Received: 26 January 2019 | Accepted: 20 June 2019

DOI: 10.1111/jvim.15551

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Time course of serum cobalamin, folate, and total iron binding capacity concentrations in pregnant bitches and association with hematological variables and survival

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Abstract

Background: Hypocobalaminemia, hypofolatemia and iron deficiency are associated with pregnancy-related anemia (PRA) and neonatal survival (NS) in women. Similar associations have not been investigated in pregnant bitches.

Objectives: To investigate time course and associations of serum cobalamin, folate and iron status indicators with hematological variables and NS in pregnant bitches.

Animals: Forty-eight pregnant bitches.

Methods: A prospective cohort study. Pregnancy was confirmed by abdominal ultrasonography twice during mid- and late pregnancy, concurrently with blood sampling. Associations among pregnancy stage, NS and laboratory variables were assessed by generalized estimating equations.

Results: Compared with midpregnancy, serum cobalamin (adjusted mean [95% confidence interval, CI]) decreased at late pregnancy (430 pg/mL [394-466] versus 330 pg/mL [303-357], respectively; $P < .001$), whereas serum folate did not. Every increment of 1 in parity number or litter size corresponded to 28.6 pg/mL (95% CI, 5.6-51.6; $P = .02$) and 20.3 pg/mL (95% CI, 10.9-29.7; $P < .001$) decrease in serum cobalamin concentration. Compared with midpregnancy, serum iron ($P < .001$) and transferrin saturation ($P = .01$) increased at late pregnancy. The decrease in red blood cell count ($P < .001$) at late pregnancy was significantly, albeit weakly, correlated with decreasing serum folate concentration ($r = 0.33$; $P = .02$). None of the measures was associated with NS.

Conclusions and Clinical Significance: Pregnancy-related anemia was common at late pregnancy. Unlike in women, in pregnant bitches, serum iron and transferrin saturation were increased at late pregnancy. Future studies are warranted to investigate the clinical ramifications of hypocobalaminemia in pregnant bitches and the utility of prophylactic folate administration in mitigating PRA.

KEYWORDS

anemia, canine, dog, inflammation, pregnancy, thrombocytosis

Abbreviations: 95% CI, 95% confidence interval; APR, acute phase reaction; GEE, generalized estimating equation; IQR, interquartile range; IR, iron deficiency; MCV, mean corpuscular volume; NS, neonatal survival; PP, postpartum; PRA, pregnancy-related anemia; RBC, red blood cells; TIBC, total iron binding capacity; TS, transferrin saturation; UIBC, unsaturated iron binding capacity.

1 | INTRODUCTION

Pregnancy-related anemia (PRA) occurs commonly in women, and it increases in prevalence and severity as pregnancy progresses.[1] Although PRA has been associated with a plethora of potential maternal, fetal, and neonatal adverse effects,[1] the association between maternal hemoglobin concentration and perinatal complications is U-shaped, with both high and low concentrations exerting pernicious effects.[1-3] A few small-cohort studies in dogs have addressed similar changes in red blood cell (RBC) indices in pregnant bitches, with conflicting results. Most commonly, PRA has been reported to develop during later pregnancy stages,[4-7] whereas 2 studies either failed to document preparturition anemia or had reported statistically significant, albeit clinically insignificant, hematocrit decrease during pregnancy.[8,9]

Several mechanisms jointly contribute to the development of PRA in women. Hemodilution from volume expansion is a physiological process that causes mild anemia, peaking in women at 20-24 weeks of gestation.[10] Disease processes, on the other hand, highly depend on dietary intake and the preconception health status, and include vitamin and mineral deficiencies. Most commonly, these include iron deficiency (ID), hypocobalaminemia, hypofolatemia and concurrent inflammatory conditions.[1,11,12]

The pathophysiology of PRA in dogs is poorly defined. Purported mechanisms are similar to humans, and include hemodilution,[5] RBC life-span alterations and possibly vitamin deficiencies and ID.[6,7,9,13,14] However, corroborative evidence is often circumstantial (ie, documented decrease of folate concentration during pregnancy, whereas correlations with RBC indices were not assessed)[14] and more commonly is lacking. Thus, concepts regarding the pathophysiology of PRA in dogs are largely derived from studies in women, and associations among development of PRA, indicators of iron status and vitamin deficiencies in pregnant bitches are lacking.

In women, the frequencies of hypocobalaminemia, hypofolatemia and ID progressively increase during pregnancy.[1,11,12,15-19] Deficiencies thereof bear direct deleterious ramifications on various tissues, including fetal central nervous system, irrespective of PRA.[16,19] Consequently, daily oral iron and folic acid supplementation is routinely recommended to pregnant women by the World Health Organization as part of the antenatal care, notwithstanding the lack of robust evidence to support this practice.[15,16,19,20] The repercussions of ID and vitamin deficiencies on fetal development have been poorly studied in pregnant bitches, excluding a possible association between folate deficiency and cleft palate.[13,21] Despite this paucity of studies, vitamins are often administered to pregnant bitches by breeders and veterinarians, owing to current recommendations in women.

Therefore, our primary hypotheses were that hypocobalaminemia, hypofolatemia, and ID develop during pregnancy in healthy bitches and that possible associations exist among these measures and PRA, other RBC indices and neonatal survival (NS).

2 | MATERIALS AND METHODS

2.1 | Animals, sample collection, and laboratory methods

The study was approved by the Institutional Ethics Committee and prospectively enrolled 48 intact adult healthy bitches, from 2 different breeders, with the breeders' consent. Pregnancy was confirmed by abdominal ultrasonography (Mindray M9 with sector and linear 3T transducers, Shenzhen Mindray Bio-Medical Electronics Co, Shenzhen, China) by a single board-certified theriogenologist (S.T.) twice during pregnancy. Dogs administered hormonal and vitamin supplementation, sulfonamides, or anticonvulsants (which might affect serum folate concentration) were excluded. The signalment, diet type, occurrence of co-morbidities and NS rate immediately postpartum (PP) and at 1, 2 and 4 weeks PP were documented.

Blood was collected from each pregnant bitch at mid- and late pregnancy. Blood samples for serum cobalamin, folate, free iron, and unsaturated iron binding capacity (UIBC) concentrations were collected in tubes containing no anticoagulant, with gel separators, allowed to clot, centrifuged, and immediately stored at −80°C. Samples were analyzed within 6 months of collection (Cobas 6000, Roche, Mannheim, Germany, at 37°C). The sum of free iron and UIBC constituted the total iron binding capacity (TIBC). Blood for CBC was collected in potassium-EDTA tubes and analyzed within 60 minutes from collection (Advia 2120c, Siemens, Siemens Medical Solutions Diagnostics, Erfurt, Germany). In addition, fresh blood smears were air-dried and stained with modified Wright's staining solution and evaluated microscopically for polychromasia, erythrocyte and leukocyte morphology, and confirming the automated platelet count. Decreased RBC count, hematocrit, and hemoglobin concentration were used to define anemia.

2.2 | Statistical analysis

Associations between pregnancy stage and outcome variables (ie, serum concentrations of cobalamin, folate, iron, UIBC, TIBC and transferrin saturation [TS]) were evaluated using generalized estimating equation (GEE) analyses. The results of cobalamin, folate, UIBC, and TIBC were normally distributed as determined by the Shapiro-Wilk test. Natural logarithmic transformation of iron concentration and TS was performed to achieve normal distribution. Breeder was entered as an additional factor, whereas parity and litter size were entered as covariates in each model, to control for their potential effects on the outcome variables. Interactions between the time point during pregnancy and the breeder were assessed in each model. The additional factor, covariates, and interaction terms for which significance level was $P > .1$ were removed from the models. Separate GEE models were analyzed, and the results were reported separately for each breeder, where significant interactions were observed. Results of the GEE analyses are presented as means and 95% confidence intervals (CIs), adjusted for the additional factors and covariates included in each analysis.

CBC analytes were compared between pregnancy stages using paired *t* test or Mann-Whitney *U* test, for normally (ie, RBC, hemoglobin, hematocrit, and mean corpuscular volume [MCV]) or nonnormally

(ie, all other CBC analytes) distributed variables. Quantitative results are presented as median and interquartile range (IQR).

The Bonferroni's method was applied to correct the significance level for multiple outcome variables. Linear correlations among serum iron, UIBC, TIBC, TS, cobalamin and folate concentrations (including temporal changes thereof) with CBC measures and NS were assessed by Pearson or Spearman correlation tests, for normally and non-normally distributed data, respectively. Frequencies of hypoferremia, hypocobalaminemia, hypofolatemia, thrombocytosis, and anemia were compared between mid- and late pregnancy using the Chi square or Fisher's exact tests, as appropriate.

Data were analyzed using commercially available statistical software (SPSS 22.0, IBM, Chicago, Illinois). All tests were 2-tailed, and for all, $P < .05$ was considered significant.

3 | RESULTS

3.1 | Signalment

The study enrolled 48 pregnant bitches, from 2 different commercial breeders (Breeder 1, 27; Breeder 2, 21), including Australian Shepherd and Shih Tzu dogs (9 each; 19%), Shetland Sheepdog and Border Collie (8 each; 17%), Maltese (4; 8%), German Shepherd, Jack Russell Terrier and Welsh Corgi (3 each; 6%), and Cavalier King Charles Spaniel (1; 2.1%). Their median age was 45 months (IQR, 30-60). Most bitches were primiparous (18; 38%). Parity numbers of multiparous bitches were 2 (15; 31%), 4 (8; 17%), 3 (6; 13%), and 5 (1; 2%). Median interval between pregnancies was similar between the 2 Breeders (12 months [IQR, 9-12] and 12 months [IQR, 9.75-12] in Breeder 1 and Breeder 2, respectively; $P = .63$).

Preventive endoparasite control regimen was similar for both breeders and included administration of a pyrantel pamoate-praziquantel-febantel combination (Drontal Plus, Bayer, Leverkusen, Germany). Preventive treatment against ectoparasites included environmental control with a pesticide (imidacloprid 10% and IGR 2% pyriproxyfen; Dorfen, Dor-Ky, Nes-Ziona, Israel) by Breeder 1, whereas Breeder 2 provided this same environmental treatment, with topical fipronil spot-on (Frontline, Merial, Duluth, Georgia) as recommended by the manufacturer. All the dogs from each breeder were fed the same balanced AAFCO-approved canine diet, but the diets differed between the 2 breeders.

3.2 | CBC analytes, serum cobalamin, folate and TIBC concentrations during pregnancy

Median (IQR) intervals between the first and second sampling, and between the second sampling and parturition were 30 days (27-34) and 4 days (2-6), respectively.

Serum cobalamin concentration decreased ($P < .001$) at late compared to mid-pregnancy (Table 1). Correspondingly, hypocobalaminemia was more frequent ($P = .03$) at late pregnancy (12/48 dogs; 25%)

TABLE 1 Serum cobalamin, folate, iron, unsaturated iron binding capacity, and total iron binding capacity concentrations at mid- and late pregnancy of 48 healthy pregnant bitches

Analyte		Pregnancy stage	Adjusted mean (95% CI)	P^a	Reference interval
Serum cobalamin (pg/mL)		Mid-P	431 (395-467)	<.001	250.0-830.8
		Late-P	330 (303-358)		
Serum folate (ng/mL)		Mid-P	13.5 (12.5-14.5)	.09	10.2-24.2
		Late-P	12.5 (11.6-13.5)		
Serum iron (mg/L)[b]		Mid-P	0.95 (0.86-1.05)	<.001	0.97-2.63
		Late-P	1.32 (1.16-1.51)		
TS (%)		Mid-P	27 (24-29)	.01	27-66
		Late-P	32 (28-37)		
UIBC (mg/L)[b]	Breeder 1	Mid-P	2.6 (2.4-2.8)	.001	1.8-2.3
		Late-P	3.0 (2.7-3.3)		
	Breeder 2	Mid-P	2.6 (2.4-2.8)	.2	
		Late-P	2.3 (2.0-2.7)		
TIBC (mg/L)[b]	Breeder 1	Mid-P	3.6 (3.4-3.9)	<.001	2.8-4.9
		Late-P	4.4 (4.1-4.7)		
	Breeder 2	Mid-P	3.6 (3.4-3.8)	.1	
		Late-P	3.9 (3.6-4.1)		

Note: The study included 27 bitches from Breeder 1 and 21 from Breeder 2.

Abbreviations: 95% CI, 95% confidence interval; Late-P, late pregnancy; Mid-P, midpregnancy; TIBC, total iron binding capacity; TS, transferrin saturation; UIBC, unsaturated iron binding capacity.

[a]General estimating equation test was used to determine the associations of independent variables (eg, breed/breeder/parity/age and pregnancy stage) with dependent variables (eg, serum cobalamin, folate iron, UIBC, and TIBC concentrations). Independent variables which had been found to have an affect were subsequently included in a separate model. Differences between the 2 pregnancy stages are presented herein.

[b]Reference intervals are based on values established by the Clinical Pathology Laboratory, Animal Health Diagnostic Center, Cornell University (https://ahdc.vet.cornell.edu/sects/clinpath/reference/chem.cfm, accessed August 14, 2018), using the same reagents and instrumentation as used in the present study.

compared to midpregnancy (4/48 dogs; 8%), while neither the frequency of decreased nor increased serum folate concentration differed between these time points (10/48 versus 11/48 and 0/48 versus 0/48, respectively). Serum iron ($P < .001$) and TS ($P = .01$) increased at late pregnancy compared to midpregnancy. Total iron binding capacity and UIBC increased at late pregnancy compared to midpregnancy in dogs of Breeder 1, but not in those of Breeder 2 (Table 1).

The neutrophil, platelet, and leukocyte count significantly increased, whereas the RBC count decreased ($P < .001$ for all) at late pregnancy compared to midpregnancy (Table 2). The frequency of anemia was higher ($P < .001$) at late pregnancy (37/48 dogs; 77%) compared to midpregnancy (18/48 dogs; 37%), as was the frequency of thrombocytosis ($P = .002$) (40/48 dogs; 83% versus 26/48 dogs; 54%, respectively), but there was no difference in the frequency of leukocytosis between these time points (16/48 dogs; 33% versus 9/48 dogs; 19%, respectively). Morphological erythrocyte abnormalities were absent in microscopic examination of blood smears.

The breeder had a significant association with serum concentrations of TIBC ($P = .02$) and UIBC ($P = .02$), and a significant interaction of breeder with the time point during pregnancy was noted for UIBC and TIBC. Therefore, results for these variables are reported separately (Table 1).

Parity had a significant ($P = .02$) association with serum cobalamin concentrations. Every increment of 1 in parity was associated with a corresponding serum cobalamin concentration decrease of 28.6 pg/mL (95% CI, 5.6-51.6).

Litter size had a significant ($P < .001$) association with serum cobalamin concentration, with every increase of 1 in litter size associated with serum cobalamin concentration decrease of 20.3 pg/mL

TABLE 2 Hematological analytes at mid- and late pregnancy of 48 healthy bitches

Analyte	Pregnancy stage	Median (interquartile range)	P[a]	Reference interval[b]
Leukocytes ($\times 10^9$/L)	Mid-P	10.6 (9.3-13.1)	<.001	5.2-13.9
	Late-P	13.2 (10.2-15.1)		
Red blood cells ($\times 10^{12}$/L)	Mid-P	5.88 (5.58-6.41)	<.001	5.7-8.8
	Late-P	5.22 (4.84-5.67)		
Hemoglobin (g/L)	Mid-P	141 (130-151)	<.001	129-184
	Late-P	122 (110-131)		
Hematocrit (proportion of 1)	Mid-P	0.40 (0.37-0.43)	<.001	0.37-0.57
	Late-P	0.36 (0.32-0.39)		
Mean corpuscular volume (fL)	Mid-P	66.85 (64.72-69.68)	.001	58.8-71.2
	Late-P	68.7 (65.6-71.1)		
MCHC (mmol/L)[c]	Mid-P	21.9 (21.6-22.5)	<.001	19.2-22.5
	Late-P	21.2 (20.5-21.8)		
RDW (proportion)[c]	Mid-P	0.13 (0.12-0.14)	<.001	0.12-0.14.
	Late-P	0.15 (0.14-0.16)		
Platelets (10^9/L)	Mid-P	413 (333-522)	<.001	143-400
	Late-P	546 (442-709)		
Mean platelet volume[d] (fL)	Mid-P	11.0 (10.2-12.2)	.02	7.0-11.0
	Late-P	11.9 (10.6-13.4)		
Neutrophils ($\times 10^9$/L)	Mid-P	6.86 (4.35-8.63)	<.001	3.9-8.0
	Late-P	8.06 (6.38-10.28)		
Lymphocytes (10^9/L)	Mid-P	2.31 (1.80-3.11)	.2	1.3-4.1
	Late-P	2.39 (1.77-3.02)		
Monocytes (10^9/L)	Mid-P	0.76 (0.57-1.01)	.3	0.2-1.1
	Late-P	0.77 (0.55-0.95)		
Eosinophils (10^9/L)	Mid-P	0.44 (0.17-0.76)	<.001	0.0-0.6
	Late-P	0.82 (0.39-1.72)		
Basophils[d] (10^9/L)	Mid-P	0.04 (0.02-0.06)	.02	0.0-0.1
	Late-P	0.05 (0.03-0.08)		

Abbreviations: Late-P, late pregnancy; Mid-P, midpregnancy; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width.
[a]Student's *t* test was used to compare differences between the 2 pregnancy stages.
[b]Reference intervals were established by the Clinical Pathology Laboratory at the authors' institution.
[c]All analytes excluding the MCHC, RDW, and the differential count were normally distributed.
[d]Results became insignificant for the mean platelet volume and basophil count after applying the Bonferroni correction for multiple comparisons.

(95% CI, 10.9-29.7). Litter size also had a significant association with serum concentrations of TIBC (P = .04) and UIBC (P = .01) in dogs of Breeder 2, but not for those of Breeder 1, with every increment of 1 in litter size resulting in 0.09 mg/L (95% CI, 0.002-0.179) decrease in serum TIBC concentration. There were no associations of litter size with serum folate or serum free iron concentrations.

3.3 | Correlations among serum cobalamin, folate and TIBC concentrations with hematological variables and NS

The decrease in RBC during pregnancy was significantly, albeit weakly, correlated with the decrease in serum folate concentration (r = 0.33; P = .02) and with the increase of serum TIBC concentration (r = –0.40; P = .004). Neither serum cobalamin nor serum folate concentrations were correlated with the MCV. Lastly, the fold decreases in both serum cobalamin and serum folate concentrations during pregnancy were positively and weakly correlated (r = 0.314; P = .03).

Neither serum TIBC nor serum cobalamin or folate concentrations were correlated with NS at parturition, and at 1, 2, and 4 weeks PP. Moreover, the RBC count, the RBC count difference, and the fold decrease in the RBC count between late and midpregnancy were not associated with NS.

3.4 | Neonatal survival

The median (IQR) litter size was 5 (3.8-7.0). Median (IQR) NS at parturition and at 1, 2, and 4 weeks PP were 100% (100%-100%), 100% (78.8%-100%), 100% (75%-100%), and 100% (74.1%-100%), respectively. Frequencies of litters with 100% survival 4 weeks PP were similar among breeders (Breeder 1, 16/27 litters, 59%; Breeder 2, 12/21 litters, 57%).

4 | DISCUSSION

This study documented previously unreported, unexpected iron status trends in healthy bitches during pregnancy, contrary to extensively reported findings in women. Notwithstanding the high frequency of PRA, neither ID nor hypocobalaminemia was associated with its development, whereas decrease in serum folate concentration was weakly and significantly correlated with the RBC count. In addition, a significant decrease in serum cobalamin concentration was documented at late pregnancy, which was exacerbated by higher parity and litter size.

Nonregenerative anemia was frequently documented in this study, and worsened with progression of pregnancy, in corroboration with previous, considerably smaller (12-23 pregnant bitches) studies,[5-7] but in contrast to 2 other studies, including a comprehensive study of 324 bitches, where PRA was not documented.[8,9] In women, the prevalence of PRA increases during pregnancy. Hemodilution is purportedly an important contributor to its development in both women and bitches.[1,5] A common, physiological phenomenon, hemodilution should have affected all pregnant bitches had it played a pivotal role in the pathogenesis of PRA, notwithstanding individual variations. Likewise, although both litter size and the breed have previously been associated with the hematocrit in pregnant bitches,[7,8] it appears unlikely these could solely account for the frequency discrepancy of PRA among studies. In women, a substantial difference in the prevalence of PRA is related to disparate frequencies of iron, cobalamin, and folate deficiencies, resulting from varying socioeconomic background and dietary history.[1] In pregnant bitches, however, the interplay between iron and vitamin status and development of PRA has not been investigated. In this study, we demonstrated that neither serum cobalamin nor TIBC was associated with the occurrence of PRA, while concomitant decreases in the RBC count and in folate concentration were positively, albeit weakly, correlated. In women, anemia is associated with deleterious maternal and neonatal complications,[1-3] whereas in our study, similar associations between PRA and NS were not found. Nevertheless, the relatively limited cohort size herein, as well as the short follow-up period precluded documentation of rare fetal (eg, fetal absorption and loss), perinatal (eg, NS and fading puppy syndrome), and long-term (eg, developmental disorders) complications. Therefore, future research is warranted to examine whether PRA and neonatal complications are indeed associated, and whether PRA adversely affects health in the bitch and fecundity in subsequent cycles.

The prevalence of hypocobalaminemia in pregnant women, in an analogy to PRA, greatly varies geographically, ranging from 5 to 72%.[22] The etiology of the progressive decline in maternal serum cobalamin concentration during pregnancy is multifactorial. During fetal development, active cobalamin transport against a concentration gradient preferentially shunts cobalamin to the placenta and fetus,[17,23-25] thereby depleting maternal reserves.[1] Cobalamin and folate tissue demand greatly increase during pregnancy in women, as reflected by elevated serum methylmalonic acid and homocysteine concentrations, even in face of normal cobalamin and folate concentrations.[16,17] Therefore, the reference intervals for cobalamin in nonpregnant women might not apply to pregnant ones, and cobalamin supplementation might help ameliorate PRA, even in normocobalaminemic pregnant women.[26] Additionally, maternal cobalamin reserves are highly diet-dependent,[16] as the diet serves as the main cobalamin intake source, and preexisting vitamin deficiencies reduce maternal-fetal cobalamin transport, thereby increasing the risk of fetal complications.[27] Irrespective of its cause, hypocobalaminemia in pregnant women is associated with many maternal, fetal, and perinatal complications and developmental defects, including neural tube defects, increased perinatal death, low birth weight, and recurrent miscarriage.[1,17,18] Pregnancy-related anemia in women, on the other hand, is infrequently associated with hypocobalaminemia,[17,28] notwithstanding the apparent beneficial effects exerted by cobalamin supplementation, regardless of maternal cobalamin status.[26] In pregnant bitches, little is known of the consequences of cobalamin deficiency. Furthermore, an analogous decline in cobalamin concentration during pregnancy has not been reported in dogs.[14,29] The present study documents a significant decrease in maternal cobalamin concentration during pregnancy, which albeit unassociated with PRA, might still have other clinical repercussions, as in women.[16] Furthermore, in the present study, incremental increases in parity number or litter size were accompanied by a

corresponding decrease in serum cobalamin concentration. This finding is suggestive of a negative effect of pregnancy on cobalamin homeostasis, with higher number of fetuses increasing cobalamin demand, and repeated pregnancies preventing repletion of exhausted cobalamin stores in the bitch.

Unlike cobalamin, folate deficiency has been linked directly or indirectly (eg, by documenting beneficial effects of folic acid supplementation) to developmental defects in dogs, and specifically cleft palate and cleft lip.[13,21] In pregnant women, folate deficiency is a well-recognized cause of neural tube defects, abortions, orofacial clefts, preeclampsia, hypercoagulability, and venous thrombosis,[16,30] as well as of PRA,[15] and in this study, similarly, decreases in serum folate concentrations were significantly and positively correlated with PRA in healthy pregnant bitches. The known causes of folate depletion in pregnancy include increased folate demand for uteroplacental and fetal growth, decreased folate absorption, increased folate catabolism, and urinary excretion and hormonal influences.[15] Folic acid supplementation during pregnancy is common practice in human medicine,[15,16,20] and in light of the beneficial effects of folic acid supplementation on the occurrence of cleft palate in predisposed dog breeds, and its association with PRA herein, prospective large-scale studies are warranted to substantiate similar recommendations in dogs, and investigate whether prophylactic folate administration mitigates PRA.

The significant increase in serum free iron, TS, and TIBC concentrations at late pregnancy noted in this study was unexpected. Iron deficiency is the leading pathological cause of PRA in pregnant women, peaking at late pregnancy, and characterized by a decrease in TS.[1,19,31] Even in absence of concurrent anemia, ID is associated with increased maternal and neonatal morbidity and mortality.[1] Several differences might account for the discrepancy between the frequency of ID in pregnant women and the iron concentration trends in pregnant bitches noted herein. First, in women, the prevalence of preconception relative ID is high, with >50% of women in the United States having preconception iron concentration below the recommended level for prevention of pregnancy-related ID.[1,32] Dogs in this study, on the other hand, were fed commercially balanced AAFCO-approved diets, were regularly treated against both ecto- and endoparasites, and had sustained no concurrent disease. Consequently, their preconception iron reserves were likely replete. Second, the considerably longer gestation period in women might partly account for the discrepancy. Third, both free iron and TIBC concentrations are inversely correlated with the acute phase reaction (APR) and decrease in inflammatory states.[33] Pregnancy in the bitch is characterized by an APR, rising at early and midpregnancy and later subsiding. This is demonstrated by the increase in C-reactive protein concentration during pregnancy in the bitch, peaking at 30-45 days postovulation, and precipitously declining at late pregnancy.[34] Additionally, a statistically significant increase in serum ceruloplasmin and fibrinogen concentrations occurs early in pregnancy, compared to the second half of pregnancy.[35] Although serum acute phase proteins were not measured in the present study, hampering our interpretation of results, there exists an apparent inverse temporal relationship between the APR, as described in pregnant bitches, and serum iron and TIBC concentration noted in this study; namely, iron concentration was lower at midpregnancy, coinciding with the APR peak, and increased at late pregnancy, when the APR reportedly subsides. This trend seemed to be offset by increased litter size, as observed in Breeder 2. Additional factors that might have caused the observed discrepancies in UIBC and TIBC concentrations among breeders included differences in nutritional plans and variations in preconception or early pregnancy cobalamin, folate, and iron status.

Additional findings of this study concern the CBC. First, in corroboration of previous reports, serum concentrations of both cobalamin and folate were not correlated with the MCV, a unique feature in dogs,[36-39] in contrast to cats and humans, where deficiency in either vitamin might cause macrocytosis.[40-43] Second, the platelet count significantly increased at late pregnancy, and thrombocytosis was commonly observed in this study. Thrombocytosis occurs in pregnant bitches, contrary to women, where thrombocytopenia, although infrequent, is more commonly encountered.[44] Thrombocytosis was noted both during pregnancy as well as in nonpregnant bitches in diestrus, implicating progesterone as a possible cause.[45] However, this was later rebutted in a different study, where thrombocytosis was only documented at late pregnancy, but not during diestrus.[4] Iron deficiency, a known etiology of thrombocytosis,[46] is an improbable cause of thrombocytosis during pregnancy, given the findings of the present study. Similarly, while inflammation is a common cause of thrombocytosis in dogs,[46] pregnancy-related inflammation is an unlikely explanation for the observed rise in platelet count during pregnancy, because of the opposite temporal trends in inflammation and thrombocytosis during pregnancy in the bitch.[34,35]

In this study, measurements were obtained only at mid- and late pregnancy, but not preconception or at early pregnancy. Therefore, additional important changes might have gone undetected, which is a limitation of this study. Additionally, the follow-up period of the bitches and their litters was limited to 4 weeks PP, precluding the assessment of potential complications related to maternal folate and cobalamin deficiencies (eg, belated puppy death, increased occurrence of infectious diseases, or developmental retardation). Additional limitations include the limited number dog breeds in the study, particularly large-breed dogs, and the inclusion of only 2 breeders. Litter size in the dog varies with breed, and in some breeds it also varies with age.[47] Consequently, the association between litter size and various analytes ideally should have been investigated for each breed separately, which was not feasible owing to the small number of dogs in each breed group herein. Although all dogs had been regularly dewormed, fecal tests had not been performed, and therefore, the presence of occult intestinal parasitism, potentially contributing to ID, could not have been ruled out. Lastly, inflammation markers were not assessed, thereby hampering our interpretation of possible interactions of inflammation with the iron status, occurrence of PRA and pregnancy-associated thrombocytosis.

5 | CONCLUSIONS

A mild nonregenerative normocytic normochromic anemia was frequently noted during pregnancy in most bitches. Unlike in women, where ID constitutes a leading pathological cause of PRA, in pregnant bitches serum iron and TS were increased at late pregnancy.

Additionally, TIBC concentration was increased at late pregnancy in this cohort, coinciding with previously reported APR trends during pregnancy in the bitch. Notwithstanding lack of association with PRA or NS, hypocobalaminemia frequently developed during pregnancy, was exacerbated by increased litter size and higher parity, and might bear clinical and therapeutic implications, based on human and animal research. The decrease in serum folate concentration during pregnancy was correlated with the RBC count, warranting future prospective studies to investigate whether prophylactic folate administration mitigates PRA.

ACKNOWLEDGMENT

An abstract (poster) of this work was presented in the 2018 ECVIM Congress, Rotterdam, the Netherlands.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by the institutional ethics committee (reference number: KSVM_VTH/20_2016).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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How to cite this article: Nivy R, Mazaki-Tovi M, Aroch I, Tal S. Time course of serum cobalamin, folate, and total iron binding capacity concentrations in pregnant bitches and association with hematological variables and survival. *J Vet Intern Med*. 2019;33: 1627–1634. https://doi.org/10.1111/jvim.15551