*Aging Cell* (2011) **10,** pp991–995 Doi: 10.1111/j.1474-9726.2011.00744.x

# A model of canine leukocyte telomere dynamics

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## Summary

**Recent studies have found associations of leukocyte telomere length (TL) with diseases of aging and with longevity. However, it is unknown whether birth leukocyte TL or its age-dependent attrition – the two factors that determine leukocyte TL dynamics – explains these associations because acquiring this information entails monitoring individuals over their entire life course. We tested in dogs a model of leukocyte TL dynamics, based on the following premises: (i) TL is synchronized among somatic tissues; (ii) TL in skeletal muscle, which is largely postmitotic, is a measure of TL in early development; and (iii) the difference between TL in leukocytes and muscle (ΔLMTL) is the extent of leukocyte TL shortening since early development. Using this model, we observed in 83 dogs (ages, 4–42 months) no significant change with age in TLs of skeletal muscle and a shorter TL in leukocytes than in skeletal muscle ($P < 0.0001$). Age explained 43% of the variation in ΔLMTL ($P < 0.00001$), but only 6% of the variation in leukocyte TL ($P = 0.035$) among dogs. Accordingly, muscle TL and ΔLMTL provide the two essential factors of leukocyte TL dynamics in the individual dog. When applied to humans, the partition of the contribution of leukocyte TL during early development vs. telomere shortening afterward might provide information about whether the individual's longevity is calibrated to either one or both factors that define leukocyte TL dynamics.**

**Key words: aging; skeletal muscle; telomere; life span; longevity; mammals.**

## Introduction

In humans, leukocyte telomere length (TL) is associated with aging-related diseases, cardiovascular disease in particular (Oeseburg *et al.*, 2010; Aviv, 2011). Furthermore, recent findings suggest diminished survival of elderly persons with relatively short leukocyte TL (Bakaysa *et al.*, 2007; Kimura *et al.*, 2008; Fitzpatrick *et al.*, 2011). As leukocyte TL reflects hematopoietic stem cell (HSC) TL (Sidorov *et al.*, 2009), it is possible that HSC TL dynamics (HSC TL at birth and its age-dependent shortening after birth) play a role in aging and longevity.

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Accepted for publication *9 August 2011*

Variations of 4–6 kb in leukocyte TL are commonly displayed among individuals at birth (Frenck *et al.*, 1998; Rufer *et al.*, 1999; Okuda *et al.*, 2002; Akkad *et al.*, 2006) and throughout the human lifespan (Rufer *et al.*, 1999; Alter *et al.*, 2007; Barbieri *et al.*, 2009). In addition, age-dependent leukocyte TL attrition is highly variable among individuals (Chen *et al.*, 2011). For obvious reasons, cross-sectional studies are more convenient than longitudinal examinations of age-dependent leukocyte TL shortening over many years, but they require large cohorts comprising individuals of a wide age range. Moreover, these studies can determine age-dependent leukocyte TL attrition only for the group but not the individual.

In humans, TL is highly synchronized (equivalent) at birth and *in utero* among cells from different organs and tissues within the individual (Youngren *et al.*, 1998; Okuda *et al.*, 2002; Kimura *et al.*, 2010a). This synchrony is largely maintained in adults (von Zglinicki *et al.*, 2000; Gardner *et al.*, 2007; Kimura *et al.*, 2010a; Granick *et al.*, 2011). In addition, reflecting proliferative history, TL in cells of the so-called postmitotic tissues is consistently longer than that of proliferative tissues and cells (Gardner *et al.*, 2007; Granick *et al.*, 2011). Neurons in the cerebellum are perhaps the only human somatic cells that hardly replicate during most of extra-uterine life (Spalding *et al.*, 2005). In this sense, cerebellar neurons are truly postmitotic. The numbers of skeletal muscle and fat cells apparently increase during growth and development, but evidently their replication during adult life is relatively small (Spalding *et al.*, 2005, 2008). For instance, the estimated annual turnover rate of fat cells during adult life is about 10% (Spalding *et al.*, 2008) and the mean TL in human skeletal muscle shows little or no change between the ages of 20 and 80 years (Ponsot *et al.*, 2008), suggesting little replicative activity. In contrast, leukocytes undergo tremendous turnover, which is particularly fast for neutrophils, the circulation life of which is 6–8 h (Summers *et al.*, 2010). Consequently, the rate of leukocyte TL shortening in humans is approximately 0.03 kilobase (kb) per year during adult life (Chen *et al.*, 2011) and is considerably faster during growth and development (Frenck *et al.*, 1998; Rufer *et al.*, 1999; Sidorov *et al.*, 2009). This has been shown not only for humans but also for monkeys (Baerlocher *et al.*, 2007), a phenomenon attributed to the higher rate of HSC replication during early life (Baerlocher *et al.*, 2007; Sidorov *et al.*, 2009).

Accordingly, we designed a model that transforms a cross-sectional analysis into a quasi-longitudinal analysis of leukocyte TL by using TL in skeletal muscle as an internal reference of TL in early life. The central postulate of the model is as follows: TL in skeletal muscle and the difference between leukocyte and skeletal muscle TLs (ΔLMTL) provide a broad account of leukocyte TL dynamics over the life course of the individual. We tested this postulate in dogs.

## Results

A total of 83 dogs (4–42 months old; 58% females) were included in the study. They comprised a spectrum of breeds from Maltese (body weight 2.4 kg) to bernese mountain dog (body weight 55.8 kg). Their general characteristics are presented in Table 1. (For a list of all breeds participating in this study, see Table S1) A few dogs did not have the complete set of tissues (blood, muscle, and fat).

There was no sex-related difference in TL in any of the tissues after adjustment for age and weight. There were too few dogs in each breed to provide any meaningful evaluation of TL by breed.





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**Table 1** General characteristics

| Parameter | All | Males | Females |
|---|---|---|---|
| *N* | 83 | 35 | 48 |
| Age (month) | 13.45 ± 8.24 | 15.34 ± 9.85 | 12.06 ± 6.60 |
| Weight (kg) | 13.69 ± 11.17 | 15.02 ± 11.27 | 12.75 ± 11.12 |
| Telomere length (kb) | | | |
| Muscle | 16.48 ± 1.21 | 16.58 ± 1.11 | 16.41 ± 1.29 |
| Fat | 16.30 ± 1.31 | 16.58 ± 1.39 | 16.13 ± 1.25 |
| Leukocyte | 15.21 ± 1.40 | 15.22 ± 1.38 | 15.21 ± 1.43 |
| Leukocyte–muscle | −1.25 ± 0.65 | −1.32 ± 0.71 | −0.98 ± 0.65 |
| Leukocyte–fat | −1.11 ± 0.74 | −1.32 ± 0.83 | −0.98 ± 0.65 |
| Leukocyte–muscle/month | −0.11 ± 0.06 | −0.10 ± 0.06 | −0.11 ± 0.05 |

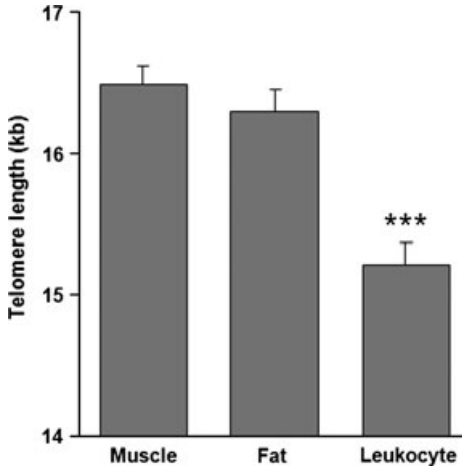


**Fig. 1** Telomere length (TL) in skeletal muscle, fat, and leukocytes. The statistical analysis is based on 68 dogs with complete set of samples (muscle, fat, and leukocytes). ***Denotes significant difference for leukocyte TL from both muscle and fat TL at $P < 0.0001$ (by paired *t*-test). The significance of comparisons using all dogs ($N = 79$ for muscle, $N = 76$ for fat, and $N = 78$ for leukocytes) by nonpaired *t*-test was also at $P < 0.0001$.

Leukocyte TL was considerably shorter than TLs in muscle or fat ($P < 0.0001$; Fig. 1). Although TL was slightly shorter in fat than in muscle, this difference was not statistically significant.

Wide inter-individual variations in TLs (~ 6 kb) were observed across dogs in all tissues, but TLs were highly synchronized within tissues of the individual dogs, so that dogs with short (or long) telomeres in one tissue displayed short (or long) telomeres in other tissues (Fig. 2). As per Fig. 1 and Table 1, the linear regression describing the relations of leukocyte TL with muscle or fat TL was below the identity line, in accordance with the shorter leukocyte TL than muscle or fat TL. While there was no significant relation between TL and age for muscle and fat, leukocyte TL shortened with age at a rate of 0.04 kb month$^{-1}$ ($R^2 = 0.06$, $P = 0.035$) (Fig. 3).

To further explore age-dependent leukocyte TL attrition, we examined the relation between ΔLMTL and age. Linear regression was the most parsimonious model describing this relation, in which the change in ΔLMTL with age was 0.052 kb month$^{-1}$ ($R^2 = 0.43$, $P < 0.00001$) (Fig. 4). That said, it is also apparent that the rate of change in ΔLMTL was much faster in younger than in older dogs. This is displayed in Fig. 5, which shows the rate of change in ΔLMTL vs. age. This relation is best fitted by a curvilinear function that describes a more rapid rate of change in ΔLMTL in younger than in older dogs (Fig. 5).

We analyzed multi-regression models of (i) ΔLMTL in which the independent variables included age, muscle TL, weight, and sex or (ii) leukocyte TL, normalized for fat (difference between leukocyte TL and fat TL), in which the independent variables included, age, fat TL, weight, and sex. Age explained most of the inter-individual variations in both models, but it accounted for 43.6% of the variation in ΔLMTL and only for 14.2% of the variation in leukocyte TL, normalized for fat TL.

## Discussion

The central and inter-related findings of this work are as follows: first, although TLs in skeletal muscle and subcutaneous fat, two poorly proliferative tissues, are longer than in leukocytes, considerable synchrony exists in TLs among these three tissues and presumably other somatic tissues. This TL synchrony was observed in other mammals, including monkeys (Gardner *et al.*, 2007) and humans (von Zglinicki *et al.*, 2000; Gardner *et al.*, 2007; Kimura *et al.*, 2010a; Granick *et al.*, 2011). Second, assessment of age-dependent leukocyte TL shortening based on leukocyte TL data across dogs of different ages is confounded by the wide inter-individual variation in leukocyte TL. Third, the change in ΔLMTL with age provides a much better account of leukocyte TL dynamics than that of leukocyte TL across dogs of different ages. Moreover, as the ΔLMTL model generates leukocyte TL dynamics data based on the individual animals rather than across animals, the inter-individual variation in

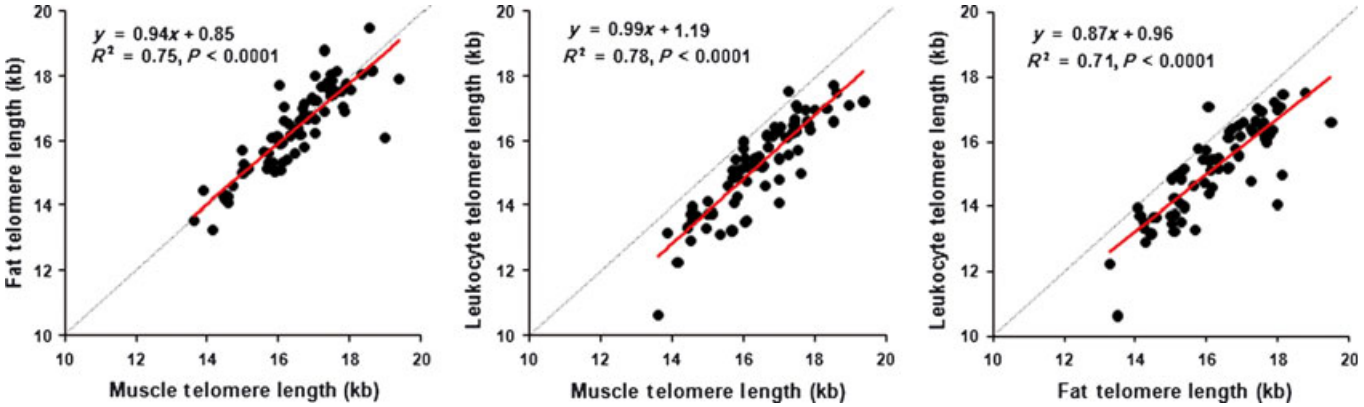


**Fig. 2** Synchrony in telomere length between skeletal muscle, fat, and leukocytes. *N* values for the left, middle, and right panels are 73, 74, and 71, respectively.

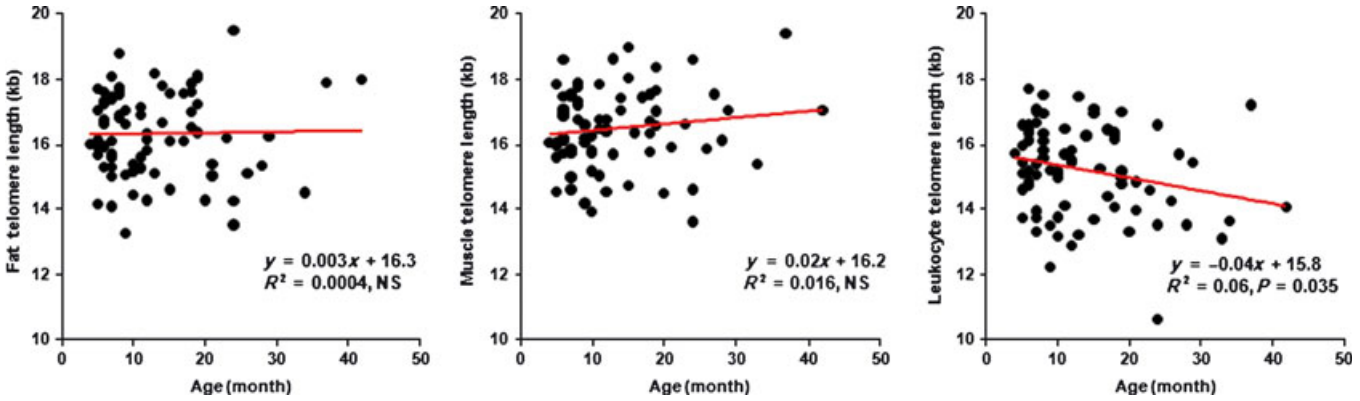


**Fig. 3** Telomere length vs. age in fat, skeletal muscle, and leukocytes. *N* values for left, middle, and right panels are 76, 79, and 78.

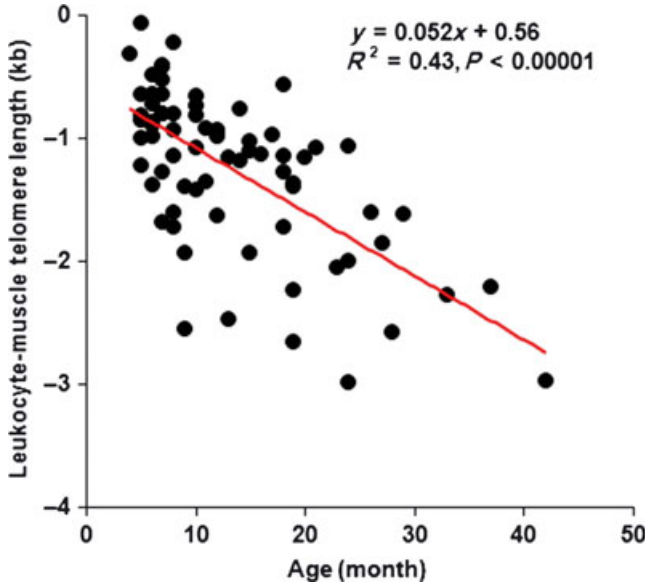


**Fig. 4** The difference between telomere length in leukocyte and skeletal muscle (ΔLMTL) vs. age. *N* = 74.

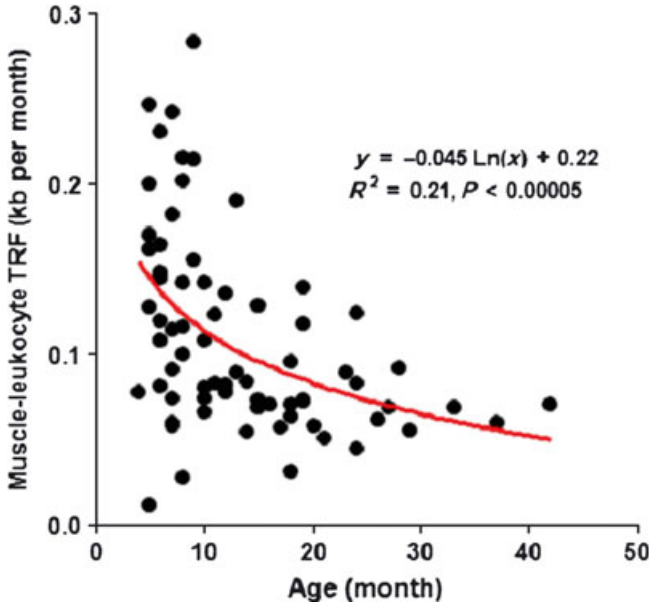


**Fig. 5** The rate of change per month in the difference between telomere length in leukocyte and skeletal muscle (ΔLMTL) vs. age. The figure displays the curvilinear nature of the relation of the rate of change in ΔLMTL (*N* = 74).

age-dependent leukocyte TL shortening can be evaluated in the context of growth, development, aging, and longevity. This cannot be accomplished based on the cross-sectional evaluation of leukocyte TL.

Previous studies were unable to convincingly show age-dependent leukocyte TL shortening in a sample of dogs of one breed or a sample of dogs of different breeds (Nasir *et al.*, 2001; McKevitt *et al.*, 2002). We confirmed the difficulty when using the conventional cross-sectional design. That said, we did detect an age-dependent shortening in leukocyte TL, where within the range of 4–42 months, age explained 6% of the inter-individual variation in leukocyte TL among dogs. However, age explained 43% of the inter-individual variation in ΔLMTL within this age range.

The growth rate of dogs, like those of most mammals, is not constant, that is, it is relatively fast early in life and gradually reaches its plateau (Hawthorne *et al.*, 2004; Trangerud *et al.*, 2007). Moreover, larger dog breeds display longer growth period than smaller ones, with an estimated attainment of adult weight at around 9 months for small breeds and up to 15 months in large breeds. Different growth phases for different breeds might explain the wide scatter of the rate of change in ΔLMTL in the younger dogs and the leveling off in this parameter in the older dogs.

It is noteworthy that normalizing leukocyte TL for fat TL provided a better account of age-dependent leukocyte TL shortening than leukocyte TL itself. However, age explained only 14.2% of the inter-individual variation in leukocyte TL, normalized for fat TL, suggesting that fat telomere dynamics might be modified by a wider inter-individual variation in the replication of fat cells than skeletal muscle cells during growth and development, which is evidently the case for humans (Arner *et al.*, 2010).

Might the findings in dogs be applicable to humans? Humans are endowed with much shorter telomeres than dogs and live much longer. Therefore, in principle, TL in HSCs, as expressed in leukocyte TL, might reach a critical length that sets a limit to the individual's life course. This is unlikely to be the case for dogs, at least based on their mean TL.

The question then is to what degree do HSC telomere dynamics define and to what extent are they defined by human aging. After all, elderly persons may die with short leukocyte TL and not because their leukocyte TL is short. Our model might help solving this puzzle by partitioning in a quantitative way the contribution of TL during early development as opposed to telomere shortening afterward to leukocyte TL at any given age. The relative contribution of these factors to the relation between leukocyte TL and aging has been intractable based on conventional cross-sectional analyses or short-duration longitudinal evaluations of leukocyte TL.

In practice, specimens of skeletal muscle (and leukocytes) for TL measurements can be obtained during surgeries in the elderly. Moreover, studies that utilize autopsy specimens might be undertaken with a view to measure leukocyte and muscle TLs and compute the ΔLMTL. It is noteworthy that little is known about the effect of injury and physical activity/exercise on TL dynamics in human skeletal muscle because most studies examining these matters were performed in a few subjects. Given the wide inter-individual variation in TL among individuals, the findings of these studies are inconclusive (reviewed in Kadi & Ponsot, 2010). It is evident, nonetheless, that little, if any, skeletal muscle TL erosion occurs in adults (Ponsot *et al.*, 2008) even though satellite cells in skeletal muscle may undergo division because of injury and perhaps other factors including exercise (Kadi & Ponsot, 2010). Certainly, even if some telomere shortening takes place after birth, it is but a fraction of leukocyte TL shortening.

In conclusion, our model assigns in quantitative terms the relative contribution of TL during early development vs. ΔLMTL to leukocyte TL dynamics in the individual. If a relatively long TL during early development, expressed in muscle TL, explains leukocyte TL in exceptionally old persons or in healthy elderly individuals, it is likely that HSC TL is a determinant in human aging and longevity. However, if short leukocyte TL in persons displaying aging-related diseases and diminished longevity is explained by a greater loss of telomere repeats since early development, expressed in ΔLMTL, it is likely that leukocyte TL shortening simply registers the pace of human aging. Given that among newborns, the inter-individual variation in TL, as expressed in leukocytes and other somatic tissues, is at least 4 kb (Okuda *et al.*, 2002; Akkad *et al.*, 2006), it is evident that at any age throughout the human life course, the main determinant of leukocyte TL, and by inference HSC TL, is TL at birth. We therefore predict that if telomere biology plays a role in human aging and longevity, it would be primarily mediated through HSC TL at birth.

Finally, there is still no coherent picture about the connection between TL and predilection to cancer in the general population, based on the measurements of leukocyte TL (Wentzensen *et al.*, 2011). This might relate in part to different methods used to measure leukocyte TL, the measurement errors of which are quite large in some laboratories, and confounding by chemotherapy and irradiation – treatments that create havoc in the hematopoietic system and might impact leukocyte TL. Measurements of muscle TL in laboratories that measure this parameter reliably would therefore provide a better account of the TL-cancer nexus.

## Experimental procedures

### Dogs and sample collections

We studied dogs of all breeds undergoing spaying or neutering. We recorded the age from dates of birth provided by the dog owners, breed, body weight, and sex of each animal. During surgery, we collected blood, subcutaneous fat, and skeletal muscle (cremaster muscle for male and rectus abdominis muscle for female dogs).

### Telomere length measurement

DNA was extracted by the phenol/chloroform method, and TL was measured by Southern blot analysis of the terminal restriction fragment length, as previously described (Kimura *et al.*, 2010b).

### Statistical analysis

Data presented in Table 1 are expressed as means ± SD. Data presented in the figures in the form of bar graphs are expressed as mean ± SEM. For each tissue, mean values of TL were compared using a paired Student's *t*-test (Fig. 1). Relationships between continuous variables were determined using Pearson's correlation coefficients. Logarithmic model provided the best fit for the relationship between the rate of ΔLMTL per month vs. age (Fig. 5). Multiple linear regression analysis was used to identify the best independent predictors of ΔLMTL and the difference between leukocyte TL and fat TL. $P$ value $< 0.05$ was considered significant.

## Acknowledgments

This work was supported by NIH grant AG030678.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Table S1** Breeds used for the study.

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