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ORIGINAL ARTICLE

The influence of sex and age on bone turnover markers in the adult to geriatric Kangal shepherd dogs

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Abstract

Objectives: The objective of this research was to learn more about bone metabolism in intact female and male Kangal shepherd dogs during the aging process following skeletal maturity. It also evaluated the potential application of biochemical bone indicators in veterinary clinical practice.

Methods: Bone markers were determined as bone alkaline phosphatase (BALP), osteocalcin (OC), C-terminal telopeptide of type I collagen (CTX), and cross-linked C-telopeptide of type I collagen (ICTP) in this study. Kangal shepherd dogs of different age (adult, senior, and geriatric) and sex (male and female) groups were split into six groups of equal numbers ($n = 8$/group).

Results: In this study, the effect of age was observed on serum BALP, OC, CTX, and ICTP concentrations. Specifically, BALP was highest in geriatric female Kangal shepherd dogs, while serum OC, CTX, and ICTP concentrations were highest in geriatric male Kangal shepherd dogs. However, no effects of sex and age–sex interactions were identified. Moreover, the effects of age, sex, and age–sex interactions had no significant effect on serum creatinine, CK, LDH, Mg, and P concentrations or ALT activities. However, only sex was found to affect serum AST activities and gradually decreased with age in females. The effect of age and age–sex interactions on serum Ca concentrations was significant (the lowest serum Ca concentrations were in geriatric females), but the effect of sex was not.

Conclusions: These results show the effect of age and sex on bone turnover in Kangal shepherd dogs and provide information about bone biomarkers.

KEYWORDS
age, bone turnover, Kangal shepherd dogs, sex

1 | INTRODUCTION

It is well-known that bone metabolism is negatively affected by age and sex hormone deficiency in humans; however, the information in dogs is less clear. Phenomena associated with age and sex hormone deficiency in humans include estradiol deficiency in postmenopausal women and hypogonadism in older men and are the main causes of decreased bone mineral density (BMD).[1] Bone metabolism in both dogs and humans changes from modeling to remodeling as they become older.[2] It has been reported that the composition of mature dog bone is quite like that of human bone.[3] It has been shown that cortical and cancellous bone cell volume in adult and aged dogs gradually decreases with advancing age, similar to that in humans.[4,5] However, the lack of substantial responses in histomorphometric,

bone mass, and biochemical measurements may restrict the use of dogs in studies of cancellous bone loss during ovarian dysfunction osteoporosis.[6] The hierarchical organization of canine and human compact bone is comparable; however, the exact causes for this species difference are unknown. A study of sex variation in bone metabolism indicators of dogs found substantial differences in both sexes.[7] However, research on the link between bone metabolism and sex in dogs has shown mixed results. Furthermore, no studies have evaluated the effects of age and sex due to limited sample sizes and narrow age ranges.

Bone is a dynamic, active, and constantly changing tissue. Biochemical indicators measured in serum and urine can be used to monitor the activity of bone-forming cells (osteoblasts) and bone resorption cells (osteoclasts).[8] Bone remodeling is a gradual process that involves the removal of mineralized bone by osteoclasts and the rebuilding of bone matrix by osteoblasts. Osteoblasts and osteoclasts produce substances that can be detected in the blood or urine, some of which are clinically relevant in the evaluation of bone metabolism. With this continuous renewal mechanism, the mechanical integrity of bone tissue is maintained. The commonly used bone formation markers are the amino and carboxy propeptides of bone-specific alkaline phosphatase (BALP), osteocalcin (OC), and collagen type I (PINP and PICP), produced by osteoblasts, and required for the osteoid formation and matrix mineralization. Pyridinoline (PYD), cross-linked C-telopeptide of type I collagen (ICTP), deoxypyridinoline (DPD), tartrate-resistant acid phosphatase (TRAP), and the amino and carboxy telopeptides of collagen type I (NTX and CTX) are all bone resorption indicators. Moreover, bone resorption and formation processes are coupled through the RANK/receptor activator of the osteoclast regulatory proteins NF-kappa B ligand (RANKL)/osteoprotegerin (OPG) system. As a result, each cycle of bone resorption is followed by a wave of bone creation, resulting in the protection of skeletal integrity.[9] To keep records of remodeling activities, bone formation and resorption markers have been established. Humans and animals are affected by daily changes and other causes[10,11]; it is usually recommended that blood or urine samples be obtained at the same time.[12] The impact of physiologic alterations on bone metabolism in dogs is currently being studied and targeted at detecting possible high values.[7,12–15] Similarly, we targeted the physiologic effects of age and sex in Kangal shepherd dogs over bone-related analytes (BALP and OC for osteoblastic activity; CTX and ICTP for osteoclastic activity) that are frequently investigated in animals.[7,15,16]

The measurement of biochemical markers for the evaluation of bone metabolism in veterinary clinical practice has not yet been fully established. In veterinary medicine; however, preclinical and certain clinical investigations can be used to assess bone responses to bone biomarkers.[15] The biological variability of bone markers is the primary problem in their use. Age, sex, nutrition, exercise, and systemic diseases are factors that affect biological variability.[7] In addition, daily and seasonal changes have been detected in animals and humans.[7] The effects of age,[16] diurnal variability,[12,15,17] and the effect of breed[18] on bone turnover biomarkers have been studied in dogs. Although there is increasing interest in the use of biomarkers in small clinical applications, especially in dogs, horses, and cats,[8,14,16,18,19] it is currently only used as a scientific method in research.

In veterinary medicine, plasma and/or serum biochemical profiles have been frequently employed to assess the clinical and metabolic status of individual animals as well as groups or herds. Previous research has shown that serum biochemical analytes in dogs change with physiologic factors, including age and sex.[20,21] The effects of age and sex on hormones, which regulate serum Ca concentrations, have also been demonstrated to affect bone metabolism in dogs.[22] However, the effects of age and sex on serum biochemical changes related to skeletal metabolism in Kangal shepherd dogs remain unclear.

Because of changes in the skeletal and muscular systems as Kangal shepherd dogs age, their ability to perform herd protection duties is diminished, resulting in retirement from the herd protection duties. However, changes in bone tissue metabolism that occur during the aging process in these dogs are unknown. No study has been found examining the changes in bone biomarkers with age and sex in Kangal shepherd dogs. This is the first study to comparatively evaluate bone metabolism in adult, senior, and geriatric intact male and female Kangal shepherd dogs.

2 | METHODS AND MATERIALS

The study was conducted with the permission of the Sivas Cumhuriyet University Animal Experiments Ethics Committee (confirmation number:65202830-050.04.04-505). This study was carried out in Kangal District Governorate Dog Breeding Farm (Altitude: 1533, Latitude: 39.233334, Longitude: 37.383331) in Kangal District of Sivas Province in March 2021. Animals used in the study were housed in the same care and feeding conditions. All the animals included in the study were clinically healthy. None of the dogs had a history of preexisting or ongoing orthopedic diseases or any bone lesions. Additionally, no drug administration was performed that could affect bone metabolism. All animals underwent clinical examination throughout the study period. The study was conducted on adult, senior, and geriatric male, and female Kangal Shepherd dogs. The female dogs included in the study were in the anestrus period. Adult male and female groups were between 3 and 5 years old, senior male and female groups were between 6 and 8 years old, and geriatric male and female animals were ≥9 years old.[23] To prevent the effect of diurnal rhythm, blood samples were taken from the vena cephalica antebrachii in BD Vacutainer SST II gel tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) between 10.00–12.00 am during the study. The sera were extracted after 15 minutes of centrifugation at 3000 rpm on the blood samples. Sera were stored at −80°C for analysis.

2.1 | Serum bone turnover marker analysis

Bone-specific alkaline phosphatase (BALP) and osteocalcin (OC) from bone formation markers, cross-linked C-telopeptide of type

I collagen (ICTP), and collagen type I amino and collagen type I C-telopeptide (CTX) from bone resorption markers were determined by commercial ELISA kit (Bioassay Technology Laboratory, Shanghai, China) analysis in serum samples. The ELISA analysis was performed according to the kit manual. Briefly, serum samples, biotinylated antibodies, and Streptavidin-HRP were added sequentially to the plate coated with canine-specific antibodies and allowed to incubate for 1 hour. Then, the washing process was performed, the substrate solutions were added, and the reaction was started, respectively. The reaction was stopped by adding stop solution and reading in an ELISA plate reader (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer, USA) at 450 nm within 10 minutes. Analyzes were performed in duplicate from all samples. The standard curve range of bone turnover markers was as follows: BALP: 0.2–60 ng/mL, OC: 0.05–20 ng/mL, CTX: 0.05–15 ng/mL, and ICTP:0.2–70 ng/mL. Results are expressed in ng/ml.

2.2 | Serum biochemical markers analysis

In serum samples, creatine kinase (CK), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities, and lactate dehydrogenase (LDH), calcium (Ca), magnesium (Mg), and phosphorus (P) concentrations were determined by an autoanalyzer (Mindray BS 200, China). Canine reference intervals for these biochemical analytes were from those used at the Sivas Cumhuriyet University Veterinary Faculty Animal Hospital and are as follows: CK: 64–314 μ/l, AST: 18–56 μ/l, ALT: 17–95 μ/l, LDH: 24–388 μ/l, Ca: 9.4–11.1 mg/dL, Mg: 1.6–2.4 mg/dL, P: 2.9–5.3 mg/dL.

2.3 | Statistical analysis

The sample size ($n = 8$ per group) was calculated with the G*Power Version 3.1.9.6 (Germany) program according to the data in a preliminary study. The effect size (based on serum BALP concentrations from a pilot study of 40 dogs), α error probability, and the power (1 - β error probability) were selected as 0.56, 0.05, and 0.80, respectively. The conformity of the data to the normal distribution was performed using the Shapiro–Wilk test. The mean and SEM values of the data were calculated using the average absorbance values of the SPSS version 26 software. Statistical analysis of the main effect of sex and age and interactions was analyzed using the two-way ANOVA test in GraphPad Prism 8 software. In cases where there was a statistical difference, the main and interaction effects between the groups were determined by performing the post hoc Tukey test. Statistical significance was defined as P values less than 0.05.

3 | RESULTS

The main effect of sex and the age–sex interaction on serum BALP concentrations were not statistically significant ($P > 0.05$). However, the main effect of age was statistically significant on serum BALP ($P < 0.001$). Serum BALP concentration in geriatric female dogs was significantly higher than in adult male and female dogs in addition to senior male and female dogs ($P < 0.05$) (Figure 1).

While the main effect of age on serum OC and ICTP values was statistically significant ($P < 0.01$), the main effect of sex and the interaction of age and sex were not statistically significant ($P > 0.05$). When the effect of the age difference between the groups was shown serum OC and ICTP concentrations in geriatric male dogs were higher than adult females and adult and senior male dogs ($P < 0.05$) (Figure 1).

It was determined that there was no statistically significant effect of sex and age–sex interaction on serum CTX concentrations ($P > 0.05$). However, the effect of age on serum CTX concentrations was statistically significant ($P < 0.01$). The CTX concentration was highest in geriatric male dogs ($P < 0.05$). However, there was no statistical difference between other age and sex groups ($P > 0.05$) (Figure 1).

The effects of age, sex, and age–sex interaction on creatinine, CK, ALT, LDH, Mg, and P were statistically insignificant ($P > 0.05$). However, the effect of sex on serum AST activities was significant ($P < 0.01$). Senior males had the highest serum AST activities, while geriatric females had the lowest serum AST activities ($P < 0.05$). The main effect of sex did not affect serum Ca concentrations ($P > 0.05$). However, the effects of age and age–sex interactions on serum Ca concentrations were found to be statistically significant ($P < 0.05$). Serum Ca concentration was lowest in geriatric females and highest in adult females ($P < 0.05$). However, there was no statistical difference between the dog groups except for the geriatric female ($P > 0.05$) (Figure 2).

4 | DISCUSSION

In this study, we investigated the physiologic effects of age and sex on bone metabolism in Kangal shepherd dogs, a specific genetic resource. To the best of our knowledge, this study is the only research evaluating bone cell functions in male and female Kangal shepherd dogs during the aging process (adult, senior, and geriatric). We targeted serum BALP and OC concentrations as bone formation biomarkers, and CTX and ICTP as bone resorption biomarkers in this study to investigate the effects of age and sex on bone metabolism in Kangal shepherd dogs.[7,14,18] We also investigated several serum biochemical analytes (creatinine, CK, AST, ALT, LDH, Mg, P, and Ca) in the study.

The loss of bone mass is a prevalent aspect of age-related bone alterations, which increases the risk of fracture. The processes that cause bone aging are complicated, involving both systemic and local influences. Experimental and human studies emphasize that aging occurs as a result of a series of changes in the cell, tissue, and structural concentrations in the organism.[24] With aging, deterioration in all bone functions is observed. Changes in the

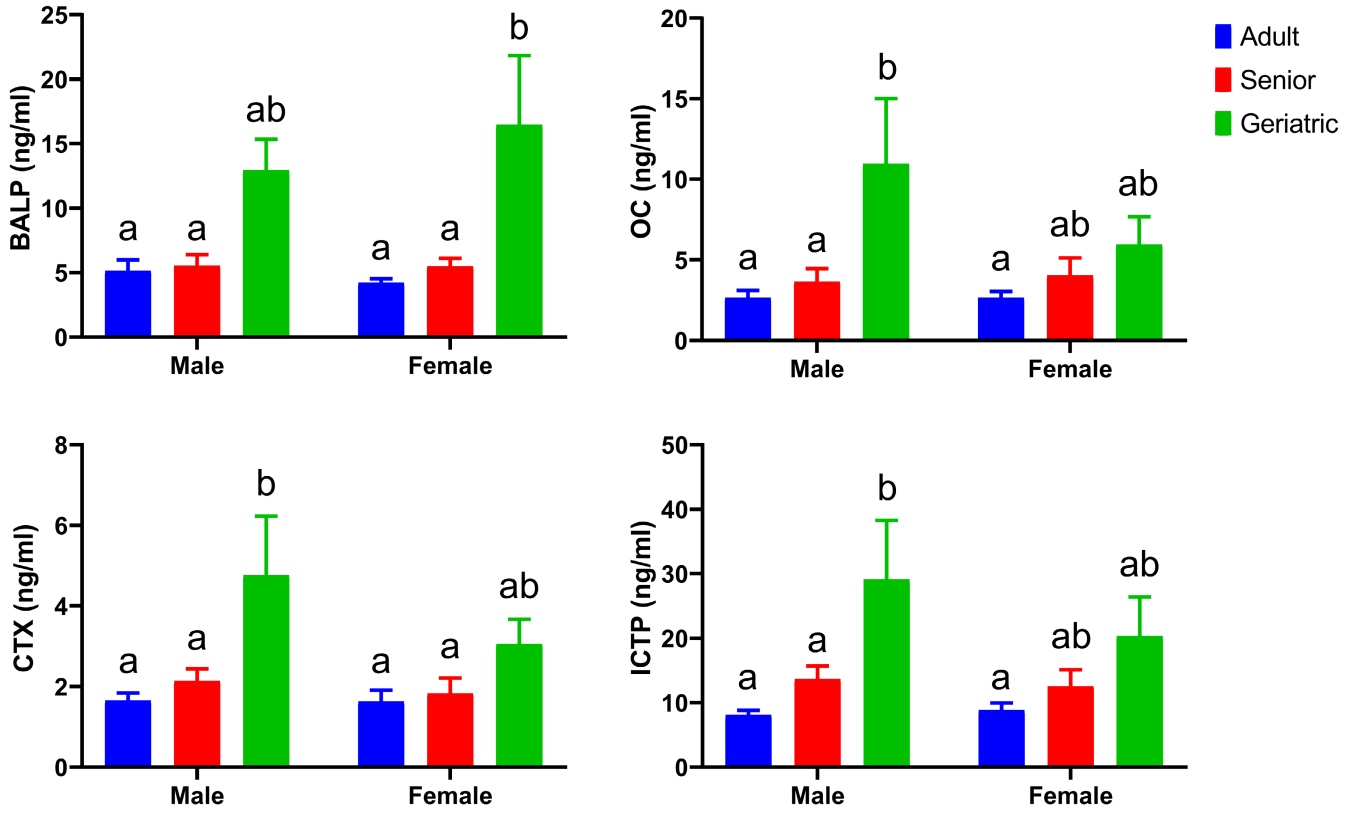

FIGURE 1 Effect of sex and age on bone turnover in Kangal shepherd dogs.[a,b]Different letters denote significance between the age groups (two-way ANOVA post hoc Tukey test). BALP—bone alkaline phosphatase; OC—osteocalcin; CTX—carboxyterminal cross-linking telopeptide of type I collagen; ICTP—carboxyterminal cross-linked telopeptide of type I procollagen.

morphological and geometric characteristics of the skeleton, as well as a decrease in bone density, load-bearing capacity, and responsiveness to systemic humoral stimuli, all contribute to aging. These changes can result in increased osteoclastic bone resorption and loss of skeletal mass over a long period, as previously shown in dogs.[25] Future research on canine bone metabolism may represent new findings.

BALP, a bone mineralization protein released by osteoblasts, is a highly specific marker of osteoblast activity.[18] In a previous study on Beagle dogs, it was stated that serum BALP concentration decreased with age.[26] In addition, it has been reported that serum BALP concentrations decrease with aging in elephants, which is due to the inactivation of osteoblast functions and bone mineralization.[27] However, in the study conducted on dogs, it was stated that sex did not have an effect on serum BALP concentration, which was due to the heterogeneity between study groups.[7] While the main effect of age on serum BALP value was observed in our study, no effect of sex and age–sex interaction was observed. In the previous study, serum BALP concentration was found to be higher in geriatric female dogs, similar to our study.[28] Furthermore, it has been shown in a previous report that serum BALP concentration declines with age but increases following 8 years of age, which supports our present findings.[16]

Only osteoblasts and megakaryocytes synthesize OC, a vitamin K-dependent protein. Although it is one of the most common non-collagenous proteins in bone, little is known about its involvement in bone development. In general, there is a tight link between blood OC concentrations and bone formation rates evaluated by bone histomorphometry, as well as between serum OC concentrations and other serum bone formation indicators.[15] Although sex and age–sex interaction did not affect serum OC concentrations in our study, age was found to be the main effect. Interestingly, the serum OC concentration was highest in geriatric male dogs in our study. In addition, numerical increases were detected in geriatric female dogs. Studies have reported decreases in serum OC concentrations in dogs and some other animal species with aging.[16,28,29] In previous studies on dogs, low Ca concentrations and high PTH concentrations were reported in aged dogs.[25] In addition, it was reported in this study that bone formation and resorption decreased with age, and resorption increased in aged dogs.[20] On the other hand, in studies conducted in humans, an increase in serum OC and parathyroid hormone (PTH) concentrations and a decrease in 25 hydroxy-vitamin D were reported in geriatric females, and it was reported that this contributed to the formation of bone diseases in geriatric patients.[30] Although the mechanism of this increase in serum OC concentrations in geriatric Kangal shepherd dogs cannot be fully explained, in addition to the increase in serum PTH concentrations and the decrease in serum 25 hydroxy-vitamin D (25(OH)D3), it may be associated with bone loss as a result of aging-induced lack of Ca absorption

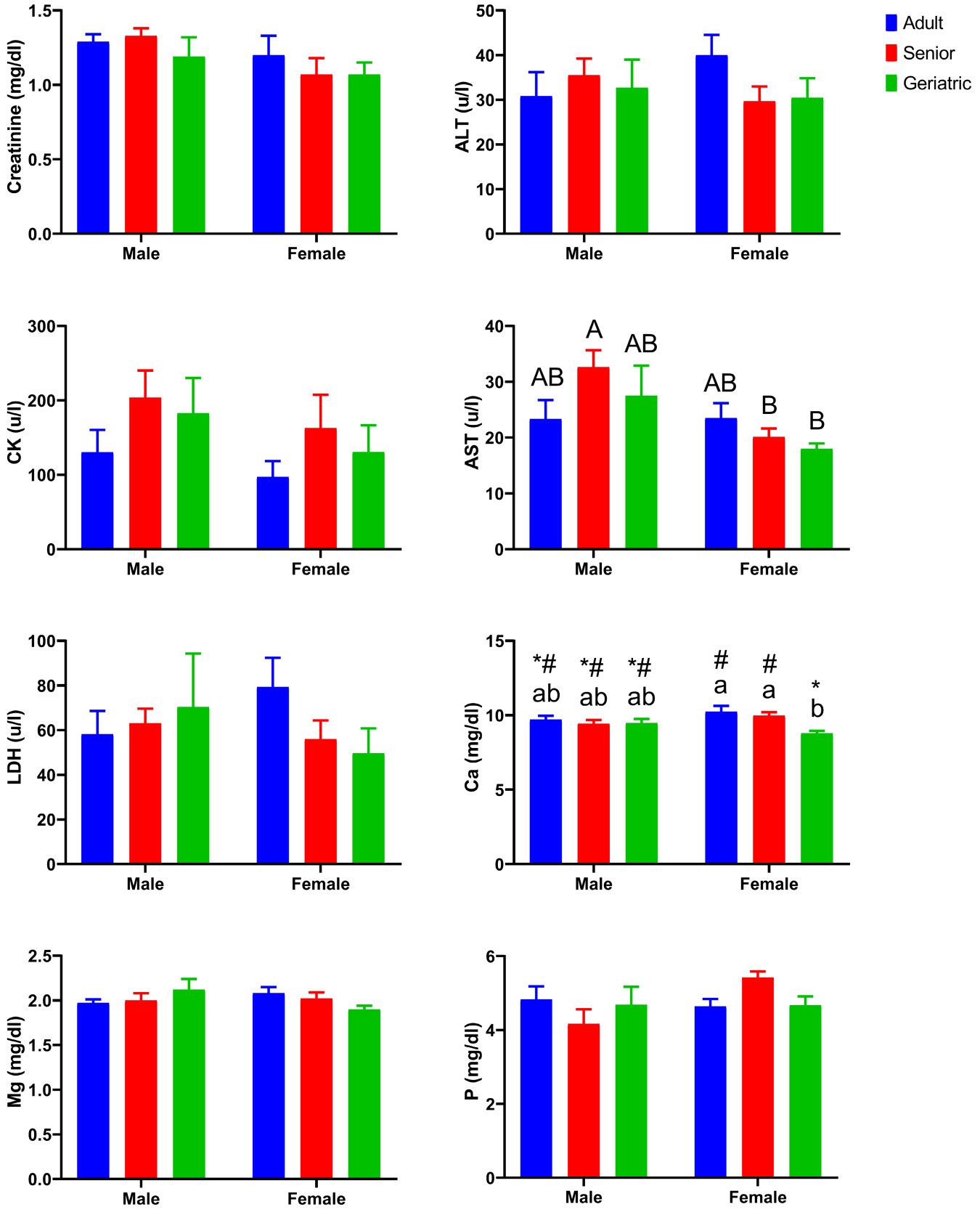

FIGURE 2 Effect of sex and age on biochemical changes in Kangal shepherd dogs. [a,b]Different letters in the figure denote significance between the age groups (two-way ANOVA post hoc Tukey test). [A,B]: Different letters in the figure denote significance between the sex groups (two-way ANOVA post hoc Tukey test). [*,#]Different symbols in the figure denote significant age–sex interactions between the groups (two-way ANOVA post hoc Tukey test).

in the kidney and intestine.[25,31,32] There is a need for further OC studies with a larger number of Kangal shepherd dogs.

CTX is a short peptide fragment that is a particular marker of bone resorption that is produced following type I collagen breakdown and does not cross-react with other collagens. It has been reported that serum CTX concentrations decrease with age in female dogs and the lowest is in geriatric female dogs.[28] In a study investigating the effect of sex in dogs, it was stated that the serum CTX concentration was higher in females than males, because females were more prone to bone resorption.[7] Interestingly, only the main effect of age was significant on serum CTX concentrations in our study. The highest serum CTX concentration was seen in geriatric male dogs. However, numerical increases were seen in geriatric females compared to other age and sex groups. Elevated serum CTX concentrations in geriatric Kangal shepherd dogs may be associated with increased bone turnover, decreased serum 25(OH)D3, and increased PTH concentrations.[25,31]

In our study, while the main effect of age–sex interaction and sex did not influence serum ICTP concentrations, the main effect of age was found to be significant. Serum ICTP concentrations were found to be the highest in geriatric male dogs. However, numerical elevations were seen in geriatric female dogs. Contrary to our findings, a previous study in dogs reported that serum ICTP concentrations decreased with age, reaching the lowest concentrations, especially in dogs older than 8 years of age.[16] The increase in serum ICTP concentrations is an indicator of increased bone resorption activity. These findings are in line with the fact that as people become older, their androgen concentrations (free or available testosterone) decline.[33] Impaired testosterone is linked to bone loss in men,[34] which might be linked to a drop in vitamin D concentrations, leading to calcium malabsorption and reduced bone production.[35] Conversely, estrogen is also known to control bone resorption in elderly male individuals.[36]

In a previous study on healthy small-breed dogs, it was reported that sex had a significant effect on plasma ALT and AST activities, and that age had a significant effect on serum Ca concentrations.[37] In the same study, age and gender were not reported to affect serum creatinine concentrations.[37] However, in another dog study, it was reported that while sex did not affect serum AST and ALT activities, or LDH and creatinine concentrations, age did.[20] In our study, there was no significant effect of age, sex, and age–sex interaction on serum creatinine, CK and ALT activities, or LDH, Mg, and P concentrations in serum biochemical analytes in Kangal shepherd dogs. These variables are within reference limits, and most variables (creatinine, ALT, P) are consistent with previous studies in healthy-apparent senior and geriatric dogs.[21] Serum AST concentrations were within reference intervals,[21] and only the effect of sex was found to be significant. Interestingly, serum AST concentrations decreased with aging in female dogs. This result is consistent with the finding of a previous study in dogs.[37] Furthermore, this may be because females have a lower lean body mass than males. While the main effect of sex on serum Ca concentrations was not observed, the effect of age and age–sex interaction was significant. It was observed that serum Ca concentrations decreased with aging in female Kangal shepherd dogs. In addition, the lowest serum Ca concentration was determined in geriatric females and the highest in adult female Kangal shepherd dogs. In a previous study, the effects of age and sex on serum Ca-regulating hormone concentrations were investigated in dogs.[22] It has been reported that serum 25(OH)D3 concentrations decrease and serum PTH concentrations increase in old dogs compared with younger dogs. Moreover, serum calcitonin concentrations were shown to be higher in older male dogs than in younger male dogs.[22] This study also reported that serum 25(OH)D3 concentrations were higher in female than male dogs.[22] Serum Ca concentrations may have decreased as a result of low serum estrogen and 25(OH)D3 concentration concentrations in the female Kangal shepherd dogs of our study.

Our research has several limitations. Controlling bone biomarker sampling conditions is the initial step. Bone biomarkers can reveal circadian variation and biological diversity in dogs and humans.[17,38] Therefore, sampling time may affect serum concentrations of bone biomarkers. Although attention was paid to the sampling time in this study, no study was found showing the effect of circadian rhythm on bone turnover and the best sampling time in Kangal shepherd dogs. Secondly, hormones such as 25(OH)D3, PTH, and calcitonin, especially sex hormones (testosterone and estrogen), which affect bone metabolism, could not be measured in this study. Finally, puppies and young Kangal shepherd dogs were not included in the study.

In conclusion, it was observed that there was an increase in bone turnover related to aging in male and female Kangal shepherd dogs, especially in geriatric male and female Kangal shepherd dogs. Furthermore, serum AST and Ca concentrations were shown to decline with age, particularly in female Kangal shepherd dogs. As a result of the age and sex dimorphism in bone metabolism, more research on Kangal shepherd dogs is required, with many pups and young dogs participating.

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DISCLOSURE

All authors have read the study, and there is no conflict of interest between the authors.

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