0 mL H_2O_2 and 3.0 mL milli-Q water (18.2 MΩ cm) (Fisher Scientific UK Ltd, Loughborough, UK). Hair samples were prepared and run in batches. To control for sources of contamination (e.g. in acids or water) two blank tubes were run with each batch (containing all liquids but no sample). Additionally, a certified reference material (CRM) was run in duplicate for each batch. For hair digests, the CRM was powdered human hair (ERM-DB001; Sigma-Aldrich, UK) certified (all as μg/g DM^{-1}) for As (0.044 ± 0.006), Cd (0.125 ± 0.007), Cu (33 ± 4), Se (3.24 ± 0.24) and Zn (209 ± 12).

Elemental analysis by ICP-MS: Elemental analysis was by inductively coupled plasma-mass spectrometry (ICP-MS; iCAPTM Q, Thermo Fisher Scientific Inc., Waltham, MA, USA) using a He collision cell with 'kinetic energy discrimination' to reduce polyatomic interference in the analysis of Ag, Al, As, B, Ba, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Rb, S, Sr, Tl, U, V and Zn. Lithium, Be and P were determined in standard (vacuum) mode and Se in 'hydrogen-cell' mode, with 'in-sample switching'. Internal standards were Ge, Rh and Ir. Final hair elemental composition is presented after correction for blanks and batch variation (using the CRM as reference) as μg/g of dry matter [DM; 1 μg/g = 1ppm]. For major and trace elements, recovery was >95% (e.g. Ca, 97%; Zn, 106%; Cu, 100%) with <5% coefficient of variation for each (n=11 separate analyses). Operational blanks (n = 10) were run to determine the operational Limit of Detection (LOD; 3*SD) and Limit of Quantification (LOQ, 10*SD). Intra-assay variability for all elements was <2%.

159 **Statistical Analysis**

160 Continuous data (e.g. age of the dogs, elemental composition) was analysed by one-way analysis of
161 variance (ANOVA; Healthy Controls *vs.* Developmental Skeletal Disease). Appropriateness of each
162 statistical comparison was assessed by visualizing histograms of residuals and further residual (on
163 y-axis) plots of 1) fitted-values and 2) expected normal quantiles. Non-parametric data (i.e. those
164 with skewed residual errors, common for elemental analysis) were statistically analysed after $\log_{10}$
165 transformation. Data are reported as means ± 1 standard deviation (S.D.), unless otherwise
166 indicated in the text. 95% confidence intervals around the mean may be approximated from the
167 data as ×2 S.D. We considered $P<0.05$ as indicating statistical significance, but adjusted P-values to
168 account for the number of comparisons (0.05/17 minerals present above LOQ). Multivariate, linear
169 discriminant analyses were used to identify patterns in complex (i.e. multiple variates), non-
170 independent data (e.g. all elemental data for hair) using principal component analysis (PCA) and
171 orthogonal partial least squares discriminant analysis (OPLS-DA) using SIMCA-P v12 (Umetrics,
172 Umea, Sweden). The method is an objective means to effectively demonstrate statistically
173 significant patterns in complex, non-independent datasets. All data were otherwise analyzed using
174 Genstat v17 (VSNi, Rothampsted, UK).

175

176 ***Power and sample size*****:** We estimated that over the period of study (June 2015 – November 2015)
177 we would collect hair from approximately five:one healthy dogs (i.e. no clinical signs of
178 developmental skeletal disease) *vs.* dogs with developmental skeletal disease. In a previous study
179 (Forte and others 2005), the response within each subject group was normally distributed with a
180 standard deviation of 3.41 and 47 (for copper and zinc, respectively). If the true difference in our
181 control *vs*. disease means is 4 (Cu) and 56 (Zn) μg/g DM^{-1} (>25% mean difference) we will need to
182 recruit at least 33 control subjects and 7 experimental subjects to be able to reject the null

hypothesis that the population means of the experimental and control groups (for Cu and Zn) are equal with probability (power) 0.80. The Type I error probability associated with this test of the null hypothesis is 0.05.

188 **Results**

189 The elemental composition of dog hair is presented in Table 1. A number of elements were
190 measureable, but without high confidence i.e. above limits of quantification (LOQ) and whilst we
191 provide pooled averages below, we do not report further on these elements. For information,
192 elements at low levels in hair samples (median [IQR] μg/g DM^{-1} for pooled estimates) were: Arsenic
193 (As, 0.040 [0.01-0.17]), Beryllium (Be, 0.001 [0.000-0.001]), Cadmium (Cd, 0.009 [0.004-0.033]),
194 Cobalt (Co, 0.014 [0.010-0.031]), Caesium (Cs, 0.002 [0.001-0.005]), Lead (Pb, 0.18 [0.10-0.37]),
195 Lithium, (Li, 0.040 [0.025-0.080]), Molybdenum (Mo, 0.071 [0.041-0.130]), Rubidium (Rb, 0.044
196 [0.025-0.094]), Silver (Ag, 0.047 [0.028-0.076]), Titanium (Ti, 0.60 [0.32-1.00]), Thallium (TI, 0.001
197 [0.000-0.002]), Uranium (U, 0.002 [0.001-0.004]) and Vanadium (Va, 0.080 [0.045-0.150]).
198 Nevertheless, it is of interest that for a few isolated cases some values were well above LOQ in hair.
199 For example, three cases had hair arsenic of 3.85, 4.60 and 27.6 μg/g DM^{-1}.

200

201 Comparison of 'Other Breeds' (i.e. not including Labrador) indicated that hair from dogs with MCPD
202 contained significantly lower amounts (μg/g DM^{-1}) of copper (27% lower, P=0.03) and zinc (21%,
203 P=0.01) (Table 1 & Figure 1). Comparison of Labrador retrievers only (Healthy Labradors *vs.*
204 Labradors with MCPD) also indicated that Labradors with MCPD had significantly lower hair (μg/g
205 DM^{-1}) copper (17% lower, P<0.001), zinc (23%; P=0.01) and also sulphur (18%, P=0.009). Hence, hair
206 zinc and copper composition may biomark MCPD regardless of breed, whereas hair sulphur content
207 may only biomark MCPD in Labradors, suggesting a breed pre-disposition to altered sulphur
208 metabolism and consequent incorporation into hair. Correction for age, sex and neutered status
209 had no effect on the significance of these results.

210

211 Multivariate analysis of the whole elemental dataset (normalised to account for marked variation
212 in elemental composition) according to 1) disease status (MCPD Disease = 'Yes') and 2) sex of dog
213 (Male/Female) clearly discriminates disease status as the largest contributor (linear discriminant
214 vector 1, 68.1%) to variation in the dataset, with gender also being important (25.6% of the
215 variation) but not with respect to disease status (Figure 2A). Whilst affected animals (blue dots in
216 Figure 2C) retained significant variation in overall hair mineral composition, in that the group are
217 not distinct from unaffected animals, the loadings attributable to individually elements in those dogs
218 with *vs.* those without disease clearly distinguish reduced hair zinc >>copper>sulphur (Figure 2D).
219

Discussion

In this study, we report for the first time a significant difference in accumulated mineral concentrations in hair from dogs with MCPD compared to dogs without MCPD, suggesting that mineral status – as reflected in the long-term through hair analysis – could be used as a biomarker for this disorder.

It was to be expected that Labradors would be overrepresented in the MCPD group, since Labradors are currently the most popular breed in the UK (www.thekennelclub.org.uk) and they are a breed predisposed to develop MCPD (Lewis and others 2013). Lower hair concentrations of copper, sulphur and zinc may be explained by one of several mechanisms including malnutrition, malabsorption or high mineral presence competing for bioavailability or utilisation in the gastrointestinal tract. In a previous study, 61% of dogs with skeletal abnormalities had consumed unnecessarily high calcium (Dobenecker and others 1998). High calcium intake may chelate other divalent minerals in the gastrointestinal tract, reducing their bioavailability (Schoenmakers and others 2000).

Metalloenzymes (matrix metalloproteinases or MMPs) are vital for normal endochondral ossification (Page-McCaw and others 2007). Both zinc and copper, two minerals found to be at lower levels in hair from dogs with MCPD, are fundamental to proper functioning of matrix metalloenzymes (Page-McCaw and others 2007). It is therefore possible that diet-induced interference with metalloenzyme activity, engendered by dietary mineral imbalance during endochondral ossification may underpin MCPD in dogs. Indeed, in a separate study, we have recently reported that over 90% of UK 'complete', wet, pet foods examined did not comply with EU guidelines for mineral content (Davies, Davis and Gardner - unpublished observations). Many diets

contained either less than the nutritional minimum or exceeded the nutritional or legal maximums. Furthermore, many had gross mineral imbalance such as an inappropriately low (1:10) or high (10:1) Ca:P ratio, the optimum being 1:1. Such an imbalance, together with other specific deficiencies – copper for example – suggests that nutrition of companion animals may be an important contributory factor to variable mineral status and development of MCPD.

One particular target of interest for further study is the zinc-containing matrix metalloproteinase MMP-13, as it is expressed in the skeleton when restructuring of the collagen matrix is required for bone mineralization (Inada and others 2004; Takaishi and others 2008). MMP-13 null mice show profound developmental delays in formation, ossification and vascularization of the primary endochondral ossification centres (Inada and others 2004). Suppressed MMP-13 activity, as may occur with inadequate zinc bioavailability, could explain the histopathological features of OCD. Furthermore, a form of human chondrodysplasia – the Missouri variant of spondyloepimetaphyseal dysplasia – is caused by a mutation in the MMP-13 gene (Patel and others 1993). In a study involving 50 explants from dogs with OCD, no MMP-13 staining was present in control samples but in dogs with OCD 42% (21 of 50 specimens) were positive for MMP-13 (Kuroki and others 2005). MMP-13 therefore appears integral to non-physiologic, extra-cellular matrix turnover resulting in, or from, abnormal endochondral ossification.

The scientific basis of using hair as a biomarker of mineral or disease status (e.g. accuracy, reliability, repeatability) has met with controversy (Barrett 1985; Shamberger 2002; Zlotkin 1985). Few scientific studies have evaluated the concept of hair as a marker of underlying disease status. In one, Forte *et al* found little association between mineral composition of hair and progression of Parkinsons Disease (Forte and others 2005). To our knowledge, no study has analysed canine hair

samples and directly linked mineral composition to a disease such as MCPD. Many factors may affect canine hair growth and shedding which could confound interpretation of hair mineral analysis (Shamberger 2002; Zlotkin 1985). Most concern has centred over variability in methodologies used between different laboratories such that results from the same individual analysed at different laboratories give different outcomes (Seidel and others 2001). In a recent pilot study, two samples of hair taken from one healthy volunteer were submitted to three different commercial hair mineral analysis laboratories (Namkoong and others 2013); results were consistent for hair mineral composition, with variability only introduced by laboratory-specific differences in reference ranges. As reported in the current manuscript, use of a standardised and appropriate (e.g. hair) Certified Reference Material (CRM) obviates this inherent study-study and batch variability. Further, for a technique as sensitive as ICP-MS it is equally important to include within-run controls to account for machine drift and suitable operational blanks to address potential sources of contamination during acid-digestion. In summary, hair is an accessible biomarker of long-term mineral status but has received a bad press, largely through poor experimental practice in order to maximise its commercial potential. Indeed, in 1979, the U.S. Environmental Protection Agency conducted a meta-analysis of over 400 studies reporting hair as a biomarker of a given disease state and concluded that, "*if hair samples are properly collected and cleaned, and analyzed by the best analytic methods, using standards and blanks as required, in a clean and reliable laboratory by experienced personnel, the data are reliable*".

A limitation of this study is that some dogs in the control group may have had underlying, asymptomatic skeletal disease without any mention of such in their clinical records. Age-matched controls were not specifically selected and examined. Furthermore, the precise timing of hair-shaft

formation to shedding is not known for many different breeds and ages, although the available evidence would suggest it to likely be months or even years, as oppose to weeks.

Conclusions

This study demonstrates for the first time that dogs with MCPD have lower levels of copper, sulphur and zinc in their hair compared to dogs without MCPD. Sulphur is a major constitutive element of hair and an important constituent of cartilage. Zinc and copper are important dietary elements with known roles in endochondral metalloenzyme expression and function. Dietary mineral imbalance may therefore contribute toward MCPD. We propose mineral analysis of hair as an informative, relatively cheap, non-invasive adjunctive tool of diagnostic value for MCPD. Further prospective studies are warranted to determine a causal pathway from variation in the mineral content of diet to mineral content of hair and development of MCPD, or other DSDs. A number of factors are likely to influence hair mineral composition; dietary intake, mineral interactions in the foods, or factors involved in the digestion, absorption, assimilation and distribution of minerals. Further studies in this area are warranted. Hair analysis may offer a mechanism for early screening of puppies to perhaps identify those at risk of developing MCPD or other DSDs. Preventive dietary intervention may then be possible.

Clinical relevance

The underlying aetiopathogenesis of canine medial coronoid process disease (MCPD) has not been fully elucidated. This study suggests that mineral status during development of the skeletal system in dogs may be a contributory factor for MCPD. Our study reinforces the need for owners, and for the manufacturers of diets, to ensure that the ration provided to growing puppies contains the correct balance of minerals. Mineral profiling of hair might provide a useful, non-invasive screening

315 tool to identify individual puppies at risk of developing MCPD or other DSDs early in life, so that
316 appropriate dietary intervention can be made to help prevent onset of the disease.
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318

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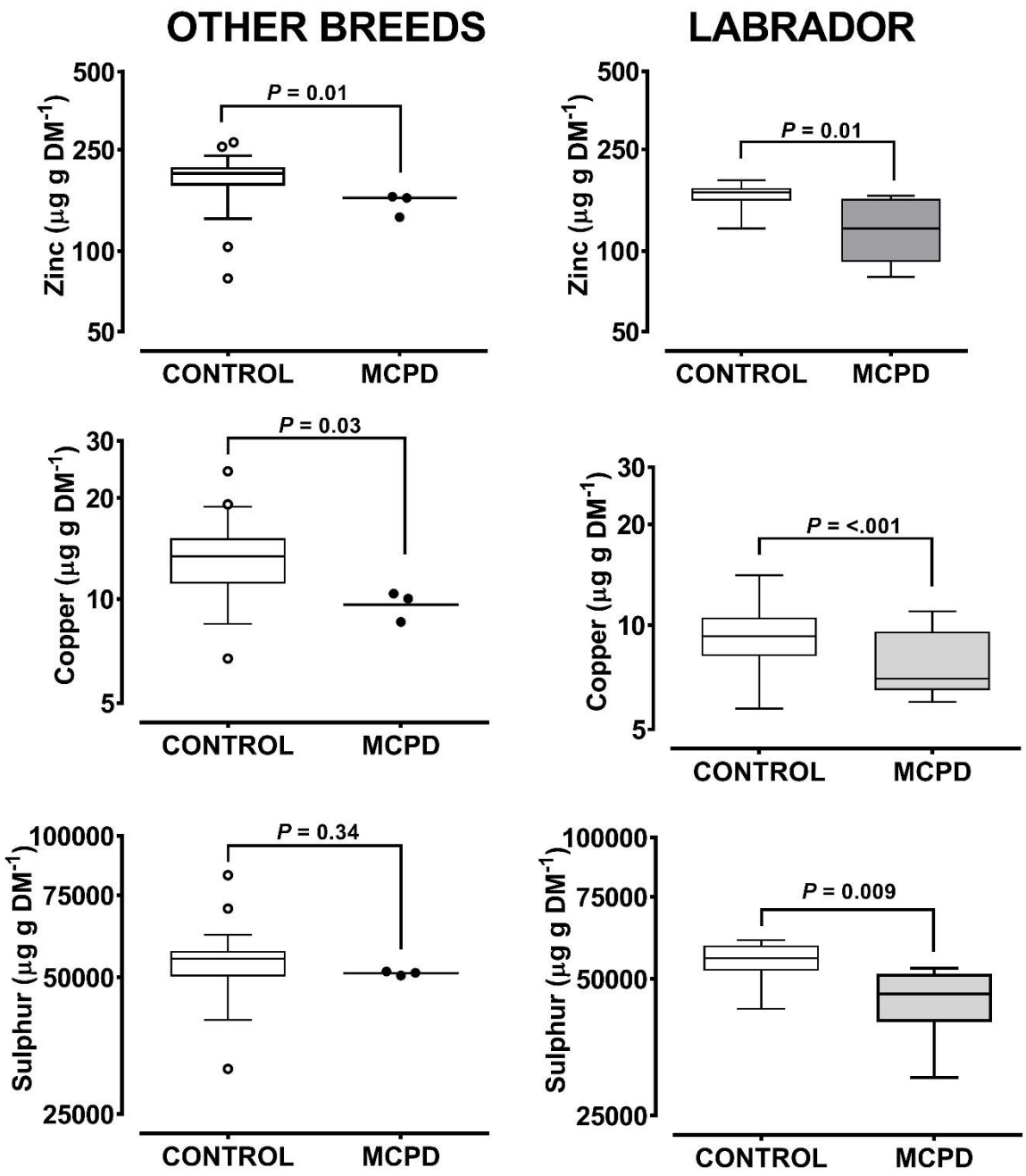
Table 1: Hair elemental composition of Healthy dogs or dogs with Medial Coronoid Process Disease.

Major element (µg g DM^{-1})	Control Healthy Dogs (n=70)		Medial Coronoid Process Disease (n=9)		*P*-value Control *vs.* MCPD		
	Other Breeds (n=60)	Labrador (n=10)	Other Breeds (n=3)	Labrador (n=6)	Other Breed	Lab	AB × Lab
Sulphur (mgs)	53.7 (49.1,55.9)	54.3 (51.8,57.7)	50.1 (49.6,50.3)	45.5 (43.2,49.7)	0.34	**0.009**	0.26
Calcium	520 (229,1242)	1360 (757,1618)	627 (600,1242)	516 (362,994)	0.97	0.29	0.50
Phosphorus	248 (195,305)	305 (267,329)	310 (278,369)	235 (200,272)	0.43	0.37	0.07
Sodium	80.9 (45.8,160)	260 (205,678)	131 (119,338)	107 (68.2,382)	0.46	0.22	0.05
Magnesium	68.2 (35.6,160)	254 (134,301)	116 (107,299)	121 (61.7,264)	0.41	0.31	0.09
Potassium	10.3 (3.43,18.8)	37.0 (27.7,89.6)	16.6 (12.8,37.4)	11.0 (8.80,12.9)	0.65	0.04	0.03
Boron	0.27 (0.20,0.76)	0.79 (0.43,1.10)	0.51 (0.46,0.51)	0.29 (0.16,0.50)	0.75	0.20	0.35
Trace element (all µg g DM^{-1})							
Zinc	199 (179,210)	167 (155,173)	160 (141,161)	123 (94,157)	**0.01**	**0.01**	0.64
Nickel	99.4 (0.42,245)	0.14 (0.07,0.39)	76.3 (19.1,103)	83.6 (38.0,274)	0.42	0.16	0.22
Iron	28.4 (17.1,42.7)	27.7 (22.6,59.4)	31.8 (24.3,35.2)	35.6 (19.8,75.7)	0.54	0.26	0.19
Aluminum	23.1 (13.0,44.1)	6.91 (3.70,50.4)	34.0 (19.8,34.4)	21.6 (13.0,63.8)	0.65	0.284	0.11
Copper	13.3 (11.1,15.1)	9.27 (8.14,10.4)	10.0 (8.9,10.2)	6.93 (6.58,9.14)	**0.03**	**<.001**	0.60
Manganese	0.99 (0.44,2.82)	0.97 (0.48,1.56)	0.75 (0.62,0.86)	2.58 (1.05,3.61)	0.38	0.29	0.37
Strontium	0.68 (0.32,1.70)	0.71 (0.58,1.05)	0.71 (0.58,1.05)	1.10 (0.86,2.26)	0.57	0.78	0.54
Selenium	0.69 (0.60,0.85)	0.69 (0.67,0.97)	0.69 (0.67,0.97)	0.66 (0.58,0.88)	0.67	0.85	0.97
Barium	0.56 (0.31,1.13)	0.55 (0.36,0.92)	0.26 (0.23,0.78)	1.54 (0.50,3.81)	0.49	0.10	0.22
Chromium	0.20 (0.12,0.33)	0.53 (0.19,0.65)	0.16 (0.13,0.32)	0.22 (0.18,0.33)	0.59	0.50	0.34

Table 1. Major and trace elements were measured in dried hair samples by inductively-coupled plasma mass spectrometry (ICP-MS). Data are presented as medians (1st to 3rd interquartile range). 'Other breeds' includes all breeds of dog other than Labrador. Due to a breed pre-disposition to DSD in Labradors, we analysed data as a 2 (Control vs MCPD Disease) × 2 (Other Breeds vs Labrador) factorial ANOVA design together with a pre-specified main effects interaction in order to determine if being a Labrador *per se* alleviated or exacerbated select mineral deficiencies in hair. Significance was accepted at a P-value of ≤ 0.01 (to adjust for multiple comparisons).

429 **Figure 1. Selected minerals in hair of healthy dogs with or without Medial Coronoid Process Disease.**



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431 **Figure 1.** Selected minerals were measured in freeze-dried hair samples by inductively-coupled plasma mass
432 spectrometry (ICP-MS). Data are presented as box or scatter-plots. For boxes; line is at median, box
433 represents 1st to 3rd interquartile range and whiskers are 5th to 95th percentile. 'Other Breeds' includes all
434 breeds of dog other than Labrador. Data was first analysed as a 2 (Breed; Other vs Labrador) × 2 (Disease,
435 Healthy vs MCPD) factorial ANOVA. Significance was accepted at a P-value of ≤0.002 (to adjust for multiple
436 comparisons) and if indicated, further post-hoc comparison was made using Mann-Whitney U-test ('Other
437 breeds') or one-way ANOVA.

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Figure 2: Multivariate analysis of hair mineral composition in dogs with or without medial coronoid process disease

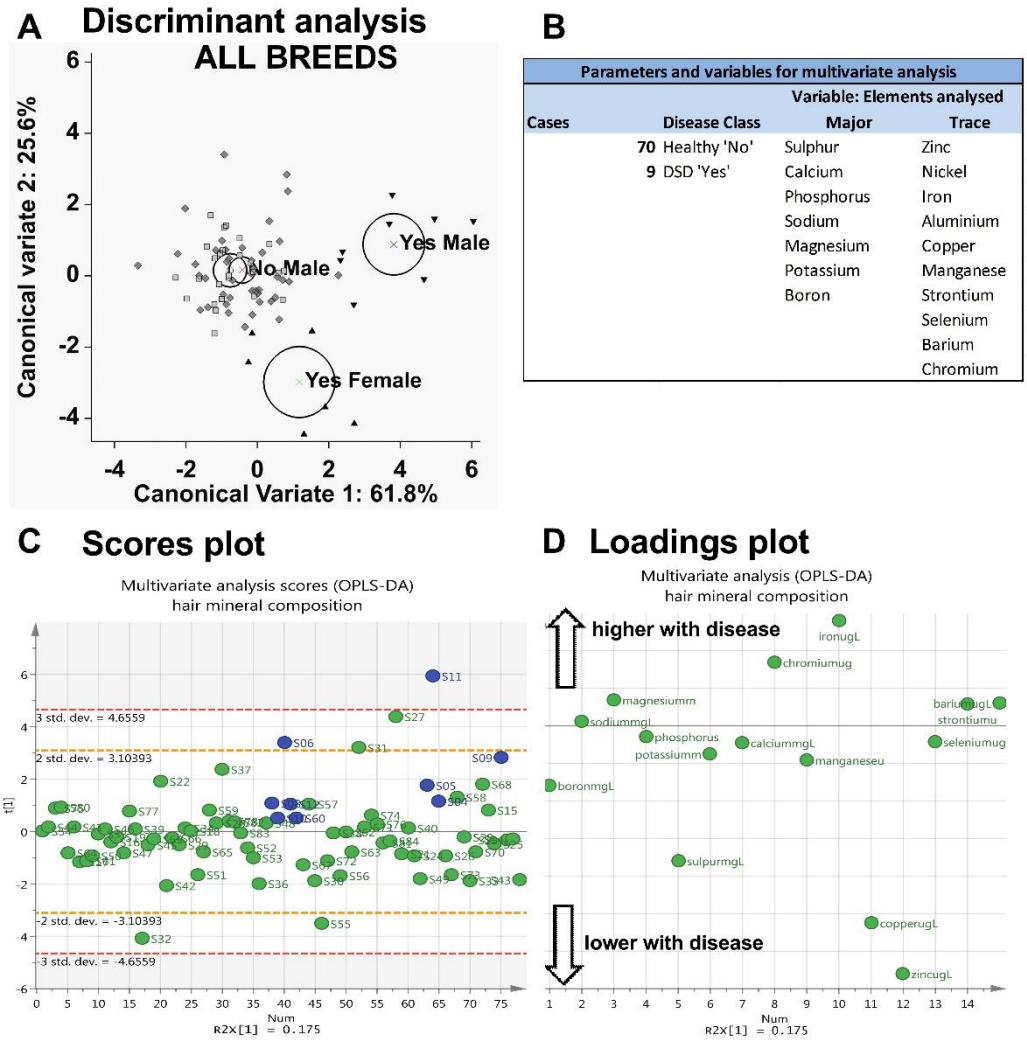

Figure 2. **Multivariate analysis of hair mineral composition in dogs with or without medial coronoid process disease**. **A**) linear discriminant plot after multivariate analysis of all cases, according to disease class (MCPD = 'Yes', Control = 'No') and including all variables as described in (**B**). Each point is an independent sample with circles representing 95% confidence interval around group means. Groups are healthy dogs (i.e. No Disease, 'No') or dogs with MCPD ('Yes'). Gender of the individuals is indicated. 61.8% of the variation in the whole dataset may be attributed to the first principle component, disease status (i.e. along x-axis). A further 25.6% may be attributed to the second principle component, gender (i.e. along y-axis). C. In SIMCA, with all parameters and variables included according to (B) indicates that the mineral composition of hair is broadly similar amongst all dogs; dogs with MCPD ('Yes') are indicated in blue, healthy dogs in green ('No'). D). In SIMCA, loading scores from orthogonal partial least squares – discriminant analysis (OPLS-DA) indicate elements contributing toward most variability in the mineral composition of hair. Scores at 0 do not contribute toward variability in hair mineral content. Highly positive (e.g. iron) or negative (e.g. copper and zinc) scores indicate higher or lower incorporation into hair respectively, in animals with MCPD. Arrows indicate direction of change and are for clarity.