Association between shorter leukocyte telomeres and multiple sclerosis

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ABSTRACT

Relative telomere length (TL) is regarded as a biomarker of biological age. Accelerated immune aging, as represented by TL reduction, has been demonstrated in autoimmune diseases, including multiple sclerosis (MS). However, it is still unresolved whether telomere shortening is the cause or the consequence of the pathogenic events underlying autoimmunity.

Assessing TL in whole blood DNA samples in 138 MS patients and 120 healthy controls showed reduced TL in patients as compared with controls There seems to be a prelude of accelerated telomere shortening, which may increase the risk for development of MS.

1. Introduction

Telomeres comprise highly conserved, noncoding, repetitive sequences of DNA that, together with a number of shelterin proteins, form caps at the ends of eukaryotic chromosomes, that play an essential role in maintaining the stability and integrity of the chromosomal architecture during duplication (Turner et al., 2019). After each round of normal somatic cell division, a small part of these terminal repetitive sequences is lost. When the telomeres reach a critical length, the cell either dies through apoptosis or it enters a state of senescence by undergoing a *permanent cell cycle arrest*. Relative telomere length (TL) shows a wide inter-individual variability, which is the result of interactions between multiple environmental and genetic factors. Heritability accounts for up to 70% of the variance in TL, but only a fraction of this variance can be explained by the identified genetic variants in candidate gene and genome-wide association studies (Broer et al., 2013; Hjelmborg et al., 2015). Sex differences have also been repeatedly observed in telomere length with females having longer telomeres compared to males. A meta-analysis carried out in 2014 could confirm this, although this difference was not universally found in studies that did not use Southern blot methods (Gardner et al., 2014).

TL has been an important topic of research because of its association with cellular senescence, the process of aging and the pathogenesis of many diseases, including different autoimmune and/or inflammatory diseases, including multiple sclerosis (MS).

MS is an inflammatory neurodegenerative disease of the central nervous system that leads to demyelination, axonal degeneration, accompanying neurological impairment and disability. The disease typically affects individuals in their early adult life, with an onset between 20 years and 40 years of age. MS occurs more frequently in women than men, and is considered the most common progressive cause of non-traumatic neurological disability in young adults. The clinical manifestation is heterogeneous; including motor impairments, sensory and visual disturbances, fatigue, pain, mood disturbances and cognitive deficits, in relation to the spatiotemporal dissemination of pathological lesion sites in the CNS (Dendrou et al., 2015). MS typically begins with a relapsing remitting phase (RRMS), which can later develop into a secondary progressive phase (SPMS). The initial relapsing remitting course is marked by inflammatory events, both in the periphery and the CNS, and full or partial recovery. Conversion to SPMS is mostly diagnosed in retrospect, based on a progressive deterioration after an initial relapsing disease course. However, a small proportion of patients directly present with a progressive loss of neurological functions, without experiencing initial relapses, designated as primary progressive MS (PPMS) (Compston and Coles, 2008; Disanto et al., 2010; Lassmann et al., 2012). MS is likely caused by a complex interplay between multiple genetic and environmental factors, leading to inflammatory-mediated central nervous system deterioration (Gourraud et al., 2012). The strongest genetic risk factors for MS are genes within the HLA complex, with the HLA class II variant HLA-DRB1*15:01 showing the

most striking association with an up to threefold increased risk for developing MS (Lincoln et al., 2005). Additionally, GWAS data substantiate the assumption that susceptibility to MS is affected by the action of common variants in multiple genes (Baranzini and Oksenberg, 2017). Environmental and lifestyle contributors to the risk of MS are high latitude, female sex, vitamin D deficiency, obesity during adolescence and cigarette smoking (Alfredsson and Olsson, 2019). The so-called hygiene hypothesis postulates that high incidence of infections in early childhood reduces the risk of developing autoimmune and allergic diseases (Fleming and Fabry, 2007). Conversely, the development of MS has also been associated with specific infections; in particular, Epstein-Barr virus (EBV) infection has been implicated in the autoimmune mechanisms and epidemiology of MS (Dobson and Giovannoni, 2019).

Shortened TL has been described in a small cohort of primarily progressive MS patients when compared to normal subjects, a phenomenon according to the authors caused by oxidative stress which in turn can lead to various inflammatory responses. Thus, the authors suggest that decreased TL and increased oxidative stress reflect the severest state of the disease (Guan et al., 2015). Another recent study demonstrates that shorter TL is associated with greater disability and brain atrophy independent of chronological age and MS disease duration (Krysko et al., 2015).

Based on these findings, we aimed to further evaluate the relationship between TL and MS.

2. Materials and methods

DNA samples were obtained *via* isolation from peripheral white blood cells from 138 unrelated German MS patients (52 males with a mean age at blood withdrawal of 39.22 ± 11.51 years, 86 females with a mean age at blood withdrawal of 39.46 ± 13.42 years). Male and female patients were compared regarding the age of blood withdrawal. The effect failed to reach significance ($p = .91$). The patient cohort contained patients with relapsing remitting (RRMS, $n = 102$), secondary progressive (SPMS, $n = 27$) and primary progressive MS (PPMS, $n = 5$), all of whom fulfilled the McDonald diagnostic criteria (McDonald et al., 2001). Disease course was evaluated clinically by standardized neurological assessments, including the Expanded Disability Status Scale (EDSS). The EDSS was performed by specialized neurologists from the Department of Neurology of the St Josef-Hospital, Ruhr University, Bochum (EDSS median 2,5, range 1–8,5, $n = 128$).

DNA was also obtained from 120 geographically- and age-matched healthy German control subjects (65 males with a mean age of 43.67 ± 15.74 years, 55 females with a mean age of 45.63 ± 20.36 years). The study was approved by the Ethics Committee of the Medical Faculty of the Ruhr-University Bochum, Germany. All patients and controls gave written consent for their participation.

PCR reactions were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) and analyzed using StepOne Software v2.3. (Applied Biosystems) to determine the relative telomere length (Cawthon, 2009). Our reference was a pool of DNA of 6 healthy individuals in different decades of age (26, 37, 43, 48, 56 and 67 years old). The cell lines were established at the Department of Human Genetics of the Ruhr-University Bochum. Peripheral blood mononuclear cells (PBMCs) were isolated by Pancoll (PAN biotech, Aidenbach, Germany, lymphocytes separation medium) density gradient centrifugation. B-lymphoblastoid cell lines (BLCL) were derived from blood cells by transformation of PBMC with Epstein-Barr virus. The blood cell lines were propagated in IMDM medium (3.024 g/L NaHCO3 and L-glutamine supplement) and 10% Fetal Bovine Serum. For 5 ml medium 0.599 g penicillin G-sodium salt and 1 g streptomycin were added. Cells were grown in incubators maintained at 37 °C and 5% CO2.
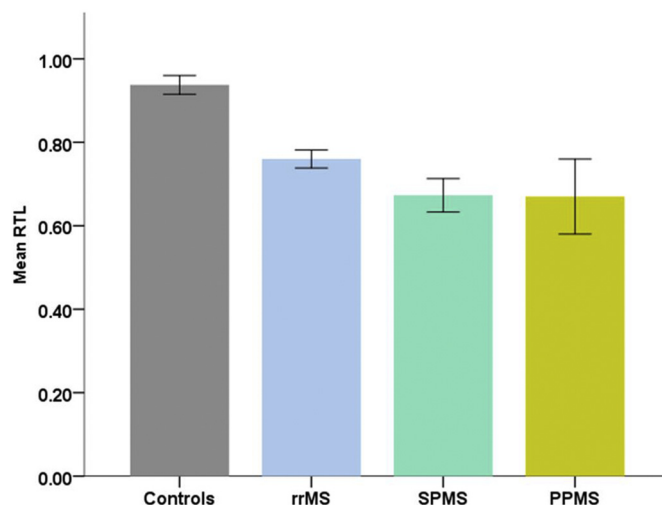

Fig. 1. Mean telomere lengths for healthy controls and different MS subtypes. Error bars show standard error.

3. Results

3.1. Telomere length in vivo

MS patients ($n = 138$) showed significantly shorter TL (0.74, SD = 0.22) than controls ($n = 120$; average TL: 0.94, SD = 0.25) ($t_{(256)} = 6.89; p < .001$). Fig. 1 shows the average TL for the different groups in our cohort.

To further explore this effect and to determine whether the type of MS affects this result, we performed a univariate ANCOVA with group (controls, RRMS, SPMS, PPMS) as between-subjects factor and age as covariate. Age was included in the model as we observed a significant age difference in our cohort ($F_{(3, 254)} = 13.42; p < .001$). Patients with relapsing-remitting MS were youngest (average age: 35.23 years), followed by healthy controls (average age: 44.55), patients with PPMS (average age: 49 years) and patients with SPMS (average age: 51.82 years).

The ANCOVA revealed a significant main effect of group ($F_{(3, 254)} = 34.37; p < .001$), indicating that TL differed between the four groups. On average, all three groups of MS patients showed shorter TL (RRMS: $0.76 +/- 0.22$; SPMS $0.67 +/- 0.21$; PPMS $0.67 +/- 0.27$) as compared to controls ($0.94 +/- 0.25$). Bonferroni-corrected post-hoc tests showed that this effect reached significance for all three comparisons between controls and MS patients (all p's < 0.01). However, all comparisons between two MS patient groups failed to reach significance (all p's > 0.95).

In addition, we combined the two progressive groups (PPMS and SPMS) and compared them to the RRMS group, since effects in the previous analysis might have been affected by the small sample size in the PPMS group. Here we found that the progressive group showed significantly shorter TL ($0.67 +/- 0.22$) than the RRMS group ($0.76 +/. 0.22$) ($p < .05$).

The effect for the covariate age also reached significance ($F_{(1, 256)} = 116.94; p < .001$), indicating a strong negative relationship between age and TL ($r = -0.45; p < .001$). To further investigate the observed age effects, we correlated age and TL using Neyman-Pearson correlation coefficients in both MS patients and controls. The correlation reached significance for both controls ($r = -0.65; p < .001$) and MS patients ($r = -0.46; p < .001$), indicating that older individuals had shorter TL, independent of the group (see Fig. 2).

In order to specifically investigate whether there was a difference between RRMS and SPMS, we also recalculated the ANCOVA with only these two groups, excluding controls and PPMS patients. However, in this comparison, the group effect failed to reach significance ($p = .22$)
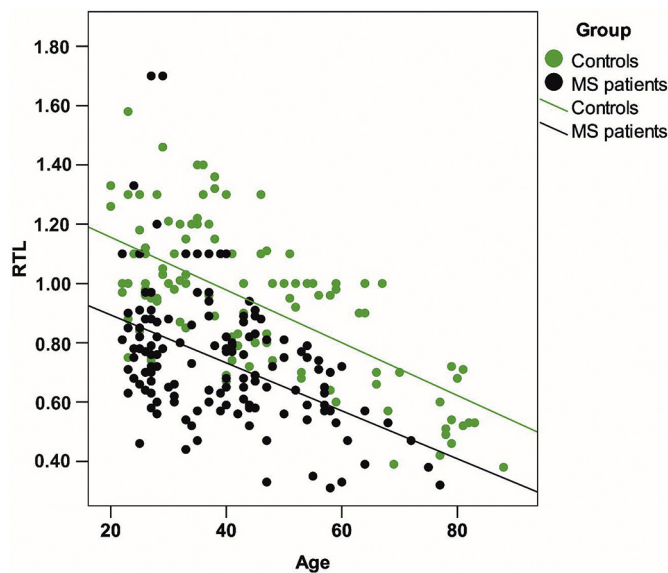
Fig. 2. Correlation of relative telomere length (TL) with age at blood draw in MS patients and healthy controls. The correlation coefficients for the relation of age and RTL was $r = -0.46$ ($p < .001$) for MS patients and $r = -0.65$ ($p < .001$) for controls.
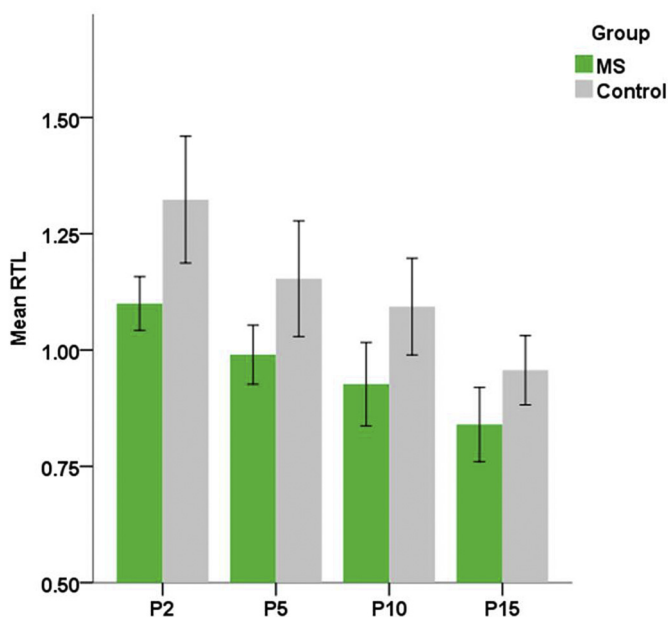

Fig. 3. Telomere length in lymphoblastoid cell lines after different passages. Each column represents the TL sum of three individuals.

(Fig. 3).

3.2. Sex differences in telomere length

In order to assess sex differences in TL, we first assessed whether there was a significant age difference in male and female patients/controls, since age-dependent TL shortening has been frequently observed in cross-sectional studies on human blood cells (Iwama et al., 1998). For controls, there was no significant age difference between males (average age: 43.67 years) and females (average age: 45.63 years, $p = .55$). Similarly, patients did not show any significant age difference between males (average age 39.22 years) and females (average age: 39.46 years, $p = .91$). Thus, there was no systematic co-variation between age and sex in our sample.

In order to test whether there were sex differences in TL, we used a univariate ANCOVA with TL as dependent variable and sex (male, female) and group (patients, controls) as between-subjects-factor. Age was used as covariate. While the effects of age ($p < .001$) and group ($p < .001$) reached significance as reported above, both the main effect of sex ($p = .22$) and the interaction sex by group ($p = .25$) failed to reach significance.

Since we had uneven gender distributions in both groups (patients: 87 females, 51 males; controls: 54 females, 66 males), we also analyzed the two subgroups of our cohort separately, as the significant group effect might have masked potential weaker sex effects. For patients, the sex effect failed to reach significance ($p = .96$), as it did for controls ($p = .09$). Thus, there were no significant sex effects on TL in our cohort.

3.3. EDSS and telomere length

We found a significant negative association between EDSS and TL ($r = -0.296$; $p = .001$), indicating that shorter TL was associated with higher EDSS. To assess, whether this effect was independent of age, we performed linear regression analysis with EDSS as dependent variable and sex, age and TL as predictors. While the overall model reached significance ($R = 0.624$; $F(3,127) = 26.30$; $p < .001$), TL as a predictor did not. This indicates that this effect was likely to be driven by chronological age ($p < .001$) and that there is no independent contribution of TL in our sample.

3.4. Telomere lengths in vitro

TL was measured in blood cells derived from six cases after different passages (P2, P5, P10 and P15): three MS-patients age 26, 34 and 54 years at the time of blood drawn and three controls at the same age.

As only three individuals were tested per group, non-parametric statistics had to be used to investigate these effects. We first used a Friedman test to investigate whether there was a significant difference between the four time points in the overall sample. The effect reached significance ($\chi^2 = 17.59$; $p = .001$), indicating that TL shortened over time. When only controls were analyzed, the effect still reached significance ($\chi^2 = 8.79$; $p = .032$). Similarly, it also reached significance ($\chi^2 = 8.79$; $p = .032$) when only MS patients were analyzed.

Then, in order to investigate whether there were differences between controls and MS patients at the four different time points, we used Mann-Whitney U tests to compare the two groups. However, the effect failed to reach significance at all four time points (P2: $p = .20$; P5: $p = .40$; P10: $p = .20$; P15: $p = .40$). Thus, patients and controls do not show significant differences at any time point, despite the fact that patients show lower absolute TL at all four time points.

4. Discussion

Many studies have found shorter TL in autoimmune and/or inflammatory diseases that are characterized by immune system dysfunction and premature senescence of immune cells. Since chronic stress exposure, inflammation and increased oxidative stress as well as increased leucocyte renewal are features accompanying these illnesses, longer and/or more severe exposure to these conditions might result in accelerated telomere shortening (Jose et al., 2017). Also in the case of MS, it has been suggested that shorter telomeres found in primary progressive MS patients reflect the most severe state of the disease and biological aging contributes to MS progression as shorter TL was associated with greater disability and brain atrophy (Guan et al., 2015; Krysko et al., 2015).

We can also show in our cohort, that the combined progressive groups (PPMS and SPMS) showed significantly shorter TL than the RRMS group, however, the shortening was not correlated with the progression of clinical disability as recently demonstrated by Krysko

et al. Our data therefore do not provide any evidence for a chronological age-independent effect of TL in MS disability progression. Furthermore, there were no significant sex effects on TL in our cohort, which fits to the heterogeneous data situation from Real-time PCR studies (Gardner et al., 2014).

Rather we show that the age- and sex-adjusted TL in MS cases is significantly shorter than in controls and declines in a linear fashion in both MS cases and controls which could point to a similar decrease of TL per year in both cohorts and not an accelerated TL shortening in MS patients (Fig. 2).

Therefore, it is unclear whether telomere shortening is the cause or the consequence of MS. Of course, it should be noted that only a few PPMS patients were examined. But also in the case of rheumatoid arthritis (RA), a variety of studies report shorter telomeres in patients when compared to healthy controls, but no correlation with disease duration or severity (Colmegna et al., 2008; Fujii et al., 2009; Koetz et al., 2000; Steer et al., 2007). It is widely supposed that the TL reflects the cumulative exposure to inflammation and oxidative stress, which are often features accompanying autoimmune and/or inflammatory diseases. It is therefore conceivable that disease duration and severity might be reflected by accelerated telomere shortening.

On the other hand, if TL shortening precedes the onset of illness, it could be regarded as risk factor for certain diseases which together with still unknown trigger mechanisms leads to the first symptoms. A meta-analysis estimated a 70% heritability of TL, calculated from individual studies that quote values between 34 and 82% (Broer et al., 2013). A complex phenotypic trait like TL is very likely the result of the action of many genes of small effect. These genetic effects could alter TL in various ways, such as the initial TL in the fertilized egg, individual resistance to telomere attrition or the extent of telomerase expression (Dugdale and Richardson, 2018). Beside genetic factors, it is clear that a substantial amount of variability in TL is also explained by environmental factors. In fact, there is increasing evidence that shorter TL is associated with stress, including childhood adversity, stressful life events, and chronic stressors (Price et al., 2013).

Patients may already have congenitally shorter telomeres with decreased functionality. It is known that TL progressively shortens linearly with age and interestingly, most of the inter-individual variation in TL among adults seems to establish very early in life with environmental factors during adulthood only having a limited impact (Benetos et al., 2013; Factor-Litvak and Susser, 2015; Heidinger et al., 2012). Thus, TL in adulthood may largely reflect TL at birth and TL shortening during childhood (Okuda et al., 2002).

A number of studies have shown that already maternal stress during pregnancy is associated with reduced TL in the offspring (Send et al., 2017; Suh et al., 2019; Werlang et al., 2018). Beside psychosocial stress during pregnancy also critical life events experienced by the mother during the prenatal period are associated with shorter telomeres in the newborns' cord blood (Entringer et al., 2013; Marchetto et al., 2016). Furthermore, reduced TL was associated with a history of perinatal complications, such as preterm pre-labor rupture of the membrane, maternal diabetes, low birth weight, and low Apgar scores (Menon et al., 2012; Salihu et al., 2015; Shalev et al., 2014). At least some of these effects seem to be more pronounced in females (Entringer et al., 2011).

Incidents in early life may therefore have implications on TL that could have lasting effects throughout the life course (Entringer et al., 2018; Factor-Litvak and Susser, 2015). Therefore, as yet unidentified or neglected determinants on TL *in utero* or during childhood could underlie the associations between early life exposures and late life outcomes like autoimmune diseases such as MS or RA. However, these hypotheses need not be mutually exclusive, since it is conceivable that individuals who have shortened telomeres prior to the onset also show further acceleration of TL shortening with greater exposure to the respective pathology.

5. Conclusion

This study demonstrated that understanding whether telomere shortening precedes MS onset, and how early prior to MS it occurs, could be crucial in distinguishing primary from secondary events involved in the pathogenesis of MS. Further studies on the telomeres and the related telomerase system could open up new perspectives in the field of MS research. In particular, the extension of this research at birth and early childhood could help to better understand the role of telomere biology in age-related disorders.

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