

Mitochondrial DNA Copy Number as a Biomarker of Multiple Sclerosis

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Abstract

Multiple sclerosis (MS) is a chronic autoimmune disease that affects the central nervous system (CNS). In its early stages, it results in inflammation, demyelination, and axonal loss. Egypt has the highest rates in the Middle East region. The pathogenicity of MS involves mitochondrial function. Damage to mitochondrial DNA (mtDNA) can produce variation in the copy number (CN) and decline in mitochondrial function. Our goal was to determine the potential of mtDNA-CN as a biomarker of MS and the progression of the disease. The study included 25 patients with relapsing remitting MS (RRMS) and 25 age and sex matched apparently healthy control. Two peripheral blood samples were collected from each patient, one during the remission phase and the other during the phase of relapse. A quantitative real-time polymerase chain reaction (qPCR) was performed to assess CN of mitochondrial DNA. There was a statistically significant decline in the number of mtDNA copies during the remission phase as compared to controls ($p < 0.01$), yet no difference was seen between mtDNA-CN in relapsing subjects versus controls. Moreover, the copy number of mtDNA during the relapse phase was significantly higher than the remission phase suggesting the ability of mtDNA to differentiate between remission and relapse phases ($p < 0.05$). Our study observed that mtDNA-CN at a cut off (0.75), can differentiate between RRMS patients in the remission phase and controls with a sensitivity of 56%, specificity 84%, positive predictive value (PPV) 65.6% and negative predictive value (NPV) 77.8%, and at a cut off (1), mtDNA-CN can differentiate between remission and relapse MS patients with a sensitivity 72%, specificity 56%, PPV 62.1% and NPV 66.7%. In conclusion, mtDNA-CN can be proposed as a biomarker of MS.

Keywords: RRMS, qPCR.

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Introduction

The central nervous system (CNS) is affected by the well-known chronic autoimmune disease known as multiple sclerosis (MS), which also results in demyelination, focal inflammation,

and axonal injury.¹ In young adults and those whose onset occurs between the ages of 20 and 40, it is regarded as one of the most prevalent neurological diseases that causes disability and affects more than 2.5 million people worldwide.² Egypt has the highest rates of MS

patients in the Middle East, with an average patient population of 59,671 subjects.³

Immune dysregulation, which results from complex interactions between numerous infectious exposures, genetic predispositions, and other factors, including obesity, smoking, and inadequate sun exposure, is what causes multiple sclerosis.⁴ Primary progressive MS (PPMS), secondary progressive MS (SPMS), progressive relapsing MS (PRMS), and relapsing remitting MS (RRMS) are its four clinical forms. Acute attacks (or relapses), which were experienced by about 87% of RRMS patients, were followed by full or partial recovery (or remission).⁵

Mitochondrial DNA (mtDNA), an extra-chromosomal genome, is both separate and different from nuclear genome.⁶ Each mitochondrion has got many copies of the mtDNA genome, with their number strictly regulated.^{7, 8} Mitochondrial DNA encodes many essential components in the chain of oxidative phosphorylation, which is crucial for adenosine triphosphate generation.⁹ The copy number of mtDNA represents the activity of the mitochondrial that gives an idea about mitochondrial number. So, mtDNA is considered to be an efficient measure of the mitochondrial function.¹⁰

Chronicity of the MS neuroinflammatory process causes secondary damage to the mitochondria and macromolecules, such as proteins from the electron transport chain, mtDNA, and lipids, as well as an increase in reactive oxygen species and oxidative stress.^{11,12} This harm impairs mitochondrial function, which in turn increases reactive oxygen species production in a vicious cycle.¹³ This results in failure to give the required levels of energy inside the demyelinated axons due to decrease in the production of adenosine triphosphate resulting in the activation of the apoptosis mechanisms and neurodegeneration.^{14,15}

The aim of our study was to assess peripheral blood mtDNA copy number in relapsing remitting MS patients and control subjects to evaluate its value as a biomarker of MS, and to assess its prognostic value regarding disease duration and its progression.

Subjects and Methods

Study participants

This case-control study was carried out during the period from December 2021 to February 2022 at the Department of Clinical Pathology in association with the Department of Neurology, Ain Shams University Hospitals. Two groups of participants were included in the study.

Group I: a patient Group, included 25 RRMS patients. They were recruited from the multiple sclerosis clinics in the Department of Neurology, Ain Shams University Hospitals. Patients were identified using the *McDonald's criteria 2017* for diagnosis with at least 2 preceding relapses. Relapse is the sudden onset of focal neurologic symptoms lasting more than 24 hours (such as transverse myelitis, brainstem or cerebellar syndrome, optic neuritis, or new cerebral lesions). The onset of symptoms had to be at least 30 days after a previous exacerbation and without evidence of a secondary etiology (such as fever or a systemic infection) in different CNS areas in agreement with clinical history, neurological examination, and magnetic resonance imaging¹⁶. The patient group included 13 females and 12 males, and their ages ranged from (26.4- 41) years.

Group II: a control Group, included 25 normal individuals with similar ages and sexes who were free of any inflammatory or neurological conditions as well as active infections.

Exclusion Criteria

According to clinical history, the neurological examination and medical records, subjects with autoimmune diseases, other neurodegenerative diseases, malignancy, and type 2 diabetes mellitus were excluded from our study.

Samples collection

Venous blood samples (2 mL) were collected from each subject twice [once during remission which is the period of the greatest improvement in both subjective and objective (examination) findings following the peak deficit of a relapse¹⁶ and the other during relapse]. Blood samples were collected under strict aseptic conditions and put into sterile

containers with ethylenediamine tetra acetic acid (EDTA) as an anticoagulant. For the purpose of measuring the copy number of mtDNA, samples were kept at -80°C. For the extraction of the genomic DNA, blood samples were used. We avoided repeatedly freezing and thawing.

For each study participant, we obtained demographic data (age and gender) and complete medical history, focusing on relevant prior history of MS, and drug usage. This was followed by detailed clinical examination, especially neurological assessment. For the patient group only, the Expanded Disability Status Scale (EDSS score) for multiple sclerosis was used to assess disability.¹⁷ Quantitative real-time polymerase chain reaction (qRT-PCR) was used for measurement of mtDNA copy number.

Genomic DNA extraction and PCR amplification

Commercial whole blood genomic DNA purification mini kits (Gene Jet TM, Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA), were used for genomic DNA extraction, according to the manufacturer's instructions.

To measure the copy number of mitochondrial DNA (mtDNA-CN) in relation to nuclear DNA, the RT-PCR assay was carried out. We employed a rapid and accurate measurement of mtDNA copy number as the ratio of mtDNA per cell (mtDNA/nDNA). The single copy genes in the mitochondria and the nucleus were targeted simultaneously. For a single-copy nuclear gene, commercial RT-PCR assay kits [catalogue number 4403316, TaqMan Copy Number Reference assay human RNase P (RNP), Applied Biosystems; Thermo Fisher Scientific, Inc. USA), and other kits (catalogue number 440291, TaqMan assay ND1 mitochondrial, Applied Biosystems; Thermo Fisher Scientific, Inc. USA) were used, according to the manufacturer's instructions. NADH-ubiquinone oxidoreductase chain 1 (ND1), coding for the nicotinamide adenine dinucleotide, designed to target the mitochondrial MT-ND1 gene, was used for the mitochondrial gene.

Each PCR reaction consisted of 20 µl, contained 1 µl mtDNA encoded ND1 TaqMan probe, 1 µl nuclear DNA (nDNA)- encoded RNP TaqMan probe, 2 µl DNA, and 10 µl Master Mix and 6 µl RNase free water. Reaction tubes were placed into the RT-PCR instrument system (DT lite real-time PCR, DNA Technology, Russia) after the reaction mix was prepared and carefully mixed.

Copy number calculation

The cycle threshold (Ct) values of ND1 were used as the target assay and the Ct values of RNP were used as the reference assay to calculate the relative amount of mtDNA. The $2^{-\Delta\Delta Ct}$ method was used to determine the mtDNA copy number.¹⁸ For each sample, the following calculations were made:

$(\Delta Ct) = Ct \text{ target gene} - Ct \text{ reference gene.}$

The average (mean) ΔCt was calculated for the sample of control groups.

$(\Delta\Delta Ct) = \Delta Ct \text{ of the patient or control samples} - \text{mean of } \Delta Ct \text{ of control samples.}$

Finally, relative estimation of mtDNA copy number in each sample was calculated using ($2^{-\Delta\Delta Ct}$) method.

Statistical Analysis

Data analysis was done using the Statistical Package for the Social Sciences (SPSS) statistics software (version 26.0, IBM Corp., USA, 2019). Quantitative non-parametric data are presented as numbers and percentages, and categorical data expressed as median and Interquartile range (IQR). When comparing two independent mean groups for non-parametric quantitative data, the Wilcoxon's rank sum test was used, and the Chi-Square test used to study associations between two qualitative independent variables. For a repeated measure design where the same subjects were assessed under two different circumstances, the Wilcoxon Signed Rank Spearman was used. We used correlation analysis to identify the direction of the linear relationship between two variables as well as the strength of the association between two quantitative variables. Probability values (p values) <0.05 were regarded as significant. The diagnostic effectiveness was evaluated using the receiver operating characteristic (ROC) curve analysis.

Results

In our study, the age and gender of RRMS patients and controls were comparable ($p>0.05$), Table 1.

Table 1. Descriptive and comparative statistics of demographic data in relapsing remitting multiple sclerosis (RRMS) patients' group and the control group.

Parameters		Group		p value
		RRMS Patients group (n=25)	Control group (n=25)	
Age (years)	Median	33	30	NS
	(Q1-Q3)	(26.5- 41)	(24.5- 35)	
Sex	Male	12 (48%)	14(56%)	NS
	Female	13 (52%)	11(44%)	

Q1-Q3: 25th - 75th percentile (interquartile range), χ^2 : Chi square test, Z: Wilcoxon Rank sum test.

$P > 0.05$ is not significant (NS).

Patients with RRMS were further divided into two groups based on the length of the disease in years: less than 6 years and more than 6 years. Table 2 displays comparative statistics using the Wilcoxon rank sum test along with descriptive statistics of their demographic data regarding age, EDSS, and time in months

between relapse and remission. There was no difference in age, EDSS, and the number of months between relapse and remission in RRMS, between RRMS with disease duration less than 6 years and those with disease duration greater than 6 years ($p>0.05$).

Table 2. Descriptive and comparative statistics of demographic data in relapsing remitting multiple sclerosis (RRMS) patients with duration less than 6 years and more than 6 years.

Parameters		Group		p value
		RRMS Patients with disease duration <6 years n=14	RRMS Patients with disease duration >6 years n=11	
Age (years)	Median	34.5	33	NS
	(Q1-Q3)	(26- 42)	(29-42) NS	
EDSS	Median	3.5	4	NS
	(Q1-Q3)	(3-5.25)	(2-5)	
Time between relapse and remission in months	Median	4.62	5	NS
	(Q1-Q3)	(3.25-6.25)	(4.25-6)	

Q1-Q3: 25th- 75th percentile (interquartile range). RRMS: Relapsing Remitting multiple sclerosis. EDSS: expanded disability status scale. Z: Wilcoxon Rank sum test. $P > 0.05$ is not significant (NS).

Table 3 displays descriptive and comparative statistics of the number of mtDNA-CN during remission and relapse phases in the RRMS patient group compared to the control group using the Wilcoxon rank sum test. There was no difference in mtDNA CN between the group of

RRMS patients in the relapse phase and the control group ($p>0.05$). However, mtDNA CN decreased significantly in the RRMS patient group during the remission phase in comparison to the control group ($p<0.01$), as shown in Figure 1.

Table 3. Descriptive and comparative statistics of mitochondrial DNA copy number (mtDNA-CN) during relapse and remission in relapsing remitting multiple sclerosis (RRMS) patients and the control Group.

Parameters	Group				^z <i>p</i> value
	RRMS Patient group (n=25)		Control group (n=25)		
	Median	Q1-Q3	Median	Q1-Q3	
mtDNA-CN in relapse	1	0.6-1.35	0.917	0.77-1.17	NS
mtDNA-CN in remission	0.7	0.45-1	0.917	0.77-1.17	<0.01

Q1- Q3: 25th- 75th percentile (interquartile range). Z: Wilcoxon Rank sum test. *P* > 0.05 is not significant (NS).

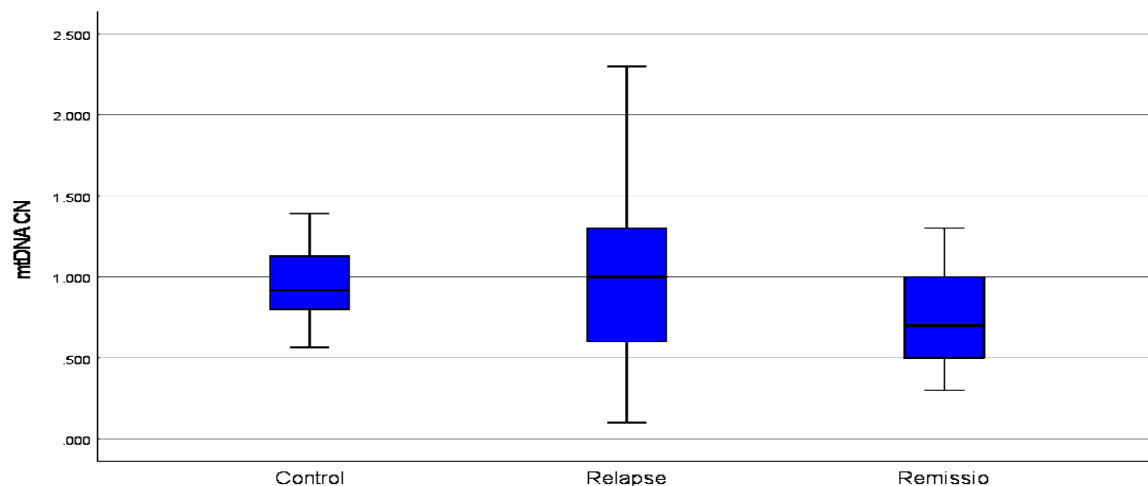


Figure 1. Box Blot, showing median levels of mitochondrial DNA copy number (mtDNA-CN) in relapsing remitting multiple sclerosis (RRMS) patients in remission phase, relapse phase and control groups.

Paired comparison using Wilcoxon signed rank test showed significantly high value of mtDNA-CN in RRMS patient groups in relapse phase

compared to remission phase (*p* < 0.05) Table 4 and Figure 2.

Table 4. Descriptive and comparative statistics of mitochondrial DNA copy number (mtDNA-CN) during relapse and remission in relapsing remitting multiple sclerosis (RRMS) Patients.

Parameters	Group				^z <i>p</i> value
	RRMS Patient group in relapse (n=25)		RRMS Patient group in remission (n=25)		
	Median	Q1-Q3	Median	Q1-Q3	
mtDNA-CN	1	0.6-1.35	0.7	0.45-1	<0.05

Q1- Q3: 25th - 75th percentile (interquartile range) Z: Wilcoxon signed Rank test. *p* < 0.05: significant statistical difference.

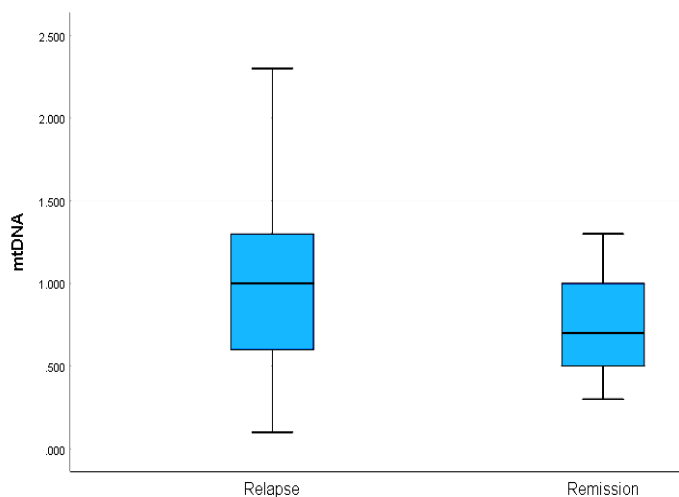


Figure 2. Box Blot, showing median levels of mitochondrial DNA copy number (mtDNA-CN) in relapsing remitting multiple sclerosis (RRMS) patients during relapse and during remission.

Patients with RRMS who were diagnosed less than six years during relapse displayed mtDNA-CN values comparable to those of normal control individuals and patients diagnosed for more than six years ($p>0.05$, for all) (Tables 5 & 6, Figure 3). Patients with disease duration <6 years showed significantly lower mtDNA CN when compared to control subjects ($p<0.01$) and when compared to patients with disease

duration >6 years ($p<0.05$), (Tables 5 & 6, Figure 3). During the relapse and remission phases, mitochondrial DNA CN was comparable in patients with disease durations of <6 years and >6 years ($p>0.05$, for all) (Table 7, Figure 3).

Mitochondrial DNA CN in remission and relapse phases showed a significant positive correlation ($p<0.01$) (Table 8, Figure 4).

Table 5. Descriptive and Comparative Statistics of mitochondrial DNA copy number (mtDNA-CN) during relapse and remission in relapsing remitting multiple sclerosis (RRMS) patients less than 6 years disease duration of multiple sclerosis and the control group.

Parameters	Group				^z <i>p</i> value
	RRMS Patients with disease duration <6 years n=14		Control group (n=25)		
	Median	Q1-Q3	Median	Q1-Q3	
mtDNA CN in relapse	0.95	0.47-1.5	0.917	0.77-1.17	NS
mtDNA CN in remission	0.65	0.4- 1.02	0.917	0.77-1.17	<0.01
Q1- Q3: 25 th - 75 th percentile (interquartile range); Z: Wilcoxon Rank sum test. <i>P</i> > 0.05 is not significant (NS).					

Table 6. Descriptive and comparative statistics of mitochondrial DNA copy number (mtDNA-CN) during relapse and remission in relapsing remitting multiple sclerosis (RRMS) patients more than 6 years duration of multiple sclerosis and control subjects.

Parameters	Group				^z <i>p</i> value
	RRMS Patients with disease duration		Control group		
	>6 years		(n=25)		
	n=11				
	Median	Q1-Q3	Median	Q1-Q3	
mtDNA-CN in relapse	1.1	0.7-1.2	0.917	0.77-1.17	NS
mtDNA-CN in remission	0.8	0.5- 1	0.917	0.77-1.17	<0.05
Q1- Q3: 25 th - 75 th percentile (interquartile range). Z: Wilcoxon Rank sum test. <i>P</i> > 0.05 is not significant (NS).					

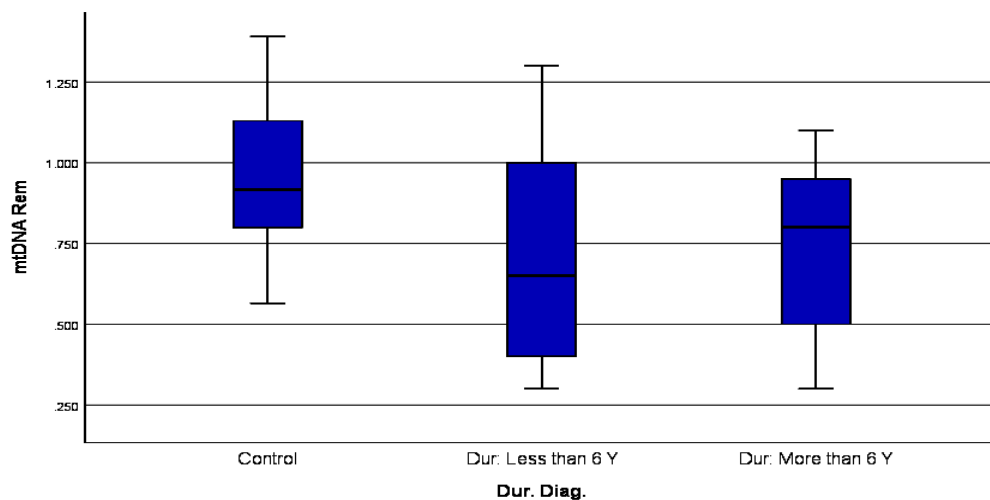


Figure 3. Box Blot, showing median levels of mitochondrial DNA copy number (mtDNA-CN) in relapsing remitting multiple sclerosis (RRMS) patients less than 6 years duration and more than 6 years duration during remission versus control group.

Table 7. Descriptive and Comparative Statistics of mitochondrial DNA copy number (mtDNA-CN) during relapse and remission in relapsing remitting multiple sclerosis (RRMS) patients less than 6 years duration and more than 6 years duration.

Parameters	Group				^z <i>p</i> value
	Patient group with disease duration <6 years (n=14)		Patient group with disease duration >6 years (n=11)		
	Median	Q1-Q3	Median	Q1-Q3	
mtDNA-CN in relapse	0.95	0.475-1.5	1.1	0.7-1.2	NS
mtDNA-CN in remission	0.65	0.4-1.025	0.8	0.5-1	NS

Q1- Q3: 25th- 75th percentile (interquartile range); Z: Wilcoxon Rank sum test. $P > 0.05$ is not significant (NS).

Table 8. Correlation between mitochondrial DNA copy number (mtDNA-CN) in remission and in relapse in patients with relapsing remitting multiple sclerosis (RRMS) group.

Parameters	r_s	⁸ p value
mtDNA-CN in relapse	0.574	<0.01

r_s : Spearman's rank correlation. * $P \leq 0.05$ is significant.

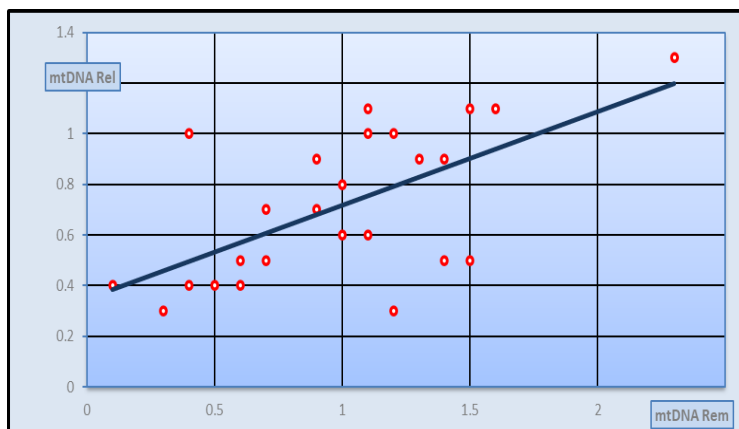


Figure 4. Correlation study between mitochondrial DNA copy number (mtDNA-CN) in relapse and tDNA-CN in remission among patients with relapsing remitting multiple sclerosis (RRMS) group.

In order to evaluate the diagnostic performance of mtDNA-CN in the RRMS patients' group during remission and the control group, the ROC curve analysis was used as shown in Table 9 and Figure 5. The cutoff that discriminates between RRMS patients versus control group was 0.75, at which diagnostic sensitivity was 56%, 84%

specificity, negative predictive value (NPV) 65.6%, positive predictive value (PPV) 77.8%, diagnostic efficiency 70% and at area under the curve (AUC) of 0.598, suggesting that patients with mtDNA-CN values equal to or below the cutoff of 0.75 are more likely to be RRMS patients.

Table 9. Diagnostic performance of mitochondrial DNA copy number (mtDNA-CN) in remission in relapsing remitting multiple sclerosis (RRMS) patients' group versus the control group.

Parameter	Cutoff	Diagnostic Sensitivity (%)	Diagnostic Specificity (%)	NPV (%)	PPV (%)	Diagnostic Efficiency (%)	AUC
mtDNA CN in Remission	≤ 0.75	56	84	65.6	77.8	70	0.598

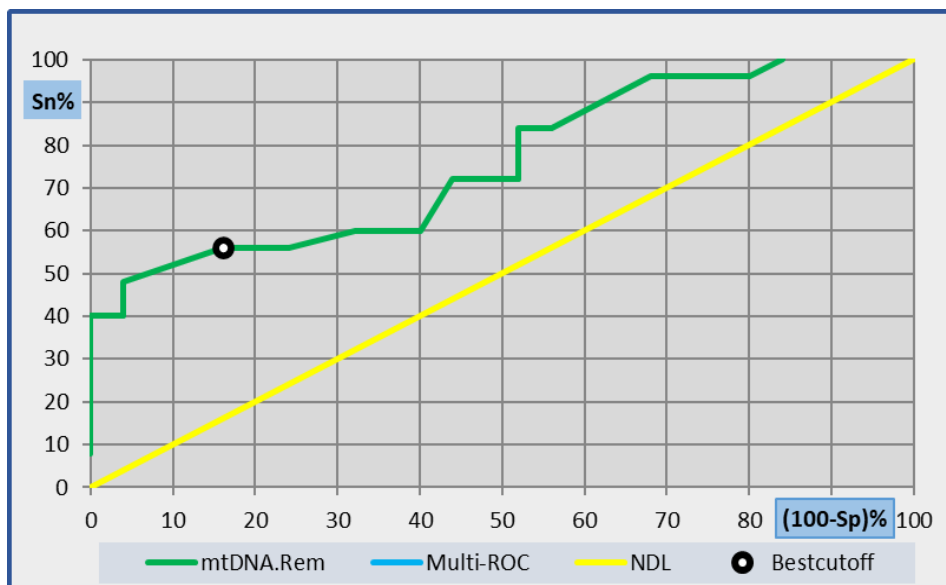


Figure 5. Receiver Operating Characteristic (ROC) curve analysis showing the diagnostic performance of mitochondrial DNA copy number (mtDNA-CN) in remission versus control.

In order to assess the effectiveness of mtDNA-CN as a diagnostic indicator in the group of RRMS patients, the ROC curve analysis was used during remission and during relapse phases as shown in Table 10 and Figure 6. The cutoff that discriminates between both remission and

relapse phase was 1.0, at which diagnostic sensitivity was 72%, 56% specificity, NPV 66.7%, PPV 62.1%, and diagnostic efficiency 64% at area under the curve (AUC) of 0.643, suggesting that mtDNA-CN values were elevated in relapse phase at the cut off value of one.

Table 10. Diagnostic performance of mitochondrial DNA copy number (mtDNA-CN) in relapsing remitting multiple sclerosis (RRMS) patients' group during remission phase versus relapse phase.

Parameter	Cutoff	Diagnostic Sensitivity (%)	Diagnostic Specificity (%)	NPV (%)	PPV (%)	Diagnostic Efficiency (%)	AUC
mtDNA-CN	≥ 1	72	56	66.7	62.1	64	0.643

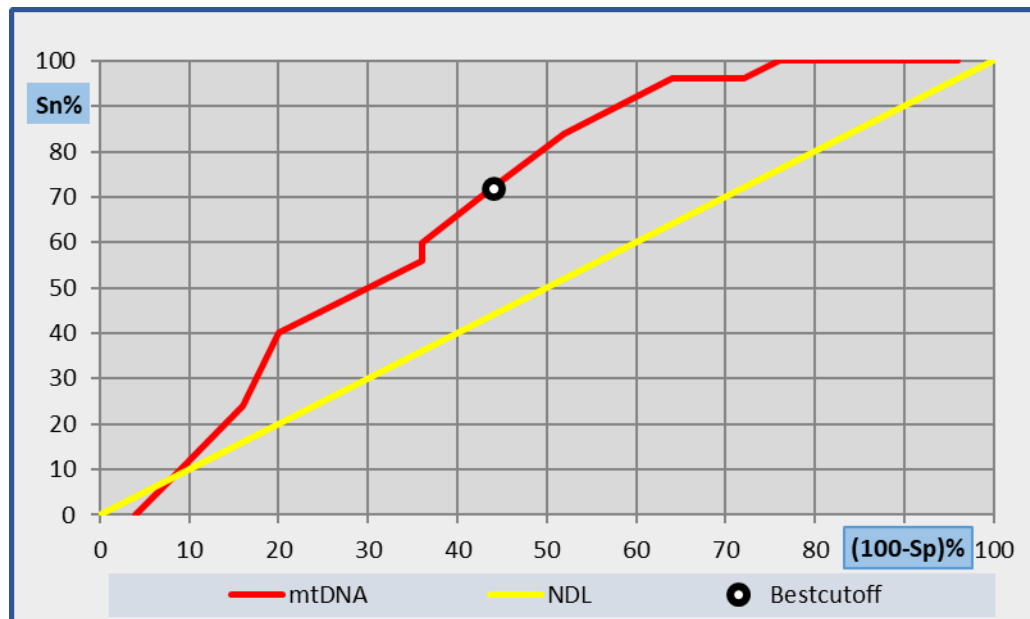


Figure 6. Receiver Operating Characteristic (ROC) curve analysis showing the diagnostic performance of mitochondrial DNA copy number (mtDNA-CN) in RRMS patients for discriminating between remission phase versus relapse phase.

Discussion

The current study aimed to assess peripheral blood mtDNA-CN in relapsing remitting MS subjects and control subjects to evaluate its rule as a biomarker of MS, and to assess its prognostic value regarding disease duration and its progression.

This study included 25 patients complaining from relapsing remitting MS and 25 sex and age matched apparently healthy controls who have no history of neurological or autoimmune diseases. Patients with RRMS were identified using McDonald's 2017 criteria.¹⁶ Each patient was evaluated twice: once during the remission phase and again during the relapse phase.

The results of our study showed that, when RRMS patients were in remission, their copy number of mtDNA in peripheral blood decreased significantly more than it did in the control group. In contrast to the controls, RRMS patients who were in remission had a median mtDNA-CN value that was about 24% lower. When Al-Kafaji et al., 2020, measured the mtDNA-CN in peripheral blood of 60 RRMS patients, they found that it was significantly lower in the RRMS patients than in the healthy

controls.¹⁹ In comparison to healthy subjects, the mean value of mtDNA-CN was about 19% lower in RRMS patients.

Different studies revealed controversial findings as regards mtDNA-CN in neurodegenerative diseases including MS. Lowes et al., 2019, reported decreased mtDNA mean value in cerebral spinal fluid (CSF) of postmortem individuals with progressive multiple sclerosis 15% lower than matched controls.²⁰ Also, it was reported that mtDNA content of the brain tissues of MS patients reduced and depleted²¹. Both Alzheimer's disease²² and Parkinson's disease²³ were linked to decreased levels of mtDNA, and it was hypothesized that this might be a regular finding in neurodegenerative diseases.²⁰ These findings suggested that MS and other neurodegenerative diseases may share a common risk factor for low mtDNA-CN. Finally, we observed similar decreases in mtDNA-CN, which may indicate that impaired mitochondrial function is directly responsible for the decreased copy number of mtDNA in RRMS patients' peripheral blood.

On other hand, elevated mtDNA levels were found in the CSF of MS patients²⁴⁻²⁶ by Varhaug et al., 2017, Leurs et al., 2018 and, Fissolo et al., 2019. The authors hypothesized that the mtDNA-CN could be a sign of an early disease activity and reflect early, active inflammation.²⁴

In the context of these findings, we assessed mtDNA-CN in peripheral blood during relapse phase, and our results revealed that mtDNA-CN increased during relapse phase and achieved statistically significant difference in comparison to remission phase mtDNA-CN values. In comparison with normal controls, mtDNA-CN values were higher in RRMS subjects in relapse but did not reach statistical significance.

The previous studies²⁴⁻²⁶ explained that elevated mtDNA-CN can be a consequence of cellular and mitochondrial stress because of persistent neuro-axonal damage that develops during relapse and is well known to be more severe in progressive forms of MS.²⁷ Although maintaining normal mitochondrial function requires mtDNA, which encodes essential components of the electron transport chain, mtDNA damage is more severe and lasts longer than damage to nuclear DNA under oxidative stress.²⁸ A compensatory response mechanism by increasing the number of copies of the mtDNA may be triggered by such mtDNA damage in order to safeguard the mitochondrial genome and maintain normal function.²⁹ Demyelination raises axonal energy demand, which could compound the neurodegenerative effects of MS and explain why mtDNA levels are higher in MS.^{25,27}

Keeping in mind that greater mtDNA damage leads to lower mtDNA-CN, loss of mitochondrial genomic integrity, and ultimately mitochondrial dysfunction,³⁰ which is the hallmark of neurodegenerative disorders.³¹ Moreover, mtDNA and mitochondrial damage result in energy failure, which increases neuronal inflammation and demyelination. Degeneration of the neurons results from this in turn.³² In the acute phases (relapse) of RRMS, elevated mtDNA-CN is therefore seen during acute inflammation, which predicts the neurodegenerative process. Furthermore, an increase in inflammatory cells in the afflicted areas is anticipated to contribute to mtDNA

release into the CSF and an increase in copy number in the CSF and plasma.³³

On the other hand, decreased mtDNA-CN is accompanied by advancing cell dysfunction in neurodegenerative diseases like multiple sclerosis. This suggests that altered neuronal mtDNA levels (impaired mtDNA replication with degradation, and depletion) at the beginning of neurodegeneration result in reduced mtDNA release before actual cell death happens.^{11,33}

Correlation analysis was done in an attempt to evaluate the effect EDSS on mtDNA-CN. In our study, we noticed a negative correlation between EDSS and mtDNA-CN in remission and relapse patients; however, this negative correlation did not achieve a statistical significance. In this context, Armon-amer et al., 2020, showed significant difference between EDSS and mitochondrial dysfunction. By assessment of mitochondrial activity, the study revealed lower mitochondrial activity levels with higher disability (higher EDSS).³⁴ On other side, Leurs et al., 2018, revealed non-significant correlation between EDSS and mtDNA-CN.²⁵ In our study, EDSS in patients with disease duration more than 6 years was higher than in patients with disease duration less than 6 years, but this difference also did not achieve a statistical significance.

Varhaug et al., 2017, reported inversely statistically significant correlation between mtDNA-CN and the length of time since the onset of the disease,²⁴ Al-Kafaji et al., 2020, demonstrated that RRMS patients with illnesses lasting longer than 10 years had significantly lower mtDNA-CN levels than RRMS patients with illnesses lasting less than 10 years¹⁹. These results might shed light on how low mtDNA-CN and disease progression are related.

Damage to myelin starts from the first days of disease³⁵ and over time, neurodegeneration worsens. Repetitive relapse/remission attacks may promote irreversible axonal injury after years in RRMS patients, where the disability steadily deteriorates.³⁶ Based on this data, we stratified RRMS patients into two groups according to disease duration to more than or less than 6 years. In the remission and relapse phases, there was no discernible difference in mtDNA-CN between RRMS patients with

disease duration less than 6 years and more than 6 years.

In our study, Spearman's rank correlation was performed between mtDNA-CN in remission and relapse and showed significant positive correlation. Also, the ROC curve analysis was applied to discriminate between mtDNA-CN of RRMS patients in remission and their mtDNA-CN in relapse, at a cut off value of one AUC was 0.643 with (95% CI 0.522– 0.764).

By using the ROC curve analysis, our results showed that at a cut off value of 0.75, mtDNA-CN discriminated between RRMS patients in remission phase and normal controls with a sensitivity of 56%, specificity 84%, NPV 65.6% and PPV 77.8%. The (AUC) was 0.598 (95% CI: 0.469-0.726). Al-Kafaji et al., 2020, also reported that mtDNA-CN can distinguish between RRMS patients and healthy controls. Although the authors regrettably did not provide a cutoff value, the AUC of peripheral blood mtDNA-CN for differentiating between RRMS patients in remission and controls was 0.882 (95% CI: 0.826-0.938).¹⁹

In conclusion, the present study demonstrated that mtDNA-CN reduction in peripheral blood from RRMS patients may be a significant event in the pathogenicity of MS. These finding suggested that the number of copies of circulating mtDNA in peripheral blood may be a noninvasive biomarker for MS.

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Author Contributions

MMZ: designed and approved the whole research protocol and amended the final paper version to be published. HMAZ: contributed to the protocol design, revised laboratory work, and revised the manuscript draft version to be published. SSK: supervised sample collection according to inclusion criteria, revised clinical data, diagnosis, and patient classification. MEM and SHH: monitored data collection process and the laboratory work,

interpreted the data, and provided clinical support. RMS: collected the samples and patient's clinical data, carried out the laboratory work and statistical analysis of the results, and wrote the manuscript draft. The manuscript was read and approved by all authors.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The protocol of the study was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Ain Shams University (reference number: FMASU MD 242/2020).

Informed consent

All study participants provided written informed consent before included in the research.

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