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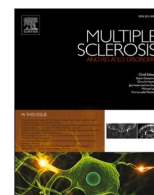

Cerebrospinal fluid mtDNA concentrations are increased in multiple sclerosis and were normalized after intervention with autologous hematopoietic stem cell transplantation

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ABSTRACT

Background: Mitochondrial DNA (mtDNA) is a pro-inflammatory damage-associated molecular pattern molecule and could be an early indicator for inflammation and disease activity in MS. Autologous hematopoietic stem cell transplantation (aHSCT) is a potent treatment for MS, but its impact on mtDNA levels in cerebrospinal fluid (CSF) remains unexplored.
Objectives: To verify elevated CSF mtDNA concentrations in MS patients and assess the impact of aHSCT on mtDNA concentrations.
Methods: Multiplex droplet digital PCR (ddPCR) was used to quantify mtDNA and nuclear DNA in 182 CSF samples. These samples were collected from 48 MS patients, both pre- and post-aHSCT, over annual follow-ups, and from 32 healthy controls.
Results: CSF ccf-mtDNA levels were higher in patients with MS, correlated to multiple clinical and analytical factors and were normalized after intervention with aHSCT. Differences before aHSCT were observed with regard to MRI-lesions, prior treatment and number of relapses in the last year prior to aHSCT.
Conclusion: Our findings demonstrate elevated CSF mtDNA levels in MS patients, which correlate with disease activity and normalize following aHSCT. These results position mtDNA as a potential biomarker for monitoring inflammatory activity and response to treatment in MS.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease marked by inflammation and neurodegeneration in the central nervous system (CNS). Initially, the disease is driven mainly by inflammation, but over time, neurodegenerative processes take precedence (Dendrou et al., 2015). While several proteins have been investigated as potential biomarkers of disease activity and treatment response with varying reliability and reproducibility (Yang et al., 2022), few studies have explored circulating nucleic acids in blood and CSF as biomarkers for MS.

Mitochondrial (mt-) DNA is a circular, double-stranded molecule carrying 37 genes, of which 13 encode proteins involved in the energy metabolism of the eukaryotic cell. Unlike nuclear (*n*-) DNA, mtDNA is not protected by histones and is, due to the highly oxidative nature of the mitochondria, subjugated to a much higher rate of oxidative stress (Gambardella et al., 2019). Furthermore, structural similarities between the CpG-rich human mtDNA and the structure of bacterial DNA additionally promotes the recognition of human circulating cell-free (ccf-) mtDNA as a danger-associated molecular pattern (DAMP) by the immune system (Zhang et al., 2010). Multiple cells of the immune system have been described to release mtDNA with various functions. mtDNA webs released from innate immune cells contain antimicrobial peptides within the webs (Brinkmann et al., 2004; Yousefi et al., 2008). Lymphocyte released mtDNA webs are devoid of antimicrobial peptides and have been suggested to have a signalling function, acting as both para- and autocrine pro-inflammatory stimulants (Costanza et al., 2019;

Abbreviations: mtDNA, mitochondrial DNA; nDNA, nuclear DNA; ccf, circulating cell free; aHSCT, autologous hematopoietic stem cell transplantation; ddPCR, droplet digital polymerase chain reaction; EIDA, evidence of inflammatory disease activity.

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Ingelsson et al., 2018). Increased concentrations of ccf-mtDNA in the CSF have been suggested to be associated with inflammation (Gambardella et al., 2019; Varhaug et al., 2017). In a small cohort of people with relapsing-remitting (RR) MS, CSF ccf-mtDNA was decreased by 50 % after initiation of lymphocyte-targeting disease-modifying treatment (DMT) with fingolimod (Leurs et al., 2018).

Autologous hematopoietic stem cell transplantation (aHSCT) is a treatment intervention that has been in use for MS since the mid 1990s (Fassas et al., 1997). The purpose of the treatment is to ablate the immune system with high-dose chemotherapy and then rebuild a novel immune system with the aid of autologous hematopoietic stem cells (Muraro et al., 2017). Recently it was reported that 63 % of patients treated with aHSCT in Sweden maintained a state of no evidence of disease activity (NEDA) 10 years following aHSCT, requiring no additional treatments (Silfverberg et al., 2023).

This study had two aims. First to confirm previous reports of increased CSF ccf-mtDNA concentrations in people with MS. Second, to investigate if CSF ccf-mtDNA concentrations were affected by intervention with aHSCT.

2. Subjects and methods

2.1. Ethical approval

The study was approved by the Regional Ethical Board of Uppsala (Dnr 2008/182 and 2012/080/1). All participants provided informed and written consent in accordance with the Declaration of Helsinki.

2.2. Patients and controls

All patients diagnosed with relapsing-remitting (RR) MS according to the revised 2017 McDonald criteria (Thompson et al., 2018) planned for intervention with aHSCT using a cyclophosphamide based conditioning regimen at Uppsala University Hospital from December 2011 to February 2020 were invited to participate in the study (Fig. 1). Fifty, out of the total 77, patients volunteered to participate and undergo lumbar puncture. Two patients were excluded from analysis, when their samples failed the quality control (QC).

The remaining 48 patients, mean age 31 (SD 6.7), were included in the study along with 32 healthy controls (HC), mean age 25 (SD 7.2).

Patients were divided based on previous treatments: *1st line, 2nd line*

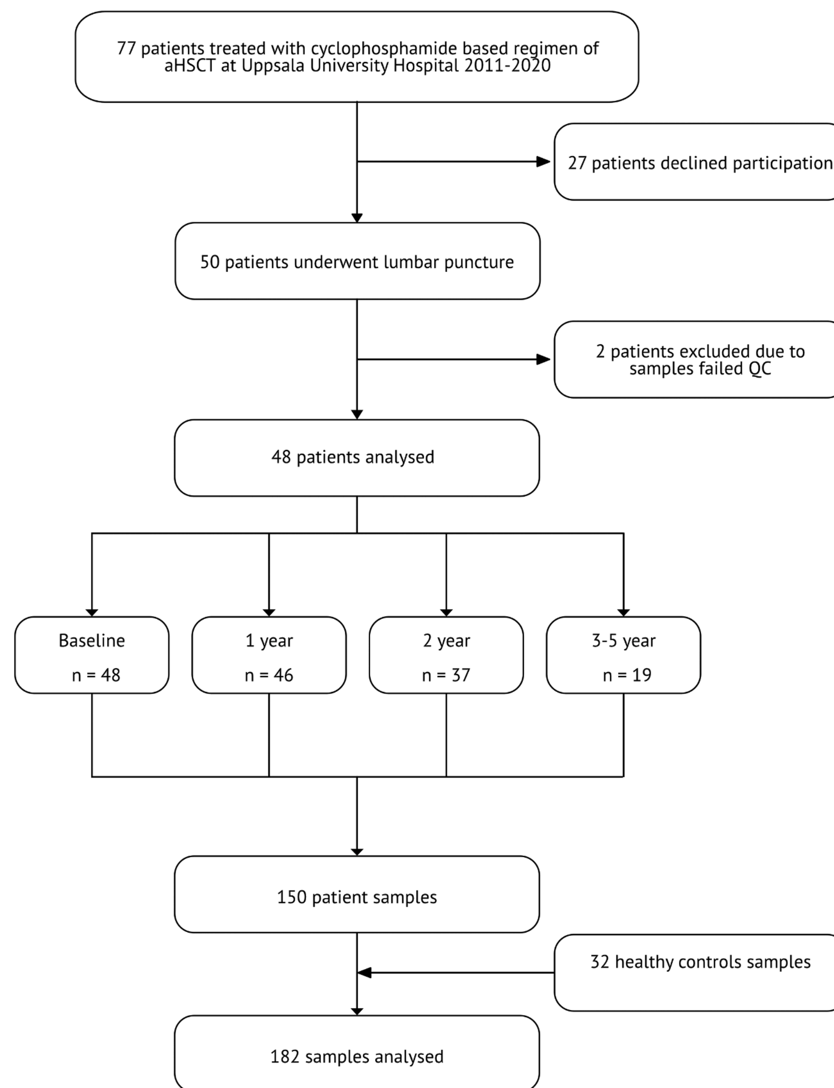

Fig. 1. Patient inclusion chart. All patients diagnosed with RRMS according to the revised 2017 McDonald criteria planned for intervention with aHSCT using a cyclophosphamide based conditioning regimen at Uppsala University Hospital from December 2011 to February 2020 were invited to participate in the study. Fifty, out of the total 77, patients volunteered to participate and undergo lumbar puncture. Two patients were later excluded from analysis, when their samples failed the quality control (QC).

and *treatment-naïve*. Treatments considered as 1st line treatments were dimethyl fumarate, glatiramer acetate, interferon and teriflunomide; while the 2nd line treatments consisted of fingolimod, rituximab and natalizumab. (Table 1).

NEDA-2 was defined as absence of new MRI events and clinical relapses, while patients not maintaining NEDA-2 after aHSCT were considered to have evidence of inflammatory disease activity (EIDA).

2.3. CSF collection

Patients were offered to undergo lumbar puncture before aHSCT and thereafter at 1, 2 and 5 years post-aHSCT. If, for some reason (e.g., pregnancy), patients were unable to undergo lumbar puncture they were asked to undergo lumbar puncture at a later follow-up visit instead. Some patients underwent extra lumbar punctures to assess disease status in the case of suspected relapse. Healthy controls donated CSF at a single timepoint. All CSF samples were handled according to consensus guidelines (Teunissen et al., 2009).

2.4. Procedures

Autologous hematopoietic stem cells were mobilized with a single dose of 2 g/m^2 cyclophosphamide followed by filgrastim 5–10 μg/kg/day for 6–7 days and then harvested approximately 10 days after the start of the mobilization regimen. No *ex-vivo* graft manipulation was performed. Patients were conditioned with a combination of cyclophosphamide and rabbit anti-thymocyte globulin (cyclophosphamide 200 mg/kg; rATG 6 mg/kg). Prophylaxis for fungal, viral and bacterial infection was administered during neutropenia. Prophylaxis for herpes viruses and *Pneumocystis jiroveci* continued for a minimum of 3 months.

2.5. Analysis of ccf-mtDNA

Concentrations of ccf-mtDNA were determined in 4.5 μL of CSF with a droplet digital polymerase chain reaction (ddPCR) in a QX200 platform (Bio-Rad, Hercules, CA, USA) as previously described by Podlesniy et al. (Podlesniy and Trullas, 2018) The primers and hydrolysis probe sequences for mtDNA and nDNA can be found in the supplementary text.

The ddPCR multiplex assay, allowing simultaneous detection of mt- and nDNA, was performed in 20 μL reactions. If nDNA concentration exceeded 2 copies/μL, the sample along with any related follow-up samples were excluded due to cellular contamination.

Table 1
Demographics and clinical characteristics of the cohort.

			MS	Healthy control
N			*(n = 48)*	*(n = 32)*
Age at inclusion		Mean (SD)	31 (6.7)	25 (7.2)
Sex		F/M (% women)	32/16 (67)	15/17 (47)
EDSS		Median [IQR]		
	Baseline		4.0 [2.0–4.0]	NA
	1 year		2.0 [1.0–3.0]	NA
	2 years		2.0 [1.0–3.0]	NA
	3–5 years		2.0 [0.0–3.0]	NA
Annual Relapse Rate		Mean (SD)	1.4 (1.5)	NA
Disease duration (years)		Mean (SD)	6.1 (5.7)	NA
Number of previous treatments		Median [IQR]	2.0 [1.0–3.0]	NA
All treatments				
	Naïve		9	NA
	1st Line		12	NA
	2nd Line		27	NA
NEDA-2/EIDA			39/9	NA

2.6. Statistical analysis

Statistical analyses were performed using R version 4.2.3 (R Development Core Team, 2010). D'Agostino's normality test was used to assess distributions of variables. Data were log-transformed in order to reduce skewness. Student's *t*-test was used when assessing differences in biomarker concentrations between two groups. Welch's *t*-test was used when comparing groups with unequal variances. To assess concentration differences compared to baseline, mixed multilevel linear regression models were fitted with sex and age as covariates using the lme4 package. Student's *t*-tests with Bonferroni correction were used to assess differences between different treatment groups. Spearman's ranked correlation analysis in GraphPad Prism version 9.5.0 for macOS (GraphPad Software, http://www.graphpad.com) was used to determine correlations between non-transformed ccf-mtDNA concentrations and various clinical and analytical factors. Correlations were classified according to the British Medical Journal guidelines (Wechsler, 1997). A two-tailed *p*-value < 0.05 was considered significant. Figures were made in GraphPad Prism version 9.5.0 for macOS and graphically processed in Affinity Designer (v.1.10.5, Serif (Europe) LTD., https://www.affinity.serif.com).

3. Results

CSF samples from 50 patients with RRMS and 32 HC were analyzed with a multiplex ddPCR assay. Two baseline samples breached the cut-off ($\geq$ 2 copies/μL of nDNA) and were therefore excluded from further analyses along with any follow-up samples from the same patients. This resulted in a total of 182 samples being analyzed (Fig. 1).

3.1. Age and sex did not influence ccf-mtDNA concentrations

A multiple linear regression model was built to assess the effects of age and sex, however, neither age ($p = 0.60$) nor sex ($p = 0.41$) affected ccf-mtDNA concentrations in this model.

3.2. Characterization of CSF samples

Some basic analyses of the blood and CSF were conducted as part of health care (Table 2). CSF cell counts and CSF albumin were also quantified in HC and were within normal limits.

3.3. CSF ccf-mtDNA in multiple sclerosis compared to healthy controls

Median ccf-mtDNA concentrations were significantly higher in RRMS patients (16 copies/μL [IQR 5.0 – 41]) compared with HC (5.6 copies/μL [3.4 - 8.9]) ($p < 0.0001$) (Fig. 2).

3.4. ccf-mtDNA concentrations and clinical factors

Correlations between CSF ccf-mtDNA concentrations and clinical variables were first assessed in samples obtained before aHSCT. At this timepoint, CSF ccf-mtDNA correlated moderately with the number of relapses in the year prior to intervention with aHSCT ($r = 0.54$, $p < 0.0001$) and weakly to the expanded disability status scale (EDSS) ($r = 0.34$, $p < 0.05$). Concentrations of ccf-mtDNA were negatively correlated with disease duration ($r = -0.35$, $p < 0.05$). Thereafter a similar analysis was made, analyzing samples obtained after aHSCT, were only disease duration correlated negatively to ccf-mtDNA 1 year after aHSCT ($r = -0.29$, $p < 0.05$).

Table 2
Characterization of the CSF samples of the cohort.

Factor	*Unit*		HC (*n* = 32)	**Baseline**	*n*	**1 year**	*n*	**2 year**	*n*	**3–5 year**	*n*
CSF-Mononuclear cells	million cells/L	Median [IQR]	<5.0	2.0 [1.0–5.3]	*n* = 42	1.0 [0.0–2.0]	*n* = 40	1.0 [0.0–2.0]	*n* = 30	1.0 [0.0–3.0]	*n* = 11
CSf-Polynuclear cells	million cells/L	Median [IQR]	<1.0	0.0 [0.0–0.0]	*n* = 42	0.0 [0.0–0.0]	*n* = 40	0.0 [0.0–0.0]	*n* = 30	0.0 [0.0–0.0]	*n* = 11
CSF-Albumin	mg/L	Median [IQR]	<320	220 [150–310]	*n* = 42	200 [150–290]	*n* = 40	220 [160–310]	*n* = 30	260 [160–320]	*n* = 11
Albumin quota	CSF/S	Median [IQR]	NA	4.9 [3.7–7.6]	*n* = 42	4.7 [3.3–6.4]	*n* = 40	5.0 [3.2–8.5]	*n* = 30	6.1 [4.5 - 7.3]	*n* = 11
P-IgG	g/L	Median [IQR]	NA	9.2 [8.0–11]	*n* = 42	9.2 [7.8–11]	*n* = 40	9.2 [7.6–10]	*n* = 30	9.8 [8.0–11]	*n* = 11
CSF-IgG	mg/L	Median [IQR]	NA	38 [26–61]	*n* = 42	29 [21–40]	*n* = 40	28 [19–47]	*n* = 30	34 [16–40]	*n* = 11
IgG index		Median [IQR]	NA	0.7 [0.6–0.9]	*n* = 42	0.6 [0.5–0.8]	*n* = 40	0.6 [0.5–0.7]	*n* = 30	0.5 [0.4–0.6]	*n* = 11
CSF-IgG OCB	Yes/No	Median [IQR]	NA	39/3	*n* = 42	34/5	*n* = 39	20/2	*n* = 22	6/5	*n* = 11
CSF-NfL	ng/L	Median [IQR]	NA	840 [380–3000]	*n* = 42	430 [290–580]	*n* = 39	360 [230–550]	*n* = 30	350 [290–440]	*n* = 11
S-IgM	g/L	Median [IQR]	NA	0.8 [0.6–1.5]	*n* = 38	0.8 [0.5–1.1]	*n* = 36	0.7 [0.5–1.1]	*n* = 21	0.7 [0.5–0.8]	*n* = 11
CSF-IgM	mg/L	Median [IQR]	NA	0.7 [0.2–1.2]	*n* = 38	0.3 [0.1–0.6]	*n* = 36	0.2 [0.1–0.4]	*n* = 21	0.2 [0.1–0.3]	*n* = 11
IgM index		Median [IQR]	NA	0.1 [0.07–0.2]	*n* = 38	0.07 [0.05–0.15]	*n* = 36	0.06 [0.05–0.11]	*n* = 21	0.06 [0.05–0.08]	*n* = 11
CSF-IgM OCB	Yes/No	Median [IQR]	NA	7/31	*n* = 39	2/36	*n* = 38	2/18	*n* = 20	0/10	*n* = 10

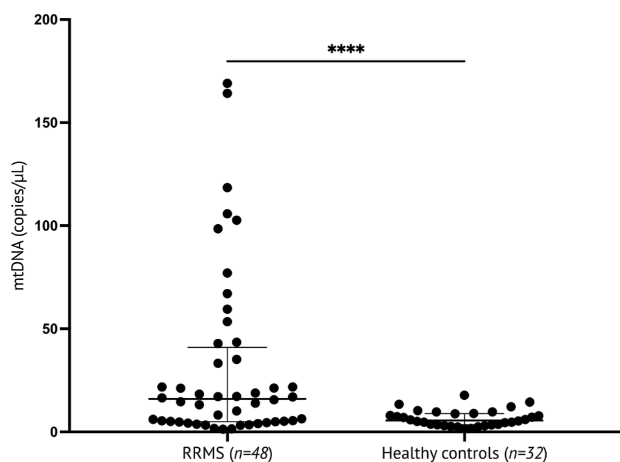

Fig. 2. CSF ccf-mtDNA concentrations in patients with relapsing-remitting MS compared with healthy controls. Ccf-mtDNA levels were quantified with ddPCR in CSF from patients with RRMS (before aHSCT) and healthy controls. The data was log-transformed to reduce skewness and then Student's *t*-test was used to establish statistically significant difference between the groups. Each dot represents a separate sample and the error bars mark the (non-transformed) median concentration with interquartile range. $p < 0.0001$ (****).

3.5. ccf-mtDNA concentrations and multiple routine CSF analyses

Correlations between CSF ccf-mtDNA concentrations and multiple routine CSF analyses were initially assessed in samples obtained before aHSCT. A strong correlation was observed between CSF ccf-mtDNA and NfL concentrations at baseline ($r = 0.62$, $p < 0.0001$). Correlations of moderate strength were observed between baseline ccf-mtDNA concentrations and mononuclear cell concentrations ($r = 0.55$, $p < 0.0001$), albumin concentration ($r = 0.40$, $p < 0.01$), IgG- ($r = 0.56$, $p < 0.0001$) and IgM concentrations ($r = 0.55$, $p < 0.001$) in CSF, as well as to the IgM index ($r = 0.41$, $p < 0.05$).

Furthermore, correlation analyses were performed between the routine analyses and CSF ccf-mtDNA concentrations in samples obtained during yearly follow-ups after aHSCT. At 1 year after aHSCT, ccf-mtDNA concentrations in the CSF correlated moderately to IgG ($r = 0.44$, $p < 0.01$) and IgM concentrations ($r = 0.41$, $p < 0.05$) and weakly to mononuclear cell concentrations ($r = 0.39$, $p < 0.05$). However, no statistically significant correlations were discerned in samples from the later follow-ups (Table 3).

3.6. CSF ccf-mtDNA concentrations and gadolinium-enhancing lesions at baseline

Patients with gadolinium-enhancing lesions at baseline ($n = 17$) exhibited significantly higher concentrations, median 35 copies/μL [IQR 12 - 90], of CSF ccf-mtDNA compared to patients without enhancing lesions ($n = 31$), median 10 copies/μL [4.3 - 21] ($p < 0.01$). See Fig. 3.

The number of gadolinium-enhancing lesions at baseline did not correlate to ccf-mtDNA concentrations ($r = 0.16$, $p = 0.53$).

3.7. CSF ccf-mtDNA concentrations and DMTs

To investigate if baseline levels of ccf-mtDNA were influenced by DMTs, the patients were divided into three groups based on their last treatment prior to intervention with aHSCT. Patients receiving 2nd line treatment ($n = 27$) had significantly lower ccf-mtDNA concentrations at baseline, median 5.4 copies/μL [IQR 4.1 - 17], compared with both the 1st line treated ($n = 12$), median 39 copies/μL [18 - 75] ($p < 0.001$) and treatment-naïve patients ($n = 9$), median 21 copies/μL [12 - 102] ($p < 0.01$). No significant differences were observed between the 1st line-treated group and the treatment-naïve patients (Fig. 4.).

3.8. CSF ccf-mtDNA concentrations after aHSCT

The median concentration of CSF ccf-mtDNA at baseline was 16 copies/μL [IQR 5.0 – 41]. After aHSCT, the median concentration decreased, approaching the level of HC (Fig. 5). At the 1-year follow-up, the median concentration was 5.9 copies/μL [3.5 – 12] ($p < 0.0001$), at the 2-year follow-up 8.0 copies/μL [3.0 – 16] ($p < 0.0001$) and in the

Table 3
Correlation analyses of ccf-mtDNA concentrations and clinical- and analytical factors.

Analysis	Baseline		1 year		2 year		3–5 year	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
EDSS	**0.34**	**0.0168**	0.07	0.6495	0.00503	0.9764	0.21	0.3964
Disease Duration	**−0.35**	**0.0160**	**−0.29**	**0.048**	−0.18	0.2917	0.08	0.7398
Number of Relapses last year prior to aHSCT	**0.54**	**<0.0001**	NA	NA	NA	NA	NA	NA
Number of Gd+ lesions last year prior to aHSCT	0.16	0.5296	NA	NA	NA	NA	NA	NA
Mononuclear cells	**0.55**	**0.0001**	**0.39**	**0.014**	0.1303	0.4925	0.075	0.8341
Polynuclear cells	0.005	0.9739	0.29	0.0616	0.2832	0.1294	−0.60	0.0727
Albumin	**0.40**	**0.0091**	0.29	0.0745	0.22	0.2426	0.42	0.203
Albumin quota	0.28	0.0693	0.22	0.1759	0.2031	0.2819	0.20	0.5517
P-IgG	0.16	0.3139	0.25	0.1153	−0.1659	0.381	0.36	0.2731
CSF-IgG	**0.56**	**0.0001**	**0.44**	**0.0045**	0.3382	0.0676	0.33	0.3211
IgG index	0.29	0.0604	0.06	0.6939	0.2308	0.2198	0.17	0.6175
IgG OCB	0.13	0.4271	−0.007	0.9672	0.08133	0.6749	0.35	0.329
S-IgM	0.19	0.2585	−0.006	0.9744	−0.003698	0.9848	0.26	0.4348
CSF-IgM	**0.55**	**0.0003**	**0.41**	**0.0121**	0.01011	0.9585	0.35	0.2862
IgM index	**0.41**	**0.0103**	0.12	0.4718	−0.09639	0.6189	−0.077	0.8228
IgM OCB	0.27	0.1002	−0.27	0.1029	0.03434	0.8623	NA	NA
CSF-NfL	**0.62**	**<0.0001**	0.17	0.2811	−0.1423	0.4531	−0.036	0.9241

Both clinical and analytical factors routinely used in clinical practice were analyzed with Spearman's rank correlation coefficient for associations to concentrations of CSF ccf-mtDNA. Factors with associations of statistical significance are shown in **bold text** with the *r*- and *p*-values for each timepoint. The *r*- and *p*-values are shown in **bold text** at the timepoint where statistical significance was reached.

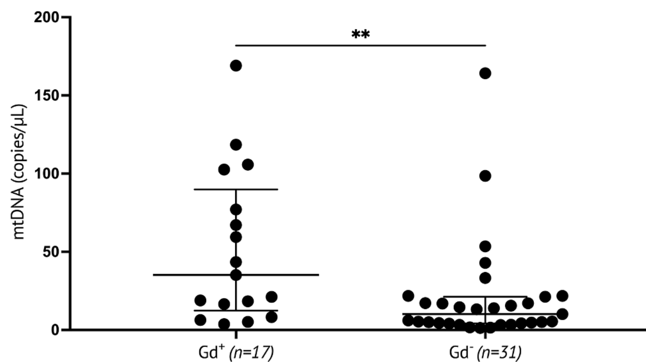

Fig. 3. CSF ccf-mtDNA in patients with and without gadolinium-enhancing lesions. Patients with gadolinium-enhancing lesions at baseline (Gd^+) had higher concentrations of CSF ccf-mtDNA compared to patients with no lesions (Gd^-). The data was log-transformed to reduce skewness and then Welch's *t*-test was used to establish statistically significant difference between the groups. Each dot represents a separate sample and the error bars mark the (non-transformed) median concentration with interquartile range. $p < 0.01$(**).

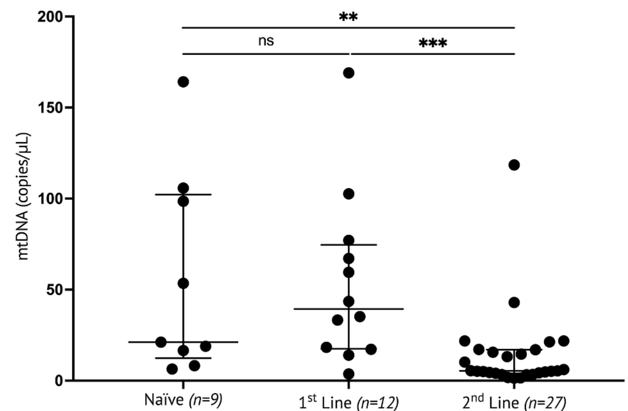

Fig. 4. CSF ccf-mtDNA concentrations by DMT class at baseline. Patients were divided into three groups based on their last treatment prior to intervention with aHSCT: treatment-naïve patients and patients treated with either 1st- or 2nd line treatment. The data was log-transformed to reduce skewness and then multiple *t*-tests with Bonferroni correction were used to investigate differences in CSF ccf-mtDNA concentrations based on DMT class. Each dot represents a separate sample and the error bars mark the (non-transformed) median concentration with interquartile range. $p < 0.01$ (**), <0.001 (***).

combined 3–5-years follow-up group the median concentration was 7.2 copies/µL [4.8 – 11] ($p < 0.05$). No differences were observed between HC and either of the follow-up timepoints.

3.9. CSF ccf-mtDNA concentrations after aHSCT and previous DMTs

To investigate the impact of previous DMT on CSF ccf-mtDNA concentrations after aHSCT, paired non-parametric analyses were performed between the baseline samples and each patient's first available follow-up sample (Supplementary Figure 1).

The median ccf-mtDNA concentration for patients treated with 1st line DMTs before aHSCT was 39 copies/µL [18 – 75) and decreased to 6.0 copies/µL [3.5 – 9.0] ($p < 0.001$). The treatment-naïve group had a median concentration of 21 copies/µL [12 – 102], which decreased to 6.6 copies/µL [5.1 – 31] ($p < 0.05$). Despite low median ccf-mtDNA concentrations at baseline (5.4 copies/µL [4.1 – 17]), a significant decrease, to 5.1 copies/µL [3.2 – 11], was observed in the 2nd line treated group as well ($p < 0.05$).

3.10. CSF ccf-mtDNA concentrations and evidence of inflammatory disease activity after aHSCT

Patients who had EIDA ($n = 9$) were compared with patients who maintained NEDA-2 ($n = 39$). At some timepoints, but not all, patients with EIDA had higher concentrations compared to patients who maintained NEDA-2 (Fig. 5). At 1 year after aHSCT, the median concentrations were 10 copies/µL [IQR 7.7 – 19] for patients with EIDA and 5.2 copies/µL [3.3 – 10] for patients remaining in NEDA-2 ($p < 0.05$). At 2 years after aHSCT, the median concentration was 18 copies/µL [6.2 – 29] for patients with EIDA and 7.1 copies/µL [2.3 – 13] for patients remaining in NEDA-2 ($p < 0.05$). No differences in ccf-mtDNA concentrations were observed at baseline or 3–5 year after aHSCT (Supplementary Fig. 2).

4. Discussion

Two key findings of this study are that CSF ccf-mtDNA

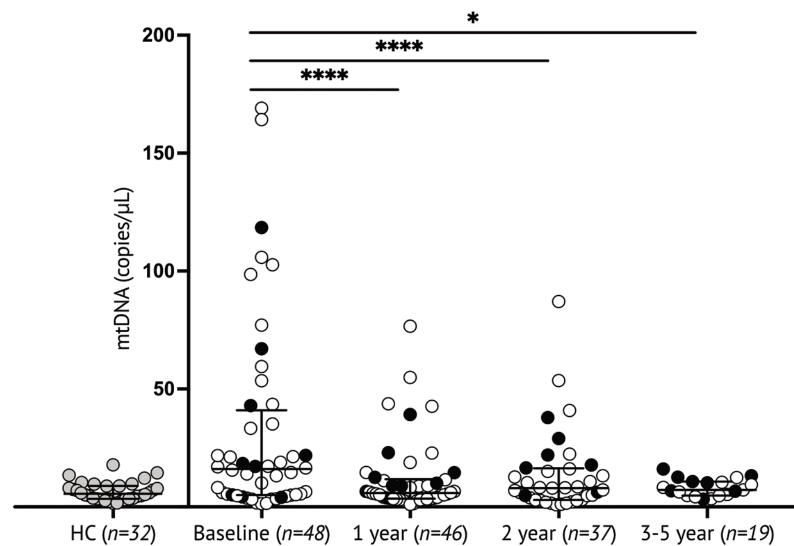

Fig. 5. CSF ccf-mtDNA before and after aHSCT. CSF ccf-mtDNA was quantified in 150 patient samples from 48 patients with RRMS collected before (baseline) and at annual follow-up visits after aHSCT, along with 32 samples from healthy controls (HC). Utilizing a mixed multilevel linear regression model, fitted with age and sex as covariates, differences between baseline and the yearly follow-up visits were investigated. The data was log-transformed to reduce skewness. Each dot represents a separate sample and the error bars mark the (non-transformed) median concentration with interquartile range. $p < 0.05$ (*), <0.0001 (****). Data from HC (marked in grey) is included in the figure as a reference. Black dots represent patients ($n = 9$) who, at any point experienced evidence of inflammatory disease activity (EIDA) after aHSCT. White dots represent patients ($n = 39$) who remained in NEDA-2 throughout the follow-up period. Welch's *t*-test was used to establish statistically significant differences between NEDA-2 and EIDA (1 & 2 year: $p < 0.05$; Baseline & 3–5 year: not significant).

concentrations were increased in people with MS compared with healthy controls and that the levels were normalized following intervention with aHSCT. Furthermore, CSF ccf-mtDNA concentrations correlated moderately with various clinical and analytical factors. Therefore, mtDNA could serve as a new biomarker of inflammation that could be used to assess treatment responses.

The precise source of the elevated mitochondrial DNA (mtDNA) measured in this study remains uncertain. A plausible explanation is that the increased ccf-mtDNA concentrations are related to CNS infiltration of autoreactive lymphocytes. Supporting this hypothesis, Costanza et al. reported that $CD4^+$ *T* cells release mtDNA extrusions both in vitro and in vivo in an experimental autoimmune encephalomyelitis model and that these cells were responsive to a mitochondrial reactive oxygen species (mtROS) inhibition. Notably, blocking of mtROS significantly reduced the secretion of pro-inflammatory cytokines (Costanza et al., 2019). Current evidence, including findings from treatments such as fingolimod (Leurs et al., 2018) and aHSCT, indicates a potential lymphocytic origin for the ccf-mtDNA detected in CSF from people with MS. The lymphocytic origin hypothesis is further reinforced by the correlation between mononuclear cell concentrations and CSF ccf-mtDNA concentrations at baseline observed in this study. ccf-mtDNA concentrations were notably higher in CSF from people with MS compared with HC, corroborating earlier findings. The ccf-mtDNA concentrations in CSF from people with MS were consistent with those reported in other studies (Varhaug et al., 2017; Leurs et al., 2018). However, research involving different control groups, such as those with inflammatory or non-inflammatory neurological diseases, did not observe an MS-specific elevation in ccf-mtDNA levels in CSF (Leurs et al., 2018; Fissolo et al., 2019). This suggests that elevated CSF ccf-mtDNA may not be a unique marker for MS, as it is also present in other inflammatory brain disorders. Furthermore, the phenomenon of 'inflammaging' — a term for the chronic low-grade inflammation occurring with age in healthy individuals — might influence ccf-mtDNA levels (Varhaug et al., 2017). One characteristic of inflammaging is the gradual increase of ccf-mtDNA concentrations with increasing age (Pinti et al., 2014). In support of this view, Fissolo et al. (2019) reported higher median ccf-mtDNA concentrations in the HC group compared to our findings, possibly attributable to age differences between the cohorts. Consequently, the presence of higher ccf-mtDNA levels in older individuals with non-inflammatory neurological conditions could explain the lack of observed differences between these control groups and people with MS in some studies. We did not observe an effect of age on ccf-mtDNA, but the healthy controls in our cohort were quite young, with a mean age of 25 years. Inflammaging is neither a uniform nor linear process, so this should come as no surprise (Franceschi et al., 2018). This age-related variance emphasizes the need for careful consideration of control group characteristics when interpreting ccf-mtDNA concentrations in neurological conditions.

Aligning with our own results, the findings by Varhaug et al. (2017) demonstrate that ccf-mtDNA concentrations correlate with disease duration in MS. However, in contrast to previous research results, we observed associations between ccf-mtDNA levels, mononuclear cell counts and NfL concentrations in CSF. Additionally, we found higher CSF ccf-mtDNA concentrations in patients with gadolinium-enhancing lesions, a finding which was not observed previously (Leurs et al., 2018; Fissolo et al., 2019). Subtle differences in the study populations and how their data were handled may have contributed to these discordant findings. E.g. in the referenced studies, differences with regards to presence or absence of gadolinium-enhancing lesions, were assessed in combined groups made up of all disease phenotypes (i.e. relapsing-remitting, secondary progressive and primary progressive), while in our study, all patients had a relapsing-remitting disease course.

The ccf-mtDNA levels decreased after aHSCT and were normalized after 3–5 years. Previously, only one study explored the ccf-mtDNA response to DMT (Leurs et al., 2018). From this small study of 23 patients, it was reported that median ccf-mtDNA concentrations decreased by about 50 % (from 18 to 9.6 copies/μL) within 6–12 months of fingolimod treatment. In contrast, patients in our cohort showed a more prominent decrease of 63 % (from 16 to 5.9 copies/μL) 12 months after aHSCT. Some patients were stable on 2nd line treatment and were shifted to aHSCT for other reasons than breakthrough disease activity (e.g. conversion to JC-virus positive status). Nevertheless, a statistically significant decrease was seen in all groups, regardless of previous treatment, suggesting that aHSCT has an additional effect for patients with 2nd line treatment as well. Only four patients required DMT after aHSCT and nine patients had EIDA after aHSCT. These patients had higher ccf-mtDNA concentrations in samples taken 1- and 2 years after

aHSCT than patients who maintained NEDA-2. This suggests that it may be worthwhile to monitor ccf-mtDNA during follow-up, although this needs to be confirmed in a prospective study.

The primary limitations of this study include a small number of participants and the inconsistent representation of patients at each follow-up timepoint, which further reduced the sample sizes for each group. A crucial aspect of biomarkers is their practical applicability in clinical settings. Although serum and plasma are more easily obtained, CSF is more directly related to the CNS environment, making it a more relevant matrix for investigating MS. A notable methodological limitation is the need for stringent quality control, particularly concerning nDNA, which may lead to that some samples cannot be analyzed. Improper sample handling, such as deviation from centrifugation protocols or delays in freezing, can lead to cellular contamination, potentially causing artificially high levels of ccf-mtDNA. On the other hand, the use of ddPCR for analyzing ccf-mtDNA offers considerable advantages, including high assay specificity and sensitivity, minimal sample volume and manipulation requirements, and the capacity for multiplexing. Compared to RNA molecules or proteins, the circular structure of mtDNA offers greater resistance to degradation by endonucleases and inhibitory molecules present in CSF, enhancing its stability through freeze-thaw cycles. While cost is a significant limitation for implementing novel biomarkers in clinical practice, the expense of quantifying CSF ccf-mtDNA is not significantly different from other analyses commonly performed in clinical practice.

5. Conclusions

Overall, our results suggest that ccf-mtDNA in CSF could serve as a valuable biomarker for inflammation and treatment response in MS. The study reinforces existing evidence that ccf-mtDNA levels are elevated in the CSF of people with MS. We could also demonstrate that baseline CSF ccf-mtDNA concentrations are associated with various clinical parameters, including CSF mononuclear cell counts, IgG and IgM levels, the EDSS score and the number of relapses in the preceding year. Moreover, we could show that ccf-mtDNA concentrations in CSF decreased after aHSCT regardless of prior DMT. In conclusion, our study adds to the growing evidence base for the therapeutic efficacy of aHSCT.

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Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

CRediT authorship contribution statement

Ivan Pavlovic: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Christina Zjukovskaja:** Formal analysis, Investigation, Visualization, Writing – review & editing. **Faisal Hayat Nazir:** Writing – review & editing. **Malin Müller:** Writing – review & editing. **Anna Wiberg:** Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. **Joachim Burman:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors have no disclosures to report.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2024.105482.

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