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Reference intervals for serum macro- and microminerals in clinically healthy horses in Northwestern Spain: Influence of age, sex, breed and diet

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ABSTRACT

Background: Serum mineral concentrations are key indicators of nutritional status, metabolic function and health in horses; however, contemporary reference intervals (RI) derived from large, well-characterised populations remain scarce.
Objectives: To establish serum RI for macro- and microminerals in clinically healthy horses in Northwestern Spain and to evaluate the influence of sex, age, breed and diet on mineral status.
Methods: Blood samples were collected from clinically healthy horses (n = 211), and serum concentrations of essential macrominerals (calcium [Ca]; phosphorous [P]; magnesium [Mg]; sodium [Na], potassium [K] and sulphur [S]) and microminerals (cobalt [Co]; copper [Cu]; iron [Fe]; iodine [I]; manganese [Mn]; molybdenum [Mo]; selenium [Se] and zinc [Zn]) were determined using precise, accurate multielement techniques (Inductively Coupled Plasma: ICP-OES and ICP-MS).
Results: Reference intervals were established for all minerals in accordance with the clinical and laboratory standards institute (CLSI) C28-A3 guidelines. Biological factors (age, sex, breed) contributed minimally to variability in concentrations, with extensive overlap between groups, indicating partitioning was unnecessary. Diet significantly influenced the concentrations of several elements, particularly Se; pasture-fed horses had lower levels of this element, often close to the lower limit of the RI, than horses receiving commercial concentrate diets.
Conclusions: This study provides robust serum RI for macro- and microminerals in horses, providing valuable data for clinical assessment, nutritional monitoring and research on mineral metabolism.

1. Introduction

Minerals are essential nutrients that act as structural components and as cofactors in key metabolic pathways involved in skeletal development, neuromuscular activity, immune competence and antioxidant defence [1–3]. When mineral homeostasis is disturbed, a wide range of clinical and subclinical alterations can arise, affecting growth, reproduction, metabolism and athletic performance [4,5]. Numerous disorders—including reproductive problems [6,7], allergic syndromes [8], viral infections [9], chronic inflammatory or respiratory diseases linked to oxidative stress [10] and gastrointestinal disorders such as equine colic [11] —have been associated with imbalances in mineral status in horses, illustrating the physiological importance of these elements.

Despite this importance, serum mineral evaluations are performed far less routinely in horses than in livestock species, for which nutritional surveillance has traditionally been more intensive due to the importance for productivity and herd health [12]. In horses, mineral analysis is generally restricted to specific diagnostic assessments rather than being incorporated into routine health monitoring, and comprehensive datasets characterising the natural variability of macro- and microminerals in healthy equine populations remain scarce.

Serum mineral concentrations can vary widely across regions due to

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environmental and geochemical influences. Soil composition, forage mineral content, rainfall patterns and underlying geology all affect the availability of trace elements such as selenium (Se), copper (Cu) and zinc (Zn), potentially leading to marginal deficiencies even in clinically healthy animals [1,13]. Intrinsic characteristics such as age, sex and breed further modulate mineral metabolism through differences in bone turnover, metabolic rate, hormonal regulation and protein turnover [14–17]. Researchers must consider these sources of variation when establishing reliable and biologically meaningful reference intervals (RI).

In regions with acidic soils and high rainfall, such as the Atlantic biogeographical zone, the geochemical environment can strongly limit the natural availability of several essential trace elements, particularly Se, Cu and iodine (I) [2,18]. Such soil–forage constraints have consistently been reported in northwestern Spain and influence the mineral status of grazing animals across the region [12,19]. However, comprehensive reference data are not available for horses living under these environmental conditions. Recent work by Kędzierski et al. [20] suggests that serum mineral concentrations in horses often fall below current reference intervals and supports the need to reassess the existing reference ranges for these trace elements. Establishing robust, region-specific RI is therefore essential to ensure accurate clinical interpretation and appropriate dietary planning for equine populations in this area.

The aims of this study were to determine serum concentrations of macrominerals (calcium [Ca]; phosphorous [P]; magnesium [Mg]; sodium [Na], potassium [K] and sulphur [S]) and microminerals (cobalt [Co]; Cu; iron [Fe]; I; manganese [Mn]; molybdenum [Mo]; Se and Zn) in a well-characterised population of clinically healthy horses in Northwestern Spain and also to evaluate the influence of age, sex, breed and diet. The RI for these minerals were established using precise, accurate multielement analytical techniques (Inductively Coupled Plasma: ICP-OES and ICP-MS), thus providing a framework for improved nutritional assessment and diagnostic interpretation in horses.

2. Material and methods

2.1. Ethical approval

Sampling and handling of animals in this study was carried out in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes (European Parliament, 2010). The trial complied with Spanish legislation on animal care (Royal Decree 53/2013 and 1083/2025). The procedures were supervised by the Bioethics Committee of the Rof-Codina Veterinary Teaching Hospital, University of Santiago de Compostela, Spain.

2.2. Animals

Between 2021 and 2025, blood samples were collected from 211 clinically healthy horses presented for routine veterinary care either to a private equine practitioner or to the Rof Codina Veterinary Teaching Hospital (Lugo, Spain). Horses were being seen for routine health check-ups or, in the case of males, for elective castration procedures. All animals underwent a full clinical examination by a veterinarian and were considered healthy at the time of sampling.

For each animal, detailed information on sex, age, breed and feeding management was recorded (Table 1). Three age categories were considered, ≤4 years, 5–14 years and ≥15 years, and the horses were classified as mares, stallions or geldings.

A wide range of breeds was represented in the study population. The main groups included crossbred horses (n = 53), Purebred Arabian Horses (n = 49), Purebred Spanish Horses (n = 39), Spanish Sport horses (n = 17), Anglo-Arabian Horses (n = 12), European warmblood horses (n = 13), Speed-selected horses (n = 13). Other breeds (n = 15), including native Iberian breeds (Asturcón and Galician Horse), Quarter Horse, Friesian, Draft horses, and Hispano-Arabian Horses, were each represented by fewer than 10 animals.

Table 1
Characteristics of the studied horses according to sex, age, feeding system, and breed.

		Age	n
Sex	Mares	≤4	17
		5-14	73
		≥15	28
		Unknown	4
	Stallions	≤4	46
		5-14	18
		≥15	2
		Unknown	3
	Geldings	≤4	1
		5-14	12
		≥15	7
Feeding system		Only forage	25
		Forage+concentrate	183
		Unknown	3
Breed		Crossbred horses	53
		Purebred Arabian Horses	49
		Purebred Spanish Horses	39
		Spanish sport horses	17
		Anglo-Arabian Horses	12
		European warmblood horses	13
		Speed-selected horses	13
		Other breeds (<10 animals)	15

Information on feeding practices was also collected, including access to pasture, type of forage, use of commercial concentrate, and mineral supplementation. For statistical analysis, two dietary groups were considered: (1) forage-only, consisting of horses maintained exclusively on pasture, hay, silage, or combinations thereof, without concentrate or mineral–vitamin supplements; and (2) forage plus concentrate/supplementation, consisting of horses receiving commercial concentrate feed and/or mineral premixes in addition to forage. This classification captured the presence or absence of exogenous mineral inputs beyond forage-based feeding and was used to examine the potential influence of diet on serum mineral status.

2.3. Sample collection and processing

Blood samples (10 mL) were collected by jugular venipuncture, using sterile syringes and needles, into two glass tubes without anticoagulant. All samples were immediately refrigerated and transported to the laboratory. Serum was separated from the whole blood samples, within 3 h of collection, by centrifugation at 3000 rpm for 15 min. The serum was stored in labelled plastic Eppendorf tubes at −20°C pending analysis.

2.4. Sample analysis

Serum mineral concentrations were determined using two different analytical techniques, depending on the expected concentration range of each element. Macrominerals (Ca, P, Mg, Na, K and S), which are typically present in serum at relatively high concentrations (exceeding 100 mg/kg or ppm), were quantified using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Agilent 5900 ×ICP-OES system; Agilent Technologies, Tokyo, Japan). This technique is appropriate for the analysis of elements found in relatively large amounts, offering high precision and accuracy for major constituents. By contrast, microminerals or trace elements (Co, Cu, Fe, I, Mn, Mo, Se, and Zn), which occur at much lower concentrations (often in the μg/kg range), were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Agilent 7900×ICP-MS system; Agilent Technologies, Tokyo, Japan). ICP-MS provides the necessary sensitivity and detection limits for trace-level analysis, making it the preferred method for assessing microminerals in biological samples. This dual-method approach

ensured accurate quantification across the broad concentration range of essential minerals present in equine serum. Serum samples were subjected to acid digestion (all trace minerals except I) and alkaline extraction (I) prior inductively coupled plasma (ICP-OES and ICP-MS) analysis, as previously described [21]. Briefly, the serum samples (1 mL) were digested by mixing with 1 mL of hyperpure nitric acid (69%) and 0.5 mL of hydrogen peroxide (33%) in propylene tubes, which were then held at 60°C for at least 2 hours. The resulting digests were diluted with 2.5 mL of Milli-Q ultrapure water. For determination of total I, samples were processed by a high temperature alkaline extraction procedure with a mixture of tetramethylammonium hydroxide 25% (w/v) in water.

2.5. Analytical quality control

The analyses were performed at the Research Infrastructures Unit of the University of Santiago de Compostela (USC, Lugo, Spain). This laboratory operates a stringent analytical quality control system and holds ISO accreditation, ensuring the reliability and traceability of the results. Analytical blanks were included, and the limits of detection (LOD) and quantification (LOQ) were calculated as respectively three and ten times the standard deviation of the blanks. All sample concentrations were above the LOQ, which was sufficiently low to allow reliable detection of all elements.

Method accuracy was assessed using certified reference material (CRM) (Seronorm™ Trace Elements Serum L-2, Billingstad, Norway) and equine serum samples spiked in the laboratory with appropriate concentrations of the elements (up to 2–10 times higher than the usual levels in the samples). Overall, good recoveries were achieved for the spiked serum samples and for certified elements in the CRM (Table 2). Both intra-sample precision (evaluated using ten replicates of the same sample) and inter-assay precision (assessed using ten independent sample preparations on different days) were measured. The results are summarized in Table 2. Precautionary measures aimed at avoiding contamination were applied during all procedural steps.

2.6. Statistical analysis

All statistical analyses were performed using SPSS v.29 (IBM Corp., Armonk, NY, USA) and Reference Value Advisor v.2.1. Prior to modelling, the distribution of each mineral was assessed by visual inspection of histograms and Q–Q plots. Because most variables displayed right-skewed distributions, the data were transformed (log10 transformation) to improve normality and homoscedasticity; all subsequent comparative analyses were performed on transformed data.

To explore the overall influence of biological factors, a general linear model (GLM) including sex and age as fixed factors, but excluding interaction terms, was fitted. Interaction was omitted because the distribution of animals was unbalanced, and all three categories were not represented across the three age groups, making an interaction model inappropriate. Type II sums of squares were used to obtain robust estimates of main effects under this unbalanced design.

Following the exploratory GLM, the effect of sex was evaluated using one-way ANOVA within the only age group (5–14 years) in which mares, geldings and stallions were all represented, ensuring a valid three-level comparison. The effect of age was assessed using one-way ANOVA in mares only, as this was the only category represented across all three age groups (≤4 years, 5–14 years, ≥15 years). For geldings and stallions, which were present in only two age categories, age differences were explored descriptively through two-group comparisons using t-tests.

The effect of diet on serum mineral concentrations was evaluated using a GLM including diet as the main factor and adjusting for sex and age. The effect of breed was assessed using a similar GLM structure, with breed entered as a fixed factor and models adjusted for sex, age and diet; only breeds represented by ≥10 individuals were included in the analysis.

Reference intervals were calculated following IFCC–CLSI (International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and the Clinical and Laboratory Standards Institute (CLSI)) recommendations and using the statistical procedures implemented in Reference Value Advisor [22]. Given the sample size (>200 observations), the RI were estimated using the non-parametric percentile method (2.5th–97.5th percentiles) with 90% bootstrap confidence intervals. Prior to RI estimation, the software automatically assessed the distribution and applied Box–Cox transformation when appropriate; it also screened for potential analytical outliers using Tukey and Dixon–Reed procedures, retaining values unless clear evidence of analytical error was identified.

3. Results

3.1. Influence of sex and age on serum mineral concentrations

A GLM was first used to evaluate the influence of sex and age on serum mineral concentrations. This approach revealed that sex significantly affected Ca, Mg, S, Cu, Fe and Se concentrations and that age significantly affected P, S, Cu and Mn concentrations (Table 3). These preliminary results motivated a more detailed, stratified analysis to better characterize the specific influence of each of the two factors on mineral concentrations. To evaluate differences related to sex under comparable conditions, one-way ANOVA was performed on the data from the 5–14-year age group, the only category with more than five

Table 2
Analytical quality programme expressed as mean ± standard deviation (SD) used in the determination of the macro and microminerals in serum in this study.

	LoD (µg/L)	LoQ (µg/L)	Seronorm ™ Trace Elements Serum L^{-1}		Spiked samples	Precision (% CV)	
			Certified value (mean ± SD)	% Recovery (mean ± SD)	% Recovery (mean ± SD)	intrasample	intraassay
Ca	0.912	3.04	94.2	98.3 ± 6.5	99.8 ± 0.9	3.33	4.58
P	3.33	11.1	65.0	91.2 ± 9.87	101.5 ± 1.8	4.56	6.63
Mg	9.10	30.3	20.6	96.6 ± 6.6	99.1 ± 1.91	4.14	4.57
Na	6.12	20.4	(2998)	90.3 ± 4.7	99.3 ± 2.1	3.25	5.63
K	1.20	4.00	(135)	108.1 ± 2.4	104.3 ± 1.8	3.98	6.32
S	2.93	9.77	(1138)	101.1 ± 3.5	104.1 ± 2.3	4.23	5.25
Co	0.002	0.007	1.04 ± 0.21	93.2 ± 6.4	99.2 ± 4.9	3.24	5.75
Cu	0.012	0.040	1050 ± 211	95.5 ± 6.3	96.8 ± 8.4	2.33	4.92
Fe	0.084	0.280	1370 ± 280	108.2 ± 11.3	105.2 ± 5.8	2.60	4.52
I	0.014	0.047	(70)	98.1 ± 7.4	94.5 ± 6.6	3.41	4.55
Mn	0.002	0.007	10.8 ± 2.0	98.3 ± 4.8	97.8 ± 3.4	4.41	8.71
Mo	0.001	0.003	(0.67)	96.6 ± 2.3	104.4 ± 3.2	1.87	4.67
Se	0.035	0.117	84 ± 17	105.1 ± 3.6	106.8 ± 4.2	2.12	4.89
Zn	0.032	0.107	1390 ± 280	100.8 ± 5.6	98.2 ± 4.1	2.14	5.05

Values shown in brackets are indicative only. LOD = limit of detection. LOQ = limit of quantification.CV: coefficient of variation. Ca, calcium; P, phosphorus; Mg, magnesium; Na, sodium; K, potassium; S, sulfur; Co, cobalt; Cu, copper; Fe, iron; I, iodine; Mn, manganese; Mo, molybdenum; Se, selenium; Zn, zinc.

Table 3
Summary of the general linear model used to evaluate the influence of sex (mares, stallions and geldings) and age (≤4 years, 5–14 years, and ≥15 years old) as main factors in macro and micromineral concentrations in serum from the study population. Statistical analysis was performed using two-way ANOVA after logarithmic transformation of the data.

	Sex		Age		
	F	*p*	F	*p*	R2
Ca	$F_{2,200}$=4.553	0.012	$F_{2,200}$=0.875	0.418	0.123
P	$F_{2,200}$=0.875	0.418	$F_{2,200}$=7.264	<0.001	0.165
Mg	$F_{2,200}$=10.495	<0.001	$F_{2,200}$=0.429	0.652	0.149
Na	$F_{2,200}$=0.615	0.542	$F_{2,200}$=0.927	0.398	0.013
K	$F_{2,200}$=1.566	0.212	$F_{2,200}$=0.040	0.961	0.017
S	$F_{2,200}$=4.148	0.017	$F_{2,200}$=4.553	0.012	0.132
Co	$F_{2,200}$=2.414	0.092	$F_{2,200}$=0.370	0.691	0.043
Cu	$F_{2,200}$=5.742	0.004	$F_{2,200}$=4.840	0.009	0.075
Fe	$F_{2,200}$=5.593	0.004	$F_{2,200}$=0.573	0.565	0.066
I	$F_{2,200}$=2.312	0.090	$F_{2,200}$=1.206	0.302	0.069
Mn	$F_{2,200}$=2.275	0.105	$F_{2,200}$=5.132	0.007	0.051
Mo	$F_{2,200}$=0.107	0.899	$F_{2,200}$=0.736	0.481	0.010
Se	$F_{2,200}$=3.943	0.021	$F_{2,200}$=0.202	0.818	0.044
Zn	$F_{2,200}$=0.270	0.763	$F_{2,200}$=0.790	0.455	0.013

Ca, calcium; P, phosphorus; Mg, magnesium; Na, sodium; K, potassium; S, sulfur; Co, cobalt; Cu, copper; Fe, iron; I, iodine; Mn, manganese; Mo, molybdenum; Se, selenium; Zn, zinc.

animals in which mares, geldings and stallions were all represented. Significant differences among sex categories were observed for Ca, Mg, S, Cu and Fe (Table 4). Mares had significantly higher serum levels of all minerals than stallions, whereas geldings exhibited intermediate values, not significantly different from mares. The geometric mean concentrations of Se were higher in geldings than in both mares and stallions, although there was a high level of within-group variability, and the differences were not statistically significant.

Age-related differences were evaluated in mares, the only category represented across all three age groups. Significant age-related effects were observed for P, S and Cu (Table 4). Phosphorus and Cu concentrations decreased gradually and statistically significantly with advancing age, whereas the S concentrations increased significantly from the youngest to the oldest group. The concentration of Mn also decreased with increasing age in mares, although the trend was not statistically significant. In both male groups, for which only two age categories were available, age-related changes followed a similar pattern for P, S and Mn, with concentrations decreasing significantly as age increased. In the case of S, the reduction in concentration was only statistically significant in geldings.

Overall, although for several minerals, statistically significant differences in minerals were attributable to sex or age, the magnitude of these effects was small, and distributions between groups overlapped extensively. These findings indicate that neither sex nor age justify partitioning of the reference interval population.

3.2. Assessment of distribution and estimation of reference intervals

Reference intervals for all macro- and microminerals were established using the non-parametric percentile method (2.5th–97.5th percentiles), with 90% bootstrap confidence intervals. Prior to RI estimation, the distribution of each mineral was evaluated through histograms and Q–Q plots, and Box–Cox transformation was applied when required by the Reference Value Advisor workflow. The transformed data showed excellent alignment with the theoretical normal distribution, with only minimal deviations at the tails (Supplementary Fig. S1), indicating the suitability of the statistical approach used.

Given the large sample size ($n = 211$) and the absence of analytically abnormal values, all observations were retained for establishing the RI. The non-parametric method was therefore applied to all mineral data, and final limits were expressed on the original measurement scale.

The resulting RI for Ca, P, Mg, Na, K, S, Co, Cu, Fe, I, Mn, Mo, Se and Zn are summarised in Table 5, together with the respective 90% confidence intervals. As expected for a clinically healthy population, the distributions were unimodal and continuous, with no evidence of physiologically distinct subgroups.

3.3. Effect of diet on serum mineral concentrations

The influence of diet was evaluated by comparing horses receiving only forage (pasture and/or hay) and those fed a diet supplemented with commercial concentrate. Geometric means and 95% confidence intervals are presented in Table 6. Sulphur and Cu concentrations were significantly higher in horses fed forage only, whereas Se concentrations were higher in horses fed a diet supplemented with concentrate. The magnitude of the differences observed for S and Cu was small; nevertheless, Se exhibited a marked difference, with no overlap between confidence intervals, representing the largest diet-associated difference observed in the dataset. For all remaining minerals (Ca, P, Mg, Na, K, Co, Fe, I, Mn, Mo and Zn), the confidence intervals for the different dietary groups overlapped and no statistically significant differences were detected.

3.4. Effect of breed on serum mineral concentrations

A GLM including breed as a fixed factor and adjusting for sex, age

Table 4
Effect of sex and age on macro and micromineral serum concentrations in horses. The effect of sex was evaluated by comparing the three sex categories within the age group 5–14 years. The effect of age was evaluated independently within each sex category if the category included >5 animals: mares were compared across the three age categories, stallions across two age categories, and geldings across two age categories. Results presented as geometric means. Different superscript letters indicate significant differences between groups.

	Ca mg/dL	tP mg/dL	Mg mg/dL	Na mmol/L	K mmol/L	S mg/L	Co µg/L	Cu µg/L	Fe µg/L	I µg/L	Mn µg/L	Mo µg/L	Se µg/L	Zn µg/L
Effect of sex														
Mares	11.9[a]	8.70	18.1[a]	127	4.04	957[a]	0.484	1074[a]	1985[a]	14.6	1.36	5.96	67.3	518
Stallions	10.7[b]	8.36	14.5[b]	124	4.18	853[b]	0.517	865[b]	1483[b]	19.2	1.07	4.24	87.6	531
Geldings	11.7[a]	8.67	17.4[a]	128	4.51	936[a]	0.612	1016[ab]	1865[a]	16.6	1.55	4.84	103.5	579
Effect of age														
Mares ≤4	11.3	9.75 [a]	17.5	128	4.10	891[b]	0.441	1140[a]	1877	16.7	1.51	5.70	66.3	478
Mares 5-14	11.6	8.70 [b]	18.1	127	4.04	957[a]	0.484	1074[ab]	1985	14.6	1.36	5.96	67.3	518
Mares ≥15	12.0	8.59 [b]	18.3	130	4.22	971[a]	0.494	1002[b]	2008	15.3	1.26	6.23	67.8	500
Stallions ≤4	11.0	9.92[a]	15.1	126	4.13	869	0.701	1008	1507	18.7	1.60[a]	4.67	72.3	526
Stallions 5-14	10.7	8.36[b]	14.5	124	4.18	853	0.517	865	1483	19.2	1.07[b]	4.24	87.6	531
Geldings 5-14	11.7	8.67[a]	17.4	128	4.51	936[a]	0.612	1016	1865	16.6	1.55[a]	4.84	103.5	579
Geldings ≥15	11.1	6.78[b]	15.7	124	3.82	875[b]	0.405	916	929	13.0	1.06[b]	8.93	118.1	429

Ca, calcium; tP, total phosphorus; Mg, magnesium; Na, sodium; K, potassium; S, sulfur; Co, cobalt; Cu, copper; Fe, iron; I, iodine; Mn, manganese; Mo, molybdenum; Se, selenium; Zn, zinc.

Table 5
Reference intervals and descriptive statistics for serum concentrations of macro and micromineral elements in healthy horses.

	Reference interval	90% CI lower limit	90% CI upper limit	Mean ± SD	Median	Range
Ca(mg/dL)	9.85-13.23	9.46-10.04	13.02-13.30	11.59 ± 0.95	11.52	9.44–13.65
tP (mg/dL)	6.26-12.33	5.71-6.45	11.81-12.91	8.98 ± 1.98	8.96	5.37-13.21
Mg (mg/dL)	1.18-2.18	1.15-1.24	2.14-2.20	1.71 ± 0.26	1.70	1.09–2.26
Na (mmol/L)	118-141	114-120	137-144	127 ± 7	128	117-145
K (mmol/L)	2.94-5.61	2.52-3.04	5.36-5.96	4.15 ± 0.09	4.17	2.38–6.18
S (mg/L)	743-1115	626-768	1096-1133	927 ± 98	931	581-1142
Co (μg/L)	0.173-1.355	0.080-0.220	1.121-1.925	0.601± 0.305	0.555	0.055–2.170
Cu (μg/L)	695-1652	666-723	1517-1850	1073 ± 247	1041	654-2184
Fe (μg/L)	741-2280	525-864	2687-3269	1790 ± 542	1783	475-3280
I (μg/L)	11.1-23.9	11.1-11.1	23.9-25.9	16.4 ± 3.8	15.5	11.1–25.9
Mn (μg/L)	0.736-2.924	0.655-0.815	2.507-5.035	1.442 ± 0.669	1.291	0.410-6.85
Mo (μg/L)	0.72-42.54	0.55-1.04	33.11-68.76	9.62 ± 11.5	5.47	0.50-74.98
Se (μg/L)	10.8-192.1	7.9-13.9	177.3-224.5	93.7 ± 7.86	87.8	7.86-295.6
Zn (μg/L)	308-874	253-339	795-995	534 ± 130	525	225–998

SD: standard deviation. Ca, calcium; tP, total phosphorus; Mg, magnesium; Na, sodium; K, potassium; S, sulfur; Co, cobalt; Cu, copper; Fe, iron; I, iodine; Mn, manganese; Mo, molybdenum; Se, selenium; Zn, zinc.

Table 6
Serum macro and micromineral concentrations in horses fed exclusively forage (pasture and hay) versus forage plus concentrate. Geometric means (GM) and 95% confidence intervals (CI).

	Only forage		Forage + Concentrate		
	GM	CI 95%	GM	CI 95%	*P value*
Ca(mg/dL)	11.5	11.3–11.9	11.3	11.2–11.5	0.836
tP (mg/dL)	8.98	8.39–9.57	8.80	8.56–9.09	0.689
Mg (mg/dL)	1.66	1.57–1.74	1.66	1.62–1.71	0.694
Na (mmol/L)	127	125–129	127	126–128	0.545
K (mmol/L)	4.40	4.01–4.83	4.01	3.90–4.15	0.205
S (mg/L)	976	952–1000	918	900–937	0.003
Co (μg/L)	0.523	0.436–0.627	0.492	0.448–0.540	0.738
Cu (μg/L)	1153	1073–1244	1017	981–1056	0.010
Fe (μg/L)	1813	1605–2058	1697	1588–1812	0.798
I (μg/L)	15.4	14.1–16.7	16.6	15.7–17.4	0.087
Mn (μg/L)	1.57	1.31–1.62	1.41	1.34–1.47	0.150
Mo (μg/L)	5.31	3.24–8.74	5.87	4.91–6.97	0.531
Se (μg/L)	26.8	18.8–38.4	76.7	69.1–84.7	<0.001
Zn (μg/L)	500	457–549	506	486–527	0.454

Ca, calcium; tP, total phosphorus; Mg, magnesium; Na, sodium; K, potassium; S, sulfur; Co, cobalt; Cu, copper; Fe, iron; I, iodine; Mn, manganese; Mo, molybdenum; Se, selenium; Zn, zinc.

and diet did not identify any statistically significant breed effects for any macro- or micromineral (all $P > 0.05$). Although small numerical differences were observed among breeds, these did not persist after adjustment of the data, and the confidence intervals indicated substantial overlap across groups (Supplementary Table S1). Overall, breed-related differences in the mineral concentrations were not of sufficient magnitude in any case to justify further subgroup analyses.

4. Discussion

4.1. Mineral status

Establishing reliable RI for serum macro- and microminerals is essential for the clinical assessment and nutritional management of equine populations. Despite the recognised influence of environmental, dietary and physiological factors on mineral status [23–27], available datasets for horses are often limited in size or scope, with many studies analysing relatively small numbers of animals or focusing on a small subset of elements [4,8,13,17,23,24,28–31] (Tables 7–8). The present study provides a comprehensive evaluation of mineral concentrations in a large cohort of clinically healthy horses in northwestern Spain, providing region-specific RI derived from a robust sample size and using standardised analytical methods. The data provided contribute to the refinement of clinical interpretation and support evidence-based nutritional decision-making in areas with comparable management systems and environmental conditions.

In general, the serum macromineral profile observed in our study is consistent with the adequacy ranges proposed by Puls [32], although this classical reference—while still widely used—relies on historical datasets and is not geographically tailored. Our reference interval for Ca (9.85–13.23 mg/dL) falls within Puls' "adequate" category [32] (10–13 mg/dL) and is consistent with values reported in studies conducted elsewhere (Table 7), in which mean Ca concentrations typically range between 11 and 13 mg/dL. Similarly, the RI for Mg (1.18–2.18 mg/dL), Na (118–141 mmol/L) and K (2.94–5.61 mmol/L) substantially overlaps with the physiologically adequate ranges defined by Puls [32] (Mg: 1.80-3.50 mg/dL; Na: 130–143 mmol/L; K: 2.4-5.0 mmol/L) and correspond well with distributions described in other equine

Table 7
Serum macromineral concentrations in horses from multiple geographic regions. Values are presented as arithmetic means ± standard deviation and range in brackets.

Country	n	Ca (mg/dL)	P (mg/dL)	Mg (mg/dL)	S (mg/L)	Na (mmol/L)	K (mmol/L)	Sample	Method	Reference
China	12	12.8 ± 1.8	3.53 ± 0.43*	2.96 ± 0.36	—	103 ± 5	3.23 ± 0.54	Serum	ICP-MS	[31]
New Zealand	21	12.2 ± 4	7.88 ± 0.16*	1.69 ± 0.05	903 ± 13.7	138 ± 4	4.30 ± 0.10	Plasma	ICP-OES	[35]
Turkey	20	11.7 ± 4.2 (8.3–15.1)	11.6 ± 0.4* (8.8–16.0)	2.59 ± 0.4 (2.26–2.86)	—	—	—	Whole blood	ICP-OES	[17]
Italy / Turkey	16	—	—	1.83 (1.06–2.86)	—	—	—	Serum	ICP-OES	[9]
Canada	60	11.6 ± 0.4 (10.7–12.4)	2.7 ± 0.81** (1.12–4.40)	1.99 ± 0.19 (1.60–2.31)	—	—	—	Serum: Ca, P and Mg Plasma: Na and K	S-C	[33]
Italy	20	—	—	1.08 ± 0.52	—	—	—	Serum	ICP-OES	[41]
Mexico	145	15.7 ± 3.2	3.58 ± 1.15**	2.13 ± 0.41	—	136 ± 4	—	Serum	AAS/ S-C (P)	[24]
Netherland	65	12.7	—	2.07	—	—	—	Serum	AAS	[7]
Slovak Republic	21	12.75 ± 0.9	3.65 ± 0.76**	1.92 ± 0.22	—	137.5 ± 2.035	—	Serum	ICP-OES	[34]

Ca, calcium; P, phosphorus; Mg, magnesium; Na, sodium; K, potassium; S, sulfur. P: (*) = total P; (**)= inorganic P. ICP-MS: inductively coupled plasma–mass spectrometry; ICP-OES = inductively coupled plasma–optical emission spectrometry; AAS= atomic absorption spectrometry; S-C= spectrophotometry-colorimetry.

Table 8
Serum micromineral concentrations (μg/L) in horses from multiple geographic regions. Values are presented as arithmetic means ± standard deviation and range in brackets.

Country	n	Co	Cu	Fe	I	Mn	Se	Zn	Sample	Method	Reference
Ecuador	30	—	902 ± 42 (601–1230)	—	—	18 ± 2 (10–50)	—	620 ± 33 (380–860)	Serum	AAS	[28]
China	8	—	1495 ± 355	2010 ± 532	—	109 ± 32	908 ± 19	467 ± 204	Serum	ICP-MS/ AAS	[31]
New Zealand	21	—	1110 ± 41	1530 ± 202	—	—	—	490 ± 23	Plasma	ICP-OES	[35]
Italy	20	—	620 ± 40	165 ± 8	—	—	—	2050 ± 150	Serum	ICP-MS	[26]
Italy	20	8 ± 1	680 ± 220	—	—	—	—	2160 ± 640	Serum	ICP-MS	[25]
Egypt	20	—	1303 (699–2805)	2118 (1162–2880)	—	82 (30–121)	134 (87–237)	366 (78–582)	Serum	AAS	[10]
Saudi Arabia	17	—	1280 ± 185 (780–1820)	7100 ± 1730 (4100-11880)	—	790 ± 50 (660–980)	45270 ± 1290 (41310–49120)	110 ± 6 (100–178)	Serum	AAS	[29]
Canada	105	—	1625 ± 119	—	—	—	—	817 ± 52	Serum	AAS	[43]
Turkey	20	—	1037 ± 45 (632–1621)	2375 ± 247	—	—	173 ± 13 (73–332)	936 ± 66 (546–1824)	Whole blood	ICP-OES	[17]
Italy / Turkey	16	—	1290 (990–2330)	2630 (780–4800)	—	4 (0–270)	1005 (710–1290)	920 (510–1440)	Serum	ICP-OES	[9]
Brazil	20	—	1033 (270–3419)	—	—	—	—	696 (345–1235)	Serum	AAS	[44]
Chile	32	—	1049 ± 241 (635–1270)	—	—	—	150 ± 55 (89–276)	569 (196–719)	Plasma-Serum	AAS/ ICP-MS	[3]
Canada	60	—	—	1581 ± 369 (850–2307)	—	—	—	—	Serum/ Plasma	AAS	[33]
Pakistan	200	—	791 ± 35	3670 ± 310	—		301 ± 79	1620 ± 110	Serum	AAS/S-C	[23]
Turkey	23	—	370 ± 74	1950 ± 740	—	—	—	517 ± 63	Serum	AAS	[40]
Iran	20	—	880 (300–1880)	1660 (480–4180)	—	—	—	1220 (670–1740)	Serum	AAS	[11]
Italy	20	—	549 ± 182	1631 ± 898	—	0.3 ± 1.0	200 ± 60	339 ± 187	Serum	ICP-OES	[41]
Mexico	145	—	990 ± 110	2850 ± 640	—	—	150 ± 70	4330 ± 1910	Serum	AAS/S-C	[24]
Poland	12	—	792 ± 93	—	—	—	—	1122 ± 245	Serum	AAS	[13]
Poland	154		1312 ± 260				93.0 ± 49.6	645± 39		AAS	[20]
Finland	8	17 ± 2	1055 ± 21	3100 ± 790	—	31 ± 7	—	420 ± 28	Serum	ICP-MS	[8]
Canada	10	—	—	—	19 ± 0.5	—	—	—	Serum	ICP-MS	[38]
Japan	69	—	—	—	16.9 ± 7.9	—	—	—	Serum	ICP-MS	[39]
Slovak Republic	21	—	725.9 ± 110	393780 ± 86600	—	—	—	2045 ± 556	Serum	ICP-OES	[34]

Co, cobalt; Cu, copper; Fe, iron; I, iodine; Mn, manganese; Mo, molybdenum; Se, selenium; Zn, zinc. ICP-MS: inductively coupled plasma–mass spectrometry; ICP-OES = inductively coupled plasma–optical emission spectrometry; AAS= atomic absorption spectrometry; S-*C*= spectrophotometry-colorimetry.

populations (Table 7), indicating that these major minerals and electrolytes fall within widely accepted physiological limits.

For P and S, comparison with previous findings is restricted by methodological differences. Our reference interval for P (6.26–12.33 mg/dL) corresponds to the *total* P measured by ICP techniques, whereas many classical clinical pathology studies quantify only the inorganic fraction [24,33,34]; total P is typically 20–30% higher than inorganic P [17,35] which may partly explain discrepancies in upper limits reported across studies. The S concentrations also vary between studies, largely because measurement of this element depends on ICP-based quantification rather than routine biochemical assays. Despite these analytical differences, both P and S concentrations in our study population fall within or are close to the ranges reported in the literature (Table 7) and, in the case of P (no data available for S), remain compatible with the adequacy thresholds proposed by Puls [32] (P: 2.7-5.0 mg/dL).

For most trace elements, the agreement between the RI established in the present study and the physiologically adequate ranges proposed by Puls [32] was variable, reflecting both biological variation and the scarcity of contemporary studies using comparable analytical methods. Copper (RI: 695–1652 μg/L) falls comfortably within the "adequate" category proposed by Puls [32] (650–2000 μg/L) and the concentrations are consistent with those reported in recent publications from Europe, Asia, and the Americas (Table 8). By contrast, Zn (RI: 308–874 μg/L) and Fe (RI: 741–2280 μg/L) do not fully correspond to the broad physiological ranges suggested by Puls [32] (Zn: 500–1500 μg/L; Fe: 840–2570 μg/L), which probably reflects differences in analytical techniques, dietary regimes and the biological lability of both elements. For Fe, comparisons are further complicated by factors such as inflammation, iron mobilization, transport proteins and sampling conditions, which can strongly influence circulating concentrations [2]. Nevertheless, for both Zn and Fe, the central tendencies in our cohort are broadly consistent with the concentrations reported in healthy adult horses from multiple geographic regions (Table 8). Serum Mn concentrations in the present study (RI: 0.736–2.924 μg/L) were low and fall within the range typically reported for horses when comparable biological matrices and modern analytical techniques are considered [36]. Although, Puls [32] does not provide explicit reference intervals for Mn in horses, circulating concentrations below approximately 2.5 μg/L are described as physiologically common, in good agreement with the central tendency observed in our cohort. The wide variability of Mn concentrations reported in the literature (Table 8), ranging from <1 μg/L to >100 μg/L across studies and geographic regions, largely reflects methodological rather than biological differences. This is clearly illustrated by Theiner et al. [36], who showed that Mn concentrations in equine whole blood are approximately ten-fold higher than those measured in serum or lithium-heparin plasma, highlighting the substantial contribution of the cellular fraction to total blood Mn. In that study, serum Mn reference intervals determined by ICP-MS (1.11–2.96 μg/L) closely overlap with those obtained here, whereas whole-blood concentrations reached median values of 12.4 μg/L. Accordingly, reports describing markedly

higher Mn values often involve whole blood, different analytical techniques, or older methodologies with limited control of trace-element contamination, which may inflate circulating Mn estimates, particularly at the very low concentrations typical of serum.

Among all trace elements, Se stands out as the most clearly divergent. The reference interval established in the present study (10.8-192.1 μg/L) lies partly below the "adequate" category proposed by Puls [32] (80–200 μg/L), and the mean concentration in our study population is markedly lower than those reported in most recent international datasets (Table 8). A substantial proportion of animals in our study fall below Puls' adequacy threshold, consistent with the well-documented Se-deficient status of grazing animals in Spain [12,19] and other regions with acidic soils and high rainfall [1,4]. This pattern further confirms Se as the primary trace element of concern for horses in northwestern Spain, mirroring deficiencies observed in local livestock [12,19] and even in human populations [37] from the same area.

For Co, Mo and I—trace elements for which published equine data are particularly scarce—our results provide relevant new reference information. Puls [32] does not propose physiological ranges for Co or Mo in horses, and for Co the few available studies report highly heterogeneous and often substantially higher concentrations than those observed in the present cohort (Table 8), likely reflecting differences in analytical methodology, biological matrix, environmental exposure and dietary background. In this context, the low serum Co concentrations observed here (RI: 0.173–1.355 μg/L) are noteworthy but consistent with values reported in other species [12] and with contemporary human data [37], where circulating Co concentrations are typically in the sub-μg/L range. For Mo, direct comparisons with equine studies are currently not possible due to the absence of published reference data; however, the serum Mo concentrations observed in this study (RI: 0.72–42.54 μg/L) provide an initial benchmark and suggest that circulating Mo levels in healthy horses may be lower than previously assumed, particularly when measured using modern multielement techniques. For I, Puls [32] proposes a broad physiological interval of 20–200 μg/L; although the reference interval established in this study (11.1–23.9 μg/L) lies toward the lower end of this range, it closely matches values reported in horses from Canada [38] and Japan [39] (Table 8), suggesting that relatively low serum I concentrations may be common in clinically healthy animals depending on regional dietary practices. Overall, while none of these elements appears to represent a deficiency or excess of immediate clinical concern in our population, the limited availability of robust equine reference data underscores the need for further well-controlled studies to improve the interpretation of Co, Mo and I status in horses.

4.2. Factors of variation

In addition to geographical and environmental factors, intrinsic characteristics of the animals under study may modulate serum mineral status. In the present study, sex and age had statistically significant effects on several minerals, but the absolute magnitude of these differences was modest, and the distributions overlapped between categories. Mares tended to have slightly higher Ca, Mg, S, Cu and Fe concentrations than stallions, while geldings consistently had intermediate values, generally not differing significantly from mares. Selenium concentrations did not differ significantly among sex categories once the substantial within-group variability (particularly in geldings) was taken into account. These patterns are consistent with the limited evidence available in horses, in which sex-related effects are typically small. For Ca, previous studies generally fail to demonstrate sexual dimorphism, as sex is rarely analysed explicitly and, when included, no significant differences were detected [17,34]. Variations in Ca were more consistently linked to female reproductive status, such as lactation or periparturient disorders [7,35], consistent with the slightly higher Ca in mares observed here. For Mg, evidence of sex-related variation is limited but suggestive; higher Mg in females has been reported in whole blood [17], while most serum-based studies do not identify sex as a major determinant [24,34]. Sulfur has been scarcely studied; the only available work assessed lactation-related changes without sex comparisons [35]. Copper showed the most consistent evidence of sex-related modulation, with higher Cu in females reported in whole blood [17] and marginally higher serum values in mares [13,25], as well as increases during gestation [23]. For Fe, literature does not support a consistent sex effect [17,29]; variations are mainly linked to inflammation, disease, or reproductive status [10,35,40], so the significant effect detected here may emerge when controlling confounders. Overall, sex exert only minor influence on serum mineral concentrations in horses, consequently, sex-specific partitioning of RI is not warranted.

Age-related effects were more apparent for a subset of minerals, particularly P, S, Cu and Mn. In mares—the only sex represented across all three age categories—P and Cu decreased gradually with advancing age, whereas S levels increased significantly and Mn levels tended to decrease, although the trend was not statistically significant. Only two age categories of males were available in the present study, but age-related patterns were broadly comparable to those observed in females: P and Mn concentrations decreased with advancing age in both stallions and geldings, and the decrease in Mn was statistically significant. By contrast, S levels tended to decrease with advancing age in males—contrary to the pattern seen in females—although the reduction was statistically significant only in geldings. The decline in P with age aligns with classical reports of higher levels in young horses due to skeletal growth [33], while studies in adults showed little variation [17], suggesting attenuation after maturity. For S, literature is extremely limited; the only study focused on lactation and reported no temporal changes [35]. Copper had rarely been analyzed by age; previous work had reported no significant differences [17]. For Mn, no studies had systematically assessed age; reported changes were related to disease or nutrition [10,23,41]. Overall, the magnitude of age-related variation remained modest, and RI overlapped widely among age groups, indicating that these effects, while physiologically meaningful, are not strong enough to justify age-specific partitioning. These findings support the view that individual mineral concentrations can be modulated by age, but that the population-level impact in healthy adult horses is limited.

Dietary management also contributed to variations in several minerals, most notably Se. Horses receiving commercial concentrate displayed substantially higher serum Se concentrations than animals fed forage only, reflecting the low Se content of forage grown on acidic, high-rainfall soils and is consistent with previous reports highlighting Se deficiency as a recurrent issue in grazing animals from northwestern European regions [1,4]. In our cohort, a large proportion of horses clustered at the lower boundaries of the RI, suggesting that marginal Se status is common in this population. Selenium concentrations deserve particular attention due to the biological relevance of this element and because suboptimal status may remain clinically silent until animals face increased metabolic or inflammatory demands. As a key component of antioxidant, immune, thyroid and reproductive functions, inadequate Se supply can potentially impact health, performance and disease resilience in horses [2,42]. Under the environmental conditions of northwestern Spain, where soils are naturally poor in Se, dietary supplementation is the primary determinant of adequate Se status. This situation has long been recognised in ruminant production systems in Spain [12,19], where targeted supplementation is routinely implemented. Our findings in horses indicate that dietary management should actively consider Se provision, particularly in animals reliant on pasture or forage-based diets without commercial supplementation, and that preventive strategies may be advisable to avoid subclinical deficiency. Further research is warranted to determine optimal supplementation practices under local conditions and to evaluate whether low Se status contributes to clinical, metabolic or performance-related outcomes in equine populations.

The Cu concentrations were slightly higher in horses fed only forage than in those receiving concentrate diets. Although concentrates typically include supplemental Cu, similar findings have been described in

other studies [25,26]. Several factors may contribute to this inverse pattern. First, dietary composition differs markedly between forage and concentrate-based rations, with variations in fibre, protein and S-containing compounds that may influence intestinal mineral absorption. Second, while the classical Cu–S–Mo antagonism is strongest in ruminants [2], high dietary S or Mo concentrations can also reduce Cu availability in horses through non-ruminal mechanisms, albeit less efficiently [32,35]. These interactions may contribute to small shifts in serum Cu when concentrate formulations differ in their content of competing minerals. Nevertheless, the magnitude of this effect was modest. For S, the concentrations were slightly higher in horse fed forage only. Although serum S is seldom evaluated clinically and published data in horses are limited, such differences likely reflect variation in organic S compounds naturally present in roughage [2].

Evaluation of breed alongside sex, age and diet did not reveal any significant effect for any mineral, indicating that breed does not greatly influence serum macro- or micromineral status in this population. While numerical differences were present in descriptive statistics, these vanished after adjustment for the main environmental and physiological factors, and the distributions of all minerals showed broad overlap across breeds. Importantly, the relevant literature does not provide robust evidence of genetically driven breed differences in baseline mineral concentrations in horses, and most published datasets report similar values across diverse breeds with comparable feeding and management conditions [17].

4.3. Added value of the study and limitations

This study provides several strengths that enhance the reliability and applicability of the proposed RI. First, the sample size ($n = 211$) exceeds the minimum CLSI requirement for non-parametric RI estimation and is larger than for most published datasets, increasing the statistical robustness and representativeness. Second, serum minerals were quantified using ICP-OES and ICP-MS, high-precision multielement techniques that minimise analytical variability and permit simultaneous evaluation of macro- and microminerals. Third, the RI were established following IFCC–CLSI recommendations, with rigorous assessment of distribution, transformation procedures and bootstrap confidence intervals, thus ensuring methodological transparency and international comparability. Finally, the work provides the first region-specific RI for horses in Spain, providing veterinarians and equine nutritionists with a clinically relevant interpretive framework tailored to the environmental and management conditions in northwestern Spain.

Several study limitations should be acknowledged. The study population did not include high-performance sport horses in active training, and therefore the results may not fully reflect mineral dynamics under intense athletic demands. Dietary information was categorised into two broad groups, and detailed compositional data for individual concentrate formulations were not available, which may mask more subtle diet-related effects. In addition, although a wide variety of breeds was represented, the numbers of some minority breeds were insufficient for meaningful statistical evaluation. Despite these limitations, the study represents one of the most comprehensive surveys of mineral status in horses available to date, and the data serve as a strong foundation for clinical interpretation.

5. Conclusions

This study provides comprehensive, population-based RI for a wide spectrum of serum macro- and microminerals in horses, derived from a large and phenotypically diverse cohort. Overall, biological and management variables such as age, sex, breed and diet contributed modestly to the variations in most minerals, and extensive overlap of distributions across categories indicated that unified RI are appropriate for clinical interpretation in this population. Among the elements evaluated, Se emerged as the mineral of greatest concern, with many horses clustering near the lower end of the RI, particularly those fed exclusively forage. Given the well-established implications of suboptimal Se status for oxidative defence, immune competence, metabolic regulation and athletic performance, the findings highlight the need for proactive dietary management to ensure adequate Se supply, especially in regions where soils and forage are naturally low in this element. Overall, the findings highlight the value of multi-element serum profiling for equine health assessment and provide a foundation for improved nutritional monitoring, preventive management and future research into mineral metabolism in horses.

Institutional animal care and use committee

The sampling and handling of animals in this study was carried out in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes (European Parliament, 2010), and the trial complied with Spanish legislation on animal care (Royal Decree 53/2013 and 1083/2025). The procedures were supervised by the Bioethics Committee of the Clinical Veterinary Hospital Rof-Codina of the USC, Spain.

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Data Availability

The data that support the study findings are available from the corresponding author upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During preparation of the manuscript, the authors used ChatGPT (OpenAI) to improve the readability and grammar usage. All content generated by the tool was carefully reviewed, edited and revised by the authors, and the final manuscript was revised and corrected by a native English speaker. All scientific concepts, analyses, conclusions and interpretations were developed by the authors. The authors take full responsibility for the content of the published article.

Ethics in publishing

The authors declare that they have followed the ethical guidelines stated in Elsevier's Publishing Ethics Policy.

CRediT authorship contribution statement

C. Fernández-Villa: Conceptualization, Data curation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – original draft. **M. Miranda:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. **L. Rigueira:** Investigation, Methodology, Resources. **L. Martinez:** Investigation. **B. Villanueva:** Resources. **S. Freire:** Resources. **M. López-Alonso:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Supplementary materials

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