Journal of Autoimmunity 105 (2019) 102295

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Cellular aging over 13 years associated with incident antinuclear antibody positivity in the Baltimore Longitudinal Study of Aging

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ARTICLE INFO

Keywords:
Autoimmunity
Telomere length
Antinuclear antibodies
Epidemiology
Immune aging

ABSTRACT

Age-associated increases in antinuclear antibodies (ANA) in the general population are commonly noted but the mechanisms underlying this observation are unclear. This study aims to evaluate whether shorter peripheral blood mononuclear cell (PBMC) telomere length, a marker of more advanced *biological* age, is associated with ANA positivity prevalence and incidence in middle and older aged autoimmune disease-free individuals from the Baltimore Longitudinal Study of Aging (BLSA). Telomere length was measured by Southern Blot and categorized into tertiles. ANA was measured in a 1:80 and a 1:160 dilution of sera by immunofluorescence using HEp-2 cells (seropositive = 3 or 4). Multiple logistic regression was used to estimate the odds ratios and 95% confidence intervals of ANA positivity comparing the shorter tertiles of telomere length to the longest tertile for two cross-sectional points in time and then longitudinally to assess the association between shorter telomere length and incident ANA positivity. Cross-sectional analyses were adjusted for sex, race and BMI (N = 368 baseline, N = 370 follow-up) and longitudinal analyses were adjusted for sex, race, BMI and time between baseline and follow-up (N = 246). No statistically significant cross-sectional associations were observed at baseline or follow-up. Among those where ANA negative at baseline, individuals with shorter telomeres were more likely to be ANA positive at follow-up, an average 13 years later. Individuals with short telomeres at both time periods were more likely to be ANA positive. Findings suggest that ANA positivity in the general population may be indicative of immune dysfunction resulting from advanced cellular aging processes.

1. Introduction

Antinuclear antibodies (ANA) are a marker of immune response to self-antigens, and have been associated with autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis. They are also seen in the general population in the absence of an autoimmune diagnosis. In the absence of autoimmune disease, prevalence of ANA generally increases with age [1,2]. In the US, ANA prevalence has been estimated to be 15% in men and 22% in women over age 70, nearly twice the prevalence in those aged 12–19 [1]. However, incidence of most autoimmune diseases is not higher in the elderly [3], and the reasons for age-associated increases in ANA are not well-known.

Telomeres are protein-bound DNA complexes at the ends of chromosomes, that help to preserve genomic integrity during cell division [4,5]. The incomplete replication of chromosomal ends during cell division results in loss of a small fraction of telomeric DNA [5], thus, telomere length indicates the history of replication and could serve as a predicter of the remaining cellular replicative potential. Functionally, immune cells such as lymphocytes with shorter telomeres have reduced proliferation and function as observed in response to the seasonal influenza vaccine [6]. Therefore, telomere length is hypothesized to be a marker of biological aging [7]. Research suggests telomere shortening in patients with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis, most likely results from rather than causes disease [8–11]. It remains unknown whether the documented increases in ANA prevalence with age coincides with other

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https://doi.org/10.1016/j.jaut.2019.06.006
Received 4 April 2019; Received in revised form 18 June 2019; Accepted 19 June 2019
Available online 11 July 2019
0896-8411/ Published by Elsevier Ltd.

biological markers of age, such as telomere length, in the absence of autoimmune disease. Shorter telomere length and rapid loss of telomere length in immune cells may be the consequence of multiple immunologic and physiologic stressors, including chronic infections and inflammation, resulting in immune dysfunction [12].

Using data from the Baltimore Longitudinal Study of Aging, we describe ANA positivity prevalence at two time points and ANA positivity incidence in relation to peripheral blood mononuclear cell (PBMC) telomere length. We hypothesize that shorter telomere length, as a marker of cellular aging, is associated with ANA positivity.

2. Methods

2.1. Study population

The Baltimore Longitudinal Study of Aging (BLSA) is a continuous enrollment cohort study of normative aging sponsored and conducted by the Intramural Research Program of the National Institute on Aging (NIA). The study was approved by the National Institute of Environmental Health Sciences Institutional Review Board. Details of the BLSA have been described previously [13]. BLSA enrollment is restricted to individuals free of cognitive impairment, functional and mobility limitations and chronic diseases except for controlled hypertension. Participants undergo comprehensive health examinations, collection of biologic samples and assessment of physical and cognitive function at the NIA Intramural Research Program Clinical Research Unit in Baltimore, MD. Visits occur approximately every 4 years for individuals under age 60, biennially for individuals aged 60–79, and annually for individuals aged 80 and older. The population for this study consisted of a subsample of BLSA participants aged 48 to 103 with longitudinal data on PBMC telomere length. Individuals with reported diagnoses of rheumatoid arthritis or SLE or use of common disease-modifying antirheumatic drugs (DMARDs) were excluded from this study. The final cross-sectional samples consisted of 368 individuals at baseline and 370 at the follow-up visit. Longitudinal analyses were conducted in the sample of ANA negative individuals at baseline who also had telomere measurements at both time points (N = 246).

2.2. Exposure: measurement of telomere length of peripheral blood mononuclear cells (PBMCs)

The procedure for telomere length measurement by the terminal restriction fragment (TRF) using Southern blot has been previously described [14]. Briefly, DNA were isolated from PBMCs using the GENTRA purification kit (Qiagen) and 1 μg DNA were digested by 10 units of *RasI* and *HinfI* (NEB). The terminal restriction fragments of digested DNA were then separated by electrophoresis on an 0.6% agarose gel along with a DNA ladder (ThermoFisher) covering 1–50 Kb. The gel was dried by at 65 °C for 2–3 h under vacuum and then treated with denaturation and neutralization solution prior to hybridization. A probe ^{32}P-$(CCCTAA)_4$ was used in hybridization at 43 °C overnight. The gel was washed and exposed to a phosphoscreen for 1–2 days. The image of the screen was collected by a PhosphoImager (Typhone, GE Healthcare). The average length of telomeres was calculated based on the DNA ladder. We used DNA from Jurkat cells on every gel as a measure of gel to gel variation and used for normalization. The coefficient of variation of telomere length of Jurkat cells measured at different times was 10.6% (n = 146).

2.3. Outcome: ANA

Stored, frozen serum samples were tested for immunoglobulin autoantibodies to human nuclear antigens using indirect immunofluorescent methods under a standardized protocol at the University of Colorado Clinical and Research Laboratory and College of American Pathology/Clinical Laboratory Improvement Amendment (CAP/CLIA) certified conditions. HEp-2 cell slides (Kallestad, Bio-Rad Laboratories, Hercules, CA) were incubated with a 1:80 dilution of sera and then washed and incubated with the burro anti-human polyvalent immunoglobulin FITC conjugate (Kallestad). Using fluorescent microscopy, three blinded trained technicians scored ANA fluorescence for each participant on a 0–4 scale. ANA intensities of 3 or 4 were classified as positive for ANA and intensities less than 3 were classified as ANA negative. To evaluate truly elevated ANA intensities (high positive) verses ANA negative, consistent with clinical diagnostic laboratory standards, ANA intensities of 3 or 4 at a higher dilution (1:160) were classified as seropositive for ANA, while intensities of 0–1 were classified as seronegative. For this analysis, intensities of 2 were considered equivocal and excluded to isolate differences between individuals with high ANA immunofluorescent intensity and ANA negative. A random subsample of participant sera was re-tested for ANA quality control and we observed 91% concordance in assay results.

2.4. Covariates

Participant characteristics included age (years), sex (female referent), self-identified Black or non-Black (referent) race, body mass index (kg/m^2) (BMI), education level (categorized as college or less versus more than college), smoking status (current smoker v. never or former smoker), and BLSA entry age (< 70 years or ≥ 70 years). BMI was categorized as < 25, 25–30 and $30 +$ kg/m^2 (i.e., underweight or healthy, overweight, and obese).

2.5. Statistical analyses

Participant characteristics were compared by ANA positivity using chi-squared statistics from contingency tables. Bivariate associations between mean telomere length and participant characteristics were evaluated using F-tests. Multiple logistic regression was used to estimate the odds of ANA positivity and the 95% confidence interval (CI) for tertile of telomere length (longest tertile of telomere length referent) adjusting for sex, race and BMI cross-sectionally at two time points: “baseline” and “follow-up.” Baseline telomere length tertile was then used to predict the odds of ANA positivity at follow-up and the odds of incident ANA positivity (seroconversion) among individuals who were seronegative for ANA at baseline adjusting for sex, race, BMI and time between baseline and follow-up. Additionally, multiple logistic regression was used to estimate the odds of ANA seroconversion for those with consistently short telomeres (i.e., in the shortest tertile of telomere length at both baseline and follow-up) compared to those who did not have consistently short telomeres. All models were additionally adjusted for age to determine if controlling for chronological age attenuated the association between telomere length and ANA. As a sensitivity analysis, the interaction between telomere length and sex were examined in separate models.

3. Results

Participant characteristics at baseline and follow-up characterized by ANA positivity at 1:80 dilution of sera is shown is Table 1. The average time between the baseline visit and follow-up visit was 13 years (SD = 3.6 years, min = 4 years, max = 18 years). The distribution of participant characteristics by ANA positivity are similar for both dilutions (see Supplemental Table 1 for 1:160 dilution results), though, as expected, more individuals are positive for ANA at 1:80 dilution. A greater proportion of ANA positive individuals were women, were not overweight or obese and had lower educational attainment than ANA negative individuals. No differences in age distribution, race, smoking status or age at study entry were observed. Approximately 99% of the positive samples exhibited a fine speckled pattern of cellular fluorescence. Table 2 shows mean telomere length and standard deviations by

Table 1
Participant characteristics by ANA positivity at 1:80 dilution cut point vs. ANA negative in the Baltimore Longitudinal Study of Aging.

	Baseline Sample (N = 368)			Longitudinal Sample (N = 246)		
	ANA Negative N (%)	ANA Positive 1:80 N (%)	p-value[a]	ANA Negative N (%)	ANA Seroconverter 1:80 N (%)	p-value[a]
	299	69		216	30	
Age Category			0.12			0.32
40-49	72 (24.1)	14 (20.3)		49 (22.7)	7 (23.3)	
50-59	87 (29.1)	15 (21.7)		72 (33.3)	7 (23.3)	
60-69	75 (25.1)	17 (24.6)		49 (22.7)	10 (33.3)	
70-79	50 (16.7)	14 (20.3)		37 (17.1)	3 (10.0)	
80 +	15 (5.0)	9 (13.4)		9 (4.2)	3 (10.0)	
Sex			0.12			0.78
Male	169 (56.5)	32 (46.4)		121 (56.0)	16 (53.3)	
Female	130 (43.5)	37 (53.6)		95 (44.0)	14 (46.7)	
Race			0.90			0.29
Non-Black	245 (81.9)	57 (82.6)		182 (84.3)	23 (76.7)	
Black	54 (18.1)	12 (17.4)		34 (15.7)	7 (23.3)	
Education			0.07			0.53
College or less	128 (42.8)	38 (55.1)		88 (40.7)	14 (46.7)	
More than college	171 (57.9)	31 (44.9)		128 (59.3)	16 (53.3)	
BMI			0.09			0.52
< 25	103 (34.5)	33 (47.8)		78 (36.1)	10 (33.3)	
25-30	128 (42.8)	26 (37.7)		93 (43.1)	11 (36.7)	
30 +	68 (22.7)	10 (14.5)		45 (20.8)	9 (30.0)	
Smoking			0.14			0.37
Current smoker	24 (8.1)	2 (2.9)		18 (8.3)	4 (13.3)	
Never or quit > 10 years ago	273 (91.9)	66 (97.1)		198 (91.7)	26 (86.7)	
Study Entry Age			0.18			0.66
< 70	286 (94.3)	62 (89.9)		205 (94.9)	28 (93.3)	
70 +	17 (5.7)	7 (10.1)		11 (5.1)	2 (6.7)	

[a] Chi-square or Fisher Exact test; Bold indicates statistical significance at p = 0.05.

participant characteristics. Younger participants, women, Blacks and those with lower BMI had longer mean telomere length in both the baseline cross-sectional and longitudinal samples. No differences in mean telomere length were observed by educational attainment, smoking status or age at study entry. Based on these results, sex, race, and BMI were included as covariates in subsequent models.

Odds ratios and 95% confidence intervals (CI) modeling the longitudinal association between telomere length and ANA positivity at

Table 2
Mean peripheral blood mononuclear cell (PBMC) telomere length (TL) (kb) by participant characteristics at baseline and follow-up in the Baltimore Longitudinal Study of Aging.

Characteristic	Baseline Sample (N = 368)			Longitudinal Sample (N = 246)		
	N	Mean TL (SD)	p-value[a]	N	Mean TL (SD)	p-value[a]
Age Category						
40–49	86	5.98 (1.08)	**0.0002**	56	5.90 (1.01)	**0.03**
50–59	102	5.39 (0.96)		79	5.44 (1.03)	
60–69	92	5.43 (0.82)		59	5.36 (0.77)	
70–79	64	5.54 (0.91)		40	5.55 (0.96)	
80 +	24	5.56 (0.82)		12	5.45 (1.03)	
Sex						
Male	201	5.18 (0.81)	**< .0001**	137	5.11 (0.79)	**< .0001**
Female	173	6.06 (0.91)		109	6.08 (0.91)	
Race						
Non-Black	302	5.52 (0.95)	**0.01**	205	5.49 (0.90)	**0.046**
Black	66	5.84 (1.17)		41	5.81 (1.23)	
Education						
College or less	166	5.58 (0.92)	0.99	102	5.52 (0.94)	0.71
More than college	202	5.58 (0.99)		144	5.56 (0.99)	
BMI (kg/m^2)						
< 25	136	5.81 (0.98)	**0.001**	88	5.82 (1.00)	**0.002**
25–30	154	5.48 (0.96)		104	5.44 (0.95)	
30 +	78	5.35 (0.85)		54	5.29 (0.86)	
Smoking						
Current smoker	26	5.44 (0.86)	0.49	22	5.46 (0.89)	0.69
Never or quit > 10 years ago	339	5.57 (0.87)		224	5.55 (0.98)	
Study Entry Age						
< 70	344	5.56 (0.96)	0.49	233	5.53 (0.96)	0.51
70 +	24	5.71 (1.01)		13	5.72 (1.20)	

[a] F-test; Bold indicates statistical significance at p = 0.05.

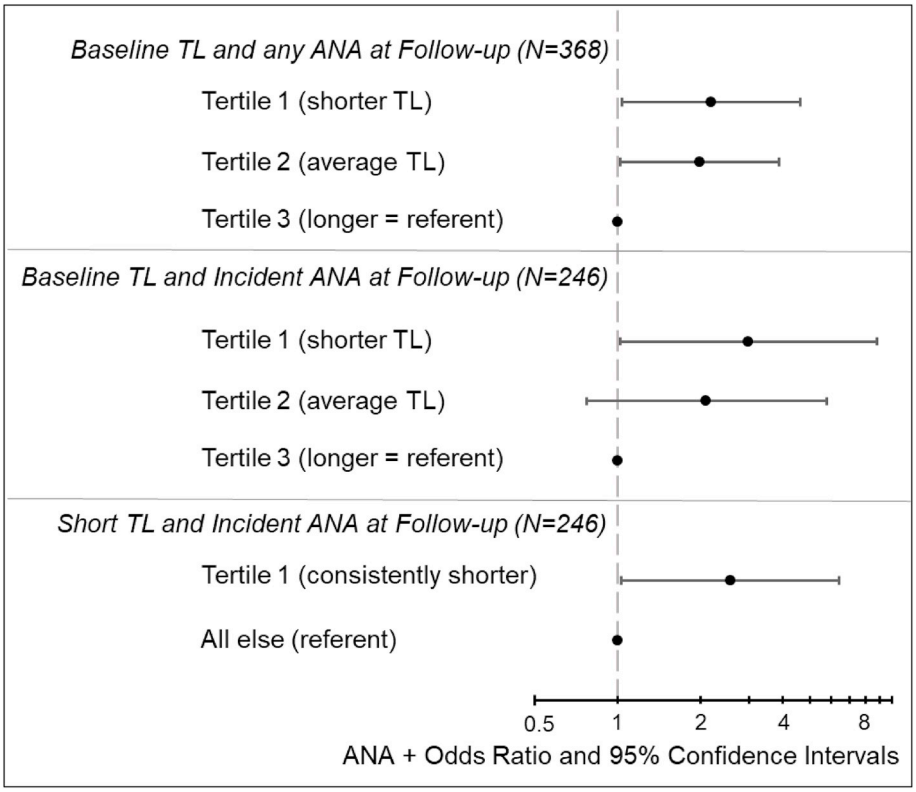

Fig. 1. Association (Odds Ratios and 95% Confidence Intervals (95% CI)) between peripheral blood mononuclear cell (PBMC) telomere length tertile (TL) and ANA positivity at 1:80 dilution adjusted for covariates in Baltimore Longitudinal Study of Aging.

1:80 dilution adjusted for covariates are reported in Fig. 1 (cross-sectional associations at 1:80 dilution and results for analysis of ANA at 1:160 dilution are available in Supplemental Table 2). No cross-sectional associations were observed at baseline or follow-up. In the longitudinal analysis, telomere length at baseline was associated with ANA positivity at follow-up with individuals in the shortest telomere length tertile having 2.19 times the odds of ANA positivity (95% CI: 1.04, 4.62) and individuals in the middle tertile having 1.99 times the odds of ANA positivity (95% CI: 1.02, 3.88) relative to individuals in the longest telomere length tertile. The association in the shortest telomere tertile was robust to different ANA positivity cut-offs (all positive and high positive).

Among participants who were ANA negative at baseline, shorter telomere length at baseline was associated with elevated odds of incident ANA positivity at follow-up ($OR_{Tertile\ 1}$ = 2.99, 95%CI = 1.02, 8.77). The magnitude of the odds ratio point estimate remained consistently high using the more stringent ANA positivity cut-off, although these models were less precise due to smaller sample sizes and 95% CIs included the null value.

Lastly, among participants ANA negative at baseline, individuals with short telomeres at both baseline and follow up (shortest tertile) had 2.57 times the odds of incident ANA positivity (95% CI: 1.03, 6.40). This association was also robust to different ANA positivity cut-offs (all positive and high positive). In sensitivity analyses, we saw no evidence that sex modified the telomere length-ANA relationship and the addition of chronological age to all models did not alter results.

4. Discussion

In the present study, we sought to determine if the age-associated increase in ANA positivity commonly reported in the literature related to a biological marker of aging, telomere length. We found that shorter telomere length, representing more age-advanced PBMCs, was associated with ANA positivity and ANA incidence at follow-up on average 13 years later. Moreover, having short telomeres at both baseline and follow-up was associated with incident ANA positivity. Though there exists a paucity of research on biomarkers of aging and ANA, our work is consistent with the limited studies of telomere length and autoimmune diseases [8], including a recent meta-analysis that concluded telomere length was significantly shorter in patients with SLE [9]. Importantly, most previous studies are cross-sectional, rendering them unable to answer a critical question of whether advanced cellular aging precedes, is concurrent, or a consequence of autoimmune disease processes [12]. The availability of longitudinal data in our study revealed that ANA production in the general, autoimmune-disease free population, conceivably may reflect or result from advanced cellular aging, and provides evidence that immune-mediated disease may not necessarily drive cellular aging.

Although often used as a marker of systemic aging, average telomere length is a more direct measure of immune aging [15,16] as a product of telomere length in lymphocytes and myeloid derived cells. Immune dysregulation and inflammation associated with immune aging are established risk factors across a broad spectrum of aging-related diseases [17,18]. Changes to both the innate and adaptive immune system characteristics of immunosenescence result in chronic inflammation, alterations in the T and B cell repertoire and dysregulation of apoptosis processes, all of which may contribute to the generation of autoantibodies [19,20].

Immune function decline in chronologically older individuals is a hallmark of aging processes. One hypothesized pathophysiologic mechanism by which shorter telomere length may result in ANA production is through immune dysfunction. Telomere length is not only a marker of biological age, but also may represent or contribute to the underlying biological aging process. Mechanistically, the incidence of ANA (seroconversion) may be a result of telomere erosion and the resultant impaired regulatory processes that are normally maintaining the tolerance to self-antigens and/or accumulated senescent cells in the body. These impaired regulatory processes in turn lead to a greater

production of inflammatory cytokines as part of an inflammatory response [21]. Shorter telomeres, caused by DNA damage to the telomere region of the genome, may also contribute to the pathogenesis of autoimmunity and the production of ANA [22,23].

The immune system plays a key role in responding to environmental challenges, and so immune aging phenotypes such as ANA and shortened PBMC telomere length may be meaningful markers of the effects of environmental exposures and provide clues to etiologic pathways. Existing studies comparing telomere length attrition with markers of immune aging, such as cytomegalovirus (CMV) infection and elevated inflammation, are limited and findings vary. For example, one study revealed an association between prospective telomere length attrition and CMV seropositivity and CMV IgG titer robust to adjustment for age, sex, employment, body mass index and smoking status [24] confirming similar results from a cross-sectional study [25]. By contrast, initial comparisons of telomere length attrition with age-related increase of inflammation-related cytokines and *anti*-CMV IgG titer suggest that age-related trajectories of telomere attrition, elevated circulating inflammatory cytokines, and *anti*-CMV IgG may be independent within individuals in the BLSA [26]. Therefore, while the underlying mechanisms may be heterogeneous, future research should examine incident ANA in relation to cytomegalovirus infection and other markers of cellular aging associated with telomere length and immune aging, such as aging T cell profiles and “inflammaging”, to elucidate patterns of the immune system aging process and determine if ANA positivity may be a useful contribution to elaborating the immune risk phenotype [27–29].

Our work has many strengths. We are among the first to investigate the age-ANA association with a biological marker of aging. Further, studies of ANA in “healthy” (i.e., non-autoimmune) populations are often limited to clinical controls and small homogeneous populations [30]. Existing longitudinal studies of ANA are based on a relatively small number of ANA-positive individuals who are followed for a short period of time (roughly 4–5 years), lacking information on incident ANA positivity [2,31,32]. Our longitudinal study was conducted using a subset of participants from the BLSA, a well characterized study of normative aging, allowing us to control for potential confounding factors not considered by other studies [13]. In addition, PBMC telomere length was measured by southern blot, which produces a reliable telomere length measurement [33]. Further, the average time between telomere length observations in our longitudinal study, approximately 13 years, suggests that the mean telomere attrition we observed was less likely due to chance or normal variation [34]. Our study does have limitations. As a study of normative aging, the BLSA participants are healthier on average than the corresponding general population, therefore, we may be underestimating the association between telomere length and ANA positivity as we observe longer telomeres in older individuals. We are also limited to two time points for this investigation, and further testing generating additional measurements of telomere length and ANA positivity as the participants age may be useful in accounting for non-linear dynamics in telomere attrition and ANA incidence.

An estimated 32 million persons in the US have ANA, with higher prevalence in older individuals and women [1]. Advanced biological aging, a marker of immune aging and immune dysregulation, may be an important driver of ANA and ANA positivity may represent individuals susceptible to low-grade inflammation associated with many chronic diseases.

Funding

This research was funded by the Intramural Research Program of the National Institutes of Health, National Institute of Environmental Health Sciences (Z01-ES049028 to DPS) and the National Institute on Aging (Z01-AG000756 to NPW).

Acknowledgements

None noted.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.06.006.

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