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Leukocyte Telomere Length in Alzheimer's Disease Patients with a Different Rate of Progression

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Abstract.
Background: Age and short leukocyte telomeres have been associated with a higher risk of Alzheimer's disease (AD). Inflammation is involved in AD and it is suggested that anti-inflammatory interleukin-10 (IL-10) may partly antagonize these processes.
Objective: The aim is to correlate telomere length (TL) in peripheral blood mononuclear cells (PBMC) from patients with AD to disease progression rate. Moreover, we evaluated whether TL was associated with IL-10 production by unstimulated or amyloid-β (Aβ)-stimulated PBMC.
Methods: We enrolled 31 late-onset AD and 20 age-matched healthy elderly (HE). After a two-year follow-up period, patients were retrospectively evaluated as slow-progressing (ADS) (Mini Mental State Examination (MMSE) decline over the two years of follow-up $\leq$3 points) or fast progressing AD (ADF) (MMSE decline $\geq$5 points). TL was measured by flow cytometry and *in vitro* IL-10 production by enzyme-linked immunosorbent assay.
Results: TL (mean $\pm$ SD) for HE, ADS, and ADF was 2.3 ± 0.1, 2.0 ± 0.1, and 2.5 ± 0.1 Kb, respectively. ADS showed a shorter TL compared to HE ($p = 0.034$) and to ADF ($p = 0.005$). MMSE decline correlated with TL in AD ($R^2 = 0.284$; $p = 0.008$). We found a significant difference in IL-10 production between unstimulated and Aβ-stimulated PBMC from ADS (40.7 ± 13.7

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versus 59.0 ± 27.0; $p = 0.004$) but not from ADF (39.7 ± 14.4 versus 42.2 ± 22.4). HE showed a trend toward significance (47.1 ± 25.4 versus 55.3 ± 27.9; $p = 0.10$).

Conclusion: PBMC from ADF may be characterized by an impaired response induced by Aβ and by a reduced proliferative response responsible for the longer telomeres. TL might be a contributing factor in predicting the rate of AD progression.

Keywords: Alzheimer's disease, disease progression, interleukin-10, peripheral blood mononuclear cells, telomere

INTRODUCTION

Alzheimer's disease (AD) is the major cause of dementia in the elderly. It is commonly accepted that AD pathology starts years to decades before the onset of cognitive symptoms [1]. This fact explains why symptomatic AD consistently represents an advanced stage of AD pathology [2].

Amyloid plaques and neurofibrillary tangles are the pathological hallmarks of AD. However considering AD as a single, well-defined entity has become an obstacle to lucid analysis of the problem of dementia in old age. Whereas in early-onset patients, AD might be best described as a purely degenerative disease with an important role for amyloid-β (Aβ) in the pathogenesis; in late-onset patients, the probability of finding other abnormalities is increased [3–7]. To date, 90% of all patients with AD in the population are older than 75 years, and 75% of patients are over 80 years of age [8]. This data suggest that aging and pathophysiological changes it induces may be joint causes of AD onset and progression in older patients.

Telomeres are specialized sequences consisting of highly conserved TTAGGG repeats [9–11] that cap the ends of linear chromosomal DNA, protecting the genome from damage and preserving chromosome stability [10, 12–14]. However, because of their end positions, telomeres are not fully duplicated during DNA replication and thus become shorter with each cell division [15–17]. This process limits the replicative lifespan of many different cells that, eventually, enter a senescent status or trigger apoptosis [18–23]. Telomere length (TL) reflects not only cellular turnover but also the exposure to oxidative and inflammatory damage [24–29] and, accordingly, may be a marker of both biological age and mortality risk [27, 30–32] that predicts incidence of age-related diseases [33–37]. To date, investigations on blood cells have been inconsistent in relationship between TL and AD [38–47]. In particular, several studies reported that TL is associated with cognitive decline in elders [48–51] and is shorter in patients with AD [38–44], but other studies showed TL is not associated with either levels of cognitive performance or age-related cognitive change [52, 53] and cannot be used in elderly as marker to diagnose the early phase of cognitive impairment (mild cognitive impairment), to distinguish between demented and non-demented patients and/or the type of dementia [45–47].

A growing body of literature shows that inflammation is involved in the neurodegeneration process [54–58] and can furthermore accelerate telomere shortening [26–29], which, in turn, may be linked with the pathogenesis of AD [38–44, 59–61]. Interestingly, it is suggested that anti-inflammatory cytokines, like interleukin-10 (IL-10), may partly antagonize these processes [62, 63]. In particular, IL-10 has been suggested to play an important role in neuronal homeostasis and may also be able to inhibit Aβ- or lipopolysaccharide (LPS)-induced generation of proinflammatory cytokines [64].

To investigate the contribution of telomere shortening to the onset and progression rate of AD, we measured TL in peripheral blood mononuclear cells (PBMC) from late-onset patients with AD and age-matched controls, and we correlated it with disease progression rate. We also assessed whether TL was associated with IL-10 production by resting and Aβ- or LPS-stimulated PBMC. Here we report that TL is directly associated with the rate of cognitive decline during the follow-up period and may thus be considered as a predictive marker of AD progression rate. In addition, an impaired response to Aβ stimulus may contribute to cause a faster AD progression.

MATERIALS AND METHODS

Participants and setting

A total of 51 individuals were enrolled in the study: 20 healthy elderly (HE) (mean age 79.1 ± 8.4 years old) and 31 patients with AD (mean age 80.6 ± 5.1 years old). Subjects diagnosed with AD fulfilled the criteria of dementia and the criteria of AD defined by NINCDS-ADRDA [65]. All individuals were Caucasians living in Milan or Paris and belonged to larger populations of outpatients.

The criteria for the diagnosis of normal cognition were: 1) no active neurological or psychiatric disorder; 2) no ongoing medical problems or related treatments

interfering with cognitive function; 3) a normal neurological exam; 4) no psychoactive medications, and 5) the ability to live and function independently in the community.

Individuals affected by cancer, cardiovascular diseases, autoimmune disorders, inflammatory diseases, and active neurological or psychiatric disorders were excluded from the study. We also excluded subjects who exhibited signs of inflammation (as stated by hemogram and C-reactive protein level) at the time of blood draw.

All participants and their relatives gave informed consent and the study protocol was approved by the respective ethics committees.

At baseline, physical, neurological, and neuropsychological examinations were performed for all patients with AD together with clinical history, computed tomography or magnetic resonance imaging scan, and cognitive testing using Mini-Mental State Examination (MMSE). Laboratory analyses included apolipoprotein E (ApoE) genotype assessment and biochemical tests.

After a two-year period of follow-up, patients with AD were retrospectively evaluated and AD progression rate was calculated by using deltaMMSE score (MMSE score at recruitment - MMSE score after the two-year period of follow-up). Subsequently, patients were divided into slow (ADS) and fast (ADF) decliners according to their rate of decline, that is lower or higher than the median rate of decline in the total population during the two-year period of follow-up (3.5 points) [66]. As the size of our study groups is quite small, we employed more stringent classification criteria and we defined patients showing a deltaMMSE $\leq$3 points and no appearance of new cognitive disorders and/or physical deterioration as ADS and patients showing a deltaMMSE $\geq$5 points and appearance of new cognitive disorders as ADF. Noteworthy, all diagnoses of slow or fast progressing AD were confirmed by clinicians.

After the two-year period of follow-up, we repeated for each HE the tests for the diagnosis of normal cognition and all of them fulfilled the criteria aforementioned. After the follow-up period, participants were asked to fast overnight and 20 ml of venous blood was drawn from each subject between 7.30 and 9.00 a.m.

ApoE genotyping

Genomic DNA was extracted from whole blood by using a salting-out method [67] and ApoE genotype was determined as previously described [68].

PBMC isolation

PBMC were collected from whole blood by using a density gradient centrifugation procedure (Lympholyte-H kit Cedarlane Laboratories Limited, Burlington, ON) and stored at -80°C pending analysis.

Flow cytometry

TL was determined by flow cytometry using the telomere PNA kit/FITC® (Dako Italia, Milan, IT) following manufacturer's directions. This Kit allows calculating relative telomere length (RTL) of sample cells (SC) using control cells (CC) with a known TL. The tetraploid 1301 cell line (Biologic Bank and Cell Factory, Genoa, IT) with a TL of approximately 25 Kb [69] was used as CC. The employed PNA telomere probe binds solely the telomere repeat sequences and does not bind to subtelomeric regions, which appear to be in the range of 2–4 kb in length. We prepared a 1:1 mixture of SC and CC, using 500,000 cells from each subject, and included 2 positive and 2 negative samples in each evaluation. In all samples, DNA was denatured for 10 min at 82°C either in the presence of hybridization solution without probe (negative) or in hybridization solution containing fluorescein-conjugated PNA telomere probe (positive). Hybridization was carried out overnight, in the dark at room temperature. After two washes at 40°C, samples were stained with propidium iodide (PI) for 4 h at 4°C for the identification of cells in G0/G1 phases and the DNA index calculation. Samples were then acquired with a FACSCanto II (Becton Dickinson, Franklin Lakes, NJ) and analyzed with a Kaluza® software (Beckman Coulter, Indianapolis, IN). For each tube, a total of 30,000 cells were analyzed. RTL was calculated with the following formula with the correction for the DNA index of G0/1 cells:

$$\mathrm{RTL} = \frac{(\text{mean FL1 sample cells with probe–mean FL1 sample cells without probe}) \times \text{DNA index of control cells} \times 100}{(\text{mean FL1 control cells with probe–mean FL1 control cells without probe}) \times \text{DNA index of sample cells}}$$

The absolute TL was calculated by multiplying RTL for 25 Kb.

Telomere length polymerase chain reaction

Genomic DNA was extracted from PBMC [68] and stored at −80°C pending analysis. To confirm flow cytometry data, TL was reassessed by using a quantitative PCR (q-PCR) method as previously reported [70]. Briefly, we determined the relative ratio (T/S ratio) of telomere (T) repeat copy number to a single copy gene (S) copy number using a comparative quantitation approach. The adopted primer pairs, their final concentration and the thermal cycling profiles were exactly as described [71] except that the number of amplification cycles was increased to 30 and 40 for the T and S reactions, respectively. In each well, an aliquot of 10 ng (10 μl) template DNA was added containing 12 μl SYBR Select Master Mix (Applied Biosystem, Foster City, CA) and 3 μl of primers. Before running samples, the linear range of T and S assay was determined by generating a standard curve using a serially diluted DNA (from 70 to 2.2 ng in 2-fold dilutions) in triplicate. Both T and S reactions showed good linearity across this input range ($r^2 > 0.99$). A calibrator sample (a mixture of several DNAs) and a negative control were included on each plate. All q-PCR assays were performed on an ABI 7500 system (Applied Biosystem, Foster City, CA) and each sample, including the calibrator, was run in triplicate. For each T and S q-PCR assay, raw data were exported from the ABI system and imported into the LinRegPCR program. Then, the program automatically calculated the fluorescence threshold for all samples, the individual threshold cycle and the mean efficiency of the run [72]. Mean efficiency was used in calculating the T and S relative concentration of each sample relative to the calibrator sample [72]. TL was expressed as T/S ratio. To confirm TL measurements, all samples were re-run and the inter-assay coefficient of variation (CV) was <5%.

In vitro IL-10 production

PBMC were resuspended at 3×10^6 ml in complete medium (RPMI, 10% FBS, 2% PS, 1% L-Glu) and were either unstimulated or stimulated with LPS (Sigma, St. Louis, MO) or with a pool of three peptides from the Aβ protein as follows: fragment 1–16 (1 μg/ml), fragment 25–35 (10 μg/ml), and fragment 1–40 (25 μg/ml) (Innovagen, Lund, Sw) at 37°C, 5% CO_2 atmosphere. Supernatants were harvested after 48 h for LPS stimulation and after 5 days of culture for the Aβ protein peptides. Production of IL-10 by PBMC was evaluated with commercial Ready-SET-Go! ELISA kit (eBioscience, Paris, Fr). The sensitivity (limit of detection) of the employed ELISA assay was 2 pg/ml. All tests kits were used following manufacturer's directions and, for each sample, data on both PBMC IL-10 production and proliferation activity were also normalized based on the ratio of living/dead cells.

Statistical analysis

Statistical analysis was performed by using SPSS statistical package (SPSS version 20, Chicago, IL). Gender and ApoE ε4 allelic distribution across groups were assessed by the χ^2-test. Demographic data, MMSE scores, and TL were examined by ANOVA univariate, followed by Bonferroni *post-hoc* test. The correlation between TLs measured by flow cytometry (kb) and TL measured by q-PCR (T/S ratio) and between TL (kb) and deltaMMSE scores was performed by linear regression analysis. Differences in IL-10 production between groups were evaluated by the Kruskal-Wallis test or by Mann-Whitney U-test where appropriate. Differences within groups between unstimulated and Aβ stimulated IL-10 productions were assessed by the Wilcoxon signed rank test. We adopted $p < 0.05$ as the threshold value for the statistical significance.

RESULTS

Characteristic of study population

Table 1 displays the demographic and clinical characteristics of participants. Despite the non-significant result obtained for the ApoE ε4 distribution ($p = 0.095$), the groups show clear differences in the numbers of ε4 allele carriers (Table 1). No difference in gender distribution (Table 1) and in the percentage of patients treated with acetylcholinesterase inhibitors was found between ADS and ADF (75% versus 55%, respectively, $p = 0.432$). At recruitment, ADS and ADF showed a similar mean MMSE score. Conversely, after the two-year period of follow-up, ADF exhibited a significantly lower score than ADS ($p < 0.001$). Accordingly, deltaMMSE, indicating the rate of disease progression, was significantly higher in ADF ($p < 0.001$) (Table 1).

Telomere length analysis

TL evaluated by the mean of flow cytometry (mean ± S.D.) was first compared between total patients with AD and HE and no difference was found

Table 1
Demographic and clinical characteristics of healthy elderly individuals (HE), slow-progressing AD patients (ADS) and fast-progressing AD patients (ADF)

	HE ($n=20$)	ADS ($n=20$)	ADF ($n=11$)	p value
Age, mean ± S.D.	79.1 ± 8.4	80.8 ± 5.7	80.4 ± 4.1	0.919
Gender, female, %	50.0	60.0	81.8	0.104
ApoE ε4, carriers, %	30.0	55.0	54.5	0.095
ApoE ε4, homozygosity, %	0.0	5.0	0.0	0.454
MMSE at recruitment, mean ± S.D.	29.1 ± 1.4[a,b]	20.4 ± 4.7	19.9 ± 6.1	<0.001
MMSE after the follow-up period, mean ± S.D.	28.9 ± 1.5[a,b]	19.6 ± 4.6	12.3 ± 5.4[a]	<0.001
deltaMMSE, mean ± S.D.	0.2 ± 0.1[a,b]	0.8 ± 1.4	7.9 ± 3.5[a]	<0.001
TL (flow cytometry), kb, mean ± S.D.	2.3 ± 0.4[a]	2.0 ± 0.4	2.5 ± 0.4[a]	0.003

[a]$p<0.05$ versus ADS; [b]$p<0.05$ versus ADF.

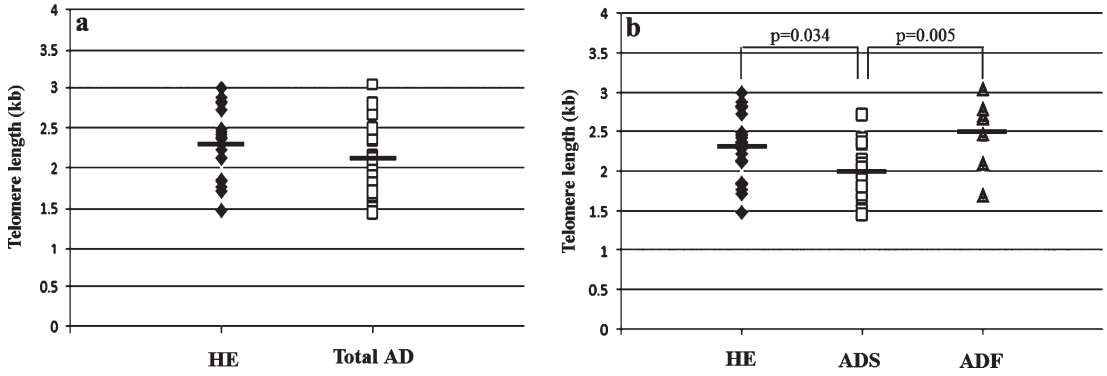

Fig. 1. Telomere length (kb) comparison between (a) HE and total AD (b) HE, ADS, and ADF.

(2.1 ± 0.5 versus 2.3 ± 0.4 Kb, respectively, $p=0.135$) (Fig. 1a).

Subsequently, patients were categorized as fast or slow progressing based on disease progression rate. This further classification led to the finding that ADS displayed shorter telomeres not only compared to HE (2.0 ± 0.4 versus 2.3 ± 0.4 kb; $p=0.034$) but also to ADF (2.0 ± 0.4 versus 2.5 ± 0.4 kb; $p=0.005$) (Table 1 and Fig. 1b).

Patients that are homozygous for ApoE ε4 have been reported to show significantly shorter TL than those with only one ApoE ε4 copy or other ApoE alleles [45]. In our study population there is only one individual homozygous for ApoE ε4 (Table 1), an ADS showing a TL of 2.02 kb, which reflect the mean TL of ADS group.

In order to confirm flow cytometry data, TL measurements obtained by flow cytometry were compared to TL measurements obtained by q-PCR, showing a very strong correlation ($R^2=0.523$; $p<0.001$).

IL-10 production in resting and Aβ- or LPS-stimulated PBMC

There was no difference in IL-10 production between the study groups (Table 2). In contrast, when unstimulated and Aβ stimulated IL-10 productions at day 5 were compared within groups, a significant

Table 2
IL-10 production from unstimulated PBMC and after stimulation with LPS or Aβ

	HE ($n=18$)	ADS ($n=20$)	ADF ($n=10$)	p value
Unstimulated IL-10 production (pg/ml) (day 2), median ± MAD	21.0 ± 6.0	23.2 ± 5.7	16.2 ± 4.9	0.629
Unstimulated IL-10 production (pg/ml) (day 5), median ± MAD	47.1 ± 25.4	40.7 ± 13.7	39.7 ± 14.4	0.820
LPS-stimulated IL-10 production (pg/ml) (day 2), median ± MAD	485 ± 249	671 ± 285	797 ± 223	0.928
Aβ-stimulated IL-10 production (pg/ml) (day 5), median ± MAD	55.3 ± 27.9	59.0 ± 27.0	42.2 ± 22.4	0.286
Number of unstimulated living PBMC (millions/ml) (day 2), mean ± S.D.	1.54 ± 0.28	1.53 ± 0.24	1.43 ± 0.22	0.430
Number of unstimulated living PBMC (millions/ml) (day 5), mean ± S.D.	1.32 ± 0.13	1.31 ± 0.26	1.29 ± 0.23	0.932
Number of LPS-stimulated living PBMC (millions/ml) (day 2), mean ± S.D.	1.49 ± 0.12	1.41 ± 0.12	1.39 ± 0.12	0.342
Number of Aβ-stimulated living PBMC (millions/ml) (day 5), mean ± S.D.	1.28 ± 0.12[a]	1.30 ± 0.26[a]	1.09 ± 0.29	0.050

MAD, median absolute deviation. [a]$p<0.05$ versus ADF. Missing data from 2 HE and 1 ADF.

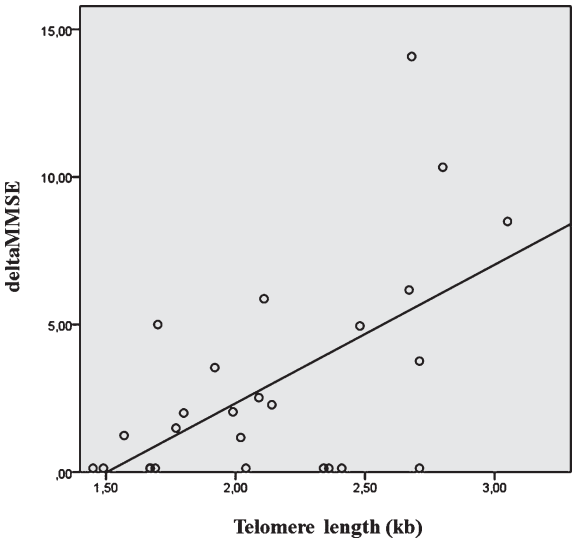

Fig. 2. Correlation adjusted for age, gender and ApoE ε4 genotype between TL and deltaMMSE score in patients with AD ($R^2 = 0.284$; $p = 0.008$).

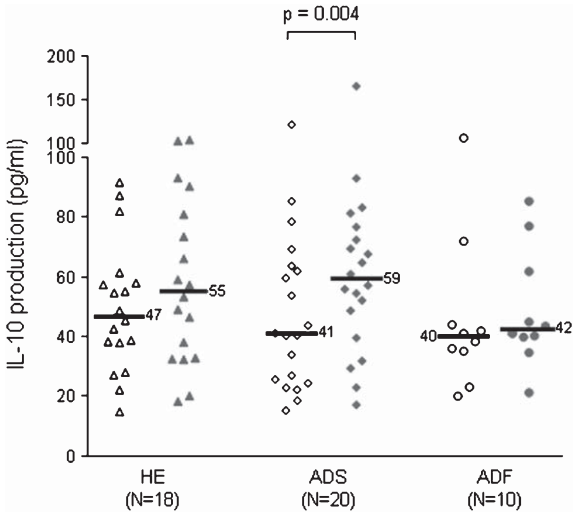

Fig. 3. IL-10 production comparison at day 5 between unstimulated (open symbols) and Aβ stimulated (solid symbols) PBMC.

positive difference was found in ADS (40.7 ± 13.7 versus 59.0 ± 27.0; $p = 0.004$) (Fig. 3). Likewise, HE showed a trend close to significance (47.1 ± 25.4 versus 55.3 ± 27.9; $p = 0.10$). Interestingly, ADF did not show such a difference, suggesting a lack of response to Aβ stimulus (39.7 ± 14.4 versus 42.2 ± 22.4; $p = 0.94$) (Fig. 3).

Moreover, the concentration of Aβ stimulated PBMC at day 5 was significantly lower for ADF compared to both ADS (1.09 ± 0.29 and 1.30 ± 0.27 million PBMC/ml respectively; $p = 0.050$) and HE (1.09 ± 0.29 and 1.28 ± 0.12 million PBMC/ml respectively; $p = 0.024$) (Table 2). Interestingly, we found no significant difference in the concentration of both unstimulated PBMC (after 2 and 5 days of culture) and LPS-stimulated PBMC between the study groups, suggesting that PBMC from ADF may have an impaired Aβ-induced but not physiologic proliferative response.

TL directly correlates with deltaMMSE score

We found a direct correlation in total patients with AD between TLs (kb) and deltaMMSE scores ($R^2 = 0.284$; $p = 0.008$) (Fig. 2). However, we found no significant correlation between TLs and Aβ-induced IL-10 production either considering all the enrolled individuals ($p = 0.448$) or considering only the patients with AD ($p = 0.530$) (data not shown).

DISCUSSION

Even though telomere shortening has been associated with neurodegeneration, dementia risk and AD etiopathogenesis, literature data have shown conflicting results. Our study showed no difference in TL between AD subjects compared to controls. Nevertheless, when patients were categorized based on disease progression rate, a difference in TL was found. In line with our results, a recent *in vivo* study showed that telomere shortening is associated with a slower cognitive decline in APP23 mice, a mouse model of AD [73].

Inflammation is considered to be involved in AD etiopathogenesis through effects on neuronal homeostasis and immune response [54–58]. In addition, the immune system of AD subjects is reported to be poorly responsive to Aβ as it exhibits a reduced ability to phagocytize amyloid peptides [74] and a severe lack of proliferative responsiveness to amyloid stimulus [75]. Our previous case-control study showed a decreased Aβ-stimulated production of IL-10 in PBMC from patients with AD [68], and the present data highlighted in ADF a lack of IL-10 increase after Aβ stimulus, suggesting an antigen-specific impairment in the production of this cytokine.

In addition, Aβ-stimulated PBMC from ADF showed a significantly lower proliferative response when compared to Aβ stimulated PBMC from both ADS and HE, also suggesting an impaired proliferative response to the stimulation induced by Aβ in ADF. We speculate that PBMC from ADF may be characterized

by an impaired capacity to respond to the inflammatory stimulus induced by Aβ. This may lead not only to a lower Aβ induced IL-10 production but also to an impaired Aβ induced proliferative response. As telomeres get shorter and shorter every time a cell divides, a reduced proliferative response to Aβ may be responsible for the maintained TL in ADF. On the contrary, PBMC from ADS may be able to better respond to Aβ. Accordingly, an overstimulation of PBMC from ADS may occurs during the progression of the pathology, leading to both a higher proliferative and telomere shortening rate compared to ADF and HE.

Our data and APP23 mice investigations [73] showed shorter telomeres in those exhibiting a slower rate of disease progression. However, considering telomere shortening as a beneficial process with regard to the rapidity of AD progression requires further experimental and clinical evidences.

The strength of the present study is the further classification of AD patients in two sub-groups, which are characterized by a different progression rate (and may display different peculiar characteristics). Conversely, its weakness is the small size of the study population. This may be responsible for the lack of significant differences in IL-10 productions between groups, notwithstanding the disparity in terms of median values between ADS and ADF. The small sample size may also be responsible for the absence of significant differences in APOE ε4 distribution between patients with AD and HE. Contributing factors to telomere shortening lie in lifestyle factors (e.g., physical activity, overall quality of life, perceived stress, smoking, nicotine and alcohol consumption) and health status. We did not compare the study groups for lifestyle factors and prevalence of age-related diseases and this may be another limitation of our study.

Our preliminary investigation confirms previous literature data suggesting PBMC as peripheral biomarkers that may mirror alterations within the diseased brain [76]. Moreover, we showed in ADF a longer TL and a lack of IL-10 increase in response to Aβ stimulus. The significant direct correlation between TLs and deltaMMSE scores indicates that TL may have a role as predictive marker of AD progression rate. The assessment of how rapidly AD is aggravating has important implications in clinical practice, since the rate of disease progression may be the most important factor in determining prognosis [77, 78].

Other investigations, with larger study groups, are necessary to clarify the mechanisms responsible for the different course of the pathology in ADS and ADF.

DISCLOSURE STATEMENT

Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/14-2808r1).

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