Article

Telomere Shortening linked to Disability and Mitochondrial DNA Copy Number in Patients with Relapsing-Remitting Multiple Sclerosis

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Abstract: Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease that affects the nervous system. Peripheral blood leukocyte telomere length (LTL) and mitochondrial DNA copy number (mtDNA-CN) are potential biomarkers of disability and neurological damage. The present work evaluated LTL and mtDNA-CN in 75 relapsing-remittent MS (RRMS) patients 50 of whom had an Expanded Disability Status Scale (EDSS) 0 to 3 (mild-moderate disability), and 25 had an EDSS of 3.5 to 7 (severe disability). Absolute LTL and absolute mtDNA-CN were measured via real-time polymerase chain reaction (qPCR). The LTL and mtDNA-CN were significantly lower in RRMS severe disability than in RRMS mild-moderate disability (3.924 ± 0.124 vs 2.854 ± 0.092, $p<0.00001$; 75.14 ± 1.77 vs 68.06 ± 1.608, $p<0.00001$, respectively). The LTL and mtDNA-CN showed a linear correlation in RRMS with mild-moderate disability ($r=0.2986$, $p=0.0351$). In addition, in a binary logistic regression model the LTL can predict severe disabil-ity ($AUC=0.697$, $p=0.0031$, cutoff ≤ 3.0875 Kb, sensitivity= 73.1%, specificity=62.5%), the prediction is improved by including age to the model ($AUC=0.765$, $p<0.0001$, sensitivity=78.26%, specificity=81.25%). Aging is closely linked to the development of disability in RRMS and can be evaluated through LTL and mtDNA-CN absolute quantification..

Keywords: keyword 1; keyword 2; keyword 3 (List three to ten pertinent keywords specific to the article yet reasonably common within the subject discipline.)

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of multifactorial origin (genetic susceptibility and environmental factors) with a heterogeneous clinical behavior featuring neurological dysfunction secondary to central and peripheral nervous system damage (1–3). Globally, MS is the leading non-traumatic cause of neuronal disability in

young adults, affecting approximately 2.8 million people (4). Demyelination and axonal loss in the central nervous system (CNS) is crucial for the episodic and irreversible progression of MS, they are mediated by an inflammatory phase followed by a neurodegenerative one (5). At the onset, patients with MS may have mild psychological and cognitive impairments, which progress to severe neurological and motor limitations (6,7). These neurodegenerative changes are assessed using the Expanded Disability Status Scale (EDSS) based on well-defined clinical and imaging parameters. Accordingly, patients are categorized from 0 (no signs, no symptoms) to 10 (death) (8–11). Patients with MS may have different phenotypes: primarily progressive multiple sclerosis (PPMS) manifests episodes of neurological dysfunction, is progressive and without recovery; relapsing-remitting multiple sclerosis (RRMS) characterized by episodes of neurological dysfunction with full or partial recovery, and finally, secondarily progressive multiple sclerosis (SPMS) results from an RRMS that acquires a PPMS-like pattern of progressive dysfunction without recovery (12). Neuroinflammation mechanisms in MS resemble those of aging but are seemingly activated early, exhibit greater intensity, and ultimately impact the course of the disease (13). Mitochondria and telomeres are closely linked to the aging process. Indeed, the core molecular mechanisms regulating aging are shared with the pathogenesis of most chronic degenerative diseases. Hence, the study of these common pathways is of current interest in MS research (14).

Telomeres are nucleoprotein structures that protect the ends of chromosomes, prevent deleterious structural changes such as intrachromosomal fusion, provide genomic stability, and regulate cellular senescence (15,16). Recently, a systematic review found that telomere length (TL) is consistently shorter in MS patients than in healthy controls. This association is independent of age and correlates with greater disability, lower brain volume, higher recurrence rate, and shorter conversion time from recurrent to progressive MS (17).

Another important factor associated with cellular aging and neurodegeneration in MS is the mitochondrial deficit (18). The mitochondrial DNA copy number (mtDNA-CN) reliably estimates the number of mitochondria in the cell (19). Studies focused on mtDNA-CN in MS, both in cerebrospinal fluid and lymphocytes, showed a significant decrease in mtDNA-CN in patients compared to controls (19,20). Furthermore, the Al-Kafaji study showed a significant decrease in mtDNA-CN in RRMS patients with more than ten years of diagnosis compared with those with less than ten years of diagnosis (19).

In the present study, we evaluated mtDNA-CN and leukocyte telomere length (LTL) as biomarkers to predict disability in patients with RRMS. Research insights into the link between aging and neurodegeneration can help classify MS patients according to prognosis and to design therapeutic strategies to prevent accelerated aging in MS.

2. Materials and Methods

2.1 Design of the study

The present research is a cross-sectional observational study. We enrolled 75 patients (50 women and 25 men) aged between 18 and 66 years old with RRMS without comorbidities (cancer, diabetes, hypertension, or other immunologic diseases), previously diagnosed, evaluated, and classified with EDSS by a neurologist. All patients fulfilled the McDonald diagnostic criteria (21). Group 1 (EDSS 0-3) with mild-moderate disability (n=50) and group 2 (EDSS 3.5-7) with severe disability (n=25). All patients were recruited from the Institute of Experimental Clinical Therapy (INTEC) MS cohort, University Center of Health Sciences (CUCS) of the University of Guadalajara (Figure 1). In addition, age, time after disease onset, disease progression index and actual treatment were recorded in both groups. This study complies with the Declaration of Helsinki guidelines.

The ethics committee of the CUCS approved the study with the number CI-03519. All participants involved in the study signed informed consent.
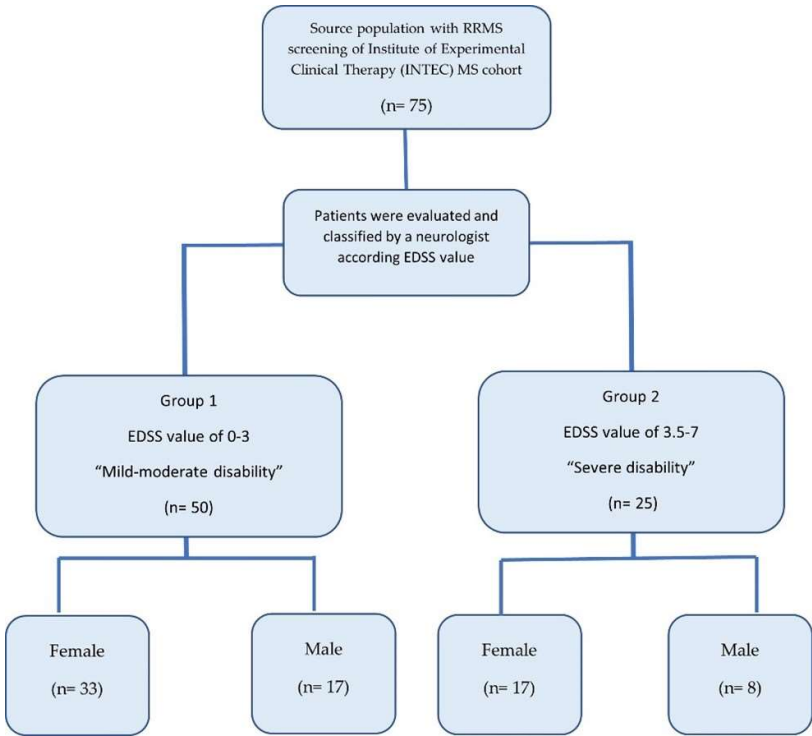
Figure 1. Flowchart of patient selection.

2.2 Absolute quantification of telomere length and mtDNA copy number

Total venous blood samples were collected for separation of leukocyte and DNA extraction by Miller method, once obtained were stored at −20 °C until analysis (39). The quantification of mtDNA-CN and LTL was assessed by the commercial assay kit Absolute Human Telomere Length and Mitochondrial DNA Copy Number Dual Quantification qPCR (ScienCell Research Laboratories, San Diego, CA, USA) according to the manufacturer's protocol. For PCR reaction (per sample): 10 µL of 2X GoldNStart TaqGreen qPCR master mix, 2 µL of primer solution (Tel, mtDNA or SCR), 7 µL of nuclease-free water, and 1 µL of DNA problem (5 ng). Initial denaturation at 95°C for 10 min, followed by 32 cycles with denaturation (95°C for 20 sec), hybridization (52°C for 20 sec), and extension (72°C for 45 sec). As a constituent region, a region of 100 bp length is used on chromosome 17, which is recognized by the first set of SCR (single copy reference). A reference DNA with a known concentration (telomere length 348 ± 11 kb per diploid cell and mt DNA of $1.27 \pm 0.03 \times 10^3$ copies per diploid cell) was used.

2.3 Statistical analysis

The statistical analysis consisted of descriptive statistics (means, frequencies and percentages) to describe the groups of patients. The Anderson-Darling (n> 30) or D´Agostino-Pearson (n <30) tests were used to evaluate the normality of the data. The outliners were identified with the Rout method. For the comparison between the groups of patients with mild-moderate disability *vs.* severe disability, inferential statistics were

used with parametric tests such as Student's T for variables with normal distribution; Mann's U test was used for variables with non-normal distribution; Chi square test or Fisher's exact test was used for the comparison of frequencies. The predictive capacity (sensitivity, specificity, PPV and NPV) was evaluated with ROC curves. To predict severe disability, binary logistic regression models were developed, which were evaluated with the Akaike corrected information criterion and the Z statistic for comparison of ROC curves (LTL *vs.* Age *vs.* LTL + Age). All analysis were performed using GraphPad Prism version 9.02 for Windows (La Jolla California USA, www.graphpad.com) and MedCalc Statistical Software version 15.8 (Ostend, Belgium; https://www.medcalc.org).

3. Results

2.1. Patients

Seventy-five patients with RRMS, one group of 50 RRMS with mild to moderate disability (EDSS from 0 to 3), and the other group of 25 RRMS patients with severe disability (EDSS from 3.5 to 7), were enrolled. Of the total number of patients, there were 50 women and 25 men. The mean age was 39.48 ± 11.52, mean years since diagnosis was 8.607 (±5.881), mean progression rate was 0.6726 ± 0.8374, and the mean EDSS 2.907 ± 1.712. There was no significant difference regarding sex, age, or time since diagnosis between the 2 study groups. There was significant difference regarding progression rate and EDSS. The EDSS of the RRMS with mild disability group (EDSS 0 to 3) was 1.91 ± 0.8846 and the EDSS of the RRMS with advanced disability group (EDSS 3.5 to 7) was 4.9 ± 1.109 (Table 1).

Table 1. Characteristics of groups of patients and their pharmacological treatments. Group 1 with mild to moderate disability (EDSS 0-3) and group 2 with severe disability (EDSS 3.5-7).

| Characteristic	Group 1, n=50 "Mild-moderate disability"	Group 2, n=25 "Severe disability"
Female	33 (66%)	17 (68%)
Male	17 (34%)	8 (32%)
Age	37.70 (± 11.409)	43.04 (± 11.110)
Years since diagnosis	7.78 (± 5.1211)	10.26 (± 6.9868)
EDSS*	1.91 (± 0.8846)	4.9 (± 1.109)
Progression rate*	0.5139 (± 0.6355)	0.9898 (± 1.0864)
Treatment:		
Glatiramer Acetate	19 (38%)	9 (36%)
Rituximab	4 (8%)	3 (12%)
Interferon β	14 (28%)	5 (2%)
Fingolimod	5 (10%)	2 (8%)
Azathioprine	0	3 (12%)
Natalizumab	1 (2%)	1 (4%)
Dimethyl fumarate	3 (6%)	1 (4%)
None	4 (8%)	1 (4%)

EDSS=Expanded Disability Status Scale

Progression rate= EDSS/years with the disease

*$p<0.05$. Progression rate comparison by student T test and EDSS by Mann Whitney test.

### 2.2 Absolute LTL and mtDNA-CN in mild-moderate disability vs. severe disability

Comparisons of LTL (3.924 ± 0.124 *vs.* 2.854 ± 0.090) and mtDNA-CN (75.14 ± 1.77 *vs.* 68.06 ± 1.60) between the groups with mild-moderate disability and severe disability were significant (Figure 2).
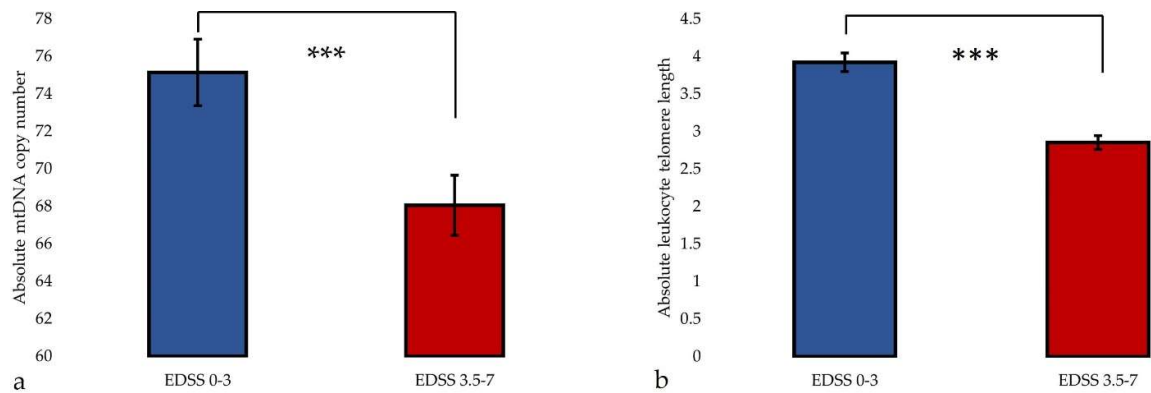
[1]   **Figure 2**. (**a**) Absolute mitochondrial DNA copy number (mtDNA-CN) from relapsing-remitting multiple sclerosis (RRMS) patients with mild-moderate disability (blue) and severe disability (red). (**b**) Absolute leukocyte telomere length (LTL) in RRMS patients with mild disability (blue) and severe disability (red). ******* = p <0.00001, unpaired student T-test, normality evaluated by D'Agostino & Pearson test.

2.3 Correlation between LTL and mtDNA-CN

Pearson's correlation test was significant in the mild-moderate disability group (Figure 3). There was no correlation in the severe disability group.
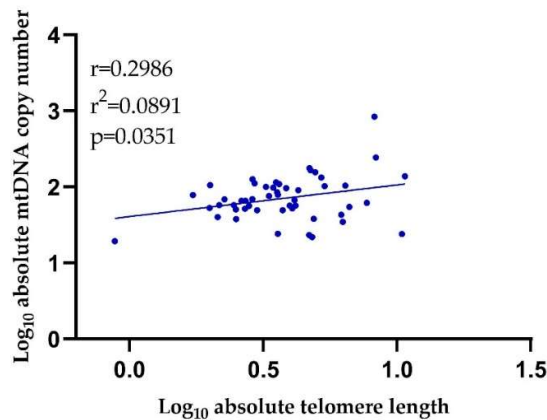

Figure 3. Correlation (Pearson) between $\log_{10}$ LTL and $\log_{10}$ mtDNA-CN in RRMS patients with mild-moderate disability.

2.4 Telomere Disability Prediction

The predictive capacity of LTL was evaluated directly (raw data), the receiver-operating characteristic (ROC) curves was significant (p = 0.003), and the area under the curve (AUC) was 0.697. The cut-off point was obtained through the Youden index,

and the associated criterion was ≤ 3.0875 Kb, which showed a sensitivity of 73.1% and specificity of 62.5%, positive predictive value (PPV) of 48.6%, and negative predictive value (NPV) of 83.3% (Figure 4).
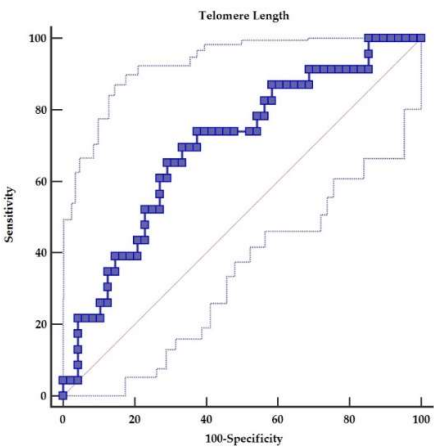
Figure 4. Receiver-operating characteristic (ROC) curves for LTL (raw data) in patients with RRMS.

2.5 Binary Logistic Regression Model of LTL and Age

Predictive models based on binary logistic regression was developed. Of the variables introduced, only LTL maintains significance when controlling for variables in Wald test (Table 2). However, the integration of age together with LTL improved the precision of the model, which did not occur with the rest of the variables.

Table 2. Features evaluated in the binary logistic regression model for the prediction of severe disability in MS.

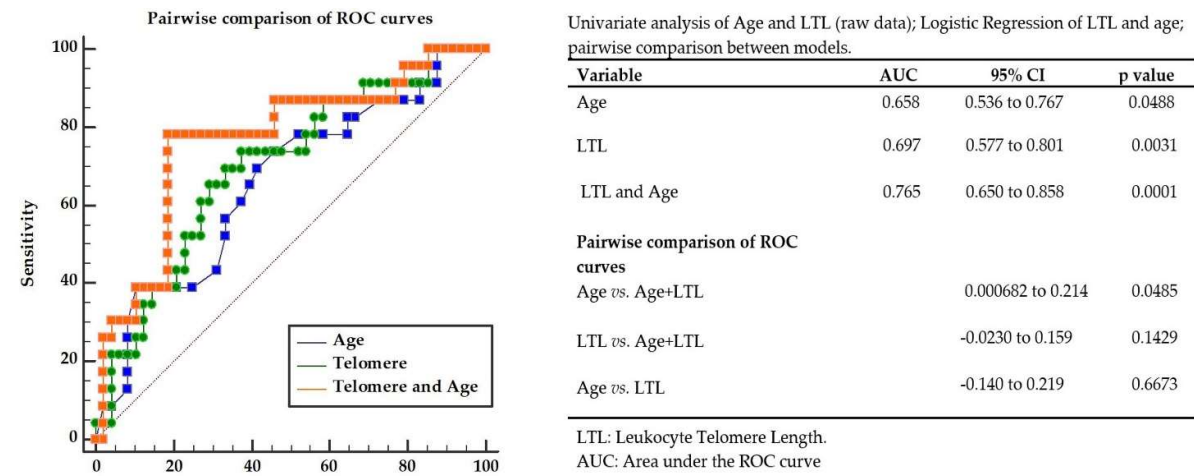| Variable	Odds ratio	95% CI	p
LTL*	0.5855	0.3672 to 0.9336	0.0245
mtDNA-CN	0.9980	0.9810 to 1.0152	0.8142
Age	1.0331	0.9789 to 1.0903	0.2362
Sex (female)	1.0040	0.3057 to 3.2974	0.8143
Years since diagnosis	1.0585	0.9572 to 1.1706	0.2678

LTL: Leukocyte telomere length; mtDNA-CN: mitochondrial DNA copy number.

* $p < 0.05$, Wald test. CI = confidence interval

LTL and age had the highest precision and was the most appropriate according to Akaike's criteria (AUC = 0.765, $p < 0.0001$, sensitivity = 78.26%, specificity = 81.25%, PPV = 66.7 and NPV = 88.6%) (Figure 5). Pairwise comparison of ROC curves, between LTL and age *versus* LTL did not reach significance (p=0.1429), so the equivalence of the models (LTL and age *vs.* LTL alone) was not ruled out (Figure 6).
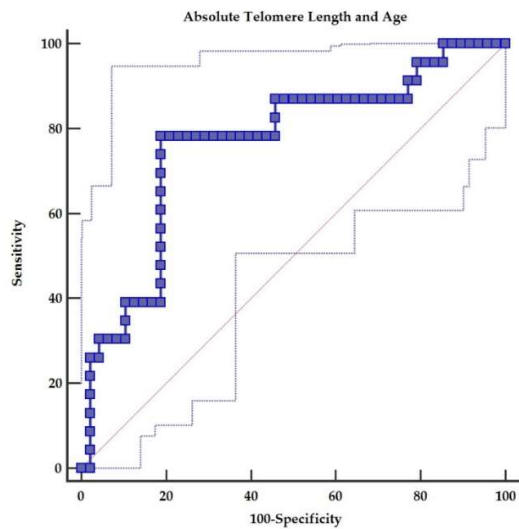
Figure 5. Receiver-operating characteristic (ROC) curve for binary logistic regression model for LTL and age in patients with RRMS.

Univariate analysis of Age and LTL (raw data); Logistic Regression of LTL and age; pairwise comparison between models.

Variable	AUC	95% CI	p value
Age	0.658	0.536 to 0.767	0.0488
LTL	0.697	0.577 to 0.801	0.0031
LTL and Age	0.765	0.650 to 0.858	0.0001
Pairwise comparison of ROC curves			
Age *vs.* Age+LTL		0.000682 to 0.214	0.0485
LTL *vs.* Age+LTL		-0.0230 to 0.159	0.1429
Age *vs.* LTL		-0.140 to 0.219	0.6673

LTL: Leukocyte Telomere Length.
AUC: Area under the ROC curve

Figure 6. Pairwise comparison of ROC curves of LTL (raw data), age (raw data) and LTL and age (binary logistic regression).

4. Discussion

In recent years, MS has been approached from the perspective of biological aging as the trigger of the cellular and immunological changes involved in the development of neuroinflammation, neurodegeneration, and disability. Several authors consider that, among the multiple factors underlying neurodegeneration, accelerated neurological aging occurs in MS. Telomeres are found at the ends of chromosomes and are made up of repeats of the hexanucleotide TTAGGG, a non-coding sequence (15,22). Telomere changes contribute to the pathogenesis of several multifactorial chronic diseases, including neurological disorders. Loss of chromosomal integrity due to shorten telomeres facilitates fusions with other chromosomes, mutations, and other events that impair cell function, decrease cell division, and lead to cell aging (23,24).

Mitochondria is an organelle that regulate intracellular calcium homeostasis, ATP generation, programmed cell death (apoptosis), reactive oxygen species (ROS) production, and aging processes, all of which affect the telomere structure and function (25). Numerical and structural alterations of mitochondria is pathogenic, especially in high-energy-demanding tissues such as muscle and the nervous system(26). Age and accelerated aging are factors of neurological deterioration in MS. Age is considered a strong independent factor to develop a progressive MS phenotype via complex and multidirectional interactions with aging and degenerative processes (27,28). In this sense, the telomeres and mitochondria are two of the most important cellular components in the regulation of aging and disease.

The patients enrolled in this study were primarily young (mean 39.48 ± 11.52 years) and selected in such a way that the distribution of MS was similar in both sexes and to the proportion of mild-moderate/severe disability observed in the general population; hence, the effect of sex and age on LTL or mtDNA-CN was minimized. The wide variability of LTL and mtDNA-CN reflects the interactions of organisms with environmental factors and the influence of endogenous factors (genes, hormones, ROS, among others); notably, up to 70% of the variance of TL is explained by heritability (29,30).

Although the variability in TL is determined by multiple factors, in the group with a mild-moderate disability, we found a low but significant linear correlation (r=0.2986, p=0.0351) between LTL and mtDNA-CN. Even if a similar finding was previously documented in elderly women (31), our study is the first to correlate these two senescence parameters in patients with MS. The lack of such a correlation in the severe disability group suggests that this correlation was lost in advanced disability stages due to greater heterogeneity in the processes of aging and neurodegeneration, as well as by the influence of other factors such as increased immune activation, chronic inflammation, and age (13,32). The bidirectional interaction between mitochondria and TL result from different mechanisms related to mitochondrial function, such as oxidative stress, apoptosis, energy efficiency of the respiratory chain, and chronic inflammation. Of note, all of these can produce telomere shortening and subsequent exposure of subtelomeric DNA, which in turn affect mitochondrial function through activation of genes that prevent genetic damage such as p53 and p21(33). The aging is fundamental for the progression of disability; actually, senescent changes have been identified in multiple cell lineages of the nervous system that are affected during the evolution of MS, such as neurons, microglia, oligodendrocytes, and astrocytes (34–38). In addition, we compared the absolute quantification of LTL and mtDNA-CN in mild-moderate disability patients *versus* patients with severe disability and found significant differences for both parameters (3.924 ± 0.124 vs 2.854 ± 0.092, p<0.00001; 75.14 ± 1.77 vs 68.06 ± 1.608, p<0.00001, respectively); thus, such discrepancies support the interdependence of LTL and mtDNA-CN in the different phases of disability in MS.

Telomere biology and age play a crucial role in health and disease. In several studies, age has been reported as one of the most important factors for conversion of a RRMS into SPMS (39–41). This clinical distinction reflects the underlying neurological damage: in the former phenotype the damage is predominantly inflammatory whereas in the progressive phenotype it is neurodegenerative (5,42). Moreover, LTL can serve as a biomarker of immunosenescence and has been linked to the progression of neurological damage (40); thereby, LTL could be useful for assessing and predicting disability in MS(43). We have found LTL to be an independent predictor of disability. After integrating possible predictors (LTL, mtDNA-CN, sex, age, and time with the disease) to the binary logistic regression model, only age and LTL were useful for prediction. The ROC curve for direct LTL values alone has moderate predictive capability (AUC = 0.697, p = 0.003, cutoff ≤3.0875 Kb, sensitivity = 73.1 %, specificity = 62.5%, PPV = 48.6%, NPV = 83.3%). The high NPV of LTL

identifies LTL as a relevant biomarker for assessing disability status. Interestingly, when LTL and age were combined in the binary logistic regression model, accuracy increased (AUC: 0.765, $p < 0.001$, sensitivity = 78.26 %, specificity = 81.25 %, PPV = 66.7 %, NPV = 88.6 %). Although LTL and age are related risk factors that predict disability, LTL remains as a disability factor even when analyzed independently from other variables. This is of great importance since age is the highest prognostic factor for MS progression (44); however, LTL can be as strong or even stronger than age as a predictor of disability status and/or disease progression in MS.

5. Conclusions

More studies, especially longitudinal ones with large samples that fully address the different MS phenotypes, are required to establish the clinical utility of LTL for the evaluation and prediction of disability, as well as to unravel the molecular mechanisms involved in the process of neuroinflammation, neurodegeneration, and aging and its relationship with the structure and function of telomeres and mitochondria in MS.

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