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■ Reference intervals for canine hematologic analytes using Siemens Advia 120

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ABSTRACT. Hematologic investigation is essential for the evaluation of health status of companion animals. Appropriate and accurate reference intervals (RIs) are required for the interpretation of laboratory results. Thus, the primary aim of the present study was to establish canine complete blood count (CBC) RIs using Advia 120, a widely used in veterinary medicine automated hematology analyzer. Additional objectives were to evaluate sex as a partitioning factor of RIs and to investigate the effect that breed size has on CBC RIs. Reference individuals were selected by indirect sampling method from the medical records of a veterinary teaching hospital. The reference population comprised 284 adult dogs of both sexes and various breeds. The reference individuals were allocated into 3 groups based on breed size (small-sized, medium-sized and large-sized breeds). Complete blood count results from the dogs that met the inclusion criteria were used for the nonparametric calculation of RIs. Statistical and nonstatistical criteria were employed in order to decide whether sex-specific RIs are needed. Depending on the data distributions, mean or median comparisons were used to determine the effect of breed size and lifestyle on CBC results. Nine outliers were detected based on CBC results. The estimated RIs were generally comparable to those previously reported in the literature. Sex-dependent partitioning of RIs was indicated by the statistical criteria for a few analytes. From a clinicopathologic point of view though, sex-dependent partitioning of RIs is questioned and seems not to be required. Breed size appears to have an effect on CBC RIs. The RIs determined in the present study can be used as a guide for the interpretation of CBC results in dogs and can potentially be adopted by veterinary laboratories using Advia 120. Finally, based on the results of this study, breed size should probably be considered when interpreting CBC results.

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ΠΕΡΙΛΗΨΗ. Η γενική εξέταση του αίματος (ΓΕΑ) θεωρείται μείζονος σημασίας για την εκτίμηση του επιπέδου υγείας των ζώων συντροφιάς. Ωστόσο, για την ορθή ερμηνεία των εργαστηριακών αποτελεσμάτων απαιτούνται ακριβείς τιμές αναφοράς. Στο πλαίσιο αυτό, ο πρωταρχικός στόχος της παρούσας εργασίας ήταν η εξαγωγή τιμών αναφοράς για τις παραμέτρους της ΓΕΑ στον σκύλο, με τη βοήθεια του Advia 120, ενός, ευρέως χρησιμοποιούμενου στην κτηνιατρική, αυτόματου αιματολογικού αναλυτή. Δευτερευόντως, διερευνήσαμε εάν απαιτείται διαχωρισμός των τιμών αναφοράς της ΓΕΑ με βάση το φύλο των σκύλων, καθώς και αν το μέγεθος της φυλής μπορεί να επηρεάσει τις τιμές αναφοράς της ΓΕΑ. Τα ζώα επιλέχθηκαν από το ιατρικό αρχείο της πανεπιστημιακής κλινικής ζώων συντροφιάς του Τμήματος Κτηνιατρικής του Α.Π.Θ. Τον παραπάνω πληθυσμό αποτελούσαν 284 αρσενικοί και θηλυκοί σκύλοι διαφόρων φυλών. Το σύνολο των σκύλων ταξινομήθηκε σε 3 ομάδες με βάση το μέγεθος της φυλής (μικρού, μεσαίου, μεγάλου). Οι τιμές αναφοράς υπολογίστηκαν μη παραμετρικά, χρησιμοποιώντας τα αποτελέσματα της ΓΕΑ των σκύλων που πληρούσαν τα κριτήρια ένταξης στη μελέτη. Προκειμένου να αποφασιστεί εάν απαιτείται ο διαχωρισμός των τιμών αναφοράς της ΓΕΑ με βάση το φύλο, εφαρμόστηκαν τόσο στατιστικά όσο και μη στατιστικά κριτήρια. Ανάλογα με την κατανομή των δεδομένων πραγματοποιήθηκε σύγκριση μέσων ή διαμέσων, με σκοπό να ελεγχθεί η πιθανή επίδραση του μεγέθους της φυλής στις τιμές της ΓΕΑ. Εννέα ζώα αποκλείστηκαν από τη μελέτη, λόγω της ύπαρξης φανερώς ακραίων τιμών στη ΓΕΑ. Σε γενικές γραμμές, οι τιμές αναφοράς που υπολογίστηκαν στην παρούσα εργασία είναι παρόμοιες με αυτές που αναφέρονται στη διεθνή βιβλιογραφία. Επιπλέον, με βάση την εφαρμογή των στατιστικών κριτηρίων, υποδεικνύεται ότι για κάποιες παραμέτρους της ΓΕΑ απαιτούνται διαφορετικές τιμές αναφοράς για τα δύο φύλα. Παρόλα αυτά, από κλινικοεργαστηριακής απόψεως, ο διαχωρισμός των τιμών αναφοράς αμφισβητείται και πιθανώς δεν είναι αναγκαίος. Επίδραση του μεγέθους της φυλής παρατηρήθηκε στις τιμές αναφοράς της ΓΕΑ. Οι τιμές αναφοράς που παρουσιάζονται στην παρούσα εργασία μπορούν να χρησιμοποιηθούν ως οδηγός για την ερμηνεία των αποτελεσμάτων της ΓΕΑ στους σκύλους και να «κιοθετηθούν» από κτηνιατρικά εργαστήρια που χρησιμοποιούν τον αιματολογικό αναλυτή Advia 120. Τέλος, με βάση τα αποτελέσματά μας, το μέγεθος της φυλής θα πρέπει, πιθανώς, να λαμβάνεται υπ' όψιν κατά την ερμηνεία των αποτελεσμάτων της ΓΕΑ.

Keywords: complete blood count; dog; partitioning; reference values, breed size; sex

INTRODUCTION

Complete blood count (CBC) is a routinely performed blood test for the evaluation of overall health status in companion animal medicine. As for every laboratory test, interpretation of the CBC results is highly dependent on appropriate and accurate reference intervals (RIs). The latter also facilitate the implementation of external quality assurance practices. Siemens Advia 120 is a widely used in veterinary medicine automated hematology analyzer that employs laser flow cytometry technology. However, to our knowledge, there is only one study reporting CBC RIs in dogs using Advia 120 (Moritz et al., 2004). In that study, the reference population was comprised of 46 dogs. According to ASVCP Quality Assurance and Laboratory Standards (QALS)

Committee guidelines for the determination of RIs in veterinary species though, the reference individuals should be ideally ≥ 120 , in order the nonparametric method to be implemented for the calculation of RIs (Friedrichs et al., 2017). Hence, the primary objective of the present study was to establish RIs for hematology analytes in a thus large population of adult dogs using Advia 120. The secondary objectives were: i) to investigate whether sex-specific RIs are required in dogs, similarly to humans, and ii) to further examine the effect of breed size on CBC RIs.

MATERIALS AND METHODS

Reference individuals were selected by *a posteriori* method. The medical records of the Companion

Animal Clinic, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Greece between 2012-2016 were retrospectively reviewed for dogs that have been presented for a routine health examination, castration or minor dental problems. The inclusion criteria were the following: no lactation, no history of illness or medication in the near past, age ≥ 6 months, complete vaccination and deworming, absence of abnormal findings during physical examination, CBC performed within the same day of sampling.

In our laboratory, blood samples are routinely being centrifuged after the completion of hematologic analysis and the plasma is macroscopically examined for the presence of hemolysis or lipemia. Additionally, when thrombocytopenia (defined in our laboratory as platelets count $<200,000/\mu\text{L}$) is identified, a blood film is always evaluated for the presence of platelet clumps. Thus, medical records reporting either hemolysis/lipemia or pseudothrombocytopenia were excluded from the study.

A total of 284 dogs fulfilled the defined inclusion criteria. The reference population was comprised of 110 male and 174 female dogs. The mean age of dogs was 6.0 years (range: 0.5-16.0 years), while the mean body weight was 14.9 kg (range: 1.6-52.0 kg). The exact distribution of canine breeds in the reference population of the study is presented in Table 1. The reference individuals were categorized into 3 groups based on breed size; 133 small-sized breeds, 80 medium-sized breeds and 71 large-sized breeds. Mixed breed dogs were allocated into the aforementioned categories based on their body weight, as previously described (Taylor et al., 2010): small-sized breeds: <10 kg, medium-sized breeds: 10-19.9 kg and large-sized breeds: >20 kg. Out of 284 dogs, 194 had an indoor lifestyle, whereas 90 were housed outdoors.

Blood samples were collected into K_3EDTA containing tubes (Deltalab, Barcelona, Spain). All hematologic analyses were performed on Advia 120 automated hematology analyzer (Siemens Healthcare Diagnostics, Deerfield, USA) with the canine setting of the multispecies software. The analytes that were available in all medical records were the following: red blood cells (RBC) count, hemoglobin (HGB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin

Table 1: Distribution of canine breeds in the reference population of this study

Canine breed	In reference population	
	Number	Percentage
Akita Inu	2	0.7
Basset Hound	1	0.4
Beagle	6	2.1
Boxer	3	1.1
Canadian Shepherd	1	0.4
Cane Corso	1	0.4
Caucasian Shepherd	2	0.7
Cavalier King Charles Spaniel	1	0.4
Chow Chow	2	0.7
Cocker Spaniel	7	2.5
Collie	2	0.7
Dachshound	1	0.4
Doberman	2	0.7
Dogo Argentino	3	1.1
English Bulldog	2	0.7
Epagnuel Breton	1	0.4
French Bulldog	6	2.1
Golden Retriever	4	1.4
Greek Pointer	5	1.8
Griffon	1	0.4
German Shepherd	6	2.1
Husky	4	1.4
Jack Russel	3	1.1
Kurzhaar	1	0.4
Labrador Retriever	4	1.4
Lasa Apso	2	0.7
Malinois	1	0.4
Maltese	12	4.2
Mongrel	124	43.7
Pekingese	6	2.1
Pinscher	11	3.9
Pitbull	7	2.5
Pointer	2	0.7
Pomeranian	2	0.7
Poodle	8	2.8
Pug	2	0.7
Rottweiler	2	0.7
Schnauzer Miniature	1	0.4
Schnauzer Standard	1	0.4
Setter	5	1.8
Shih Tzu	3	1.1
Spitz	3	1.1
Tsihuahua	3	1.1
West Highland Terrier	4	1.4
Yorkshire Terrier	14	4.9
Total	284	100

(MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cell (WBC) count, differential WBC count (neutrophil, lymphocyte, monocyte, eosinophil and basophil counts), large unclassified cell (LUC)

count, platelet (PLT) count and mean platelet volume (MPV). At least one control (Siemens Healthcare Diagnostics, Deerfield, USA) was run daily and prior to blood sample analysis to ensure the good internal quality control.

The statistical software package SPSS 19 (SPSS Inc., Chicago, USA) was used for the calculation of descriptive statistics and comparison studies between breed-specific subgroups. Depending on the raw and transformed data distribution, mean or median comparison was used in order to determine the effect of

breed size on CBC results. The RIs were determined using the statistical language R (R Foundation for Statistical Computing, Vienna, Austria) and the package *referenceIntervals*. Outliers were detected using Horn's method. The nonparametric and bootstrapping methods were used for the determination of reference and confidence intervals, respectively. Calculated RIs were at the 95th percentile, while confidence intervals were at the 90th percentile.

In order to determine whether sex-specific RIs are required in dogs, both distance and proportion statis-

Table 2: Descriptive statistics and reference intervals for hematologic analytes in dogs (n=275), using Advia 120.

Analyte	Unit	N	Mean	Median	SD	Min	Max	Data distribution	2.5th Percentile (90% CIs)	97.5th Percentile (90% CIs)
Red blood cells	10 ⁶ /μL	275	6.9	6.9	0.8	4.95	9.27	G	5.36 (5.06-5.53)	8.67 (8.40-8.80)
Hemoglobin	g/dL	275	15.8	15.8	1.9	11.5	20.8	G	12.2 (11.6-12.6)	19.4 (18.9-19.6)
Hematocrit	% L/L	275	47.5	47.4	5.7	33.1	62.2	G	37.2 (34.9-37.7)	58.7 (57.3-59.5)
MCV	fL	275	68.9	69.2	3.4	58.4	78.8	G	61.6 (59.5-62.9)	75.6 (74.5-77.4)
MCH	pg	273	22.9	23.0	1.2	18.8	26.5	NG	20.1 (19.3-20.4)	25.1 (24.9-26.5)
MCHC	g/dL	274	33.2	33.3	0.9	30.2	36.4	G	31.4 (30.5-31.7)	34.9 (34.8-35.7)
RDW	%	275	13.5	13.4	0.9	11.5	16.9	NG	12.0 (11.8-12.1)	15.7 (15.3-16.0)
White blood cells	10 ³ /μL 10 ⁹ /L	274	9.6	9.4	2.5	4.4	17.1	NG	5.5 (4.8-5.7)	15.8 (15.1-17.0)
Neutrophils	%	275	63.3	64.2	9.5	29.3	83.0	NG	42.4 (37.4-46.0)	78.8 (77.5-81.3)
Neutrophils	10 ³ /μL	273	6.1	5.8	1.9	2.8	12.3	NG	3.2 (2.9-3.3)	11.1 (10.5-12.3)
Lymphocytes	%	274	24.8	22.9	8.4	8.6	54.2	NG	11.6 (10.5-12.5)	44.4 (43.6-49.3)
Lymphocytes	10 ³ /μL	273	2.3	2.2	0.9	0.7	5.9	NG	1.0 (0.8-1.1)	4.7 (4.5-5.9)
Monocytes	%	274	5.4	4.9	2.1	2.0	16.1	NG	2.7 (2.4-2.9)	11.9 (10.2-15.9)
Monocytes	10 ³ /μL	274	0.5	0.5	0.2	0.1	1.8	NG	0.2	1.2 (1.0-1.6)
Eosinophils	%	273	4.9	4.4	3.4	0.1	18.1	NG	0.3 (0.2-0.5)	13.6 (13.1-18.1)
Eosinophils	10 ³ /μL	273	0.47	0.40	0.36	0.01	2.06	NG	0.03 (0.02-0.05)	1.45 (1.40-2.06)
Basophils	%	275	0.5	0.4	0.4	0	2.8	NG	0.1	1.5 (1.2-1.9)
Basophils	10 ³ /μL	275	0.04	0.04	0.03	0	0.29	NG	0.01	0.14 (0.11-0.16)
LUC	%	275	1.0	0.8	1.0	0	6.9	NG	0 (0-0.1)	4.0 (3.3-4.9)
LUC	10 ³ /μL	275	0.10	0.07	0.10	0	1.04	NG	0	0.37 (0.27-0.42)
Platelets	10 ³ /μL	273	354	330	115	149	844	NG	193 (170-206)	661 (630-844)
MPV	fL	274	11.7	11.1	2.3	8.2	22.5	NG	8.9 (8.5-9.0)	17.5 (17.0-21.8)

CIs, confidence intervals; G, Gaussian; LUC, large unclassified cells; Max, maximum; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; Min, minimum; MPV, mean platelet volume; NG, non-Gaussian; RDW, red cell distribution width; SD, standard deviation.

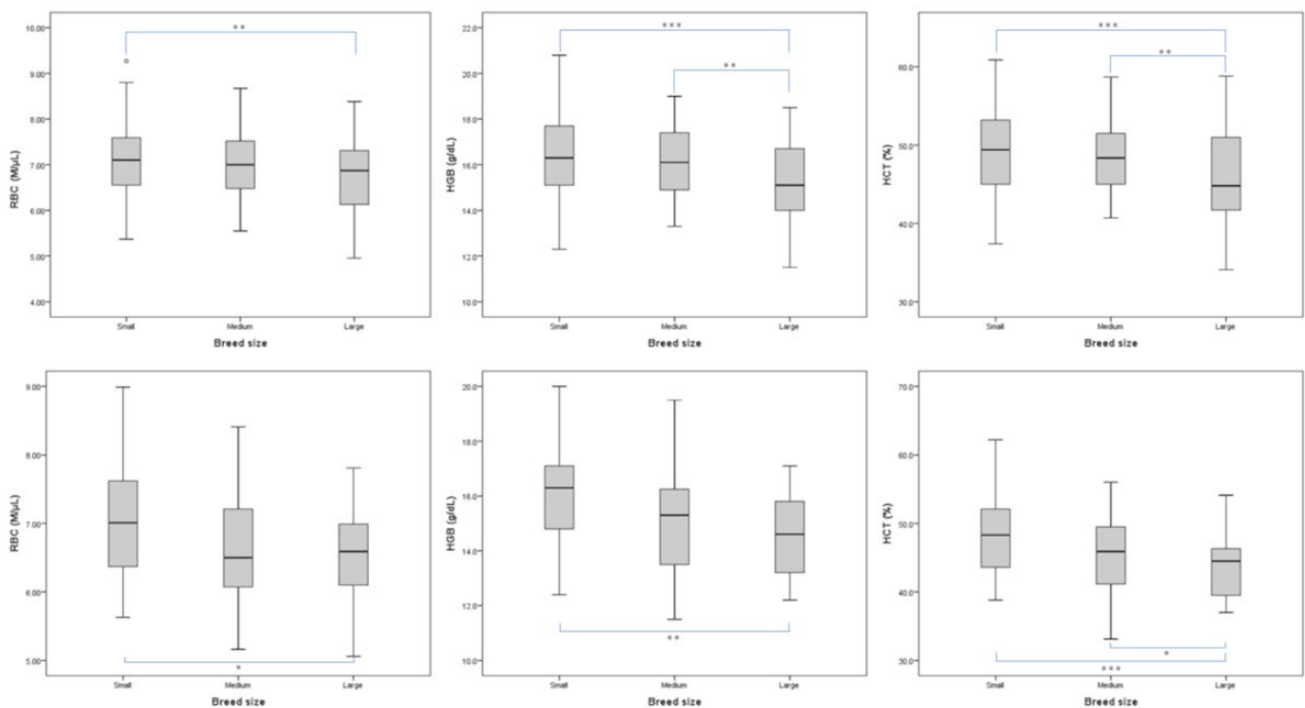


Figure 1. Boxplots of the red blood cell (RBC), hemoglobin (HGB), and hematocrit (HCT) values of the different breed size-specific subgroups. In the upper row, boxplots of RBC, HGB, and HCT values of small-, medium-, and large-sized dogs breeds with indoor lifestyle are depicted. In the lower row, boxplots of RBC, HGB, and HCT values of small-, medium-, and large-sized dogs breeds with outdoor lifestyle are presented. The lines represent the main body of data, whereas the dot represents an observed outlying point. The boxes represent the interquartile ranges; they are bisected by a line, which stands for the mean value. * $P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

tical criteria previously proposed were applied (Lahti et al., 2002; Lahti et al., 2004). However, for the final decision, non-statistical criteria were employed, laying emphasis on the clinical relevance of the reported differences between the sex-specific RIs.

RESULTS

Nine dogs were excluded from the study based on profoundly abnormal CBC results (moderate to severe leukopenia [$n=1$], moderate to severe leukocytosis [$n=1$], severe monocytosis [$n=1$], severe eosinophilia [$n=2$] and severe thrombocytopenia [$n=3$]). Few outliers ($n=1-2$) were identified for some analytes using Horn's method. The results of the statistical analysis and calculated CBC RIs are presented in Table 2.

Sex-dependent partitioning of RIs was statistically indicated for lymphocytes count (based on distance

statistical criteria) and monocytes count (based on proportion statistical criteria). Statistically significant differences were noted between small-, medium- and large-sized dog breeds in the following analytes: RBC count (ANOVA, $p=0.010$), HGB concentration (ANOVA, $p<0.001$), HCT (ANOVA, $p<0.001$), MCV (ANOVA, $p=0.020$), eosinophils count (Kruskal-Wallis Test, $p=0.020$) and PLT count (ANOVA, $p<0.001$). However, only the differences in RBC count, HGB concentration, and HCT were proven statistically significant between the different breed-specific subgroups with the same lifestyle (Figure 1). Specifically, among the dogs with indoor lifestyle, statistically significant differences were observed between small-, medium-, and large-sized dog breeds in RBC count (ANOVA, $p=0.020$), HGB concentration (ANOVA, $p=0.004$), and HCT (ANOVA, $p=0.002$). Among the dogs with outdoor lifestyle, statistically significant differences were documented

between the three breed size subgroups in HGB concentration (ANOVA, $p=0.021$) and HCT (ANOVA, $p=0.007$), while marginally insignificant difference was noted in RBC count (ANOVA, $p=0.064$).

DISCUSSION

In the present study, CBC RIs in dogs were calculated using Advia 120 by extracting the reference individuals from the medical records of a veterinary teaching hospital. Although the *a posteriori* determination of RIs is not considered ideal, it is acceptable by the ASVCP QALS Committee (Friedrichs et al., 2017). The main advantage of this method, compared with the *a priori* method, is the capacity to include a large reference population, whereas the primary disadvantage is the limited control of some preanalytic and analytic factors. In an attempt to overcome the aforementioned limitation, strict inclusion and exclusion criteria were set. Moreover, in the context of the present study, it was investigated whether sex-dependent partitioning of CBC RIs is necessitated or not, while the effect of breed size on CBC RIs was also assessed.

In general, our CBC RIs are in agreement with those previously reported by Moritz et al. (2004). From a clinical point of view, the most significant differences are noted in RIs for RBC count, HGB concentration, HCT and WBC, neutrophils and lymphocytes counts. Specifically, our RIs for RBC count, HGB concentration and HCT are generally shifted downwards. The upper reference limit of WBC count and the lower reference limit of lymphocytes count are also shifted downwards in the present study. In terms of neutrophils count, our RIs are wider than those of Moritz et al. (2004). The observed differences between our RIs and those reported in the literature (Moritz et al., 2004) may be attributed, first and foremost, to preanalytic factors (notably the size, the demographic characteristics and the selection method of the reference population) and secondarily to analytic factors (e.g. statistical analysis).

The need for partitioning of RIs is increasingly discussed in veterinary medicine (Reynolds et al., 2010; Lavoué et al., 2013; Lawrence et al., 2013; Paltrinieri et al., 2014; Hegstad-Davies et al., 2015; Oikonomidis et al., 2018)). The rationale behind

partitioning is the decrease of the variability of the reference population, which eventually leads to the narrowing of RIs. The reference population should comprise a minimum of 40 animals in order to be considered for partitioning of RIs (Friedrichs et al., 2017). Since the aforementioned criterion was met in our study, we investigated whether sex-dependent partitioning of RIs is necessitated. Based on previously proposed statistical criteria for Gaussian and non-Gaussian data distributions (Lahti et al., 2002; Lahti et al., 2004), partitioning was warranted only for lymphocytes and monocytes counts. From a clinicopathologic point of view though, sex-dependent partitioning of RIs is questioned and seems to not be required for any of the aforementioned analytes, in accordance with the relevant literature (Bourgès-Abella et al., 2011). In that study, sex-specific RIs were required for HCT and PLT count (Bourgès-Abella et al., 2011), this is not confirmed by our results. However, on the grounds of the well-known effect that breed has on hematologic analytes (Lawrence et al., 2013), we suggest that sex should be investigated as a partitioning factor in dogs of the same breed. The need for breed-dependent partitioning of RIs was not investigated in the present study, due to the insufficient number of individual canine breeds.

Several differences were observed between small-, medium- and large-sized breeds; a rather expected finding, based on the existing literature (Lawrence et al., 2013). However, when the breed size-specific subgroups were compared in the context of the same lifestyle, the differences were proven statistically significant only for RBC count, HGB concentration, and HCT, with the larger breed dogs having generally lower values.

Some limitations are recognized in the present study. Our RIs were determined *a posteriori*, by extracting the reference individuals from a database of medical records. Despite the fact that the inclusion criteria were strict, they were not set before sampling, as ideally should have been. Additionally, although the preanalytic and analytic procedures are well standardized in our laboratory, we cannot exclude a minor effect of preanalytic and analytic factors on the calculated CBC RIs. Finally, a limitation concerning the RIs for PLT count is recog-

nized, since data of the microscopic evaluation of platelets in the blood film were available only for dogs that had platelets count $<200,000/\mu\text{L}$. In this context, dogs with platelets count $\geq 200,000/\mu\text{L}$ and platelet clumps were inevitably included in our reference population, while dogs with platelets count $<200,000/\mu\text{L}$ and evidence of platelet clumps in the blood film were indiscriminately excluded from our study. Thus, our platelets count RIs should be considered somewhat biased.

CONCLUSIONS

Complete blood count RIs using Advia 120 were established based on a large reference canine population with pre-existing CBC results and in accordance with ASVCP QALS Committee guidelines for the determination of RIs in veterinary species. In addition, the necessity of sex-dependent partition-

ing of RIs was investigated, using both statistical and non-statistical criteria, while the effect of breed size of dogs on CBC RIs was also assessed. Our RIs can potentially be adopted by veterinary laboratories using Advia 120 after a proper validation. Sex-dependent partitioning of RIs seems to not be required, while the effect of breed size of the dogs should be taken into consideration when evaluating CBC results. Finally, the present study can also be used as a paradigm for the *a posteriori* determination of RIs based on the official ASVCP QALS Committee guidelines.

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CONFLICT OF INTEREST

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

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