EXTENDED REPORT

Reduced telomere length in rheumatoid arthritis is independent of disease activity and duration

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Background: Rheumatoid arthritis (RA) is associated with reduced lifespan and shortened telomere length in lymphocytes, but the mechanism underlying this is unclear. Telomere loss in white blood cells (WBC) is accelerated by oxidative stress and inflammation in vitro. It was postulated that the accelerated WBC telomere shortening in RA occurs as a result of exposure to chronic inflammation.

Objectives: To measure telomere terminal restriction fragment (TRF) length in a large cohort of RA cases and healthy controls, to explore associations of TRF length with features of disease and with RA-associated HLA-DRB1 alleles.

Methods: WBC and TRF length were measured by Southern blot in DNA from 176 hospital-based RA cases satisfying the 1987 American College of Rheumatology criteria and from 1151 controls. TRF length was compared between cases and controls, and the effects of disease duration, severity and HLA-DRB1 alleles encoding the shared epitope (SE) were assessed.

Results: Age- and sex-adjusted TRF length was significantly shorter in RA cases compared with controls (p<0.001). There was no association between age- and sex-adjusted TRF length and disease duration, C reactive protein or Larsen score. The presence of one or more SE-encoding alleles was associated with reduced adjusted TRF length in RA cases (SE positive vs SE negative cases, p=0.038), but not in controls.

Conclusion: The reduced TRF length in a large group of patients with RA compared with controls has been shown. The reduction is apparently independent of disease duration and markers of disease severity, but is influenced by HLA-DRB1 genotype.

Telomeres consist of TTAGGG tandem repeats and associated proteins at the ends of chromosomes.[1] By demarcating the ends of chromosomes from random DNA breaks, telomeres prevent inappropriate DNA repair and end-to-end fusion. Telomere length in white blood cells (WBC) declines progressively with age at rates of about 20–40 bp per year. This decline is apparently accelerated in the presence of oxidative stress and inflammation. WBC telomere length in the individual, therefore, reflects telomere length at birth and lifelong WBC replication, which may capture the cumulative burden (rather than the acute) effect of inflammation and oxidative stress.[2] Rheumatoid arthritis (RA) is characterised by an increase in both chronic inflammation and oxidative stress. What is more, the immune system of patients with RA shows features of premature senescence, with contraction of T cell receptor diversity,[3] oligoclonal proliferation and loss of expression of CD28,[4] which is associated with shortened telomere length in cultured lymphocytes.[5] Indeed, short telomeres in peripheral blood T lymphocytes as well as granulocytes have been reported in RA,[6] and it has been proposed that this is a feature specific to individuals expressing HLA-DR4 (one of the shared epitope (SE)-encoding HLA-DRB1 alleles).[7]

The aims of this work were to examine WBC telomere length in large cohorts of well-characterised outpatient RA cases and controls and to explore associations between telomere length and various clinical features of disease. Through analysis of telomere length with respect to HLA-DRB1 alleles in patients and a subset of controls, we aimed to determine whether premature telomere erosion was a feature of RA associated primarily with HLA status or with the presence or severity of the disease itself.

MATERIALS AND METHODS

Cases of RA satisfying the 1987 American College of Rheumatology diagnostic criteria were recruited consecutively from clinics at Guy's, St Thomas' and Lewisham hospitals in London, UK. Details were available on age, sex and disease duration, as well as parameters of disease severity such as rheumatoid factor status, Larsen Score of hand radiographs, disease-modifying anti-rheumatic drug (DMARD) usage (from a retrospective review of patients' records) and modified Health Assessment Questionnaire (mHAQ) scores. Single measurements of CRP and disease activity scores (DAS28) were also available. Healthy controls were obtained from the Twins UK Adult Twin Registry, which has recruited healthy twin volunteers through national media campaigns. This cohort has been shown to be similar to the general population for age-related traits.[8] For historical reasons, the cohort is predominantly female. Both ethical approval and written informed consent to participate were obtained. Genomic DNA was extracted from peripheral WBC using a standard phenol/chloroform method.[9] DNA samples were checked for quality and integrity before duplicate measurements of the length of the terminal restriction fragments (TRF), obtained by the Southern blot method as detailed elsewhere.[10] The coefficient of variation of the TRF length in this study was 0.92%. Twin DNA TRF length was measured as part of ongoing studies of WBC telomeres.[11] Measurements in cases and controls were

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performed in the same laboratory. Briefly, DNA samples were digested overnight and resolved on 5% agarose gel at 50 V. After 16 h, the DNA was depurinated for 30 min, denatured for 30 min and neutralised for 30 min. The DNA was transferred for 1 h to a positively charged nylon membrane, then hybridised with the telomeric probe (digoxigenin 3'-end labelled 5'-(CCTAAA)$_3$) overnight in 5×SSC 0.1% Sarkosyl, 0.02% sodium dodecyl sulphate and 1% blocking reagent. After washing 3 times for 15 min, the digoxigenin-labelled probe was detected by the digoxigenin luminescent detection procedure and exposed on *x* ray film. Each DNA sample was measured in duplicate. Class II human leucocyte antigen typing in RA cases and a subset of controls was performed using sequence-specific primers.[12][13]

A single twin was selected at random as a control from each of 1204 twin pairs. Standard multiple regression techniques were used to investigate associations between TRF length and age, and age-adjusted TRF length with clinical features. Associations between categorical variables and TRF length, adjusting for age and other covariates, were assessed using analyses of covariance.

RESULTS
General characteristics of the subjects
Complete data on TRF length and disease variables were available for 176 cases. The mean age of the cases was significantly greater than that of controls (63.8 vs 48.2 years; p<0.001). The proportion of males in our RA cases (16.5%) was greater than that in controls (5.6%; p<0.001). Current smokers made up similar proportions of both cases (28%) and controls (27%), but there was a higher proportion of ex-smokers among cases (39.8% vs 17.2%; p<0.001).

TRF length in cases and controls
Figure 1 shows the relationship between TRF length and age in all subjects. Overall TRF length decreased similarly with age in both groups with a correlation of −0.398 in cases and −0.382 in controls. At all age points, RA cases displayed lower mean TRF length than controls, with a mean difference of 369 bp. TRF length was longer in women than in men (6.93 vs 6.36 kb; p<0.001 after adjusting for age). This sex difference was present in both RA cases (females 6.6 kb; males 6.37 kb; p = 0.046, age adjusted) and controls (females 6.98 kb; males 6.33 kb; p<0.001, age adjusted). Therefore, we adjusted for sex as well as age in subsequent analyses. The mean age- and sex-adjusted TRF length of cases was significantly shorter than that of controls, 6.62 kb vs 6.93 kb, p<0.001 (fig 2). Adjustment for smoking status did not alter the relationship with TRF length (data not shown).

TRF length and RA features
Linear regression analysis of RA cases showed that, taken individually, greater age (p<0.001), male sex (p = 0.014) and longer disease duration (p = 0.042) were all associated with reduced TRF length, while smoking status was not. Multivariate regression analysis showed that disease status, age and male sex were all independently associated with reduced TRF length (table 1).

If telomere attrition in RA occurs as a result of cell exposure to chronic inflammation, some form of dose–response relationship with disease duration, severity or markers of the acute phase response may be expected. However, there was no association between disease duration and age- and sex-adjusted TRF length, or with CRP level, DAS28 or mHAQ score. Age- and sex-adjusted TRF length did not differ between cases seropositive and seronegative for rheumatoid factor (6.30 and 6.27, respectively; p = 0.73). The presence of erosive disease also

had no clear effect (erosive vs non-erosive (6.31 vs. 619); p = 0.36). The Larsen score was significantly associated with disease duration, and, once this was taken into account, no independent effect of Larsen score was seen. We were unable to identify any significant associations between number of DMARDs, NSAIDs or steroids used during the course of disease age- and sex-adjusted TRF length.

TRF and HLA-DRB1 genotype
HLA-DRB1 genotype information was available in 170 cases and 128 controls. The presence of one or more copies of the SE (defined by alleles *0401, *0404, *0405, *0408, *0101,*0102, *1001 and *1402) did not influence age- and sex-adjusted TRF length in controls (SE positive vs SE negative controls (6.86 vs 6.83); p = 0.80). In cases, age- and sex-adjusted TRF length was shorter in those positive for the SE (SE positive vs SE negative cases (6.35 vs 6.59); p = 0.038; fig 3A). Comparison of cases and controls by SE status showed that TRF length in SE positive cases of RA was significantly shorter than that in SE positive controls (6.35 vs 6.86 kb, p<0.001, adjusted as before), with a similar but non-significant trend in those negative for SE (p = 0.092). We found no evidence of a dose–response relationship with the number of SE-encoding alleles. Subgroup analysis by presence of DR1 or DR4 alleles showed no statistically significant differences in adjusted TRF length between DR1 positive and DR4 positive cases, or between DR1 positive and DR4 positive controls (fig 3B). In summary, disease status was the dominant influence on TRF length, but the presence of the SE seemed to compound this effect in RA cases.

DISCUSSION
Patients with RA have reduced life expectancy. Recently, this observation has been attributed to an excess of cardiovascular deaths.[14] Telomere length is a putative marker of biological age and has been shown to be reduced in WBC from patients with age-related diseases such as Alzheimer's dementia,[15] atherosclerosis[10] and hypertension,[16] and also to be associated with early mortality in the elderly in some[17] but not all[18] studies. Two previous studies have suggested that telomere length is reduced in RA.[6][19] Our data, based on a larger dataset than either of the previously published studies, show two important findings: (1) that WBC telomere length declines in a linear fashion in both RA cases and controls, in contrast with the findings of Koetz *et al*[6]; (2) that age- and sex-adjusted WBC telomere length in RA cases is considerably shorter than in controls. The difference is equivalent to 15 times the annualised rate of WBC telomere length loss in age-adjusted controls extrapolated from cross-sectional data.

Our data do not support the hypothesis that inflammation in RA is the predominant factor promoting WBC telomere shortening, but show that telomere length measured cross-sectionally is reduced independent of RA duration and severity. That the regression lines of TRF length against age for cases and controls are parallel (fig 1) suggests that this difference in telomere length is present at a young age and it seems not to increase with age. These observations are consistent with reduced WBC telomere length in RA being an early, and possibly disease-predisposing, process rather than occurring secondary to disease. We note, however, that these tentative conclusions are based on cross-sectional analysis. Given the wide variation in age-adjusted WBC telomere length, a longitudinal study focusing on WBC subsets over an extended period would be the ideal design, but for statistical reasons this study is likely to have underestimated, rather than overestimated, changes in telomere length with age.[20] All leucocyte subsets contribute to pathogenesis of RA, with both distinct and interacting pathways, and possibly we have failed to detect
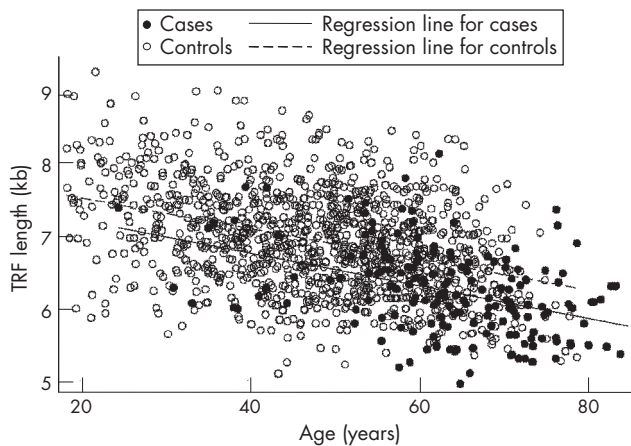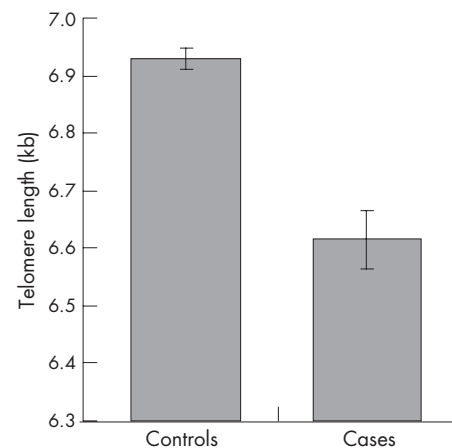
Figure 1 Relationship between terminal restriction fragment (TRF) length and age in 176 RA cases and 1151 controls.

Figure 2 Age and sex-adjusted terminal restriction fragment (TRF) length (mean (SD)) of 1151 controls and 176 cases. Difference in means (SD), 0.315 (0.11) kb; (p<0.001).

an isolated reduction in TRF length that is influenced by disease duration in one subset. Furthermore, the findings in leucocytes from the synovium and synovial fluid may be quite distinct from those in peripheral blood, reflecting perhaps selection differences during trafficking to synovial tissue, as well as the hypoxic, highly stressed conditions within the inflamed rheumatoid joint. Investigation of the differences between peripheral and tissue-specific white cell subpopulations in a longitudinal study may generate further insights into their relative rate of ageing; this combined with detailed studies of the effects of senescence on the functions of the subpopulations[5] may help advance our understanding of disease initiation and perpetuation.

WBC telomere length is partly genetically determined, as we have recently shown using the control group studied here. It has a heritability of approximately 40%.[21] A preliminary investigation in relation to class II HLA-DR antigen expression[19] showed that HLA-DR4 was associated with reduced telomere length in peripheral blood T-cells and granulocytes in a small sample of young healthy controls. Our data, from a much larger sample of older subjects, do not support an effect of HLA-DRB1 genotype on telomere length in peripheral blood WBC of healthy controls. This has many possible explanations including the potential for greater influence of environmental exposures with age; Schonland et al[19] showed maximal difference between DR4 positive and DR4 negative controls in young adulthood, with no influence of genotype on annual rate of telomere loss in older age groups. Although there are no data to suggest altered distribution of white cell subpopulations between different HLA-DRB1 genotypes, the fact that we measured the WBC TRF length in whole peripheral blood might have reduced our ability to detect a difference, and further experiments on WBC subsets will be required to draw firm conclusions. An increase in the coefficient of variation of lymphocyte TRF length with age has been shown and this might also have reduced the sensitivity of our analysis.[22] We did detect an effect of HLA-DRB1 genotype on TRF length in RA cases. Our findings are consistent with those of Schonland et al,[19] who identified short telomeres in lymphocytes and granulocytes of DR4 positive RA cases. We did not detect a difference between DR4 and DR1 subgroups, but this analysis did not have sufficient power.

As with any study, there are potential limitations. The RA cases and controls were not age matched: rather than losing information by using smaller age-matched subgroups, we adjusted for age in the analyses. Such an analysis assumes that TRF length declines linearly with age, a feature suggested

by our large cross-sectional dataset.[11] A subgroup analysis of patients and controls having similar ages, however, gave the same result. Single measurements of CRP and DAS28 scores may be criticised as poor surrogates for long-term disease activity; the Larsen score, however, is considered a good reflection of cumulative disease burden. Subgroup analysis of SE negative cases and controls was underpowered, reflecting the availability of HLA-DRB1 genotypes for only a proportion of the controls. Another potential limitation is that we did not have information on weight in RA cases. We have shown raised BMI to be associated with shorter WBC telomere length,[11] and a gain in BMI to be associated with accelerated WBC telomere attrition.[23] This is not a likely confounder in our results as patients with RA tend to be lighter than controls, which would act in the opposite direction and make the groups appear more similar. Smoking status was not a confounder in this dataset. In addition, the RA cases analysed here were all hospital attendees; the findings may be different in RA cases managed in primary care, which tend to have milder disease.

CONCLUSIONS

This study of RA cases confirms the association of disease with markedly reduced telomere length in peripheral WBC and shows no clear relationship between TRF length and disease severity or progression. Our data suggest that the difference may arise before the clinical diagnosis or during an early phase of the disease. The findings may point to an underlying pathogenic mechanism, perhaps reflecting altered WBC

Table 1 Multiple regression analysis with terminal restriction fragment length as the dependent variable in RA cases and controls

Covariate	Regression coefficient	p Value	R²
DS	−0.38	<0.001	0.37
Age	−0.022	<0.001	
Sex	0.273	0.03	
Current smoker	−0.028	0.75	
Ex-smoker	−0.093	0.293	
SE	0.043	0.581	

DS, disease status is coded as 0 and 1 for controls and cases, respectively. Current smoker and ex-smoker data are with those of subjects who never smoked, respectively.
SE represents shared epitope-encoding alleles, and is coded as absent if the subject carries no SE-encoding allele and as present if the subject carries one or more SE-encoding allele.
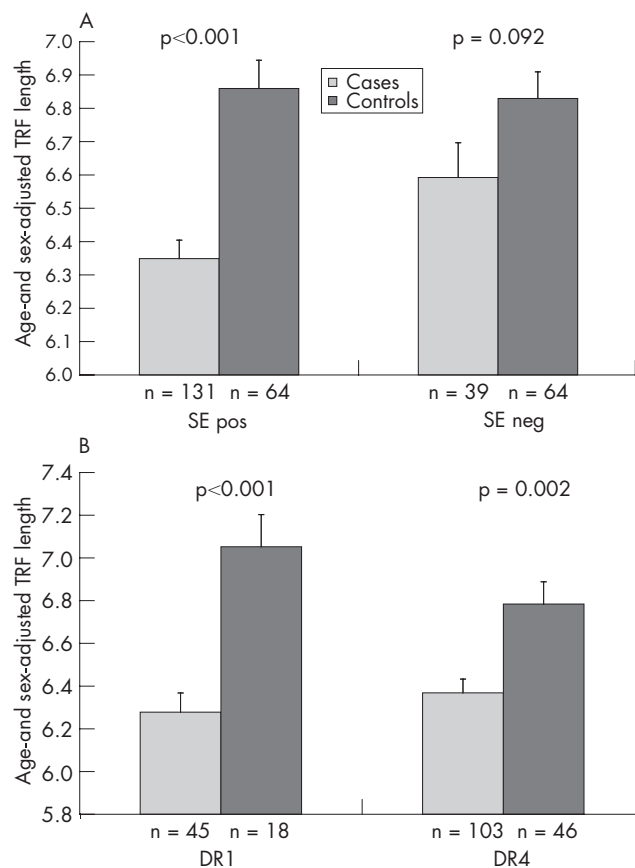
Figure 3 Comparison of age- and sex-adjusted terminal restriction fragment (TRF) length by HLA-DRB1 status in RA cases and controls. DR1, *0101, *0102; DR4, *0401, *0404, *0408; neg, negative; pos, positive; SE, shared epitope.

maturation pathways, such as thymic T cell production[6] and altered peripheral WBC turnover. In this RA population, WBC telomere length was shorter in those carrying the SE. This suggests that susceptibility to telomere erosion is at least in part determined by *HLA-DRB1* or other nearby loci in linkage disequilibrium. It remains possible, therefore, that WBC telomere dynamics (telomere length and attrition rate) will prove to be the functional basis for the association of SE with RA. Telomere length is known to be synchronised (equivalent) between tissues in the newborn, so that individuals with short telomeres in one tissue have short telomeres in other tissues.[24] Telomere length is partially synchronised in adult tissues.[25–27] It will be of interest to determine how the various types of tissue, in addition to WBC and WBC subsets, are influenced in RA. The demonstration of relatively normal TRF length in other tissues from patients with RA would support our current working hypothesis: that an early environmental influence in genetically susceptible individuals results in a prematurely aged immune system and disease. The longitudinal exploration of the role of telomere attrition in WBC subsets is likely to be a fruitful area of research in the pathogenesis of RA.

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