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Evaluation of serum C-reactive protein concentration as a marker of impending parturition and correlation with progesterone profile in peri-partum bitches

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1 **Serum C-reactive protein and progesterone profile in peripartum bitches and evaluation of**
2 **CRP as a marker of impending parturition**

3

4 **Running head:** CRP and progesterone around bitches parturition

5

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17

18 **Abstract**

19

20 C-reactive protein (CRP) is one of the major acute phase proteins in dogs; it is produced by the liver
21 and rapidly increases in response to an inflammatory stimulus. The aim of this study was to
22 measure CRP concentrations around parturition and to verify whether this protein could be useful,
23 together with progesterone (P), to detect the end of pregnancy in bitches. CRP and P concentrations
24 were measured on 66 serum samples from 28 healthy pregnant bitches, collected between -5 and +2
25 days from parturition. The effect of 'days from parturition', parity, and litter size on P and CRP
26 concentration was analyzed. P and CRP values were significantly affected by 'days from
27 parturition'. While P showed the expected decline during the last days of pregnancy, CRP
28 concentration was above the normal range from the day of parturition onwards, beginning to
29 increase at day -1. The CRP concentration profiles during the days around parturition have not been
30 previously reported in the dog. However, the late rise and the low magnitude of increase make CRP
31 difficult to use in clinical practice to assess the end of pregnancy in the bitch.

32

33

34 **Keywords**

35 Dog; Parturition; C-reactive protein; Progesterone

36

37 **1. Introduction**

38 C-reactive protein (CRP) is an acute-phase protein (APP) that is mainly produced in the liver
39 upon stimulation by proinflammatory cytokines. The acute phase response is a nonspecific reaction
40 that is triggered by any tissue injury and develops following either infectious, immunologic,
41 neoplastic, traumatic or other causes (Ceron et al., 2005). APPs are mediators and inhibitors of
42 inflammation, and express their protective effect through the opsonization of apoptotic or necrotic
43 cells, by binding to bacterial proteins and by influencing the immune response which accompanies
44 inflammation (Gabay and Kushner, 1999). CRP shows an early and strong response in dogs: a 20-
45 to 100-fold increase, depending on the cause of inflammation, can be detected after four hours, with
46 a peak concentration at 24-48 hours (Ceron et al., 2005). CRP can be evaluated in clinical exams as
47 a marker of both acute and chronic inflammatory disorders (Ceron et al., 2008). A rise in CRP
48 concentration has been detected in the first (Eckersall et al., 1993) or second (Kuribayashi et al.,
49 2003) third of pregnancy in the bitch, followed by a decline before parturition. A second increase
50 after parturition has been occasionally observed (Eckersall et al., 1993). Implantation of the
51 developing embryo in the endometrium and placental development were suggested as the likely
52 cause of an acute phase response (Eckersall et al., 1993) and it has also been ascribed to ‘the
53 influence of endocrine hormones during pregnancy’ (Kuribayashi et al., 2003). In the past, the
54 analysis of CRP and other APPs concentration was proposed as a method for early pregnancy
55 diagnosis in the bitch (Evans and Anderton, 1992; Vannucchi et al., 2002).

56 Although the exact mechanisms responsible for parturition are still to be elucidated, the presence of
57 an inflammatory response in the myometrium has been ascertained in women (Thomson et al.,
58 1999; Mendelson and Condon, 2005; Leong et al., 2008).

59 A reliable estimate of parturition date is rather difficult in the bitch when only mating dates are
60 available to the clinician. A precise prediction of impending parturition could be very useful in
61 order to avoid long observation periods and it is critical when planning a cesarean section. Serum
62 progesterone concentration declines towards the end of pregnancy (Onclin and Verstegen, 1997)

63 and some cut-off values have been calculated; when P is lower than the cut-of value, parturition is
64 likely to occur within a given time interval (Rota et al., 2015; De Cramer and Nöthling, 2018).
65 However, large individual variability exists in progesterone concentration, particularly in the last
66 days of pregnancy (Rota et al., 2015; De Cramer and Nöthling, 2018), and other easily measurable
67 parameters that could mark the end of pregnancy would be very useful in clinical practice.
68 Since CRP measurement is part of the routine biochemical profiles performed by many veterinary
69 laboratories, the aim of this study was to measure peripartum CRP concentration in the bitch and to
70 assess its use in clinical practice to detect the end of pregnancy in the bitch.

71 **2. Materials and Methods**

72 *Animals and samples*

73 Twenty-eight healthy pregnant bitches that whelped live puppies were included in the study.
74 The bitches, of various breed [Staffordshire Bull Terrier (N=6), Flat Coated Retriever (4), Boxer
75 (4), Jack Russell Terrier (3), Bouvier des Flandres (2), Australian Shepherd (2), and one each of the
76 following: American Staffordshire Terrier, Bloodhound, Bassett Hound, Labrador Retriever,
77 Golden Retriever, Samoyed, Rough Collie] and parity, ranging in age from 2 to 8 years (3.9 ± 1.6
78 mean \pm SD), had been presented to the veterinary hospitals of the University of Padova or Torino
79 for pregnancy monitoring and parturition assistance, in the period from June 2017 to October 2017.
80 Blood had been collected by cephalic venipuncture for routine progesterone assay and for routine
81 biochemistry evaluation and sera remnants had been stored frozen at -20°C .

82 The case sheet of each bitch reported the day of parturition and the number of delivered puppies. Ex
83 post selection of sera from samples collected between five days before and two days after
84 parturition was carried out. Written informed consent to use the stored samples was obtained by dog
85 owners.

86 The study was performed in accordance with the guidelines for the care and use of animals of the
87 Department of Veterinary Science of the University of Turin and of the Department of Animal
88 Medicine Production and Health of Padova.

89 *Measurement of Progesterone and CRP*

90 CRP was measured with a turbidimetric method (BT1500®, Biotechnica instruments SpA,
91 Roma, Italy); normal reference values are considered in the interval 0- 1.07 mg/dl. The assay had
92 been previously correlated with a canine CRP turbidimetric assay (Randox canine CRP reagents,
93 RANDOX, Milan, Italy) validated for the canine species (Kjelgaard-Hansen et al., 2003).
94 Repeatability was $CV < 7.5\%$; Linearity (O/A) $y = 0.9795x - 0.0074$ $R^2 = 0.9935$. The relationships
95 between the two kits was obtained by the analysis of 91 samples and described by the linear
96 regression as follows: $y = 1.5135x - 0.0123$. $R^2 = 0.9431$.

97 Progesterone was measured by Chemiluminescence immunoassay (CLIA) (Immulite 2000®;
98 Siemens Diagnostics, Flanders, NJ, USA) (Kutzler et al., 2003).

99 *Statistical analysis*

100 Statistical analysis was performed with a repeated mixed linear model where days from
101 parturition (days -5 to +2), parity (primiparous vs multiparous), and number of delivered puppies
102 (<4 vs 4-8 vs >8) were considered as fixed effects and dog was considered as random and repeated
103 effect. Hypotheses of linear model on residuals were graphically assessed. Post hoc pairwise
104 contrasts among levels were calculated using Bonferroni correction. Data were reported as least-
105 squares means \pm standard error (ls-means \pm SE). Day 0 was the day of parturition. Significance was
106 set at $P < 0.05$. Data are presented as least-squares mean \pm standard error.

107 Spearman rank correlation was calculated between CRP and Progesterone values.

108 Using the threshold of 2 ng/ml to identify bitches at term (Concannon et al., 1977), the animals
109 were divided into two groups (at term/not at term). The ability of CRP to distinguish between
110 bitches at term and not at term was evaluated by receiver operating characteristic (ROC) curve
111 analysis. Sensitivity, specificity and cut-off value of this potential marker were calculated (with
112 95% confidence interval, CI). To select the optimal cut-off value, with 95% CI, Youden's Index
113 was calculated. The value of the area under the curve (AUC) as a criterion of the accuracy of the

114 marker was defined as low (0.5-0.7), moderate (0.7-0.9) and high (>0.9) (Kjelgaard-Hansen et al.,
115 2003).

116 All analyses were performed with statistical software packages SAS V.9.3 (SAS Institute Inc., Cary,
117 NC) and MedCalc v.12.4.0 (Ostend, Belgium).

118 **3. Results**

119 P and CRP concentration were measured on a total number of sixty-six serum samples. The
120 number of samples for each of the eight days of observation was distributed as shown in Fig. 1.

121 CRP concentrations ranged from 0/0.04 mg/dl to 2.74 mg/dl and were significantly affected by
122 ‘days from parturition’ ($P=0.0195$), parity ($P=0.044$) and number of delivered puppies ($P=0.0036$).

123 Serum values showed a significant increase over time (Table 1), however pairwise contrasts did not
124 reveal significant daily differences. Mean CRP concentrations were above the normal range from
125 day 0 onwards, beginning to increase at day -1.

126 Primiparous bitches had significantly higher CRP concentrations than pluriparous ones (Fig. 2).

127 Bitches that whelped less than 4 puppies had significantly lower CRP concentrations than bitches
128 with larger litters (Fig. 3).

129 Progesterone concentration was significantly affected by ‘days from parturition’ ($P<0.0001$) and
130 showed the expected decline during the last days of pregnancy. The value at day -1 (2.74 ± 0.49
131 ng/ml) did not differ significantly from the values at days -3, -2 and 0 (4.44 ± 0.47 , 3.90 ± 0.50 and
132 0.60 ± 0.49 respectively) (Fig. 4). Postpartum values were below 1 ng/ml and did not differ between
133 days. Neither parity nor litter size significantly affected progesterone concentration.

134 An inverse correlation was found between CRP and progesterone ($r= -0.52$; $P<0.001$, Fig. 5).

135 ROC curve results are reported in Fig. 6. The area under the curve (AUC) was 0.73, with 95% CI
136 (0.591-0.835; $P=0.001$), meaning that CRP has a moderate accuracy as a marker of impending
137 parturition. The cutoff value that maximizes Youden’s Index is 1.41 mg/dl, with a sensitivity of
138 87.5% (71-96.5; 95% CI) and a specificity of 56% (34.9-75-6; 95% CI).

139 **4. Discussion**

140 Serum progesterone concentrations towards the end of pregnancy can help clinicians to
141 assess impending canine parturition (Rota et al., 2015; De Cramer and Nöthling, 2018), but our data
142 show that the progesterone concentrations are not significantly different in the last three days of
143 pregnancy. Other easy-to measure serum parameters could be useful to increase the accuracy of the
144 diagnosis and CRP, that can be assayed in any facility having instruments for biochemistry serum
145 analysis, could have represented an option. Our hypothesis was that uterine inflammation is a
146 characteristic feature of labour and parturition in bitches, as in women (Thomson et al., 1999;
147 Mendelson and Condon, 2005; Leong et al., 2008), and that CRP, which is a strong and early
148 marker of inflammation in dogs (Ceron et al., 2005; Ceron et al., 2008) could also be a marker of
149 the end of pregnancy. Serum concentrations of CRP during canine pregnancy have been reported in
150 various studies, with contrasting results, but daily values around parturition have not been
151 previously measured in the dog. Different methods for CRP determination were used, resulting in
152 different values. Our data are similar to those of Eckersall et al. (1993) who like us used the
153 technique of immunoturbidimetry, although with a different commercial kit. With a 'solid
154 sandwich' immunoassay, Ulutas et al. (2009) obtained different absolute values of CRP
155 concentration. Although technical differences may explain these differences, they hardly explain the
156 different serum profiles found in previous investigations. Some studies revealed a strong, 7-10 fold,
157 increase of CRP serum concentrations beginning around the third-fourth week after ovulation,
158 followed by a decline in the last third of pregnancy (Eckersall et al., 1993; Kuribayashi et al., 2003).
159 In six out of nine beagle bitches, an increase after parturition was also detected (Eckersall et al.,
160 1993). Other investigations (Concannon et al., 1996; Ulutas et al., 2009) did not detect such a serum
161 profile. Ulutas et al. (2009) observed a significantly higher CRP serum concentration in pregnant
162 bitches than in bitches in proestrus, but the increase in CRP concentrations was very low, both in
163 the first and in the second half of pregnancy.

164 The rate of the increase in serum CRP concentration that we detected from the day of parturition
165 onward is lower than the value reported by Eckersall et al. (1993), who observed a three- to ten-fold
166 rise.

167 Our data did not reveal any sharp increase of CRP serum values, making it difficult to use this
168 parameter as a marker of impending parturition. However this result is consistent with the trend of
169 progesterone concentration and a weak but significant negative relationship between CRP and
170 progesterone was present, meaning that CRP concentration is going to increase at the decrease of
171 progesterone concentration.

172 The peripartum pattern of CRP concentration that we observed in the bitch is similar to what has
173 been reported for other species, irrespective of different placental types and if they are unitocous or
174 polytocous. An increase of CRP serum concentrations has indeed been detected in sows, where it is
175 evident from the day of parturition until day 7 after farrowing (Wierzchosławski et al., 2018). CRP
176 increased just before delivery (≤ 2 days) in pregnant mares and decreased from 7 days after
177 parturition (Yamashita et al., 1991). In women, the CRP concentrations are generally higher during
178 pregnancy, and a significant increase can be observed in the postpartum period, approximately 10-
179 folds as high as the concentrations during the second and third trimester values (Skarżyńska et al.,
180 2018). CRP concentration was also significantly higher during the first month after calving than in
181 the last trimester of pregnancy in cows (Dębski et al., 2016).

182 These and our data show that parturition causes an increase in CRP concentrations, likely due to
183 uterine physiological inflammatory conditions (Thomson et al., 1999; Leong et al., 2008). In
184 primiparous bitches the inflammatory response can be higher because involving tissues that for the
185 first time undergo modifications correlated to the end of pregnancy. Also the influence of litter size
186 on CRP level is consistent with a higher inflammatory response provoked by a higher number of
187 feto-placental units.

188 The ROC curve analysis showed that CRP is a marker of impending parturition that has a
189 'moderate' accuracy (Kjelgaard-Hansen et al., 2003). Despite a rather good sensitivity (87.5%), it

190 has a low specificity (56%), meaning that when CRP concentration drops below the cutoff value
191 (identified as 1.41 mg/dl) a clinician can distinguish a true impending parturition in 87.5% of cases,
192 while the bitch not at term will be correctly identified in 56% of cases.

193 The late rise and the low magnitude of serum concentrations of CRP found in this study lead to
194 conclude that this parameter cannot be used alone in clinical practice to assess the end of pregnancy
195 in the bitch. However, further studies are needed in order to correlate CRP concentration with other
196 parameters of impending parturition, and especially with the physiological or pathological outcome
197 of parturition.

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203 **Conflict of Interest Statement**

204 The authors have no conflict of interest to declare.

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273 TABLE LEGENDS

274 **Table 1** Mean CRP concentration (mg/dl) from five days before to two days after parturition (day
275 0).

276 **FIGURE LEGENDS**

277 **Fig. 1.** Distribution of samples along the eight days of observation.

278 **Fig. 2.** Different mean CRP concentration (mg/dl) in pluriparous (n=14) and primiparous (n=14)
279 bitches ($P=0.044$).

280 **Fig. 3.** Different mean CRP concentration (mg/dl) in bitches with litters of different size
281 ($P=0.0036$). Different letters mean significant differences ($P<0.01$).

282 Number of bitches for each category of litter size: <4 puppies n=6; 4-8 puppies n=15; > 8 puppies
283 n=7

284 **Fig. 4.** Mean progesterone concentration (ng/ml) from five days before to two days after parturition
285 (day 0). Different letters mean significant different values ($P<0.01$).

286 **Fig. 5.** Association between CRP (mg/dl) and progesterone (ng/ml)

287 **Fig. 6.** Receiver Operating Characteristic (ROC) curve of CRP concentration to distinguish between
288 bitches at term/not at term: the area under the curve (AUC) was 0.73 with a 95% CI (0.591-0.835;
289 $P=0.001$).

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