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ORIGINAL ARTICLE

The companion dog as a model for inflammaging: a cross-sectional pilot study

Sarah M. Schmid · Jessica M. Hoffman · Jena Prescott · Holley Ernst · Daniel E. L. Promislow · Dog Aging Project Consortium · Kate E. Creevy

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Abstract Inflammaging, the chronic, progressive proinflammatory state associated with aging, has been associated with multiple negative health outcomes in humans. The pathophysiology of inflammaging is complex; however, it is often characterized by high serum concentrations of inflammatory mediators such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and C-reactive protein (CRP). Few studies have evaluated the effects of age on inflammatory cytokines in companion dogs, and most of these studies included dogs of a single breed. In this cross-sectional study, we measured multiple circulating inflammatory markers and hematological parameters in banked serum samples from 47 healthy companion dogs of various breeds enrolled in the Dog Aging Project. Using univariate linear models, we investigated the association of each of these markers with age, sex, body weight, and body condition score (BCS), a measure of obesity in the dog. Serum IL-6, IL-8, and TNF-α concentrations were all positively associated with age. Lymphocyte count was negatively associated with age. Platelet count had a negative association with body weight. IL-2, albumin, cholesterol, triglyceride, bilirubin, S100A12, and NMH concentrations were not associated with age, weight, BCS, or sex after adjustment for multiple comparisons. Our findings replicate previous findings in humans, including increases in IL-6 and TNF-α with age, giving more evidence to the strength of the companion dog as a model for human aging.

Keywords Canine · Inflammaging · Cyotokine · Aging

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S. M. Schmid (✉)
Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA
e-mail: sschmid7@utk.edu

J. M. Hoffman
Department of Biological Sciences, College of Science and Mathematics, Augusta University, Augusta, GA, USA

J. Prescott · H. Ernst · K. E. Creevy
Department of Small Animal Clinical Sciences, School of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX, USA

D. E. L. Promislow
Department of Laboratory Medicine & Pathology, University of Washington School of Medicine, Seattle, WA, USA

D. E. L. Promislow
Department of Biology, University of Washington, Seattle, WA, USA

D. E. L. Promislow
Jean Mayer USDA Human Nutrition Research Center On Aging, Tufts University, Boston, MA, USA

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Abbreviations

CRP	C-reactive protein
DAP	Dog Aging Project
IL	Interleukin
NMH	N-Methylhistamine
TNF	Tumor necrosis factor

Introduction

Aging-induced remodeling of the immune system known as immunosenesce is characterized by alterations in the composition, quantity, and function of immune organs, immune cells, and cytokines [1]. The physiological changes that drive these immune changes are complex but may involve a depletion of naïve T-cells, production of reactive oxygen species via mitochondrial dysfunction [2], and the development of senescent cells that secrete cytokines, chemokines, growth factors, and proteases [3]. The molecular mechanisms of these changes among many other age-related changes have been explored elsewhere [4–7]. This overall dysregulation of innate immunity that occurs with age can lead to a state of chronic low-grade inflammation, independent of acute illness or injury, known as inflammaging [8, 9]. Inflammaging is thought to contribute to the development of age-related comorbidities, disability, frailty, and reduced life expectancy [9]. Consequently, inflammaging and the mechanisms by which it occurs has emerged as a significant area of interest in the field of geroscience.

A hallmark of inflammaging is the altered expression of various cytokines and acute phase proteins. In aging people, studies have documented exacerbated production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-8, and IL-1β and the acute phase protein, C-reactive protein (CRP) [8, 10–13]. In contrast, the expression of IL-2, a T-cell growth factor, has been shown to undergo an age-related decline in humans and rodents [14]. IL-6 plays a pivotal role in acute phase responses by promoting the expansion and activation of T-lymphocytes, differentiation of B-lymphocytes, and synthesis of positive acute phase proteins such as CRP and serum amyloid A [15]. IL-6 has been identified as a reliable marker of inflammaging, as circulating levels increase with age and disability, and as a predictor of mortality [16–23]. In addition, IL-6 concentrations have been shown to be positively associated with age-related diseases in people including type 2 diabetes, cardiovascular disease, Alzheimer's, cancer, and arthritis [15, 24–26].

Similar to IL-6, increased levels of TNF-α and CRP have been identified as strong independent risk factors for morbidity and mortality in older people [10, 20, 21, 27]. Through its role in the initiation of the inflammatory cascade within the arterial wall, TNF-α has also emerged as an important contributor to the development of atherosclerotic lesions in people [28]. Proinflammatory cytokines such as TNF-α and IL-6 induce cellular senescence by producing reactive oxygen species, while the resulting DNA damage activates the NF-κβ signaling pathway resulting in the release of proinflammatory cytokines (such as IL-1, IL-6, and IL-8) [6]. As a result, a vicious cycle of reciprocal causation is created, aggravating inflammaging.

While evaluation of specific inflammatory cytokines is an emerging focus in the assessment of aging individuals, parameters included on routine clinicopathological assessments have also been shown to change with age. The complete blood count, which provides enumeration of platelets, red blood cells, and total white blood cell count including absolute counts of neutrophil, lymphocyte, eosinophil, and basophil subsets, and the serum biochemistry profile, which measures certain circulating proteins, fats, electrolytes, and metabolites, are two such routine assessments. Absolute counts of neutrophils have been shown to increase with age, while absolute platelet and lymphocyte counts decrease with age [29]. Biochemical markers of lipid metabolism including cholesterol and triglycerides have been shown to increase with age while other parameters such as albumin have been shown to decrease with age [30, 31].

Despite the strong evidence of a proinflammatory state associated with multimorbidity and mortality in people, the specific mechanisms to explain why inflammaging occurs and the identification of interventions, if any, that may reduce this chronic inflammatory burden remain to be elucidated.

Considering the complexity of aging, and inflammaging specifically, there is a need for multi-perspective studies, particularly those that allow longitudinal evaluation of inflammaging biomarkers. To this end, various animal models have

provided valuable insights into the biological, cellular, and molecular changes associated with the aging process [32]. The companion dog may provide an ideal model to study complex aging-related phenotypes, as dogs share the same environment as humans, but age approximately five to seven times faster than people [33]. Similar to people, dogs vary considerably in size and shape, which has been shown to impact their longevity. Furthermore, dogs demonstrate similar features of aging and frailty as humans [33–35].

Similar to humans, aging dogs have been shown to undergo changes in the relative numbers of B-lymphocytes, T-lymphocytes, and their subsets in peripheral blood [36, 37]. Patterns of pro-inflammatory cytokines in aging dogs have also been investigated; however, most studies have focused on single dog breeds or have been confined to a single geographical area. In addition, the results have been mixed. For example, in a homogenous population of German Shepherd dogs in the police service, IL-2 serum concentrations and white blood cell and lymphocyte counts were lower in the older group [38]. Another study found that across diverse breeds of dogs, young dogs have lower serum IL-6 concentrations, but no difference was found between adult, senior, and geriatric dogs [39]. In contrast, another study found no differences between young and old dogs for IL-1, IL-6, and TNF-α concentrations from peripheral blood mononuclear cells in culture, though only two breeds were analyzed (fox terriers and Labrador retrievers) [40]. Due to these conflicting reports, more study is needed to better understand how and why inflammatory markers change with age in dogs.

The purpose of this cross-sectional pilot study was to identify potential biomarkers of inflammaging in a diverse group of healthy dogs at various ages. In addition to their association with age, we also were interested in evaluating whether these biomarkers correlated with body weight, body condition score, or sex. As such, we evaluated circulating blood cell counts, biochemical parameters, serum cytokines (IL-2, IL-6, IL-8, and TNF- α), the acute phase protein CRP, and various other inflammatory markers (N-methylhistamine, S100A12) in healthy dogs of various ages, breeds, and body sizes.

Methods

Dog Aging Project and participant eligibility

The Dog Aging Project (DAP) is a nationwide community science study aimed at determining the different factors that are associated with age in the companion dog [41]. The DAP has been thoroughly explained previously [42]. Briefly, all privately owned dogs in the United States are eligible for enrollment in the Dog Aging Project, and potential participants nominate their dogs through the DAP website (www.dogagingproject.org). Participants provide informed consent and complete a ten-part Health and Life Experience Survey (HLES) on a password protected REDCap (Research Electronic Data Capture) portal [43, 44]. The HLES collects information on dog demographics, behavior, environment, physical activity, diet, and health history. Uploading of veterinary electronic medical records (VEMR) is strongly encouraged, and if completed, the dog is eligible for enrollment into smaller, nested cohorts. A subset of these DAP participants are enrolled in the precision cohort, where they receive annual biospecimen kits for the longitudinal collection of whole blood, plasma, serum, urine, hair, and feces. Each participant's primary care veterinarian assists in collecting these biospecimens. Following receipt of samples, a complete blood count, biochemical profile, and urinalysis are performed at the Texas Veterinary Medical Diagnostic Laboratory (TVMDL). The remaining biospecimen samples are biobanked at – 80 ℃ for analysis of the metabolome, microbiome, epigenome, and flow cytometry profiles, as well as for inflammaging assays.

Dogs were eligible for inclusion in the current study if they (1) were enrolled in the DAP precision cohort at the time the study was performed, (2) completed their initial biospecimen collection, (3) had no current or historical diseases reported on their Health and Life Experience Survey (HLES), and (4) had adequate banked serum samples available for inflammaging assays. For each dog, a board-certified small animal veterinary internist (SMS) reviewed the veterinary electronic medical records and laboratory results completed at the TVMDL for inclusion. Included dogs were determined to be healthy on the basis of (1) a lack of owner-reported diseases/conditions in HLES, (2) the absence of significant chronic

diseases reported in the VEMR, and (3) lack of significant abnormalities on the complete blood count, biochemistry profile, and urinalysis completed as part of the precision biospecimen kit. Dogs were excluded if they were determined to be unhealthy, had a chronic (≥ 3-week duration) history of receiving medications known to impact the immune system (lokivetmab (Cytopoint, Zoetis US, Parsippany NJ), oclacitinib (Apoquel, Zoetis US, Parsippany NJ), or corticosteroids) within 1 year of sample collection, could not be confirmed to have been fasted a minimum of 12 h prior to sample collection, or had 3 + or 4 + hemolysis, lipemia, or icterus on their screening bloodwork. Current or previous exposure to non-steroidal anti-inflammatory drugs was not exclusionary.

Sample collection and storage

Blood samples for analysis were collected by each study participant's primary care veterinarian. Each dog owner was provided a sample kit including collection vials, an insulated cooler, an ice pack, a pre-addressed shipping label, and shipping instructions. Primary care veterinarians were instructed to collect whole blood, plasma, serum, and urine. For serum samples, whole blood was centrifuged and the resultant supernatant (serum) was transferred to a clean blood tube prior to shipping. On the same day of collection, samples were shipped on ice and directly to the TVMDL for processing and testing. Upon arrival, the temperature of each sample was evaluated and a complete blood count, biochemical profile, and urinalysis were performed on each dog. Serum was aliquoted and frozen at – 80 °C for later analysis of serum inflammatory markers. Varying amounts of serum were recovered from each patient, and based on how much serum was available, aliquots for various analytes were generated in the following order: lipoprotein profile, cytokine profile, CRP, S100A12, and NMH. For the majority of dogs, these aliquots were created and frozen within 4 h of receipt, but rarely, serum was stored for up to 72 h at 4 °C before aliquoting and freezing. Samples with marked (3 + or 4 +) hemolysis, lipemia, or icterus were excluded.

Complete blood count

Hematological analysis included automated neutrophil, lymphocyte, and platelet counts (1000 cells/μL) and was performed using an Advia 120 analyzer (Siemens) at the TVMDL. Visual review of cellular morphology by laboratory personnel was also provided.

Serum biochemistry

Serum biochemical analysis included albumin (g/DL), cholesterol (mg/dL), triglycerides (mg/dL), and total bilirubin (mg/dL) among other routine parameters and was analyzed using a DxC700AU analyzer (Beckman Coulter) at the TVMDL.

Serum cytokine measurement

Serum cytokine analysis was performed at the Texas A&M University Gastrointestinal Laboratory (TAMU GI Lab). Serum cytokine concentrations (pg/mL) were determined via the canine electrochemoluminescent multiplex cytokine kit (Proinflammatory Panel 3 (4-Plex); Mesoscale Discovery, Rockville, Maryland, USA) using banked serum samples and the manufacturer's guidelines. This kit consisted of antibodies against canine TNF-α (inter-assay CV = 23.5%, intra-assay CV = 6.9%, and LLOD = 0.17 pg/mL), IL-2 (inter-assay CV = 12.2%, intra-assay CV = 9.8, and LLOD = 7 pg/mL), IL-6 (inter-assay CV = 10.6%, intra-assay CV = 10.2%, and LLOD = 2.4 pg/mL), and IL-8 (inter-assay CV = 18.6%, intra-assay CV = 5.5%, and LLOD = 1.3 pg/mL). Although the stability of canine cytokine concentrations is not known, the cytokines TNF-α, IL-2, and IL-6 have been shown to be stable in separated human serum samples when stored for up to 6 days at 4 °C prior to freezing [45–49]. IL-8 has been shown to be stable in serum at 4 °C for up to 24 h, but can increase when stored at room temperature [49]. Cytokine analysis was performed within 1 year of samples being frozen at – 80 °C and prior to any freeze–thaw cycles. The cytokines TNF- α, IL-2, and IL-6 have previously been determined to be stable in human serum for up to 2 years of storage at – 80 °C and up to three freeze–thaw cycles [50]. IL-8 has been shown to be stable for up to 1 year of storage at – 80 °C and up to one freeze–thaw cycle [50]. Each sample for each dog was run in duplicate on the sample plate, and a mean value was calculated based on standardized canine controls. All data were assessed to see if the lower limit of detection was reached. The LLOD was met in all cases.

N-Methylhistamine (NMH) measurement

Serum N-methylhistamine (NMH) concentrations (pg/μL) were measured at the TAMU GI Lab. Although the stability of NMH in canine serum samples has not been determined, plasma NMH concentration in canine whole blood has been shown to not change after 28 days of storage at 4 °C [51]. NMH concentrations were then measured in duplicate using gas chromatography–mass spectrometry as previously described [51, 52]. Briefly, following the addition of tri-deuterated NMH standards to the serum sample, NMH was extracted by use of a solid-phase method [53]. The final extract was resuspended in ethyl acetate and injected into the gas chromatography-mass spectrometer for analysis. The NMH concentration was determined by comparison of the area under each chromatographic peak for a given serum sample with the area under the chromatographic peak for the internal standard.

Measurement of CRP and serum S100A12 concentrations

Serum CRP and S100A12 concentrations were measured at the TAMU GI Lab as previously described [54]. CRP concentrations (mg/L) were measured with a previously validated commercially available ELISA (PHASE Canine CRP Assay Kit, Tridelta Development, Maynooth, County Kildare, Ireland) [55]. Serum CRP assays were run in duplicate as batches using the reagents and quality controls provided by the manufacturer. The lower limit of detection is 10 mg/L with an assay range of 10–300 mg/L. Serum S100A12 concentrations (ng/mL) were run in duplicate using an in-house ELISA validated for use in dogs [56].

Body condition scoring

Adipose tissue releases pro-inflammatory cytokines, known as adipokines [57, 58], and thus, obesity could be a large driver of circulating inflammatory markers. Body condition score (BCS) is a common method to evaluate body fat mass in animals. Among BCS scoring systems, a 9-point scale BCS is most commonly used in dogs, with 4 and 5 representing ideal weight and a BCS of 8 or 9 indicating obesity [59]. Preliminary data from DAP shows that body condition scores (BCS) are only available in 29% of VEMRs [60]. Consequently, each participant was asked to submit a lateral and dorsal photograph of their dog standing for review of body condition. BCS was assessed from these photographs by a single board-certified veterinary internist (SMS) as previously described [61].

Statistical analysis

All statistical analyses were completed in the program R version 4.2.1 (www.r-project.org). All blood markers were log transformed for normality, based on visual inspection, except for lymphocytes which were already normal and not transformed. Univariate linear models were used to evaluate the effects between age, body weight, sex, and BCS and each marker. The threshold for significance was set at $P < 0.05$, and we then applied a Bonferroni correction based on the number of tests done for each parameter, setting the critical P-value for multiple comparisons to $P < 3.85\text{E}-03$.

Results

Dogs

A total of 49 healthy dogs were included in the profiling analysis. Two dogs were removed for outlier IL-6 values (one more than tenfold higher than the rest and the other tenfold lower than the rest), for a total of 47 dogs (21 male, 26 female) analyzed in the final cohort. Twenty-nine purebred dogs and 18 mixed-breed dogs were included, representing a variety of dog breeds (Supplemental Table 1). Most dogs were neutered (44); however, 2 reproductively intact male dogs and 1 intact female dog were included. The average age was 5.6 years (range 0.7–16.4 years). When classifying by age, there were 12 young (< 3 years of age), 18 adult (3–6 years of age), 13 senior (7–10 years of age), and 4 geriatric (11 + years of age) dogs. Included dogs weighed a median of 24.1 kg (range 2.5–72.1 kg) and had a variety of BCS, with two dogs considered to be underweight (less than 4/9), 24 overweight (greater than 5/9), and 21 with an ideal (4/9 or 5/9) BCS.

Blood inflammatory markers

The results of all inflammatory markers are summarized in Table 1 and Supplemental Table 2. Five dogs did not have banked samples of SA100A12 and NMH as insufficient serum was recovered. This resulted in a sample size of 42 for both SA100A12 and NMH. All other markers were measured in all 47 dogs. Lymphocyte count was negatively associated with age (Fig. 1). IL-6, IL-8, and TNF-α were all positively associated with age (Fig. 2B–D). Platelet count was negatively associated with body weight (Fig. 3A). Neutrophil count was not associated with age or body weight, and no hematological analytes or cytokines measured were associated with sex or BCS. No biochemical analytes were associated with age, body weight, BCS, or sex, following correction. S100A12 was not associated with age, body weight, BCS, or sex. NMH was not associated with age, body weight, BCS, or sex. CRP was unable to be statistically analyzed due to all dogs having CRP values below the limit of detection (< 10 mg/L).

Table 1 Statistical associations of serum inflammatory markers and age, sex, weight, and BCS from 47 healthy companion dogs

Marker	Units	Age		Weight		BCS		Sex	
		Slope (SE)	*P* value	Slope (SE)	*P* value	Slope (SE)	*P* value	Slope (SE)	*P* value
Hematology analytes									
Neutrophil count	Log (1000 cells/μL)	0.002 (0.005)	0.729	−0.002 (0.001)	0.048	0.029 (0.013)	0.038	−0.034 (0.037)	0.352
Lymphocyte count	1000 cells/μL	−0.141 (0.027)	**3.97E−06***	0.001 (0.008)	0.744	−0.007 (0.096)	0.941	−0.039 (0.252)	0.878
Platelet count	Log (1000 cells/μL)	0.015 (0.007)	**0.022**	−0.006 (0.002)	**8.79E−04***	0.055 (0.019)	**0.006**	0.009 (0.053)	0.857
Biochemistry analytes									
Albumin	Log (g/dL)	−0.001 (0.001)	0.349	0.001 (0.001)	0.579	0.005 (0.002)	0.068	−0.009 (0.007)	0.171
Cholesterol	Log (mg/dL)	0.003 (0.005)	0.526	0.003 (0.001)	0.021	0.002 (0.014)	0.871	0.044 (0.036)	0.253
Triglyceride	Log (mg/dL)	0.026 (0.009)	**0.013**	−0.001 (0.002)	0.645	0.058 (0.024)	0.056	0.056 (0.066)	0.490
Total bilirubin	Log (mg/dL)	0.001 (0.001)	0.594	0.001 (0.001)	0.968	0.001 (0.001)	0.952	0.001 (0.007)	0.835
Cytokines									
IL−2	Log (pg/mL)	0.048 (0.016)	**0.008**	−0.003 (0.004)	0.465	0.052 (0.046)	0.330	0.149(0.121)	0.273
IL−6	Log (pg/mL)	0.069 (0.014)	**3.51E−04***	−0.002 (0.004)	0.553	0.038 (0.043)	0.508	0.023 (0.113)	0.877
IL−8	Log (pg/mL)	0.045 (0.014)	**3.79E−03***	−0.009 (0.003)	**0.008**	0.074 (0.040)	0.103	0.117 (0.107)	0.316
TNF−α	Log (pg/mL)	0.043 (0.014)	**2.92E−03***	−0.006 (0.004)	0.086	0.035 (0.042)	0.407	0.038 (0.110)	0.731
Inflammatory proteins									
S100A12	Log (ng/mL)	−0.007 (0.016)	0.651	−0.004 (0.004)	0.022	0.073 (0.043)	0.093	−0.018 (0.120)	0.877
NMH	Log (pg/μL)	−0.013 (0.008)	0.116	−0.003 (0.002)	0.128	0.032 (0.025)	0.198	0.143 (0.058)	**0.015**

A univariate linear model was performed to evaluate the effects of age, sex, weight, and BCS on each marker of interest. $n=47$ except for S100A12 and NMH ($n=42$). All markers were log transformed with the exception of lymphocytes in which raw values were used. The threshold for significance was set at $P<0.05$ (numbers in bold) and we then applied a Bonferroni correction based on the number of tests done for each parameter, setting the critical *P* value for multiple comparisons to $P<3.85E-03$ (*)

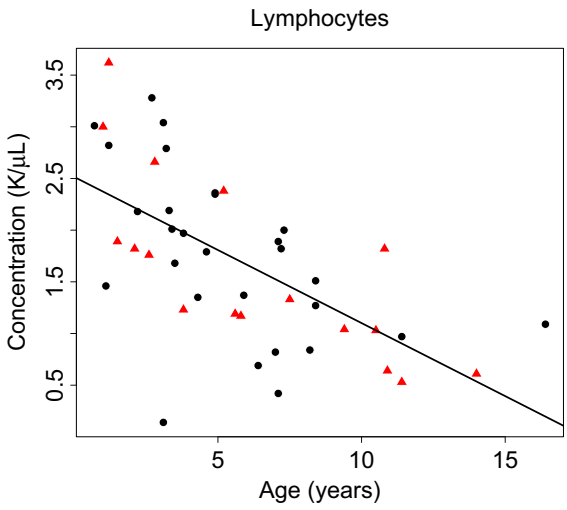

Fig. 1 Association of lymphocyte count with age. Purebred dogs are represented by red triangles and mixed-breed dogs by black circles. Linear regression statistics are in Table 1. $n=47$ (21 male, 26 female)

Discussion

Inflammaging, or the chronic, low-grade sterile inflammation that occurs with aging, appears to represent an overall dysregulation of innate immunity and unfolds through complex physiological and pathophysiological interactions among immune organs, immune cells, and the cytokines they produce [1–3, 5, 7, 8]. In people, the presence of markers of inflammaging has been associated with increased risk of frailty, morbidity, and mortality [8, 9]. Here, we demonstrate that some markers of inflammaging show patterns of age-related change in companion dogs that are similar to age-related changes described in people.

We found that IL-6 concentrations were strongly positively associated with age in dogs, similar to the increase in serum concentrations of IL-6 with age seen in people [62]. IL-6 concentrations have been shown to be positively associated with age-related diseases such as type 2 diabetes, cardiovascular disease, Alzheimer's, cancer, and arthritis [15, 24–26]. Given these associations, IL-6 has been proposed to be a reliable marker for the functional decline as a predictor of morbidity and mortality in elderly individuals [11, 19]. Only one other study has directly evaluated the association of serum concentrations of IL-6 with age in apparently healthy dogs. This study found that dogs less than 3 years of age had lower IL-6 concentrations than all other dogs, but there was no difference in IL-6 concentrations among dogs in age groups 3–6 years, 7–10 years, and 11 + years [39]. However, review of patients' medical history and clinical pathology results was not part of that prior study design. This could have resulted in the inclusion of dogs with subclinical diseases (e.g., chronic kidney disease) that had

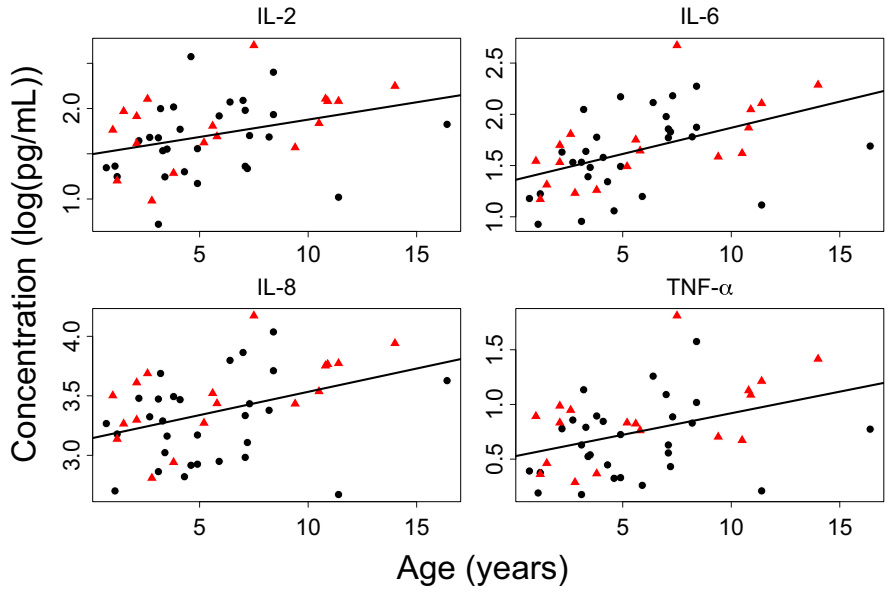

Fig. 2 Association of serum cytokine concentrations with age. Linear regression between age and **A** interleukin (IL)-2, **B** IL-6, **C** IL-8, and **D** tumor necrosis factor-α (TNF-α). Purebred dogs are represented by red triangles and mixed-breed dogs by black circles. All serum cytokine concentrations were log transformed for normality, based on visual inspection. $n=47$ (21 male, 26 female). Linear regression statistics are in Table 1

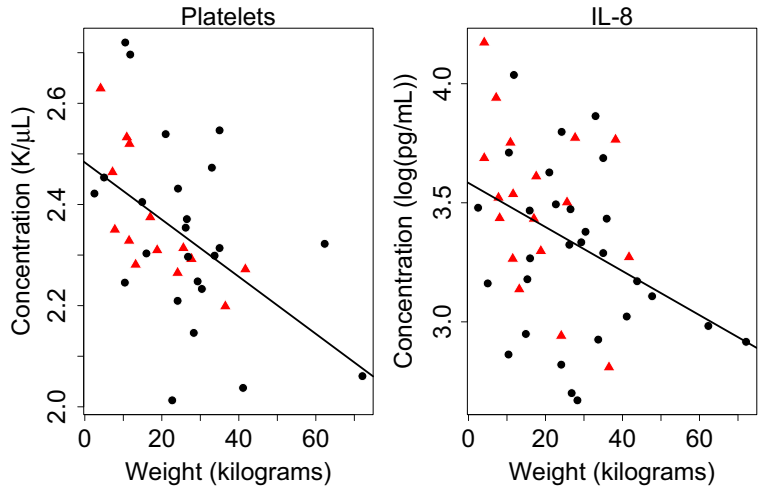

Fig. 3 Association of platelet count and serum interleukin-8 concentrations with weight. Linear regression between weight and **A** platelet count and **B** serum IL-8 concentrations. Purebred dogs are represented by red triangles and mixed-breed dogs by black circles. Platelet count and IL-8 concentrations were log transformed for normality, based on visual inspection. $n = 47$ (21 male, 26 female). Linear regression statistics are in Table 1

not been reported by owners, whereas in the study reported here, such dogs would have been excluded due to abnormalities in clinical pathology results. These differences in inclusion criteria, in conjunction with the use of a different cytokine assay, could have contributed to the different findings between the two studies.

Interleukin-8 (IL-8) is a chemoattractant cytokine that plays a key role in the recruitment and activation of neutrophils [25] and that has been associated with age-related morbidities, most notably sarcopenia and age-related macular degeneration [63]. We found that IL-8 concentrations were positively associated with age in dogs. This could have several implications in the development of disease, as IL-8 gene expression has been shown to be up-regulated in canine hemangiosarcoma [64] and mammary gland tumors [65].

Tumor necrosis factor-alpha (TNF-α) has been shown to increase with age and is associated with age-related disease, such as atherosclerosis, in humans [10], and we found evidence of a similar pattern in companion dogs. This is in contrast to two previous studies where no differences in serum TNF-α concentrations were identified between groups of young, adult, and old dogs [38, 39]. One study was of 41 German Shepherd dogs in police service, and the other consisted of dogs sampled from a single geographical area (New York). By contrast, the dogs included in our study were of a variety of sizes, breeds, and environments. In addition, in our study, age was analyzed as a continuous variable rather than dogs being grouped into age bins for analysis.

We documented a weak positive association between IL-2 and age that was lost with adjustment for multiple comparisons. Studies investigating IL-2 in aging people have yielded variable results depending on the cell subtype analyzed. In several studies, an age-related decrease in IL-2 production from stimulated peripheral blood mononuclear cells or lymphocytes, and in serum IL-2 levels, has been reported [66, 67]. Conversely, a study of CD8 + T cells obtained from 29 healthy people of different ages showed a positive association between type 1 cytokine-positive cells, IL-2 concentrations, and TNF-α concentrations and age [68].

Unexpectedly, we were unable to determine the effects of age, sex, and BCS on CRP, which is one of the most widely studied positive acute phase proteins in veterinary medicine. Healthy dogs often have CRP concentrations less than 10 mg/L, which was the lower limit of detection with the CRP assay used in this study [69–71]. The consistently low concentrations of CRP in our population may offer support that other findings in our population were not influenced by acute inflammation. To date, there is no evidence to suggest canine CRP concentrations differ by age [72]. Although S100A12 is a well-known systemic marker of inflammation in people [73] and has been associated with inflammatory bowel disease in dogs [74], our study found no association between S100A12 and age.

Similar to what has been shown in a homogenous population of aging German Shepherds in the police force [38], we found a negative association between

absolute peripheral lymphocyte count and age in a relatively heterogenous population of companion dogs. In people, differences in the number and types of lymphocytes in circulation is one of the hallmark changes seen in the immune system with age, and lymphopenia has been associated with reduced survival in adults [75]. Similar to previous studies showing platelet count increasing with age [76], our study found a weak association between platelet count and age. However, this finding was lost with adjustment for multiple comparisons.

In our study, serum triglyceride concentrations had a weak positive association with age that was lost with adjustment for multiple comparisons. In people, plasma triglyceride concentrations are higher in older adults compared to younger adults [68, 77, 78], and a similar pattern has been previously shown in dogs [79]. No other biochemical analytes were associated with age, body weight, BCS, or sex.

None of the studied inflammatory markers were found to be associated with sex, although prior to adjustment for multiple comparisons, N-methylhistamine (NMH), a metabolite that is used as a marker of mast cell degranulation [80], appeared to be higher in female dogs. Given that the majority of the dogs in this study were sterilized, associations between sex and parameters we measured might have been suppressed. Larger studies evaluating serum inflammatory markers in reproductively intact dogs are needed, though evidence suggests dogs may not show the same degree of sex differences in aging phenotypes as seen in humans [81].

Despite including a wide variety of dog breeds and sizes, few of the markers were found to be associated with weight. Platelet count was found to be negatively associated with weight, such that smaller dogs had higher platelet counts, which has not been previously described to the authors' knowledge. In addition, smaller dogs appeared to have higher IL-8 concentrations than their larger counterparts, although significance was lost with adjustment for multiple comparisons.

In people, obesity has been strongly associated with a pro-inflammatory state. Adipose tissue releases cytokines, known as adipokines, which can promote inflammation. Aging has been shown to not only increase the amount of visceral fat, but also to increase macrophage infiltration of adipose tissue, and to alter the adipokine profile produced by adipose cells [58]. We found no significant association between BCS and the inflammatory markers evaluated, despite nearly 50% of dogs included being overweight. Previous studies in dogs have shown variable results. In one study, IL-6, but not IL-8, concentrations were found to be higher in overweight dogs [82]. However, another study found IL-8, but not IL-6 or TNF-α, concentrations decreased with weight loss in obese Beagle dogs [83]. In yet another study, no differences between IL-6 and TNF-α were found between obese and normal weight healthy pet dogs [84].

While our results demonstrate an association of age with IL-6, IL-8, and TNF-α, there were several limitations to our study. Our sample size was modest, and the population of dogs included was quite heterogenous, comprising multiple ages, breeds, and body sizes. The majority of the dogs in our study were sterilized, with only three reproductively intact dogs, limiting our ability to study sex-based differences. Given the many factors that are likely to influence inflammatory markers (e.g., age, size, sex, breed, disease history, and environmental factors), a much larger sample size would be needed to evaluate the main effects of these factors and their potential interactions. We also lacked quantitative measures of body condition, including fat and muscle. Future studies, especially longitudinal studies, with a larger sample size are needed to better tease apart the effects of age, sex, weight, BCS, and body composition, and the interactions between these traits, on inflammatory parameters in dogs. As stated above, our CRP concentrations were below the threshold of detection for all dogs, making any age-related comparisons impossible. Future studies with a lower threshold of detection are required to study if CRP is an inflammaging marker in dogs. Finally, although our standard operating procedures document sample arrival conditions to exclude compromised samples, we are aware that it is still possible that alterations in temperature or time in post-collection shipping or processing could have occurred. All samples in this study underwent the same collection and storage process. Thus, if the degradation, absorption, or cellular production of cytokines occurred during shipment or storage, it should have been at random and would have been unlikely to have generated bias in favor of false positive age signatures.

In conclusion, here, we have presented one of the most comprehensive studies on age-related changes in markers of chronic inflammation in the companion dog. We replicated some previous findings in humans, including increases in IL-6 and TNF-α with age, which supports the role of these analytes as candidate biomarkers of inflammaging in dogs and provides more evidence to the potential of the companion dog as a model for human aging. Conflicting results between findings from our study and prior work in dogs indicate that much more work is necessary to understand these age-related changes in dogs. Future studies with larger sample sizes are needed to more thoroughly investigate the role of inflammatory markers in aging in the dog, and ultimately, to understand the causal effects of inflammatory burden on morbidity and mortality in dogs.

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[1]Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ, USA

[2]Department of Laboratory Medicine and Pathology, University of Washington School of Medicine, Seattle, WA, USA

[3]Department of Clinical Microbiology and Immunology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

[4]Cornell Veterinary Biobank, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

[5]Department of Small Animal Medicine and Surgery, College of Veterinary Medicine, University of Georgia, Athens, GA, USA

[6]Department of Small Animal Clinical Sciences, Texas A&M University College of Veterinary Medicine & Biomedical Sciences, College Station, TX, USA

[7]Department of Sociology, University of Washington, Seattle, WA, USA

[8]Center for Studies in Demography and Ecology, University of Washington, Seattle, WA, USA

[9]Department of Veterinary Physiology and Pharmacology, Texas A&M University College of Veterinary Medicine & Biomedical Sciences, College Station, TX, USA

[10]Department of Family Medicine, University of Washington, Seattle, WA, USA

[11]Department of Veterinary Pathobiology, Texas A&M University College of Veterinary Medicine & Biomedical Sciences, College Station, TX, USA

[12]Department of Laboratory Medicine and Pathology, University of Washington School of Medicine, Seattle, WA, USA

[13]Department of Laboratory Medicine and Pathology, University of Washington School of Medicine, Seattle, WA, USA

[14]Bioinformatics and Integrative Biology, University of Massachusetts Chan Medical School, Worcester, MA, USA

[15]Department of Biostatistics, University of Washington, Seattle, WA, USA

[16]Department of Small Animal Clinical Sciences, Texas A&M University College of Veterinary Medicine & Biomedical Sciences, College Station, TX, USA

[17]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

[18]Department of Biostatistics, University of Washington, Seattle, WA, USA

[19]Department of Laboratory Medicine and Pathology, University of Washington School of Medicine, Seattle, WA, USA

[20]Department of Population Health Sciences, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA

[21]Epidemiology Program, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

[22]Collaborative Health Studies Coordinating Center, Department of Biostatistics, University of Washington, Seattle, WA, USA

[23]School of Life Sciences, Arizona State University, Tempe, AZ, USA

[24]Department of Small Animal Clinical Sciences, Texas A&M University College of Veterinary Medicine & Biomedical Sciences, College Station, TX, USA

[25]Department of Laboratory Medicine and Pathology, University of Washington School of Medicine, Seattle, WA, USA

[26]Treuman Katz Center for Pediatric Bioethics, Seattle Children's Research Institute, Seattle, WA, USA

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Declarations

Competing interests The authors declare no competing interests.

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