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ORIGINAL ARTICLE

Telomere length in leukocytes correlates with bone mineral density and is shorter in women with osteoporosis

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Abstract
Summary Telomere length decreases with age and is associated with osteoblast senescence. In 2,150 unselected women, leukocyte telomere length was significantly correlated with bone mineral density. Clinical osteoporosis was associated with shorter telomeres, suggesting that telomere length can be used as a marker of bone aging.
Introduction The length of telomeres in proliferative cells diminishes with age. Telomere shortening and telomerase activity have been linked to in vitro osteoblast senescence and to increased secretion of pro-inflammatory cytokines. We explored whether bone mineral density correlates with telomere length in leukocytes.
Materials and methods The relationship between leukocyte telomere length, bone mineral density (BMD) and osteoporosis (as defined by the World Health Organization) was examined in a cohort of 2,150 women from a population-based twin cohort aged 18–79.
Results After adjusting for age, body mass index, menopausal status, smoking, hormone replacement therapy status, telomere length was positively correlated with BMD of the spine ($p<0.005$), forearm ($p<0.013$), but not the femoral neck ($p<0.06$). Longer telomeres were associated with reduced the risk of clinical OP at two or more sites (odds ratio=0.594 95% CI 0.42–0.84 $p<0.003$) and in women over the age of 50, clinical osteoporosis was associated with 117 bp shorter telomere length ($p<0.02$) equivalent to 5.2 years of telomeric aging.
Conclusions Shortened leukocyte telomere length is independently associated with a decrease in BMD and the presence of osteoporosis in women. Our data provide evidence that leukocyte telomere length could be a marker of biological aging of bone.

Keywords Aging · Bone mineral density · C-reactive protein · Inflammation · Osteoporosis · Telomere

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Introduction

Osteoporosis is a skeletal disorder characterized by compromised bone strength, which predisposes the affected individual to an increased risk of fragility fracture. These fractures usually occur with minimal trauma and may cause

prolonged suffering, incapacity, and premature mortality [1]. The fractures may be due to failure to achieve an adequate bone mass in early adulthood, loss of bone mass which accompanies age-related changes in bone architecture, or both [2].

Bone mass and susceptibility to osteoporotic fracture can be assessed by measurement of bone mineral density (BMD). A low BMD, estimated by dual-energy X-ray absorptiometry (DEXA), is known to be among the strongest risk factors for osteoporotic fracture [3].

With advancing age, a progressive loss of bone is observed [4]. This bone loss results, at least in part, from inadequate bone formation by osteoblasts [5]. Changes in either the replicative potential or the life span of osteoblasts may alter the rate of bone formation and osteoblast survival and these changes may influence the development of postmenopausal osteoporosis [6].

Human chromosomes terminate in telomeres, which protect chromosomes from degradation and end-to-end fusion [7]. Telomeres undergo erosion with each cycle of replication until the telomere reaches a critical length. At this stage a poorly understood signal triggers cessation of replication. Depending on the cell type, telomere shortening may trigger the loss of a cell's ability to replicate or lead to apoptosis [7, 8].

Apart from the number of cell replication cycles, other factors such as oxidative stress contribute to telomere shortening in a way not strictly dependent on the number of cell divisions [9]. Other DNA damaging agents, including alkylation or ultraviolet (UV) irradiation are also known to accelerate telomere attrition. In dividing somatic cells, although there is wide individual variation, older adults on average have shorter telomeres than younger adults with an extrapolated rate of decrease of 20–40 bp/year [10–12]. The inflammatory system appears to be an important mediator of both telomere length and bone density. Immune system activity is in fact directly linked to bone biology, both through the production of pro-inflammatory cytokines, which are higher in women with osteoporotic fractures [13] and through the secretion of receptor activator of nuclear factor-kappaB ligand (RANKL), one of the central regulators of bone resorption [14]. Indeed, in mice lacking T-cells, ovariectomy does not lead to bone loss [15], while inhibition of the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) also completely prevents ovariectomy-associated bone loss in mice [16], indicating that TNF-α may be involved in menopause-associated bone loss. Similarly, leukocyte telomere length may also be affected by exposure to inflammatory cytokines as TNF-α is able to directly inhibit the telomere lengthening enzyme, telomerase [17].

Telomeres are also shorter in several age-related conditions, including essential hypertension and atherosclerosis [11, 18–20]. Correlations between short telomeres in lymphocytes and the incidence of vascular dementia [9] and Alzheimer's disease [8] have also been reported.

Aging is known to be associated with various declines in osteoblast characteristics, such as cell growth, the mitogenic responsiveness to hormones and growth factors, and the secretion of osteoblastic markers [21]. Human osteoblasts ectopically expressing telomerase reverse transcriptase (hTERT), exhibit specific survival advantages by sustaining their replicative potential and maintaining the phenotypic and functional properties of primary osteoblasts in vitro [22]. Further, transfecting hTERT into pre-senescent human (in vitro aged) osteoblasts prevents bone loss when they are grafted into mice [23].

Based on such data we hypothesized that telomere length may be associated with bone mineral density in vivo. Given that the relationship between osteoblast telomere length and BMD cannot be explored in large-scale studies in humans and the fact that white blood cell telomere length can reflect T-cell telomere length, we examined the association between telomere length in leukocytes (an abundant replicating tissue), BMD and osteoporosis (OP) in a population-based sample. Finally, we assessed whether systemic inflammation may modify this relationship.

Materials and methods

Study participants Twins were identified from the St. Thomas' UK adult twin registry (TwinsUK) and were invited to participate in the study. Female twin pairs were 18–80 years of age and were measured for an extensive range of clinical phenotypes related to cardiovascular disease, obesity, diabetes, and osteoporosis. Here we report only the data relevant to the study of BMD. Both twins attended the clinic together for the collection of clinical data, which included age, height, and weight. Measurement of anterior-posterior projection of lumbar spine (L1-4), hip (femoral neck and forearm BMD was made using DEXA (QDR 2000W, Hologic), as described elsewhere [24]. General medical, gynecological, and lifestyle questionnaires were completed at interview. All women provided informed consent approved by the St. Thomas' Hospital Research Ethics Committee. Clinical osteoporosis was defined using WHO criteria of a DXA T-score of T-score ≤−2.5 at three separate anatomical sites: total spine, femoral neck and forearm. We also calculated the number of sites (0–3) at which an individual was affected by clinical osteoporosis [25]. Individuals who reported being on bisphosphonates were not included in the present study. Current physical activity during leisure time was recorded on a 4 point scale; 1=inactive, 2=light, 3=moderate and 4=heavy. This classification system was significantly corre-

lated with more detailed activity assessments reported in previous exercise research on a subset of these individuals several years earlier based on the Allied Dunbar Health Survey [26].

Telomere length measurement A venous blood sample was taken after an overnight fast and the mean leukocyte terminal restriction fragment (TRF) length was measured using the southern blot method as previously described [11]. Each DNA sample was resolved in duplicate (on different gels). If the difference between the duplicates was > 5%, a third measurement was performed and the mean of two results < 5% apart is taken. The coefficient of variation of the telomere terminal restriction fragment length (TRFL) assay in this study was 0.92%.

Other Laboratory Measures Urine deoxypyridinoline (DPD), corrected for creatinine, was measured using reversed-phase high performance liquid chromatography as described elsewhere [27]. Urine samples were collected as the second voided sample after an overnight fast and stored at −45°C until assayed. Bone specific alkaline phosphatase (BSAP) was measured using reagents from Quidel Co(Metra-BAP; Quidel Co., San Diego, CA). In this assay BSAP is captured by monoclonal antibody coated on a microtitre plate and the enzyme activity is then measured using p-notrophenyl phosphate as substrate. Lower detection limit of the assay is 0.7 u/L and the precision of the assay at BSAP value of 12 U/L was 5.2%. Sensitive C-reactive protein (CRP) assays were performed by an ELISA method, which has a lower detection limit of 0.15 mg/L and a coefficient of variation of 8.7% at 0.5 mg/L.

Statistical methods In this cohort we have already seen that age, body mass index (BMI), and smoking status (never smokers, ex-smokers and current smokers) influence telomere length [10] so, they were included as covariates. In addition, although hormone replacement therapy status (never use, previous or current use of HRT) is not associated with telomere length it is associated with all the bone-related measurements in this study (BMD, bone markers, osteoporosis) so it was consistently included as a covariate. Menopausal status was not associated with telomere length after adjusting for the other covariates but it affected the bone related measurements so it was also included as a covariate. Bone turnover markers (BSAP and DPD) and CRP levels were log transformed for all analyses although the natural scale value is shown in some plots.

Telomere length was regressed on age, BMI, smoking history and HRT and menopausal status and the Pearson's correlation coefficients between the multiply-adjusted TRFL and all bone-specific parameters were then computed. Linear regression techniques were used to assess the statistical significance of the correlations. Because twin-pair data are not independent observations, we examined the correlation between TRFL vs. the various factors using a linear mixed effects model which included twin pair of origin as a random effect and all other dependent variables as fixed effects. Multivariate analyses of variance were used to compare the characteristics (telomere length or CRP levels) of women with OP at one or more sites and without osteoporosis. A logistic regression model was performed to assess the effect of predictor variables on risk of OP where the outcome variable was either presence of OP at any site or OP at two or more anatomical sites and the predictor variables were serum CRP and telomere length including the covariates (age, BMI, HRT, smoking and menopausal status). S-Plus 6.0 (Insightful Corp, Seattle WA) software was used.

Results

The descriptive statistics of the study participants are presented in Table 1. Approximately one-half of study women were post-menopausal and one-third of these post-menopausal subjects (17% overall) were, or had been, on hormone replacement therapy or estrogens (HRT) at some point in time. Telomere length was strongly associated with age (Pearson's correlation coefficient $r=0.4241$ $p=2\times10^{-91}$). In this cohort the average extrapolated telomere erosion rate was 22.4 bp/year (standard error 1.1 bp) and appears to be constant with age, with no significant difference in rates of loss between various age ranges (not shown).

Without adjusting for age or any other covariates, significant positive correlations between bone mineral density at all three sites telomere length were observed as follows: lumbar spine ($r=0.1448$ $p<1\times10^{-8}$), forearm ($r=0.1667$ $p<2\times10^{-9}$) and femoral neck ($r=0.1789$ $p<2\times10^{-10}$).

The correlation remained statistically significant ($p<0.05$) at two of the sites, but not at femoral neck ($p<0.052$), after adjusting for age, BMI, smoking, menopausal and HRT status (Table 2) although the proportion of the variance in adjusted TRFL accounted for by BMD was small. The significance and magnitude of these correlations were not affected when levels of physical activity were included in the model (data not shown). Telomere length was also negatively correlated with the number of sites with osteoporosis $p<0.0142$ (Table 2), but the association with presence/absence of OP did not reach statistical significance ($p<0.07$, Table 2).

We examined the association between telomere length and two biochemical markers of bone turnover: serum

Table 1 Descriptive statistics of study cohort

Trait	Mean or percent	SD	(n with data)	% Osteoporosis [1]
Age (years)	48.28	12.94		
TRFL (kb)	7.09	0.70		
BMI (kg/m^2)	24.85	4.52		
CRP (mg/L)	3.20	6.61	(1270)	
DPD (nmol/mmol creatinine)	5.38	2.04	(1462)	
BSAP (U/L)	19.8	14.8	(1454)	
Forearm BMD (g/cm^2)	0.554	0.058	(1943)	4.4%
Neck of femur BMD (g/cm^2)	0.808	0.127	(2150)	7.2%
Spine total BMD(g/cm^2)	0.994	0.145	(2126)	7.5%
Osteoporosis in at least one of three anatomical sites	13.03%		(2150)	
HRT ever %	17.9%		(2150)	
Cigarette smoker, ever %	34.6%		(2150)	
Post-menopausal %	47.9%		(2150)	

[1] Clinical osteoporosis was defined using WHO criteria of a DXA T-score of ≤ −2.5 at each of three anatomical sites (forearm, neck of femur, total spine).

bone-specific alkaline phosphatase (BSAP) and urine deoxypyridinoline (DPD) corrected for creatinine. After adjusting for all covariates, both log-transformed markers were positively correlated with the presence of clinical osteoporosis (r=0.0748 p<0.0024 for BSAP and r=0.1104 $p<4\times10^{-5}$ for DPD). Bone mineral density at all three sites was negatively correlated with levels of these markers as follows: spine (BSAP r=−1435 $p<6\times10^{-9}$; DPD r=−0.1484 $p<3\times10^{-8}$), femoral neck (BSAP r=−0.080 p<0.002; DPD r=−0.116 $p<2\times10^{-5}$) and forearm (BSAP r=−0.096 p<0.0002; DPD r=−0.178 $p<3\times10^{-10}$). BSAP was also significantly associated with leukocyte telomere length (p<0.004, Table 2), but no association was seen between telomere length and urine DPD (Table 2).

Table 2 Correlation between telomere length and bone-related traits: bone mineral density (BMD) at three sites (forearm, femoral neck, spine), number of sites with osteoporosis, presence of osteoporosisserum bone specific alkaline phosphatase (BSAP) and urine DPD concentrations

Trait	Correlation with leukocyte telomere length	
	All N≤2150	Adjusted for CRP N=1270
Forearm BMD	**r=+0.0539 (p<0.0127)**	r=+0.0529 (p<0.0581)
Femoral neck BMD	r=+0.0400 (p<0.0517)	r=+0.0141 (p<0.55)
Spine BMD	**r=+0.0583 (p<0.0050)**	**r=+0.0640 (p<0.0166)**
Number of sites with OP	**r=−0.0520 (p<0.0142)**	**r=−0.0600 (p<0.0277)**
OP affected/unaffected	r=−0.0387 (p<0.0678)	**r=−0.0529 (p<0.0467)**
Log BSAP	**r=−0.0721 (p<0.0033)**	**r=−0.0648 (p<0.0386)**
Log DPD	r=−0.0401 (p<0.22)	r=−0.0436 (p<0.18)

The Pearson's correlation coefficient between bone biology traits and leukocyte telomere length (adjusted for age, BMI, smoking history, menopausal status and HRT status) and the p-value for the robust linear regression are shown. For the subset with information on serum CRP levels the correlation and p-value also adjusted for log (CRP) are shown.

We then investigated if levels of systemic inflammation, as measured by c-reactive protein (CRP) [28] could modify the relationship between BMD and telomere length, given that pro-inflammatory cytokines have been shown to decrease telomere length and BMD. We found that serum CRP levels (log transformed) were negatively correlated with telomere length, after adjusting for age, smoking status and BMI and non-independence between twins, (Pearson's correlation coefficient r=−0.077, p<0.019 ; Fig. 1a). In addition serum CRP levels were also associated with osteoporosis: women with OP at two or more sites had 61% higher CRP levels than women with no OP. The p-value for the linear trend of the relationship between CRP the number of sites with osteoporosis in the log scale (after adjusting for age, BMI and non-independence between twins) was 0.045 (Fig. 1b).

We hypothesized that, if inflammation is a common mechanism between leukocyte telomere shortening and osteoporosis, the association between telomere length and bone-related traits could be explained to a certain extent by serum CRP levels. We computed the correlation between telomere length- adjusted for log(CRP), age, BMI, HRT and smoking status- and BMD, number of sites with OP and markers of bone remodelling. We note that for most traits size of the sample with CRP levels was 60% less than the one available for the full set. However the correlations with serum BSAP, spine BMD and number of sites with OP remained statistically significant after adjusting for CRP. The correlation with forearm BMD was no longer statisti-

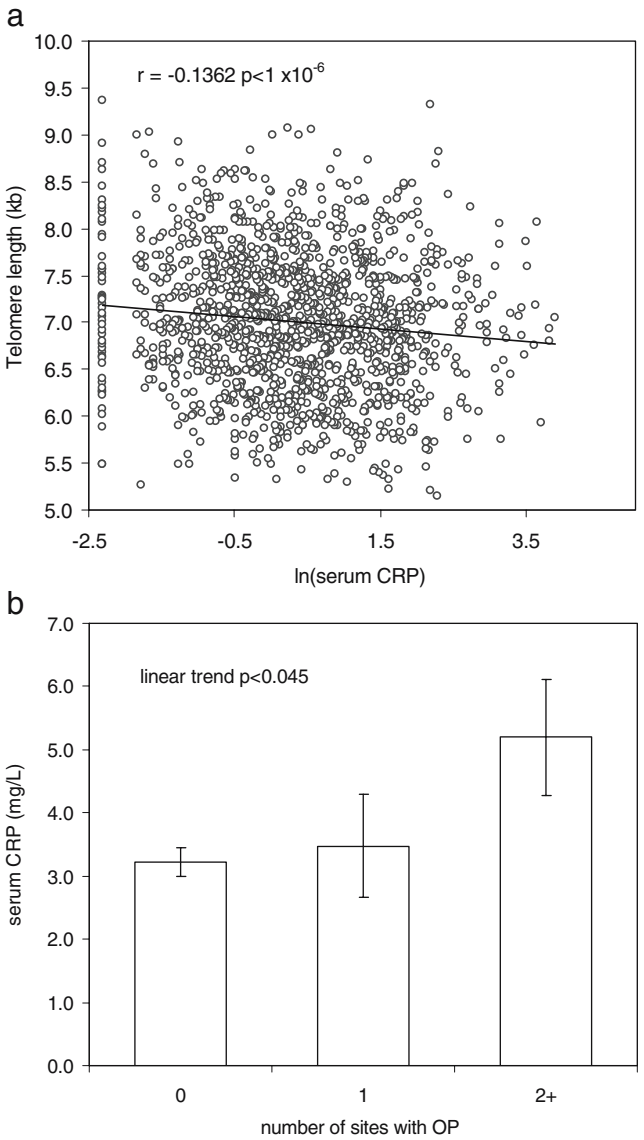

Fig. 1 (**a**) Correlation between telomere restriction fragment (TRF) length and serum C-reactive protein (CRP) unadjusted for age or other covariates. (**b**) serum CRP levels among healthy women and women with clinical osteoporosis at one and two or more sites. Means and standard errors are adjusted for age, smoking status, body mass index, HRT and menopausal status

cally significant ($p<0.058$) but the magnitude of the correlation was very similar to the one without adjustment suggesting that the smaller sample size might explain this observation. Thus, the correlations observed between telomere length and BMD, serum BSAP, and OP cannot be accounted for by low grade inflammation as measured by CRP levels. Moreover, the presence or absence of OP at any site was significantly associated with telomere length once we adjusted for CRP ($p<0.05$ Table 2).

We investigated if telomere length influenced the risk of clinical osteoporosis, and if so whether the association was dependent on inflammation. We calculated by logistic regression the effect of telomere length on the risk of OP. The odds ratio for TRFL on presence of OP was 0.795 (95% CI 0.63–1.01 $p<0.061$). The odds ratio if healthy women were compared to women with OP at two or more anatomical sites was 0.594 (95% CI 0.42–0.84 $p<0.003$) so that for each kilobase of longer telomeres a woman had >40% lower odds of having OP at two or more sites. Adjusting for CRP resulted in an OR=0.743 (95% CI 0.54–1.02 $p<0.068$) for OP and 0.522 (95% CI 0.33–0.82 $p<0.005$) for OP at two or more sites.

A comparison of the age-adjusted TRFL between women unaffected by OP at any of the three sites, with those affected revealed that women with OP at two or more sites had 206 bp shorter TRFL than unaffected women (Fig. 2a; $p<0.0034$). The difference of women affected by OP at any site and healthy women was 83 bp ($p<0.068$ n.s.)

Because most of the women in our sample with OP were post-menopausal, we assessed whether the above results

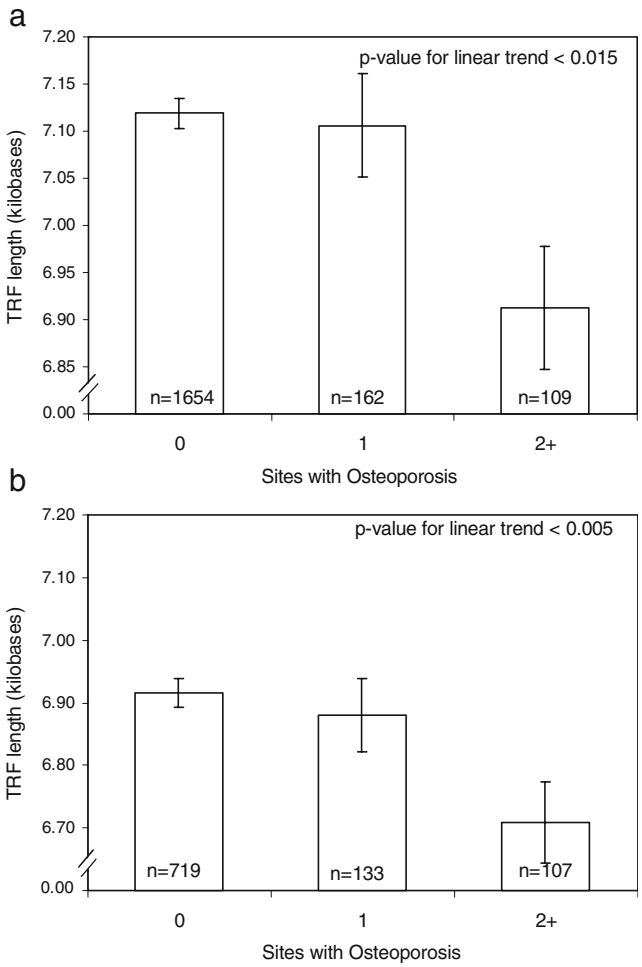

Fig. 2 Telomere restriction fragment (TRF) length among healthy women and women with clinical osteoporosis at one and two or more sites, (**a**) all women (**b**) women aged 50 and older. Means and standard errors are adjusted for age, smoking status, body mass index, HRT and menopausal status

were valid among women aged 50 and older. In this clinically relevant subset a linear trend between OP at 0, 1 and 2 or more sites (femoral neck, total spine and forearm) was also observed (Fig. 2b). The difference between women with no OP and those with OP at two or more sites was the same (207 bp $p<0.0048$) but the difference between women affected by OP at one or more sites and healthy women was 117 bp ($p<0.017$). Based on the extrapolated yearly rate of telomeric attrition after adjusting for all confounding variables, in telomeric year equivalence, women with OP had an extrapolated telomeric age 5.2 years older than their age matched peers.

Finally, we explored if a subgroup analysis of discordant twins supported the above results. In the current set there were 123 twin pairs where one twin was unaffected and the other had OP at one or more sites. The twin with OP was found to have on average 79.4 bp shorter telomeres than her healthy twin (6.86 ± 0.06 vs 6.94 ± 0.06, $p<0.08$). Although this difference did not achieve statistical significance, the results are consistent with the observation that osteoporosis is associated with shorter leukocyte telomere length after additional stratification for genetic factors.

Discussion

In this study we found a significant or nearly significant correlation between bone mineral density in women at three anatomical sites (neck of femur, spine and forearm) and leukocyte telomere length. The correlation with femoral neck BMD with telomere length than is BMD was weaker than those with lumbar spine or radius. This could explained by the femoral neck's smaller area and hence in this sense represent a slightly less robust measurement. Although these correlations were modest, they were similar in magnitude to the correlations between markers of bone turnover (DPD and BSAP) and BMD. We also observed that after adjusting for various confounders women with OP at two or more sites had significantly shorter telomeres than healthy women of the same age. The association between telomere length and OP was stronger among women aged over 50; therefore, it is unlikely that our results could have been confounded by the wide age range of the sample. The fact that twins with no OP had on average longer telomeres than their OP affected co-twins gives further support to this observation.

Our results partly concur with recent data in 84 elderly healthy males with forearm BMD data, which showed that shorter telomere length correlated with longitudinal forearm bone loss [29]. The authors found no effect with baseline BMD, although the sample size might have prevented the authors from detecting an association with cross-sectional data [30]. Our data are in disagreement with a previous study [31] which found no difference in leukocyte telomere length between controls and patients with osteoporosis. However, the study sample was less than 30 individuals suggesting that lack of statistical power might have produced that result.

We also found using serum CRP as a surrogate for inflammation, that the presence of some inflammation was associated with shorter telomeres, and that shortened telomere length was also associated with increased CRP levels. These results appear consistent with the hypothesis that inflammation is associated with both the pathogenesis of osteoporosis and telomere shortening. Shorter telomeres are associated with pronounced inflammation in specific organs [32] and in our data shorter telomeres are associated with higher serum levels of CRP. Among patients suffering with rheumatoid arthritis, BMD has been reported to be significantly and negatively correlated with serum CRP levels [33]. Adjusting for CRP serum levels, however, did not remove the association of telomere length with spine BMD, sites with OP, and serum BSAP.

Oxidative stress enhances telomere erosion [9] at each replication cycle. Leukocyte telomere length might hence register, at least in part, the cumulative burden of inflammation and oxidative stress during the individual's lifetime. Both processes have been implicated in BMD loss. For instance, plasma levels of antioxidant vitamins C, E, and A and the enzymatic activities of superoxide dismutase in plasma and erythrocytes and of glutathione peroxidase are markedly decreased in women with OP compared with controls [34]. Further, there is substantial evidence that bone remodeling is influenced by subtle changes in pro-inflammatory and inhibitory cytokines [35]. Emerging clinical and molecular data suggest that inflammation exerts significant influence on bone turnover, inducing OP [23]. Thus it is possible that telomere length and BMD are correlated because both reflect oxidative stress and inflammation. Some of our results point to a possible link between inflammation, telomere length and bone mineral density. This observation could be explained by data showing that specific T-cell populations which appear to have entered replicative senescence and have shorter telomeres produce higher amounts of pro-inflammatory cytokines including RANKL and are associated with OP [35]. However the relationship between OP and TRFL does not appear to be directly influenced by low grade inflammation.

A rate of TRF shortening was about 100 bp per population doubling has been reported in osteoblasts and cells from osteoporotic bone have been shown in vitro to have reduced proliferative capacity and characteristics of senescent cells [31]. Although there are no data in humans directly correlating osteoblast and leukocyte telomere length, telomere length is partially synchronized in adults [36, 37] and some authors have suggested that TRF

measurement in blood cells could serve as a surrogate parameter for the relative telomere length in other tissues such as skin and synoviocytes [37]. Thus it is likely that there could also be a correlation between the results seen in the leukocytes and specialized tissues such as bone.

We note potential limitations in the current study. The sample consisted of twins rather than singletons. Yet, a previous study has shown that the participants of the TwinsUK cohort are comparable to age-matched population singletons in terms of disease-related and lifestyle characteristics, including BMD [38]. Twins are also related, which could bias the levels of significance, but we have adjusted for this using robust regression methods. Another limitation is that CRP was measured at a single time point so it may not necessarily represent cumulative inflammation whereas telomere length is likely to reflect the cumulative history of oxidative stress and inflammation. In addition we are unable with the present data to suggest a clear mechanism for the observed association. We have hypothesized there could be a correlation between leukocyte and osteoblast telomere length, but no longitudinal data on rates of erosion on these tissues exist, and the two may not be synchronized. Finally, the data shown here relate to bone mineral density which, although predictive of fracture risk, remains only a surrogate, and therefore our data do not necessarily imply an association between telomere length and risk of osteoporotic fractures.

In conclusion, we observed statistically significant correlations between age-adjusted telomere length and indices of OP in a large cohort of women. Further research is needed to test the causal pathways of these possible mechanisms and such studies could lead to advances in our understanding of OP and the aging processes of bone.

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