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CD4+/CD8+ Ratio and Growth Differentiation Factor 8 Levels in Peripheral Blood of Large Canine Males Are Useful Parameters to Build an Age Prediction Model

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Purpose: To build an age prediction model, we measured CD4+ and CD8+ cells, and humoral components in canine peripheral blood.

Materials and Methods: Large Belgian Malinois (BGM) and German Shepherd Dog (GSD) breeds (n=27), aged from 1 to 12 years, were used for this study. Peripheral bloods were obtained by venepuncture, then plasma and peripheral blood mononuclear cells (PBMCs) were separated immediately. Six myokines, including interleukin (IL)-6, IL-8, IL-15, leukemia inhibitory factor (LIF), growth differentiation factor 8 (GDF8), and GDF11 were measured from plasma and CD4+/CD8+ T-lymphocytes ratio were measured from PBMC. These parameters were then tested with age prediction models to find the best fit model.

Results: we found that the T-lymphocyte ratio (CD4+/CD8+) was significantly correlated with age (r=0.46, p=0.016). Among the six myokines, only GDF8 showed a significant correlation with age (r=0.52, p=0.005). Interestingly, these two markers showed better correlations in male dogs than females, and BGM breed than GSD. Using these two age biomarkers, we could obtain the best fit in a quadratic linear mixed model (r=0.77, p=3×10^{-6}).

Conclusions: Age prediction is a challenging task because of complication with biological age. Our quadratic linear mixed model using CD4+/CD8+ ratio and GDF8 level showed a meaningful age prediction.

Keywords: Age prediction model; Aging; CD4-CD8 ratio; Dogs; Myostatin

INTRODUCTION

A typical phenomenon of aging is a time-dependent functional decline. Like other species, dogs show a decline in their physical ability with advanced age. In canines, aging-related multiple functional declines, such

as muscle weakness, and changes in blood composition have been observed [1,2]. Body size shows a negative correlation with lifespan when large and small dogs were compared [3]. But, this negative correlation can be related to a higher death rate caused by cancer in large dogs compared to that in small dogs [4]. Indeed, there is no relationship between lifespan and body size in the same breed [5].

Gradual deterioration of the immune system is a well-known phenomenon of mammalian aging. Consequently, the senescent immune system fails to protect the body against disease, and makes unable to distinguish between self and non-self antigens in the body [6]. Therefore, age-associated changes of the immune system including innate and adaptive immunity have been studied as an important risk factor, and a hallmark of aging and age-related diseases in humans [7]. Also, several anti-aging agents such as rapamycin, aspirin, curcumin are well known to affect immune function [8]. Hematopoietic stem cells (HSCs) give rise to multipotent progenitor cells including lymphoid and myeloid cells that involve in both innate and adaptive immunity [9]. The decline of proliferative potential of these HSCs was observed in conjunction with the advanced age of cell donors [10]. Moreover, total lymphocyte numbers are decreased with age, and the proportion of lymphocyte subset changes in the peripheral blood with age in humans [11]. In particular, the ratio of CD4+ T-lymphocytes to CD8+ T-lymphocytes (CD4+/CD8+ ratio) has been used as a marker of age-associated disease in several studies, including human immunodeficiency virus (HIV)-infected patients, Singapore Longitudinal Ageing Study cohort, and elderly with frailty syndrome [12-14]. In elderly dogs, the decline of immune functionality and responsiveness to vaccination was clearly observed [15]. Consequently, old dogs have poorer defense against infection and are susceptible to autoimmune and immunodeficiency diseases. In 2001, Greeley et al [16] tracked 23 Labrador Retriever dogs from birth to death to identify age-dependent changes in various lymphocyte subsets of the peripheral blood. They found that the absolute numbers of total lymphocytes, including CD3+, CD4+ and CD8+ cells, decreased over time [16]. In particular, the proportion of CD4+ cells was decreased while the proportion of CD8+ cells was increased with age, along with maintaining the proportion of CD3+ cells [17]. To date, many studies have shown a decrease in the CD4+/CD8+ ratio [15,17].

All living organisms suffer from frailty due to a loss of muscle strength and endurance as they get older. Therefore, aging and muscle seem to be closely related to each other. The skeletal muscle cells produce and release cytokines, termed as myokines, which play active autocrine, paracrine, or endocrine roles [18]. Five representative myokines play important roles in muscles and whole body [18,19]; these include interleukin (IL)-6, leukemia inhibitory factor (LIF), IL-8, IL-15, and growth differentiation factor 8 (GDF8). IL-6 is an exercise mediator with immunoregulatory, anti-inflammatory, metabolic, and hypertrophic effects [19]. IL-6 is released into the blood circulatory system after muscle contraction [20]. LIF, which belongs to the IL-6 cytokine superfamily, has diverse biological functions including platelet formation, bone formation, adipocyte lipid transport, neuronal formation, and muscle satellite cell proliferation [21]. IL-8, primarily produced by the macrophage and endothelial cell, exerts strong chemotactic activity towards neutrophils [20] and acts as an angiogenic factor [22]. IL-15 is known as an anabolic cytokine involving in the resistance exercise for muscle mass in humans [23]. IL-15 is also involved in lipid metabolism and fat oxidation [24]. GDF8, known as myostatin, plays a regulatory role in skeletal muscle growth [25]. When the GDF8 was overexpressed in the transgenic mice, muscle mass was decreased [26]. GDF8 is also involved in regulation of adipose tissue growth [25].

Recently, GDF11, a transforming growth factor β superfamily member, has been suggested as a putative anti-aging factor in mice [27]. GDF11 is a circulating factor that prevents cardiovascular disease, cardiac hypertrophy, and cardiovascular death. Indeed, it was confirmed that GDF11 concentration was lower in older mice [27]. Conversely, in a recent study in humans, GDF11 was found to be involved in the risk of comorbidity, frailty, and surgery in the elderly with cardiovascular disease [28].

Aging causes physical frailty, and frailty is associated with aged muscles [29]. Aged muscles show structural distortions [30] as well as alterations of plasma factors, including the aforementioned myokines, which might induce systemic aging [31]. Immunosenescence, including alterations of T-lymphocytes, has been suggested as a major hallmark of aging [32]. Based on these strong implication of plasma markers related to

the aging process, we measured myokine levels and the proportion of T-lymphocytes in healthy large dogs to test development of an age prediction model.

MATERIALS AND METHODS

1. Large military dogs

While we were not able to prospectively calculate an optimal sample size for this study, we utilized existing manuscripts to justify a target number: 10 dogs in [33], 25 dogs in [34], and 23 dogs in [16]. Thus, we were able to enroll 27 dogs, which were identically managed under the rules of the Military Working Dog Training Center (Chuncheon, Korea). The age range of these dogs was from 1 to 12 years old. Our dog population was maintained in the identical environment, especially in terms of regularity, especially under the military conditions. Their diet contained all essential nutrients requirements, and the proportion of nutrients contained more than 25% crude protein, 10% crude fat, 0.6% Ca, 0.6% P, and less than 4% crude fiber, 10% crude ash, and 10% moisture. All dogs were fed under an identical schedule. Among the 27 dogs, 18 belonged to the breed German Shepherd Dog (GSD) (11 males and 7 females), and 9 belonged to the breed Belgian Malinois (BGM) (5 males and 4 females).

2. Ethical statement

All blood samples were obtained in an ethical manner following guidelines for animal health and welfare at a vet clinic. Advance approval was acquired from the Institutional Animal Care and Use Committee of the National Institute of Animal Science, of the Rural Development Administration, of South Korea.

3. Body size measurement

For abdominal circumference (AC) measurement, the circumference of the narrowest region of the abdomen between the lumbar vertebrae and the pelvis was measured when the dog was in a standing position. For thoracic circumference (TC) measurements, the circumference of the widest region of the thorax between the scapula and thoracic vertebrae was measured.

4. Purification of peripheral blood mononuclear cells (PBMCs) and plasma

Peripheral blood was obtained by venipuncture and transfer into a K_2EDTA coated vacutaner tube (Bec-

ton Dickinson, Plymouth, UK) immediately. Whole blood was diluted 1:1 with phosphate buffered saline (PBS; pH 7.0–7.2) (v:v) and carefully layered into a 4-mL Ficoll-paque PLUS (GE Healthcare Life Science, Uppsala, Sweden) containing conical tube. Tubes were centrifuged at $400 \times g$ for 30 minutes at room temperature without brake. The supernatant and white layer were transferred into cryotubes as plasma and PBMCs, respectively. Collected PBMCs were fixed with 2% paraformaldehyde for 10 minutes. Plasma and fixed PBMCs were stored in liquid nitrogen and in a -80℃ deep freezer, respectively. All the processes were done in 30 minutes from the blood samples to storage.

5. T-lymphocyte analysis

Fixed and frozen PBMCs were quickly thawed at 37℃, and cells were collected by centrifugation at $400 \times g$ for 5 minutes. Cells were washed twice with PBS (supplemented with 0.7% bovine serum albumin). Washed cells were then stained with anti-dog CD3:FITC/CD4:RPE/CD8:Alexa Fluor647® (Bio-Rad, Munich, Germany) for 15 minutes [35]. Stained cells were analyzed using FACSVerse (BD, NJ, USA).

6. Enzyme-linked immunosorbent assay (ELISA) for humoral factors

We purchased and used canine-specific ELISA kits for IL-6 and IL-8 (RayBiotech, Peachtree Corners, GA, USA), IL-15 and LIF (USBiological, Salem, MA, USA), GDF11 (Blue Gene, Shanghai, China) and GDF8 (EIAab, Wuhan, China). We measured these humoral factors in accordance with the manufacturer's application notes. For standard curves, we used a four parameter logistic (4-PL) equation:

$$y = B + \frac{(A - B)}{1 + (X/C)^D} \tag{1}$$

In this equation, the dependent variable y represents optical density (450 nm), independent variable x represents the concentration of the detected protein, A is the minimum value that can be obtained, B is the maximum value that can be obtained, C is the point of inflection, and D is the Hill's slope of the standard curve. For measuring the absorbance, we used a multi-detection microplate reader (Hidex, Turku, Finland). We applied samples randomly at different positions of a 96-well plate and measured the average values of the two independent technical replicates.

7. Statistical analysis and age prediction models

For correlation analysis, Pearson's correlation coefficients and p-values were calculated using the cor.test function in the R stats package.

Simple and multiple linear regression models were constructed to test the fitness of the factors for predicting age using the lm function in the R stats package. We used the following formula:

$$y_i = \alpha + \beta x_i + \varepsilon_i \tag{2}$$

$$y_i = \alpha + \beta_1 x_{1i} + \beta_2 x_{2i} + \varepsilon_i \tag{3}$$

$$y_i = \alpha + \beta_1 x_{1i}^2 + \beta_2 x_{1i} + \beta_3 x_{2i}^2 + \beta_4 x_{2i} + \varepsilon_i \tag{4}$$

In the simple linear regression model (formula 2), each subject (i-th subject, $1 \leq i \leq n = 27$), the dependent variable y represents age, α is the intercept, β represents the coefficient value, and the explanatory variable x_i is the measured parameter (either the CD4+/CD8+ ratio or the concentration of GDF8) that is considered as a fixed effect. Finally, general error (ε) was added to the equation. For the multiple linear regression model (formula 3) of each subject, β_1 and β_2 are coefficient values, and phenotypic values are x_1=CD4+/CD8+ ratio and x_2=GDF8. Moreover, the quadratic equation was constructed to determine the nonlinear relationship between the explanatory and dependent variables, as indicated in formula 4. The logistic parameters used in formula 4 are identical to those employed in formula 3.

To apply idiosyncratic variation of a random effect and nonlinear relationship to a multiple linear regression model, a quadratic linear mixed-effect model was formed using the lme function with the restricted maximum likelihood option in the R nlme package. The basic formula of this model is as follows:

$$y_i = \alpha + \beta_1 x_{1i}^2 + \beta_2 x_{1i} + \beta_3 x_{2i}^2 + \beta_4 x_{2i} + Z_i u_i$$
$$+ \varepsilon_i; \ u \sim N_p(0, D), \ \varepsilon \sim N_n(0, R_i) \tag{5}$$

where the age of each subject (i-th subject, $1 \leq i \leq n$; max n=27) is substituted for y. Explanatory variables, x_1=CD4+/CD8+ ratio and x_2=GDF8 were added as fixed effects; β_1, β_2, β_3, and β_4 represent the coefficient values. Z is the $n \times p$ design matrix for covariates of random effects, and u is the corresponding $p \times 1$ vector of the unobservable random effect coefficients. In this study, breeds and sex were replaced as random effects with nested classification as male and female within the GSD and BGM, respectively (p=4; breed/sex in R syntax). D is the $p \times p$ covariance matrix for random effects, and ε was added as the random error. R is an $n \times n$ covariance matrix for the errors.

We used two artificial intelligence models. The first one is a supporting vector regression model using a support vector machine (SVM) with SVM function using the e1071 package in R. In this model, the CD4+/CD8+ ratio and GDF8 were set as fixed effects, using default options, including radial basis kernel, and the parameters were optimized to cost (cost for false classification)=16, gamma (kernel width)=0.1, and epsilon (tolerance of termination criterion)=0.9 by grid-based parameter tuning function. To calculate the predicted age, the predict function in the R stats package was used.

The second model we used was an artificial neural network (ANN) model. We applied this model using IBM SPSS modeler 18.0 (SPSS Inc., Chicago, IL, USA). The CD4+/CD8+ ratio and GDF8 data were used in this model, running multi-layer perceptron with backpropagation and radial basis function (RBF) strategies with the default setting.

RESULTS

1. Body weight has a strong correlation with abdominal circumference and thoracic circumference, but not with age

Body composition of dogs are known to change through aging [36], and one recognized way to estimate body composition is through measuring AC and TC of the dog [37]. To identify the relationship between estimated body composition and age, we measured weight, AC, and TC. We found that AC and TC showed a strong and significant correlation with weight (AC: r=0.83, p=6×10^{-8}; TC: r=0.84, p=3×10^{-8}) (Fig. 1A, 1B). Next, we analyzed the correlation between the AT ratio and weight. The AT ratio also showed a significant correlation (r=0.60, p=9×10^{-4}), but was less robustly than either AC or TC alone (Fig. 1C). These results showed that AC and TC could independently serve as a useful indicator of weight. To investigate the effect of age on weight, we analyzed the relationship between weight and age (Fig. 1D) and found that weight had no correlation
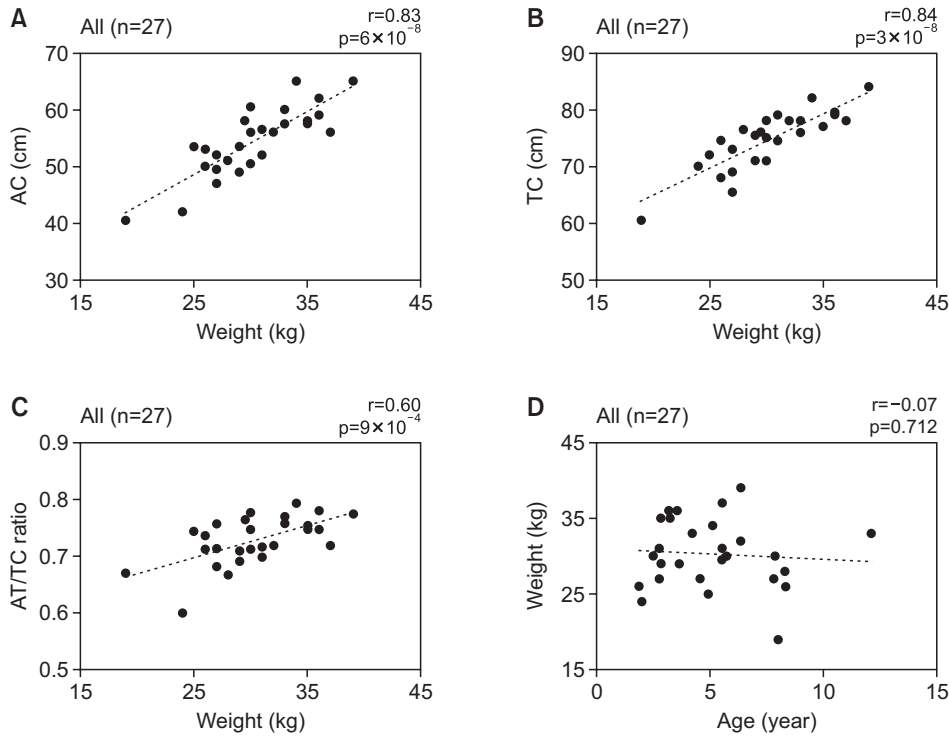
Fig. 1. Weight, abdominal circumference (AC), thoracic circumference (TC), and age. Body size, including AC and TC, showed a clear positive correlation with body weight (A, B). However, their ratio (AC/TC) showed a less positive correlation than the individual body weight parameters (C). Overall, body weight did not correlate with age (D).

with age (r=-0.07, p=0.712) in this study group.

2. CD4+/CD8+ ratio shows a significant correlation with age

To identify whether the lymphocyte subset is a suitable marker for aging in a canine model, we measured the proportion of helper (CD4+) and cytotoxic (CD8+) T-lymphocytes in the peripheral blood of 27 healthy dogs. We found that CD4+ and CD8+ cells insignificantly decreased (r=-0.29, p=0.144) and increased (r=0.30, p=0.127) in conjunction with dog age, respectively (Fig. 2A). On the other hand, the CD4+/CD8+ ratio was significantly decreased with age (r=-0.46, p=0.017) (Fig. 2A). Sex difference is well known to be associated with immunosenescence and longevity [38]. We examined whether the CD4+/CD8+ ratio is applicable to both males and females. The CD4+/CD8+ ratio was significantly decreased with age in male dogs (r=-0.56, p=0.023), but not in female dogs (r=-0.27, p=0.415) (Fig. 2B). The results indicate that the correlation between the CD4+/CD8+ ratio and age could be influenced by sex. We also observed that the lymphocyte subset was differently distributed in the peripheral blood of dogs by breed. The CD4+/CD8+ ratio showed a strong correlation with age in the breed BGM (r=-0.80, p=0.009), but not for GSD (r=-0.27, p=0.273) (Fig. 2C).

3. GDF8 levels in plasma significantly correlate with age

To discover humoral markers for age, we measured the plasma levels of five representative myokines, GDF8, IL-8, LIF, IL-15 and IL-6 levels, and GDF11. The level of GDF8 showed a significant correlation with age (r=0.52, p=0.005) (Fig. 3A). We found that this correlation was dependent on sex. The correlation was significant in males (r=0.78, p=$3×10^{-4}$), but not significant in females (r=-0.07, p=0.848) (Fig. 3B). In addition, the level of GDF8 was also dependent on breed and showed a more robust correlation significance in BGM (r=0.71, p=0.032) but no significant correlation in GSD (r=0.25, p=0.314) (Fig. 3C). With the exception of GDF8, other humoral factors did not show any correlation with age (Fig. 4). In the case of IL-6 and GDF11, we could only detect them in a few plasma samples, including 10 samples for IL-6 and 11 samples for GDF11 (Table 1, Fig. 4B, 4E).

4. Age prediction model using CD4+/CD8+ ratio and GDF8 level

To develop an age prediction model, we applied various linear models using forward selection of factors as explanatory variables including the CD4+/CD8+ ratio and GDF8 level. First, we tested how these two markers individually fit in a prediction model. We
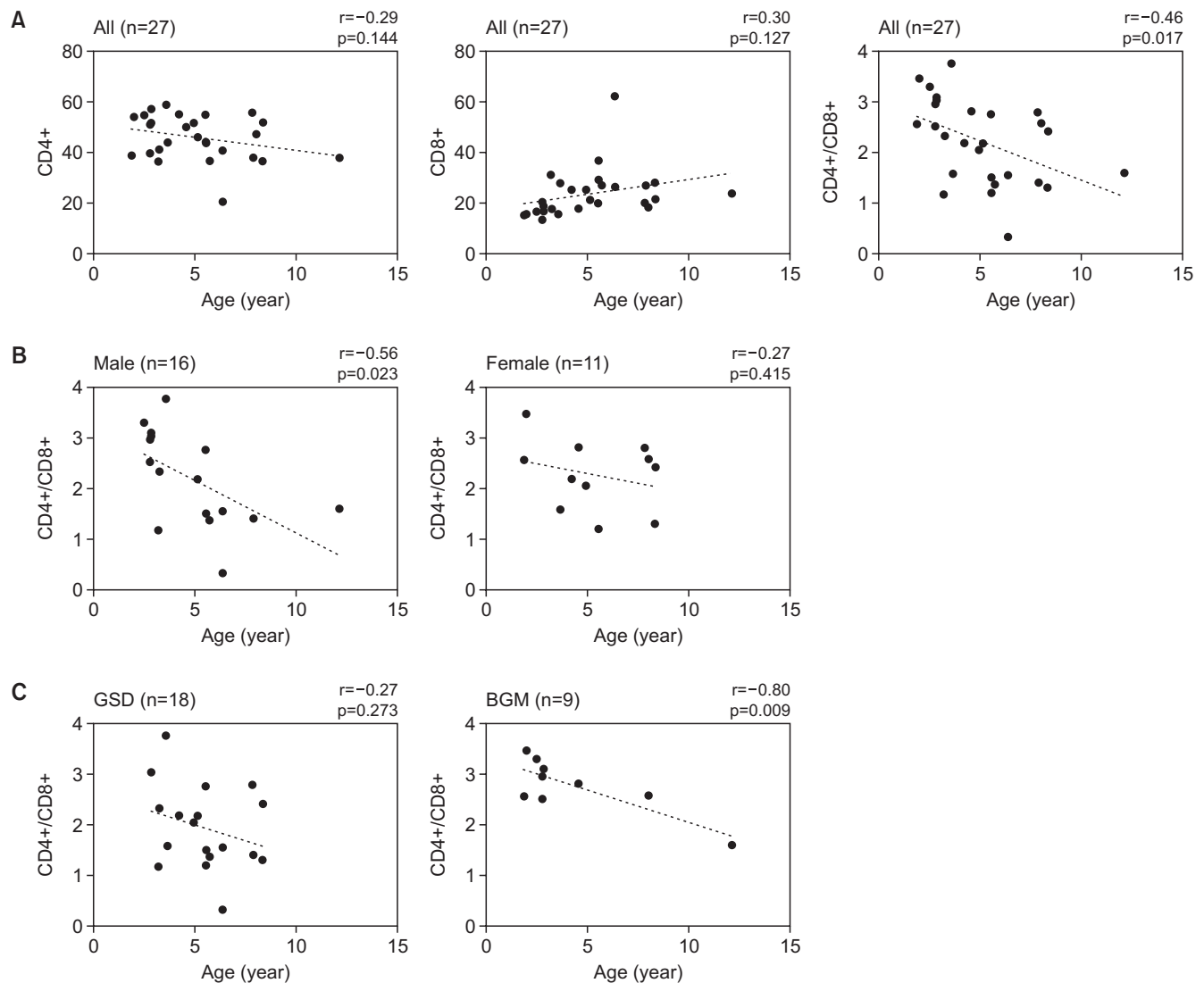
Fig. 2. Effect of age on T-lymphocyte subset in canine peripheral blood. Immunophenotyping of canine peripheral blood mononuclear cells was analyzed using a flow cytometry. Correlation of anti-CD4 positive cells (helper T cells), anti-CD8 positive cells (cytotoxic T cells), and the CD4+/CD8+ ratio with age (A). Correlation of CD4+/CD8+ ratio with age by sex (B) and by breed (C). GSD: German Shepherd Dog, BGM: Belgian Malinois.

used a simple linear model using each of these factors. Although these two markers showed a significant correlation with chronological age, simple linear model parameters poorly predicted the precise age (r=0.46, p=0.016 for CD4+/CD8+ ratio; r=0.52, p=0.005 for GDF8) (Fig. 5A, 5B). Based on these simple linear models as a reference, we tested advanced multiple linear models using two fixed effects, the CD4+/CD8+ ratio and the GDF8 level. This trial showed improved age prediction (r=0.62, p=6×10^{-4}) compared to reference models (Fig. 5C). Also, we tested several multiple linear models based on various combinations of measurement (Fig. 5D-5G). Finally, we tried a quadratic linear model for

age prediction using the two fixed effect parameters, CD4+/CD8+ ratio and GDF8 level (Fig. 5H). In our data, we already found that sex and breed influenced the age prediction power of the CD4+/CD8+ ratio (Fig. 2B, 2C) and GDF8 level (Fig. 3). To implement the effects of these variables, we set sex and breed as random effects in our quadratic linear model. These random effects were structured as a hierarchical order of male and female within each breed in a quadratic linear mixed model (see details in MATERIALS AND METHODS). Interestingly, by using this age prediction model, we identified the best fit (r=0.77, p=3×10^{-6}) for chronological age in our study group (Fig. 5I). Recently,
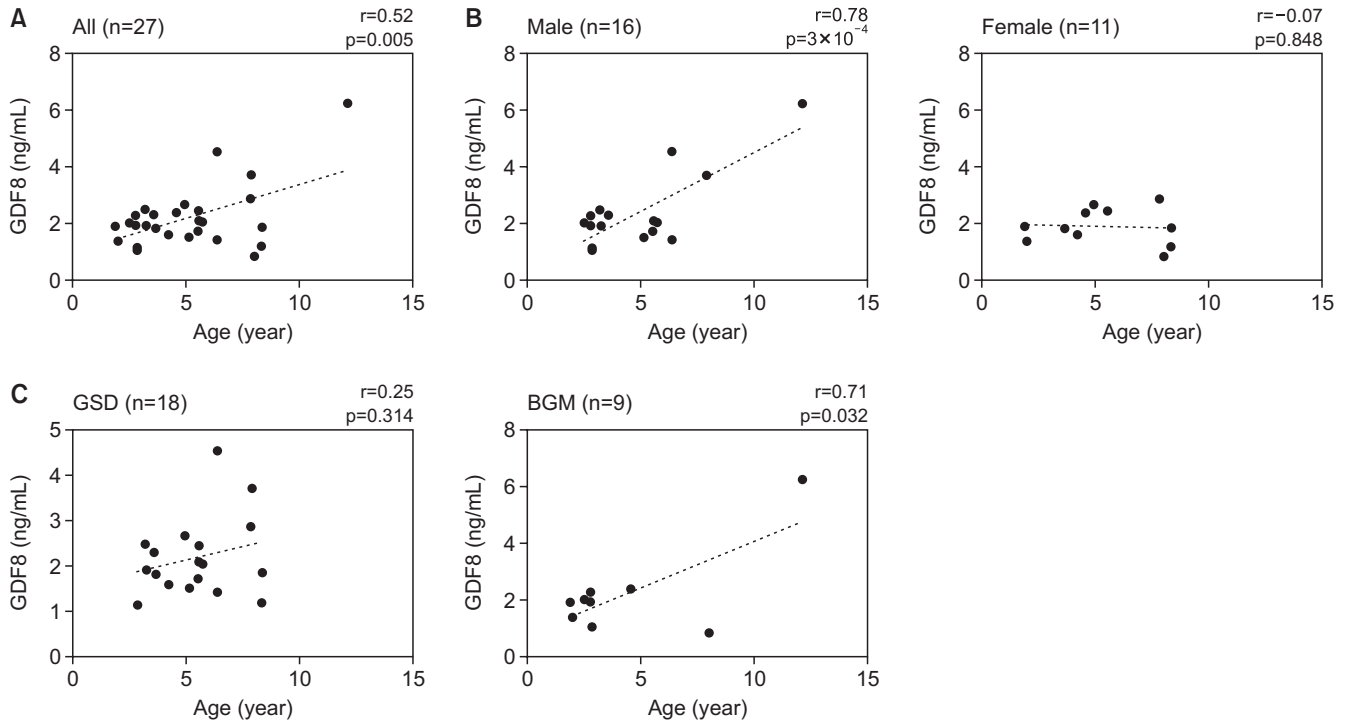
Fig. 3. Plasma level of GDF8. Among the six humoral factors, only GDF8 showed a significant correlation with age (A). Scatter plot of GDF8 with sex (B) and breed (C) effects. GDF: growth differentiation factor, GSD: German Shepherd Dog, BGM: Belgian Malinois.
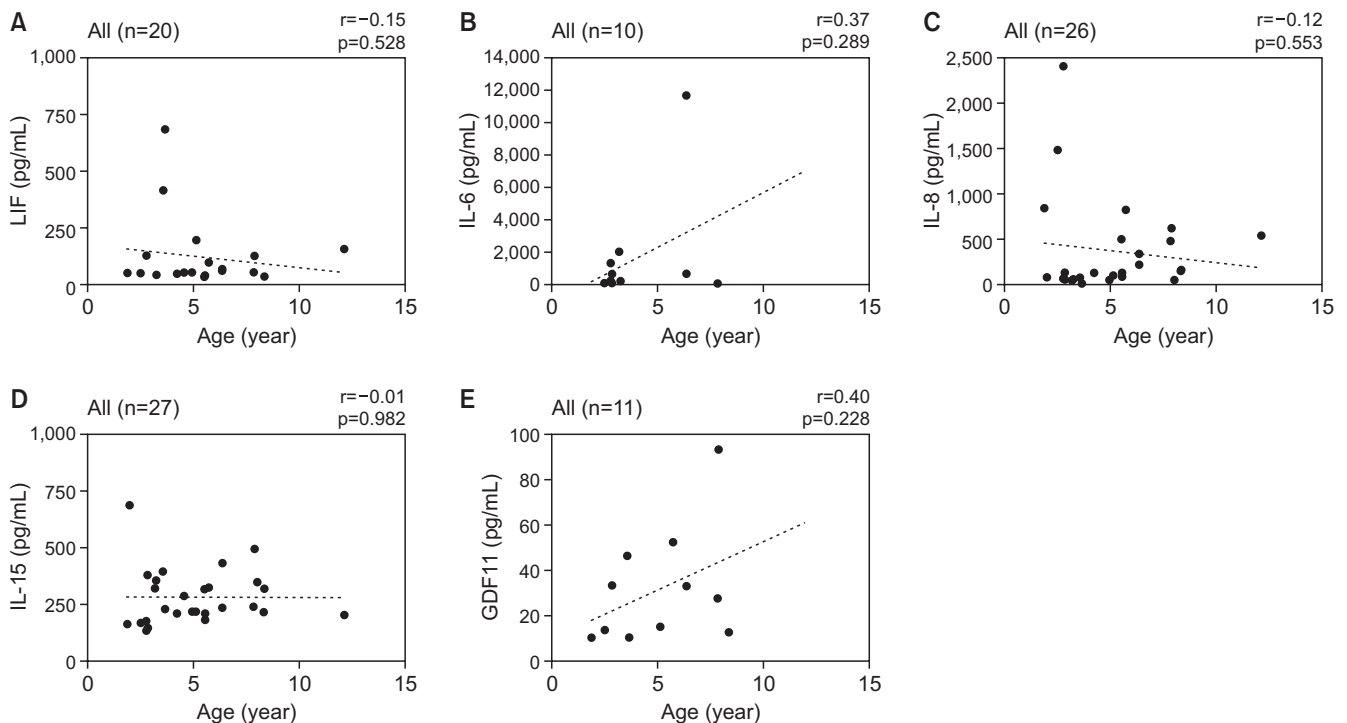

Fig. 4. Plasma level of leukemia inhibitory factor (LIF), and interleukin (IL)-6, IL-8, IL-15, and growth differentiation factor 11 (GDF11). Scatter plots of LIF (A), IL-6 (B), IL-8 (C), IL-15 (D), and GDF11 (E) showed no significant correlation with age.

machine learning algorithms have been heavily applied in biological data analysis. We also implemented some popular machine learning algorithms including ANN and SVR for age prediction model. We obtained equivalent results using ANN-RBF (r=0.72, p=2×10^{-5}) (Fig. 6A) and SVR (r=0.76, p=3×10^{-6}) (Fig. 6B) compared

Table 1. Measurements of six humoral factors using ELISA

Breed	Sex	Age (y)	GDF8 1× (ng/mL)	IL-15 1× (pg/mL)	LIF 1× (pg/mL)	IL-8 1× (pg/mL)	IL-6 1× (pg/mL)	GDF11 1× (pg/mL)
BGM	Male	2.5	2.02	169	52.2	1,480	68	13.8
GSD	Male	2.9	1.15	379	NA	134	86	33.4
GSD	Male	3.3	1.92	355	43.4	62	214	NA
BGM	Male	2.8	1.93	136	129.0	2,400	214	NA
BGM	Male	2.9	1.05	147	NA	60	656	NA
GSD	Male	6.4	4.52	431	62.2	220	684	33.0
BGM	Male	2.8	2.28	177	NA	68	1,332	NA
GSD	Male	3.2	2.48	321	NA	50	2,028	NA
GSD	Male	6.4	1.43	235	68.2	340	11,634	NA
GSD	Male	3.6	2.30	395	414.2	78	NA	46.4
GSD	Male	5.1	1.52	218	196.4	100	NA	15.2
GSD	Male	5.6	2.10	209	39.2	132	NA	NA
GSD	Male	5.5	1.72	318	36.2	500	NA	NA
BGM	Male	12.1	6.22	204	157.4	540	NA	NA
GSD	Male	7.9	3.70	492	126.0	620	NA	93.0
GSD	Male	5.7	2.04	323	98.4	820	NA	52.2
GSD	Female	7.8	2.86	240	55.0	480	70	27.6
GSD	Female	3.7	1.82	229	682.0	11	NA	10.6
GSD	Female	4.9	2.66	219	55.4	52	NA	NA
BGM	Female	8.0	0.85	347	NA	52	NA	NA
BGM	Female	2.0	1.38	686	NA	84	NA	NA
GSD	Female	5.6	2.44	183	40.6	88	NA	NA
GSD	Female	4.2	1.60	211	49.0	132	NA	NA
GSD	Female	8.3	1.20	216	NA	154	NA	NA
GSD	Female	8.4	1.86	320	36.0	162	NA	12.8
BGM	Female	1.9	1.90	163	52.4	840	NA	10.4
BGM	Female	4.6	2.38	287	53.8	NA	NA	NA

GDF: growth differentiation factor, IL: interleukin, LIF: leukemia inhibitory factor, BGM: Belgian Malinois, GSD: German Shepherd Dog, NA: not available.

to that obtained using the quadratic linear mixed model.

DISCUSSION

In this study, to identify aging-associated factors in dogs, we measured body size (weight, AC, TC, and AT ratio), the proportion of T-lymphocytes (CD4+, CD8+, and the CD4+/CD8+ ratio), the concentration of myokines (IL-8, LIF, IL-15, IL-6, and GDF8), and the concentration of an anti-aging marker, GDF11 (Table 2). In previous studies, body fat showed a correlation in the ratio of abdominal width to thoracic width [37]. In human, elderly people showed more tendency of having visceral fat than young people [39]. In addition, elderly people prone to develop obesity because of their weakened muscles, collectively called sarcopenic obesity [40].

According to these observations, we also would like to see whether there are any relationships between body composition and chronological ages in our dog study. Therefore, we measured AC, TC, and weight of the dogs to estimate body composition. We found that both AC and TC significantly correlated with weight. Also, we found a significant correlation between the AT ratio and weight. However, there was no correlation between AT ratio with age. Therefore, we could not find relationship between estimated body composition (AT ratio) with age in our study.

Helper (CD4+) and cytotoxic T (CD8+) cells play key roles in white blood cells as part of the adaptive immune system. Cytotoxic T cells recognize a specific antigen through the T-cell receptor and directly destroy infected target cells, tumor cells, and damaged cells. On the other hand, helper T cells activate other immune
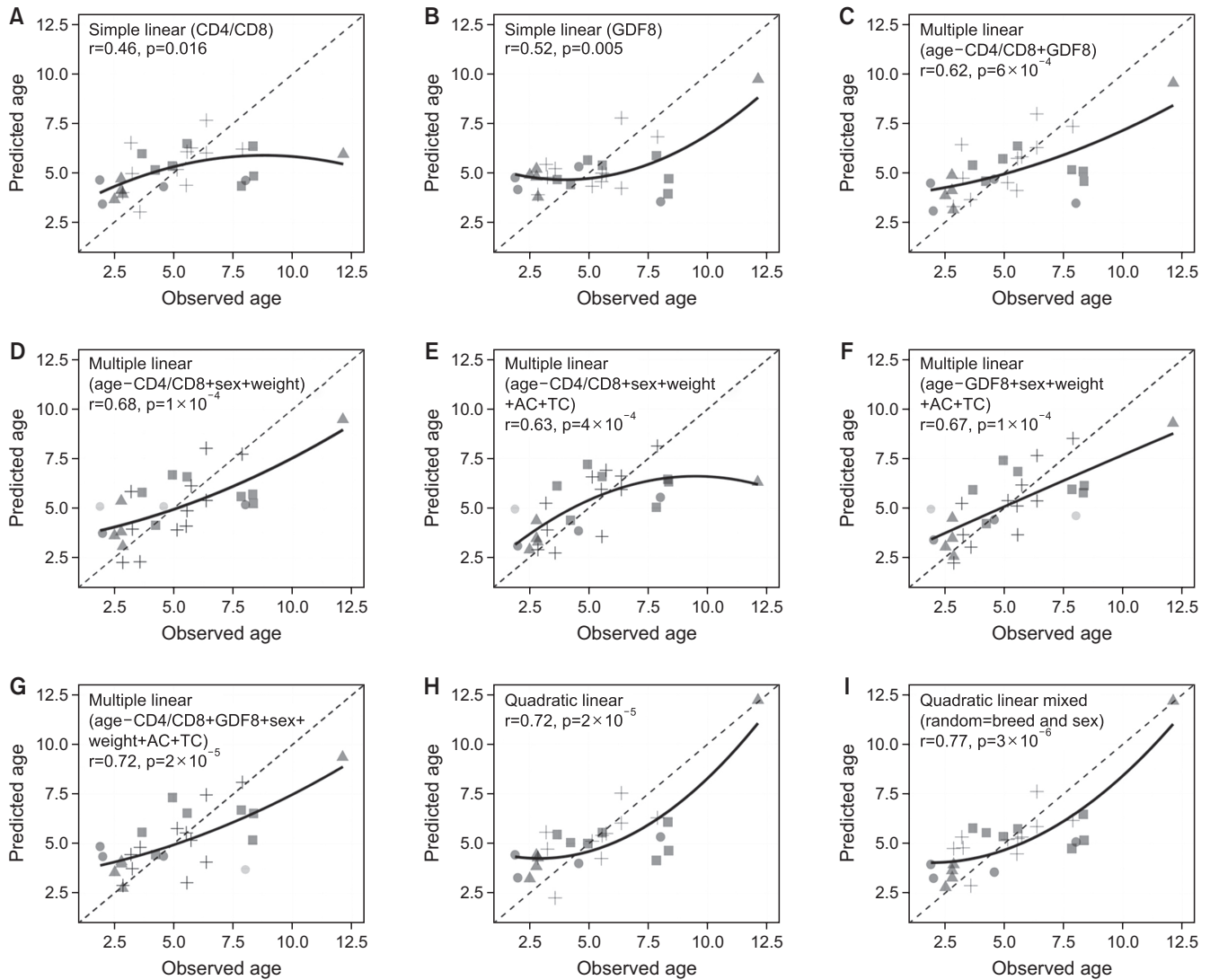
Fig. 5. Age prediction using linear regression models. Based on the chronological ages, age prediction was performed using given parameters. The black solid lines indicate second-order polynomial fits for the age prediction, and the broken lines represent the isometric reference for perfect prediction of age. Simple linear models were performed using the CD4+/CD8+ ratio (A) and GDF8 (B). Multiple linear models were performed based on the various combinations of fixed effects without considering their interaction including CD4/CD8+GDF8 (C), CD4/CD8+sex+weight (D), CD4/CD8+sex+weight+AC+TC (E), GDF8+sex+weight+AC+TC (F), CD4/CD8+GDF8+sex+weight+AC+TC (G). A quadratic multiple linear model was performed using the CD4/CD8 ratio and GDF8 (H). A quadratic mixed model was performed using the same two parameters with the breed and sex as random effects (I). Symbols: circle for BGM female; triangle for BGM male; square for GSD female; plus for GSD male. GDF: growth differentiation factor, AC: abdominal circumference, TC: thoracic circumference, BGM: Belgian Malinois, GSD: German Shepherd Dog.

cells such as B cells, cytotoxic T cells, and macrophages by releasing cytokines. Furthermore, inversion of the CD4+/CD8+ ratio (CD4+/CD8+ <1) is suggested as a hallmark of immunosenescence and age-related disease in several studies including HIV-infected patients, Singapore Longitudinal Ageing Study cohort [13] and elderly with frailty syndrome [12,14]. In our study, we also examined CD4+ and CD8+ T cells in the peripheral blood of dogs at various ages. From these results, we found that the CD4+/CD8+ ratio was significantly

correlated with dog age (Fig. 2A). However, in the case of the BGM alone (Fig. 2C), there was no correlation between CD4+/CD8+ ratio and the dog age. Although the usefulness of the lymphocyte subset was influenced by sex and breed, the CD4+/CD8+ ratio could be a useful prediction marker of age in certain cases. Several studies on lymphocyte subset analysis gave controversial experimental results in dog models. Some results showed that the levels of CD4+ and CD8+ as well as the CD4+/CD8+ ratio was a significantly correlated
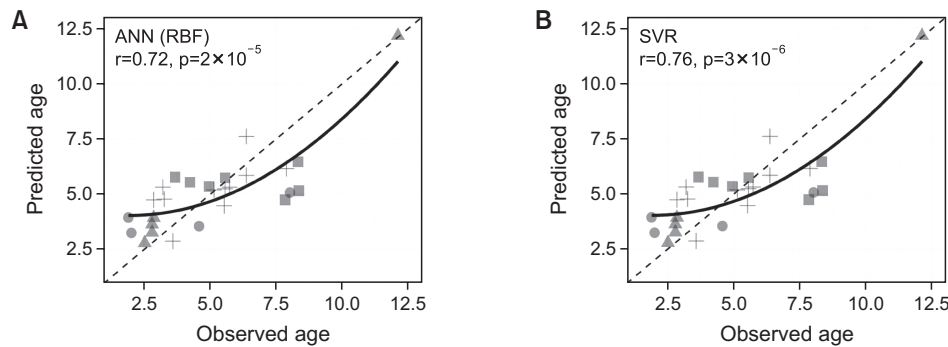
Fig. 6. Age prediction using machine learning algorithms. Age was predicted by machine learning algorithms. Predicted ages were calculated by artificial neural network (ANN) (A) and supporting vector regression (SVR) (B) algorithms. Symbols: circle for BGM female; triangle for BGM male; square for GSD female; plus for GSD male. GDF: growth differentiation factor, AC: abdominal circumference, TC: thoracic circumference, BGM: Belgian Malinois, GSD: German Shepherd Dog.

with dog age [17]. And there were some inconsistent reports including a decreased correlation of CD4+/CD8+ ratio to age [41] and no correlation between them [16] from other research groups. However, our findings showed that the lymphocyte subset distribution was changed in association with dog age (Fig. 2).

The level of GDF8 (myostatin) in the plasma of male canines significantly increased in conjunction with age (Fig. 3B). Moreover, in a study of aging in humans, GDF8 concentration was found to increase as a result of age in 70 men [28]. In both species, GDF8 level could be a reasonable marker of age in males. In contrast, in 70 women [28] and female canines (our study), GDF8 levels were not significantly changed as a result of age (Fig. 3B). GDF8 gene mutation in mice is related to a hypermuscular phenotype, which suggests that GDF8 inhibits skeletal muscle growth [26]. These findings suggest the possibility of muscle weakness when male canines get older. In fact, several studies have already shown that increased GDF8 levels are correlated with loss of muscle mass leading to age-associated sarcopenia [42], glucocorticoid-induced muscle atrophy [43], Duchenne muscular dystrophy [44], and indoxyl sulfate-induced muscle atrophy [45]. All together, these studies suggest that regulation of GDF8 concentration might be important for maintaining and recovering muscle function of individuals not only in old age but also at all ages.

IL-8 and IL-15 locally direct autocrine or paracrine activity in muscles; however, these myokines are rarely released from muscles to the circulatory system [24]. Thus, a change in IL-8 and IL-15 concentration in the plasma might not be detected. However, in our analysis of GSDs, plasma IL-8 levels were clearly detected and

increased in association with aging. Thus, we speculate that IL-8 can be released into the circulatory system in at least some dog breeds, such as the GSD. LIF has a wide variety of functions, included platelet formation, bone formation, adipocyte lipid transport, neuronal formation, and muscle satellite cell proliferation [21]. Therefore, LIF concentration in the plasma may be affected by several factors, not simply by age. We could not observe a change in the plasma LIF concentration as a result of age.

IL-6 is mainly produced in contracting muscles, and its levels are reduced at post-exercise [19,20]. We could not detect IL-6 in our experimental subjects. GDF11 is suggested to be an anti-aging humoral factor [27,28]. In addition, higher levels of GDF11 are associated with decreased risk of cardiovascular events and death in a mice study [27]. However, in humans, GDF11 is involved in sex-specific pathological weakness in older men with cardiovascular disease [28]. In our results, we could not easily detect GDF11 in healthy canine subjects.

To identify potential markers for certain phenotypes, statistical approaches including regression models have been applied [46]. In addition, statistical models for aging have been constructed based on gene expression [47], microRNA [47], proteins [47], metabolites [48], epigenetic markers [49], verbal intelligence [50], and facial patterns [51]. Although various humoral factors have been suggested for age prediction in humans [52], no studies have examined this in canines to the best of our knowledge. Here, we measured specific plasma factors from the peripheral blood of canines, which is easier to non-invasively obtain than other tissue samples. We found that the T-lymphocytes ratio (CD4+/

Table 2. Summary of measurements between male and female

Sex	Statistics	Age (y)	GDF8 1x (ng/mL)	IL-15 1x (pg/mL)	LIF 1x (pg/mL)	IL-8 1x (pg/mL)	IL-6 1x (pg/mL)	GDF11 1x (pg/mL)	Abdominal circumference (cm)	Thoracic circumference (cm)	AC/TC (ratio)	CD8+ (%)	CD4+ (%)	CD4/CD8 (ratio)
Male	Mean	4.9	2.4	282	89	475	1,057	17.9	57	77	0.74	24	44	2.17
	Standard deviation	2.5	1.3	109	105	641	2,879	27.1	5.3	3.9	0.03	11.4	10.0	0.94
Female	Mean	5.4	1.9	282	93	187	6	5.6	51	72	0.71	23	48	2.26
	Standard deviation	2.4	0.6	145	197	253	21	9.0	5.3	5.5	0.05	6.5	6.5	0.69

GDF: growth differentiation factor, IL: interleukin, LIF: leukemia inhibitory factor, AC: abdominal circumference, TC: thoracic circumference.

CD8+) as well as myokine, GDF8, and IL-8 levels were significantly correlated with canine age. Moreover, the significance of the correlation was influenced by both sex and breed difference. Consequently, we could form various models (*e.g.*, linear models, quadratic linear models, ANN, and SVR) of age prediction that were composed of two variables, the CD4+/CD8+ ratio and GDF8 concentration, as fixed effects. In addition, both breed and sex were substituted as random effects to form linear mixed models. We confirmed the fitness of age prediction by established models using a correlation test between chronological age and model-predicted age (Fig. 5, 6). The best model to fit the observed ages was the quadratic linear mixed model (Fig. 5I).

Dogs offer an opportunity to serve as a translational animal model of people, particularly given that they share similar chronic conditions such as obesity, arthritis, diabetes, and age-specific mortality patterns related to metabolic dysfunction and cancer [53]. Relevant to the current work, immunosenescence is one of the major aging traits occurring in both dog and human [6,15,16]. Levels of total lymphocytes and their proportions also seem to be important indicators of age in both species [11,12,15-17]. Furthermore, some myokines, IL-6, TNF-α and NCP-1, have been identified as common players in inflammation of both human and dog [24,54,55]. Given this, the chronological age prediction model established in this work may hold value in studying the influence of age-related treatment attempts on shifting parameters relevant to biological aging. To increase our sample size and include additional breeds in our work, we are currently repeating this work in small dogs and developing more sensitive ELISA methodology for use with smaller sample volumes. Additionally, we recognize that longitudinal *versus* single peripheral blood samples may be needed to determine whether circulating myokines levels fluctuate throughout the day. Finally, future work can be done to characterize T lymphocytes beyond CD4+/CD8+.

CONCLUSIONS

In conclusion, the CD4+/CD8+ ratio and GDF8 concentration in canine blood were useful factors in developing an age prediction model. However, genetic background and sex increased the complexity and limited the ability to predict age (the best r=0.77). Although

the aging process varies among individual canines having a diverse distribution of biological age, our age prediction model could be very promising to estimate both biological age and chronological age.

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: CKL, BHC. Investigation: HJL, SJH, SSK, YYK. Methodology: HJL, SJH, SSK, YYK, KMC, SHS, MJL, SYH. Project administration: CKL, BHC. Resources: BHC, KP, YJ, HS. Supervision: CKL. Visualization: HJL, SJH, SSK, YYK. Writing – original draft: HJL, SJH, SSK, YYK, CKL. Writing – review & editing: CKL, BHC, KMC.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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