Association of clinical characteristics and lifestyle factors with fecal S100/calgranulin concentrations in healthy dogs

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Abstract

Background: Fecal S100/calgranulin (S100A12 and calprotectin) concentrations are useful markers of gastrointestinal inflammation in dogs. In people, fecal S100/calgranulin concentrations are affected by age, obesity, diet and other lifestyle factors. Knowledge about the effects of such factors on fecal S100/calgranulin concentrations in dogs is currently scarce.

Objective: To evaluate the association between several factors and fecal S100/calgranulin concentrations in a large cohort of healthy adult dogs.

Methods: Single-spot fecal samples from 181 healthy pet dogs and data derived from a standard questionnaire served to evaluate the effect of age, sex, reproductive status, body weight and body condition, breed type and size, vaccination, endoparasite treatment, diet, environment and travel history on fecal S100/calgranulin concentrations and the fecal calgranulin ratio (fCaR).

Results: Univariate analysis showed a significant association of reproductive status (in female dogs) and breed size with fecal S100A12, fecal calprotectin and fCaR. Breed type was linked to fecal S100A12 concentrations and fCaR; recent vaccination (particularly with a vaccine against canine parvovirus) to fCaR. In multivariate models, breed size was linked to fecal S100A12 and calprotectin concentrations, and recent vaccination affected S100A12 concentrations.

Conclusions: Breed size, recent vaccination and reproductive status in female dogs can affect fecal S100/calgranulin concentrations, and these biomarkers should be interpreted in light of those confounding factors. The utility of reference intervals for fecal canine S100/calgranulin concentrations might be improved through stratification by sex/reproductive status and breed size. Fecal canine S100/calgranulin concentrations are not confounded by age, body condition, deworming, diet, environment or travel history.

Keywords: calprotectin, canine, inflammatory marker, obesity, reproductive status, S100A12, vaccination.
1 | INTRODUCTION

S100A12 (calgranulin C) and the S100A8/A9 (calprotectin or calgranulin A/B) complex belong to the S100/calgranulin subfamily of Ca²⁺-binding proteins that play a central role in the innate immune responses (Foell et al., 2007). S100A12 and calprotectin are released from activated macrophages and/or neutrophils and accumulated at sites of inflammation (Heilmann & Steiner, 2018). Recent studies suggest fecal S100A12 and fecal calprotectin concentrations to be clinically useful markers of gastrointestinal inflammation in dogs (Heilmann & Steiner, 2018). Increased fecal S100A12 concentrations are associated with clinical and endoscopic disease severity in dogs with chronic intestinal inflammation, can help to distinguish subgroups of canine chronic enteropathy and might be useful to predict the response to immunomodulatory treatment (Heilmann, Volkman, et al., 2016). Increased fecal calprotectin concentrations are also associated with the severity of clinical disease and can aid in predicting the response to immunomodulatory treatment (Heilmann, Berghoff, et al., 2018). The role of the fecal S100/calgranulin concentrations as biomarkers of localized inflammation such as inflammatory bowel disease (IBD) is also an area of intensive investigation in human medicine (Foell et al., 2007; Lopez et al., 2017; Mendall et al., 2016; Wright et al., 2014). Evaluation of the calgranulins as markers of inflammation further showed that the expression of S100A12 relative to that of the calprotectin complex (calgranulin ratio) has potential to characterize better and to offer further insight into the mechanism of particularly localized inflammatory disease processes (e.g. by differentiating acute and chronic diseases) (Heilmann et al., 2017; Lorenz et al., 2008). The calgranulin ratio has been evaluated in bronchoalveolar lavage fluid from people with inflammatory respiratory conditions (Lorenz et al., 2008) and in urine specimens from dogs with inflammatory or neoplastic urinary or urogenital diseases (Heilmann et al., 2017). The calgranulin ratio in feces (fCalR) has not been evaluated or reported in humans or dogs with gastrointestinal conditions.

Fecal S100/calgranulin concentrations were shown to be affected by increasing age, obesity, diet and also other lifestyle factors in people (Mendall et al., 2016; Park & Kim, 2018; Poullis et al., 2004). However, knowledge about the effects of patient factors (e.g. body condition) or lifestyle factors on fecal inflammatory markers, such as the S100/calgranulins, is currently scarce in small animal medicine. One investigation found healthy research colony dogs to have significantly higher fecal calprotectin concentrations than healthy pet dogs (Heilmann et al., 2008). Very young age was linked to higher fecal calprotectin concentrations in puppies (Grellet et al., 2016). However, there was no age effect on fecal calprotectin concentrations in healthy adult dogs (Heilmann et al., 2008). Fecal S100A12 concentrations were not associated with age in healthy puppies and adult dogs (Heilmann, Grellet, et al., 2018; Heilmann, Lanerie, et al., 2011). Sex-related differences in fecal S100A12 concentrations were also not observed in that study (Heilmann, Lanerie, et al., 2011). Shedding of enteropathogens (viral and/or parasitic agents) did not affect fecal S100A12 or fecal calprotectin concentrations (Grellet et al., 2016; Heilmann, Grellet, et al., 2018), but the fecal score and breed size affected fecal S100A12 concentrations in healthy puppies (Heilmann, Grellet, et al., 2018).

To the authors’ knowledge, the effect of other patient (e.g. reproductive status, body condition, vaccination status—particularly, vaccination against canine parvovirus type 2 [CPV2]) and lifestyle factors (e.g. primary environment, travel history) on fecal S100/calgranulin concentrations and the fCalR have not been evaluated or reported in a large number of healthy adult dogs. Thus, more work is needed to determine factors influencing fecal S100/calgranulin concentrations in normal dogs, and evaluation of the possibility of such associations is an important prerequisite for further clinical validation of the fecal S100/calgranulins as biomarkers of gastrointestinal disease in dogs. To fill this knowledge gap, the effect of age, sex, reproductive status, body weight and body condition, breed, vaccination, endoparasite prophylaxis, diet, environment and travel history on fecal S100A12 and fecal calprotectin concentrations was evaluated in a large cohort of healthy adult dogs. We hypothesized that in dogs, the body condition, but not any other variables, is associated with fecal S100/calgranulin concentrations—but not their relation to each other expressed as fCalR. A second aim of this study was to establish reference intervals for fecal S100A12 and fecal calprotectin concentrations using single-spot fecal samples.

2 | MATERIALS AND METHODS

2.1 | Sampling population

Single fecal samples (spot samples collected immediately following natural defecation) were collected from 202 apparently healthy pet dogs from five different countries (USA/Texas: \( n = 126 \), Sweden: \( n = 32 \), Brazil: \( n = 23 \), Germany: \( n = 11 \) and France: \( n = 10 \)) between January 2007 and October 2011. All dogs were clinically healthy, with no clinical signs of gastrointestinal disease (i.e. diarrhoea, vomiting, anorexia, weight loss, abdominal pain and depression). A study questionnaire evaluating the general health status, including historical information and all characteristics evaluated in this study, was completed for each dog by the owner and/or attending primary care veterinarian. Consent from the owner of each dog was obtained prior to enrolling a dog into the study and fecal sample collection. Based on the Institutional Animal Care and Use Committee (IACUC) guidelines at the Texas A&M University College of Veterinary Medicine and Biomedical Sciences, collecting naturally passed fecal samples from dogs for the measurement of biomarkers does not require an independent review and ethical approval of the study.

To be included in the study, dogs had to be healthy as assessed by the information provided above. Exclusion criteria were the previous diagnosis or a suspicion of a gastrointestinal disorder and the administration of medications that are known to affect the gastrointestinal tract (e.g. non-steroidal anti-inflammatory drugs). Twenty-one dogs (all recruited in the USA/Texas) were retrospectively excluded from the study due to the lack of sufficient information about the dog,
leaving 181 dogs included in the study (Table 1). Information gathered about each dog included: dog and owner identification; time and date of fecal sample collection; the age of the dog and/or date of birth; sex and neuter status; breed and breed size (small breed dogs: mean adult body weight of the respective breed ≤10 kg; medium-sized breed: 10–25 kg average adult body weight; and large-breed dogs: >25 kg average adult body weight); body weight and body condition score (BCS, using the 5-point scale (Kant et al., 2013)); housing primarily indoor (with or without outdoor access) or housing outdoor; travel history (travel outside the country of residence); diet (premium diet or supermarket brand); medication, supplements and nutraceuticals given within 6 months prior to the dog being enrolled into the study; last vaccination and type of vaccine; last endoparasite treatment and dewormer used; and any medical condition. Dogs were considered to be current on vaccines if vaccinated within 12 months of study enrolment, and recent vaccination within 4 weeks of study enrolment and fecal sample collection. Dogs were considered to be current on their deworming schedule if endoparasite treatment was administered within 3 months of inclusion.

2.2 | Sample collection and analyses

Fecal samples were stored frozen (at −20°C) within 24 hr after defecation until further processed within 12 weeks. Fecal samples were then thawed and extracted as described (Heilmann et al., 2008). Fecal S100A12 (in ng/g) and calprotectin concentrations (in μg/g) were measured in fecal extracts (stored at −20°C until assayed) using established and validated species-specific immunoassays (Heilmann, Cranford, et al., 2016; Heilmann et al., 2008). The lower detection limits of the assays are 1 ng S100A12/g feces and 2.9 μg calprotectin/g feces respectively (Heilmann, Cranford, et al., 2016; Heilmann et al., 2008). Fecal extracts with canine S100A12 or calprotectin concentrations that exceeded the assay working range were diluted (1:10 and 1:2, respectively) and re-assayed (Heilmann, Cranford, et al., 2016; Heilmann et al., 2008). The fCalR was calculated as [fecal calprotectin concentration (in μg/g) × 100/fecal S100A12 concentration (in ng/g)].

2.3 | Data analysis

Continuous data were tested for the presence of normal distribution using a Shapiro–Wilks test. All summary statistics are reported as medians and ranges (for continuous and ordinal data) or numbers and percentages (nominal data). Possible associations between variables and statistical differences between or among groups were first tested in univariate analyses (Wilcoxon rank-sum or Kruskal–Wallis test for non-parametric two-group or multiple-group comparisons, respectively; Spearman ρ correlation coefficient for non-parametric correlation analysis and likelihood ratio or Fisher’s exact test for assessment of associations between nominal variables). Variables with p < .2 in univariate analysis were then included in a multivariate

| TABLE 1  | Characteristics of the dogs (n = 181) included in this study |
|-----------|-------------------------------------------------------------|
| Group characteristic | Value |
| Total number | 181 |
| Age | |
| Age in years, median (range)a | 3.4 (0.3–15.1) |
| Age groups, n (%)a | |
| Less than 1 year of age | 14 (8%) |
| 1–3 years old | 67 (39%) |
| 4–7 years old | 54 (31%) |
| More than 7 years of age | 38 (22%) |
| Sex distribution | |
| Male/female, n (%) | 76 (42%)/105 (58%) |
| Reproductive status: neutered male/spayed female, n (%)b | 54 (72%)/74 (73%) |
| Body condition | |
| Bodyweight in kg, median (range)c | 22.7 (1.9–83.9) |
| Body condition score (5-point scale), median (range)d | 3 (2–5) |
| BCS = 2 of 5, n (%) | 2 (1%) |
| BCS = 2.5 of 5, n (%) | 18 (12%) |
| BCS = 3 of 5, n (%) | 92 (61%) |
| BCS = 3.5 of 5, n (%) | 19 (12.5%) |
| BCS = 4 of 5, n (%) | 16 (11%) |
| BCS = 4.5 of 5, n (%) | 3 (2%) |
| BCS = 5 of 5, n (%) | 1 (0.5%) |
| Breed | |
| Breed size, n (%)e | |
| Small breed | 36 (20%) |
| Medium-size breed | 51 (29%) |
| Large breed | 92 (51%) |
| Pure-bred dogs, n (%) | 124 (69%) |
| Labrador Retriever | 16 (9%) |
| Australian Shepherd dog | 8 (4%) |
| German Shepherd dog | 6 (3%) |
| Dachshund | 5 (3%) |
| Rottweiler | 5 (3%) |
| Mixed-breed dogs, n (%) | 57 (31%) |
| Primary dietf | |
| Premium food, n (%) | 116 (69%) |
| Supermarket diet, n (%) | 53 (31%) |
| Lifestyle | |
| Primary environment, n (%)g | |
| Indoor | 133 (86%) |
| Indoor with outdoor access | 11 (7%) |
| Outdoor | 10 (7%) | (Continues)
statistical software package (JMP® v13.0; SAS Institute) was used
practical importance (Hojat & Xu, 2004). A commercially available
of 0.2 was interpreted as small with the difference having negligible
and the difference to be of moderate practical important and an ES
considered as a random effect) and fecal calprotectin concentrations
In our study population was assessed by calculation of the ef-
tus, breed type and size and recent vaccination as fixed effects and
considered as a random effect). Statistical significance was set
kAvailable from 142 dogs.
jDocumented in 120 dogs.
iDocumented in 99 dogs.
hDocumented in 145 dogs.
gDocumented in 154 dogs.
fDocumented in 169 dogs.
eDocumented in 179 dogs.
dDocumented in 151 dogs.
cDocumented in 167 dogs.
bDocumented in 176 dogs.
aDocumented in 181 dogs.

Note: Abbreviation: CPV2, canine parvovirus type 2.

TABLE 1

| Group characteristic | Value |
|----------------------|-------|
| Travel history, n (%)<sup>h</sup> | 31 (21%) |
| Vaccination          |       |
| Time since last vaccination in months, median (range)<sup>g</sup> | 6 (0–35.7) |
| Vaccination included CPV2, n (%)<sup>g</sup> | 72 (73%) |

Endoparasite treatment

| Time since last deworming in months, median (range)<sub>f</sub> | 3 (0–68.5) |

| Fecal calgranulin concentrations |       |
|----------------------------------|-------|
| Fecal S100A12 concentration in ng/g, median (range)<sup>i</sup> | 9 (1–3,150) |
| Fecal calprotectin concentration in μg/g, median (range)<sup>j</sup> | 4.2 (2.9–158.7) |
| Fecal calprotectin/S100A12 ratio (fCalR), median (range)<sup>g</sup> | 79 (0–3.164) |

Note: Table 1 (Continued)

| Group characteristic | Value |
|----------------------|-------|
| Time since last deworming in months, median (range)<sub>f</sub> | 3 (0–68.5) |

| Fecal calgranulin concentrations |       |
|----------------------------------|-------|
| Fecal S100A12 concentration in ng/g, median (range)<sup>i</sup> | 9 (1–3,150) |
| Fecal calprotectin concentration in μg/g, median (range)<sup>j</sup> | 4.2 (2.9–158.7) |
| Fecal calprotectin/S100A12 ratio (fCalR), median (range)<sup>g</sup> | 79 (0–3.164) |

3 RESULTS

The dogs included in this study (n = 181) comprised of 61 different
dog breeds. The characteristics of the dogs included in the study are
summarized in Table 1.

Fecal S100A12 concentrations (measured in all 181 dogs) ranged
from 1 to 3,150 ng/g (median: 9 ng/g) and fecal calprotectin concen-
trations (measured in 142 of the dogs; not analysed for 39 dogs due
to shortness of extract material) ranged from 3 to 159 μg/g (median:
4 μg/g). Both fecal S100A12 (ρ<sub>167</sub> = −0.28; p = .0003) and fecal
calprotectin concentrations (ρ<sub>130</sub> = −0.25; p = .0040) were weakly
correlated with the dogs' body weight. Age was weakly correlated
with fecal calprotectin (ρ<sub>136</sub> = 0.17; p = .0444) but not with fecal
S100A12 concentrations (ρ<sub>172</sub> = 0.05; p = .5604). BCS was neither
correlated with fecal S100A12 (ρ<sub>151</sub> = 0.05; p = .5488) nor with
fecal calprotectin concentrations (ρ<sub>122</sub> = 0.13; p = .1638).

Three of 39 (8%) overweight or obese dogs had an increased
cecal S100A12 concentration (>463 ng/g) compared to 5/112 dogs
(4%) with ideal or thin body type (Table 2). However, this association
did not reach statistical significance (likelihood ratio test: χ<sup>2</sup> = 0.61,
df = 1, p = .4347). Similarly, 3/25 (12%) overweight or obese dogs had
increased fecal calprotectin concentrations (>39 μg/g) compared to
9/97 (9%) of lean dogs (Table 2), which was also no significant

TABLE 2

| Fecal S100A12/calgranulin status | Overweight or obesity<sup>d</sup> | Ideal or thin body condition<sup>b</sup> |
|----------------------------------|-------------------------------|---------------------------------|
| Increased fecal S100A12 concentration<sup>c</sup> | n = 3 (8%) | n = 5 (4%) |
| Fecal S100A12 concentration within RI<sup>d</sup> | n = 36 (92%) | n = 107 (96%) |
| Increased fecal calprotectin concentration<sup>e</sup> | n = 3 (12%) | n = 9 (9%) |
| Fecal calprotectin concentration within RI<sup>f</sup> | n = 22 (88%) | n = 88 (91%) |

Note: RI, reference interval.
<sup>d</sup>BCS ≥ 3.5 (5-point scale).
<sup>b</sup>BCS ≤ 3 (5-point scale).
<sup>c</sup> > 463 ng S100A12/g feces.
<sup>d</sup> ≤ 463 ng S100A12/g feces.
<sup>e</sup> > 39 μg calprotectin/g feces.
<sup>f</sup> ≤ 39 μg calprotectin/g feces.
association (likelihood ratio test: \( \chi^2 = 0.20, df = 1, p = .6570 \)). Only 3/8 dogs (38%) with increased fecal S100A12 were overweight/obese (compared to 25% of the dogs with a normal fecal S100A12 concentration), and 3/12 (25%) with increased fecal calprotectin were overweight/obese (compared to 20% of the dogs with a normal fecal calprotectin concentration) (Table 2).

There was no correlation between fecal S100A12 and fecal calprotectin concentrations (\( \rho_{143} = 0.01; p = .9155 \)). The fCalR ranged from 0.4 to 3.164 (median: 79) and was not correlated with age, body weight, or with BCS (all \( p > .05 \)).

Reproductive status (particularly in female dogs) (Figure 1), breed type and breed size (Figure 2) were significantly associated with fecal S100A12 concentrations in univariate analyses (Table 3), with diet and recent vaccination (particularly with a vaccine including CPV2) showing a trend for an association (\( p < .20 \)), but only breed size and recent vaccination were significant in the multivariate model. The association of fecal S100A12 concentrations with breed size (ES calculated as 0.71 for the difference between small and medium-sized breeds, and as 0.87 for the difference between small and large breeds) and reproductive status in female dogs (ES = 0.72 for the difference between intact and spayed female dogs) was found to be of crucial practical relevance, whereas the association with recent vaccination was moderately important (ES = 0.50 for the difference between recently vaccinated and not recently vaccinated dogs). Breed type (ES = 0.32 for the difference between purebred and mixed breed dogs), reproductive status in all dogs (ES = 0.30 for the difference between all intact and neutered/spayed dogs) and diet type (ES = 0.24 for the difference between dogs fed a premium diet and those fed a supermarket brand diet) were of negligible relevance.

Reproductive status (particularly in female dogs) and breed size were significant determinants of fecal calprotectin concentrations in univariate analyses, with diet showing a trend for an association (\( p < .20 \)), but only breed size remained significant in the multivariate model (Table 3). Only the difference in fecal calprotectin concentrations between small and large breed dogs was of major practical relevance (ES = 0.82). In contrast, the difference between small- and medium-sized breeds (ES = 0.55) was only moderately important, and the effect of reproductive status in female dogs (ES = 0.34) and that of the type of diet (ES = 0.20) were considered as negligible.

Reproductive status (particularly in female dogs), breed size and type and recent vaccination were significantly associated with fCalR in univariate analyses. However, none of these remained significant in the multivariate model (Table 4).

Fecal S100A12 concentrations were outside the upper inner Tukey fence (\( Q_3 + 1.5 \times IQR = 68 \text{ ng/g} \)) in 23 dogs and were outside the upper outer Tukey fence (\( Q_3 + 3 \times IQR = 106 \text{ ng/g} \)) in 16 dogs of which four severe outliers (2.2% of the data set) were removed. The single-sample reference interval for S100A12 concentration was established as 2–463 ng/g (using the standard method for Box-Cox transformed data) (Figure 3a).

Fecal calprotectin concentrations were outside the upper inner Tukey fence (\( Q_3 + 1.5 \times IQR = 48 \text{ pg/g} \)) in 9 dogs, and were outside the upper outer Tukey fence (\( Q_3 + 3 \times IQR = 75 \text{ pg/g} \)) in 5 dogs all of which (3.5% of the data set) were removed due to being severe outliers. The single-sample reference interval for calprotectin concentration was established as 3–39 pg/g (using the standard method for Box-Cox transformed data) (Figure 3b).

4 | DISCUSSION

Patient and environmental factors have the potential to affect the specificity of fecal markers of gastrointestinal inflammation, and the specificity of fecal S100/calgranulin concentrations for the diagnosis of chronic inflammatory enteropathies in dogs might be improved when such factors are considered. Thus, this study aimed at evaluating several clinical characteristics and environmental factors and their potential to affect fecal S100/calgranulin concentrations (fecal S100A12 and fecal calprotectin concentrations as well as their relation to each, expressed as the fCalR) in a large number of clinically healthy dogs.

Being overweight or obese was not associated with increased fecal S100/calgranulin concentrations in this study. We found increased fecal calprotectin concentrations in only 12% of overweight or obese dogs (BCS ≥ 3.5), which is only about half of the 20% rate.
consistent with a human study showing age not to affect fecal calprotectin concentrations in adults (Kant et al., 2013). In contrast to people (Kant et al., 2013; Poullis et al., 2004; Verdam et al., 2013) concentrations as a marker of gastrointestinal inflammation in adult previously shown to be associated with increased fecal calprotectin several factors including race) (Kant et al., 2013). Obesity was previously shown to be associated with increased fecal calprotectin concentrations as a marker of gastrointestinal inflammation in adults (Brignardello et al., 2018; Heilmann, Lanerie, et al., 2011). These results are also expected to be less accurate than the body mass index (BMI) system used in human medicine (Kant et al., 2013; Poullis et al., 2004).

Age was not linked to fecal S100/calgranulin concentrations in this study, which agrees with our previous results showing a lack of an effect of age on fecal calprotectin concentrations in a small number of healthy adult dogs (Heilmann et al., 2008) and on fecal S100A12 concentrations in healthy puppies and adult dogs (Heilmann, Grelet, et al., 2018; Heilmann, Lanerie, et al., 2011). These results are also consistent with a human study showing age not to affect fecal calprotectin concentrations in adults (Kant et al., 2013). In contrast to the findings of the present investigation, we previously found age to be linked to fecal calprotectin concentrations in very young puppies (Grelet et al., 2016), and higher fecal S100/calgranulin concentrations have also been reported in very young healthy children (Day et al., 2013; Garg et al., 2017; Joshi et al., 2010) and also an increase in fecal calprotectin concentrations in older people (>60 years) (Ayling & Kok, 2018; Joshi et al., 2010). Thus, only a very young age appears to be a potential confounder for increased fecal S100/calgranulin concentrations in dogs and humans.

Similar to our findings, gender was shown not to affect fecal calprotectin (Fall et al., 2017; Poullis et al., 2004) or fecal S100A12 concentrations (Day et al., 2013) in people. However, an interesting finding in our study is an effect of reproductive (i.e. neuter) status on fecal calprotectin and (based on the ES), even more relevant, on fecal S100A12 concentrations (but both showing opposite trends), particularly in female dogs, in univariate analyses. This has not previously been reported, likely because the size of subgroups of dogs in previous studies (Heilmann, Lanerie, et al., 2011; Heilmann et al., 2008) precluded further analyses and thus the possibility of finding such an effect. Interpolation of results from other species is also very difficult because the effects of patient characteristics and environmental factors on fecal S100/calgranulin concentrations have only been evaluated in people. The possibility of an effect of postmenopausal status or oral contraceptive use on fecal S100/calgranulin concentrations in women has not been investigated or reported (and would probably not be directly comparable with the situation in a spayed dog). Postmenopausal hormone changes are associated with altered immune profiles (Au et al., 2016) and have been reported to affect IBD development in women (Mendall et al., 2016). Sex hormones have been linked to innate and adaptive immune responses through alterations in the intestinal microbiome (Choi et al., 2017; Khalili, 2016), and an experimental study in ovariecetomized mice showed oestrogen imbalances to contribute to changes in the intestinal microbial environment that are similar (yet distinct) to those changes associated with diet-induced obesity (Choi et al., 2017). Thus, possible explanations for finding higher fecal S100A12 but lower fecal calprotectin concentrations in intact females than spayed female dogs could be a low-grade inflammatory...
| Variable                  | Fecal S100A12 concentration | Fecal calprotectin concentration |
|---------------------------|-----------------------------|----------------------------------|
|                           | n                           | Fecal concentration, median [range] | P (univariate analysis) | P (multi-variate analysis) | n | Fecal concentration, median [range] | P (univariate analysis) | P (multi-variate analysis) |
| Age                       |                             |                                  |                          |                          |    |                                  |                          |                          |
| ≤1 year                   | 14                          | 11 [3–1,286] ng/g               | 0.9973 (χ² = 0.048, df = 3) ns |                          | 13 | 3 [3–89] µg/g                     | 0.3227 (χ² = 3.485, df = 3) ns |
| 1–3 years                 | 67                          | 8 [1–3,150] ng/g                |                            |                          | 55 | 4 [3–159] µg/g                    |                          |                          |
| 4–7 years                 | 54                          | 9 [2–2,408] ng/g                |                            |                          | 41 | 4 [3–73] µg/g                     |                          |                          |
| ≥7 years                  | 38                          | 10 [2–1,96] ng/g                |                            |                          | 28 | 10 [3–112] µg/g                   |                          |                          |
| Sex                       |                             |                                  |                          |                          |    |                                  |                          |                          |
| Male                      | 76                          | 9 [2–3,150] ng/g                | 0.7151 (Z = 0.365) ns     |                          | 60 | 4 [3–159] µg/g                    | 0.8928 (Z = 0.135) ns    |
| Female                    | 105                         | 9 [1–2,488] ng/g                |                            |                          | 82 | 4 [3–132] µg/g                    |                          |                          |
| Reproductive status       |                             |                                  |                          |                          |    |                                  |                          |                          |
| Intact                    | 48                          | 12 [2–2,488] ng/g               | 0.0090 (Z = 2.613)       | 0.6909 (F(0.139) = 0.159) | 35 | 3 [3–89] µg/g                     | 0.2164 (Z = -1.236)      | 0.7574 (F(1.63) = 0.096)  |
| Neutered/spayed           | 128                         | 7 [1–3,150] ng/g                |                            |                          | 102| 4 [3–159] µg/g                    |                          |                          |
| Males: reproductive status|                             |                                  |                          |                          |    |                                  |                          |                          |
| Intact                    | 21                          | 10 [2–637] ng/g                 | 1.0000 (Z = 0.000)       |                          | 15 | 9 [3–73] µg/g                     | 0.7232 (Z = 0.354)       |                          |
| Neutered                  | 54                          | 8 [2–3,150] ng/g                |                            |                          | 44 | 4 [3–159] µg/g                    |                          |                          |
| Females: reproductive status|                          |                                  |                          |                          |    |                                  |                          |                          |
| Intact                    | 27                          | 22 [3–2,488] ng/g               | 0.0007 (Z = 3.380)       |                          | 20 | 3 [3–159] µg/g                    | 0.0413 (Z = -2.041)      |                          |
| Spayed                    | 74                          | 6 [1–873] ng/g                  |                            |                          | 58 | 6 [3–132] µg/g                    |                          |                          |
| Body condition (based on BCS) |                  |                                  |                          |                          |    |                                  |                          |                          |
| Underconditioned a         | 2                           | 29 [27–31] ng/g                 | 0.2212 (χ² = 3.018, df = 2) ns |                          | 1  | 3 µg/g                            | 0.4722 (χ² = 1.501, df = 2) ns |
| Ideal body condition b     | 110                         | 6 [1–3,150] ng/g                |                            |                          | 96 | 4 [3–89] µg/g                     |                          |                          |
| Overconditioned c          | 39                          | 9 [2–2,196] ng/g                |                            |                          | 25 | 5 [3–159] µg/g                    | 0.7409 (Z = -0.331)      | ns                          |
| Breed size                |                             |                                  |                          |                          |    |                                  |                          |                          |
| Small breed d              | 36                          | 23 [2–3,150] ng/g               | 0.0003 (χ² = 16.003, df = 2) | 0.0006 (F(2, 130) = 7.777) | 27 | 13 [3–159] µg/g                   | 0.0015 (χ² = 13.020, df = 2) | 0.0304 (F(2, 124) = 3.592) |
| Medium-size breed e        | 51                          | 9 [1–2,408] ng/g                |                            |                          | 36 | 4 [3–112] µg/g                    |                          |                          |
| Large breed f              | 92                          | 6 [2–1,886] ng/g                |                            |                          | 78 | 3 [3–132] µg/g                    | 0.7409 (Z = -0.331)      | ns                          |
| Breed                     |                             |                                  |                          |                          |    |                                  |                          |                          |
| Pure-bred dogs             | 124                         | 10 [2–3,150] ng/g               | 0.0204 (Z = -2.320)      | 0.1512 (F(1, 140) = 2.083) | 98 | 4 [3–132] µg/g                    | 0.7409 (Z = -0.331)      | ns                          |
| Mixed-breed dogs           | 57                          | 6 [1–2,408] ng/g                |                            |                          | 44 | 4 [3–159] µg/g                    |                          |                          |
| Diet                      |                             |                                  |                          |                          |    |                                  |                          |                          |
| Premium diet               | 116                         | 10 [1–2,488] ng/g               | 0.0680 (Z = -1.825)      | 0.7790 (F(1, 139) = 0.079) | 84 | 5 [3–159] µg/g                    | 0.1789 (Z = -1.344)      | 0.2361 (F(1, 124) = 1.418) |
| Supermarket diet           | 53                          | 5 [2–3,150] ng/g                |                            |                          | 46 | 4 [3–132] µg/g                    |                          |                          |

(Continues)
| Variable                      | n     | Fecal concentration, median [range] | P (univariate analysis) | P (multi-variate analysis) | n     | Fecal concentration, median [range] | P (univariate analysis) | P (multi-variate analysis) |
|-------------------------------|-------|-------------------------------------|-------------------------|---------------------------|-------|-------------------------------------|-------------------------|---------------------------|
| Primary environment           |       |                                     |                         |                           |       |                                     |                         |                           |
| Indoor                        | 133   | 8 [1–3,150] ng/g                   | 0.8022 (χ² = 0.441, df = 2) ns |                           |       | 110                                | 4 [3–159] μg/g          | 0.8230 (χ² = 0.390, df = 2) ns |
| Indoor + outdoor access       | 11    | 6 [2–873] ng/g                     |                         |                           |       | 11                                | 6 [3–89] μg/g           |                           |
| Outdoor                       | 10    | 8 [3–36] ng/g                      |                         |                           |       | 4                                 | 4 [3–23] μg/g           |                           |
| Travel history                |       |                                     |                         |                           |       |                                     |                         |                           |
| Yes                           | 31    | 10 [2–2,408] ng/g                  | 0.4904 (Z = 0.690) ns   |                           |       | 23                                | 6 [3–159] μg/g          | 0.9446 (Z = 0.070) ns   |
| No                            | 114   | 7 [1–3,150] ng/g                   |                         |                           |       | 84                                | 4 [3–132] μg/g          |                           |
| Current vaccination           |       |                                     |                         |                           |       |                                     |                         |                           |
| Yes^a                         | 126   | 9 [1–3,150] ng/g                   | 0.4975 (Z = 0.678) ns   |                           |       | 92                                | 4 [3–112] μg/g          | 0.9418 (Z = 0.073) ns   |
| No^b                          | 25    | 9 [3–1,886] ng/g                   |                         |                           |       | 22                                | 3 [3–159] μg/g          |                           |
| Yes + CPV2 included           | 60    | 8 [2–873] ng/g                     | 0.2267 (Z = -1.209) ns  |                           |       | 55                                | 6 [3–89] μg/g           | 0.3601 (Z = -0.915) ns  |
| Yes + CPV2 not included       | 22    | 5 [1–3,150] ng/g                   |                         |                           |       | 21                                | 3 [3–47] μg/g           |                           |
| Recent vaccination            |       |                                     |                         |                           |       |                                     |                         |                           |
| Yes^c                         | 20    | 11 [2–2,408] ng/g                  | 0.1551 (Z = 1.422)     | **0.0090 (F^1 139 = 7.010)** |       | 16                                | 6 [3–33] μg/g           | 0.7493 (Z = -0.320) ns  |
| No^d                          | 131   | 8 [1–3,150] ng/g                   |                         |                           |       | 98                                | 4 [3–159] μg/g          |                           |
| Yes + CPV2 included           | 11    | 11 [3–873] ng/g                    | 0.1191 (Z = -1.559)    |                           |       | 11                                | 6 [3–24] μg/g           | 0.4254 (Z = 0.797)    |
| Yes + CPV2 not included       | 3     | 2 [2–20] ng/g                      |                         |                           |       | 3                                 | 14 [3–33] μg/g          |                           |
| Recent endoparasite treatment |       |                                     |                         |                           |       |                                     |                         |                           |
| Yes^e                         | 63    | 9 [2–2,408] ng/g                   | 0.5597 (Z = -0.583) ns  |                           |       | 49                                | 4 [3–60] μg/g           | 0.3418 (Z = -0.951) ns  |
| No^f                          | 57    | 8 [2–1,886] ng/g                   |                         |                           |       | 36                                | 4 [3–159] μg/g          |                           |

Note: BCS, body condition score; CPV2, canine parvovirus type-2.

^aBody condition score (BCS): <2.5 of 5.
^bBCS = 2.5 or 3.
^cBCS: >3 of 5.
^dAverage adult body weight: <10 kg.
^eAverage adult body weight: 10–25 kg.
^fAverage adult body weight: >25 kg.
^gWithin 12 months of fecal sample collection.
^hLast vaccination >12 months ago.
^iWithin 4 weeks of fecal sample collection.
^jLast vaccination >4 weeks ago.
^kLast endoparasite treatment within 3 months of fecal sample collection.
^lLast endoparasite treatment >3 months ago.
| Variable | Fecal concentration, median [range] | P (univariate analysis) | P (multi-variate analysis) |
|----------|---------------------------------|------------------------|---------------------------|
| **Age**  |                                 |                        |                           |
| ≤1 year  | 33 [3–3.164]                    | 0.6283 ($\chi^2 = 1.739, df = 3$) | ns                        |
| 1–3 years| 73 [0–2.503]                    |                        |                           |
| 4–7 years| 97 [1–1.091]                    |                        |                           |
| ≥7 years | 84 [2–1.785]                    |                        |                           |
| **Sex**  |                                 |                        |                           |
| Male     | 76 [2–1.205]                    | 0.8704 ($Z = −0.163$)  | ns                        |
| Female   | 82 [0–3.164]                    |                        |                           |
| **Reproductive status** | |                        |                           |
| Intact   | 52 [0–3.164]                    | 0.0635 ($Z = −1.856$, $F(1, 83) = 2.116$) | 0.1496 ($F_{(1, 83)} = 2.116$) |
| Neutered/spayed | 97 [1–2.503]                        |                        |                           |
| **Males: reproductive status** | |                        |                           |
| Intact   | 94 [6–1.205]                    | 0.2766 ($Z = 1.088$, $F_{(1, 83)} = 2.116$) |                           |
| Neutered | 76 [2–1.046]                    |                        |                           |
| **Females: reproductive status** | |                        |                           |
| Intact   | 17 [0–3.164]                    | 0.0012 ($Z = −3.233$, $F_{(1, 83)} = 2.116$) |                           |
| Spayed   | 113 [1–2.503]                   |                        |                           |
| **Body condition (based on BCS)** | |                        |                           |
| Underconditioneda | 11                          | 0.4023 ($\chi^2 = 1.821, df = 2$) | ns                        |
| Ideal body conditionb | 92 [2–3.164]                        |                        |                           |
| Overconditionedc | 71 [1–2.503]                        |                        |                           |
| **Breed size** | |                        |                           |
| Small breedd | 36 [0–910]                        | 0.0462 ($\chi^2 = 6.150, df = 2$) | 0.6800 ($F_{(2, 108)} = 0.387$) |
| Medium-size breedc | 120 [5–3.164]                        |                        |                           |
| Large breedf | 75 [2–1.785]                        |                        |                           |
| **Breed**  |                                 |                        |                           |
| Pure-bred dogs | 74 [0–3.164]                        | 0.1178 ($Z = 1.564$, $F_{(1, 107)} = 2.200$) |                           |
| Mixed-breed dogs | 130 [3–2.503]                        |                        |                           |
| **Diet**   |                                 |                        |                           |
| Premium diet | 92 [0–3.164]                        | 0.9650 ($Z = −0.044$, $F_{(1, 107)} = 2.200$) |                           |
| Supermarket diet | 74 [2–2.503]                        |                        |                           |
| **Primary environment** | |                        |                           |
| Indoor   | 91 [2–2.503]                    | 0.7425 ($\chi^2 = 0.595, df = 2$) | ns                        |
| Indoor + outdoor access | 73 [1–3.164]                        |                        |                           |
| Outdoor  | 106 [66–645]                   |                        |                           |
| **Travel history** | |                        |                           |
| Yes      | 67 [2–3.164]                    | 0.6355 ($Z = −0.474$)  | ns                        |
| No       | 103 [2–2.503]                   |                        |                           |
| **Current vaccination** | |                        |                           |
| Yesg     | 92 [1–3.164]                    | 0.2873 ($Z = −1.084$)  | ns                        |
| Noh     | 22 [2–770]                      |                        |                           |
| Yes + CPV2 included | 55 [91 [1–3.164]                        | 0.9352 ($Z = −0.081$)  | ns                        |
| Yes + CPV2 not included | 21 [80 [2–1.430]                        |                        |                           |
status or altered gastrointestinal permeability in spayed female dogs (Khalili, 2016), but this hypothesis warrants further study.

Small breed size was significantly associated with higher fecal S100A12 and fecal calprotectin concentrations, but there was no difference in fCalR. This clinically important finding is consistent with a significant effect of breed size on fecal S100A12 concentrations in a large group of healthy puppies (Heilmann, Grellet, et al., 2018). However, it contrasts with the fecal calprotectin results in these puppies (Grellet et al., 2016). Higher fecal S100/calgranulin concentrations in small breed dogs might be explained by differences in the digestive physiology of dogs associated with breed size (increased fecal moisture and numbers of defecation in dogs of larger breeds) (Grellet et al., 2016). However, fecal scores were not obtained in our study. Thus, a potential effect of fecal consistency on the difference in fecal S100/calgranulin concentrations among the different breed sizes cannot be evaluated. However, in healthy puppies, the effect of fecal consistency on S100A12 concentrations was independent of the effect of breed size (Heilmann, Grellet, et al., 2018).

Recent vaccination (within 4 weeks of fecal sample collection)—particularly with the use of a vaccine including CPV2—but not having a current vaccination status (vaccinated within 12 months of fecal sample collection), was associated with higher fecal S100/calgranulin concentrations. This result is of moderate clinical relevance (Hojat & Xu, 2004) and contrasts with the lack of an effect of a vaccine, including diphtheria, tetanus, pertussis, polio and Hemophilus influenzae type B and also a vaccine against measles, mumps and rubella (MMR) on fecal calprotectin concentrations in the post-vaccination period in children (Thjodleifsson et al., 2002). However, vaccination types and strains differ significantly between species, which might explain

![Figure 3](image-url)
the discrepancy of the results. Parenteral administration of a vaccine might also be more associated with a systemic response. However, a trivalent vaccine against canine distemper virus, canine adenovirus-2 and CPV was shown to have good immunogenic properties but to be a poor inducer of a systemic inflammatory response based on systemic markers of inflammation (Romiszewski et al., 2018). Specifically modified live virus (MLV) vaccines against CPV can infect vaccinated dogs and replicate in the intestinal mucosa (Decaro et al., 2014). However, whether intestinal mucosal replication and fecal shedding of CPV-MLV is associated with CPV vaccine-induced (i.e., transient) intestinal inflammation or permeability changes has not been reported and warrants further study.

Finally, RIs for single-sample fecal S100/calgranulin concentrations were established in this study. The RI for fecal S100A12 concentrations in single-spot fecal samples (2–463 ng/g) was similar but slightly narrower compared with the RI previously reported for the 3-day sample mean fecal S100A12 concentrations in a smaller group (n = 53) of healthy pet dogs (2–484 ng/g) (Heilmann, Cranford, et al., 2016), suggesting that a single-day sampling strategy might be sufficient to determine fecal S100A12 concentrations accurately and that the 3-day mean fecal S100A12 concentration might need to be evaluated if values in single-spot samples are increased. However, more data—particularly in dogs with gastrointestinal disease—are needed to determine the optimal sampling strategy and provide a general recommendation for collecting fecal specimens. Similarly, the single-sample RI for fecal calprotectin concentrations in this study (3–39 µg/g) aligned with the RI for the 3-day sample mean fecal calprotectin concentration previously established from a smaller group (n = 52) of clinically healthy pet dogs (3.2–65.4 µg/g) (Heilmann et al., 2011) and also with the single-fecal sample cut-off calprotectin concentration reported in another study in dogs with chronic diarrhoea (48.9 µg/g) (Grellet et al., 2013). Severe outliers (three for fecal S100A12 concentration and five for fecal calprotectin concentration) were removed from the dataset for RI calculation after careful investigation of the dataset (Geffré et al., 2011). The possibility of an occult disease process (e.g. subclinical gastrointestinal infection, primary inflammation or potentially even neoplasia) cannot be excluded in these few dogs, given the non-invasive assessment of health in this study.

We acknowledge that this study had some limitations. Detailed information about the dietary composition and the proportions of wet and canned food was not available for the dogs included in this study. Dietary fibre content has been reported to affect fecal calprotectin concentrations in people (Mendall et al., 2016; Poullis et al., 2004), and the possibility of a similar association in dogs warrants further study. Another limitation is the non-invasive assessment of health in the dogs included in this study. With this approach, the possibility of an occult gastrointestinal disease process (e.g. occult neoplasia) cannot be definitively ruled out in all dogs. However, more invasive diagnostics would not have been feasible or considered ethical. Other lifestyle factors (e.g. walking distance per day, number of other pets in the same household) were not evaluated and need to be studied further. Based on the results of this investigation, prospective studies are also needed to evaluate fecal S100/calgranulin concentrations for potential changes from before to immediately after vaccination, and also to assess the effect of non-steroidal anti-inflammatory drug (NSAID) administration (associated with the risk of NSAID-induced gastropathy or enteropathy (McLean & Khan, 2018)) on fecal S100/calgranulin concentrations in dogs. Further research is also needed into the effect of reproductive (neuter) status on fecal S100/calgranulin concentrations in female dogs.

In conclusion, some clinical characteristics (i.e. breed size, vaccination and reproductive status in female dogs) can affect fecal S100/calgranulin concentrations. Thus, these biomarkers of inflammation should be interpreted in light of those confounding factors. The utility of reference intervals for fecal S100/calgranulin concentrations in dogs might be improved through stratification by sex/reproductive status and breed size. Fecal canine S100/calgranulin concentrations were not confounded by age, body condition, endoparasite treatment, diet, environment or travel history in this study, but further research is needed to validate those results. Measuring fecal S100/calgranulin concentrations in single-spot samples appears reliable, but this should be confirmed in dogs with gastrointestinal disease. This study’s results provide an important basis for further evaluation of the clinical utility of measuring fecal S100/calgranulin concentrations as biomarkers of gastrointestinal disease in dogs.

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CONFLICT OF INTEREST

Niels Grützner serves as Associate Editor for the Journal of Veterinary Medicine and Science. He was not involved with review of this manuscript.

AUTHOR CONTRIBUTION

Melissa M Guard: Conceptualization; Data curation; Investigation; Resources; Writing—original draft. Linda Toresson: Conceptualization; Data curation; Investigation; Resources; Writing—review & editing. Stefan Unterer: Data curation; Investigation; Resources; Writing—review & editing. Aurélien Grellet: Data curation; Formal analysis; Methodology; Resources; Validation; Writing—original draft. Niels Gruetzner: Conceptualization; Data curation; Formal analysis; Writing—original draft.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to. Based on the Institutional Animal Care and Use Committee (IACUC) guideline at Texas A&M University College of Veterinary Medicine and Biomedical Sciences, collecting naturally passed fecal samples from
dogs for the measurement of biomarkers does not require an independent review and ethical approval of the study.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.469.

DATA AVAILABILITY STATEMENT

The data sets analysed during the current study is available from the corresponding author upon reasonable request.

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