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Research Paper

Biomarking MELAS with neurofilament light chain and circulating cell free mitochondrial DNA

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ABSTRACT

Mitochondrial diseases are genetic disorders caused either by nuclear or mitochondrial DNA (mtDNA) alterations and characterized by high genetic and phenotypic variability. The common mtDNA m.3243 A > G variant in the *MT-TL1* gene leads to clinical manifestations ranging from the classical MELAS (myopathy, encephalopathy, lactic acidosis and stroke-like episodes) syndrome to milder phenotypes such as MIDD (maternally inherited diabetes and deafness) or a spectrum of clinical features of intermediate severity defined as MELAS-Spectrum. The heterogeneous disease course makes the identification of biomarkers for monitoring disease progression challenging, particularly if we consider the occurrence of stroke-like episodes (SLEs), which remain unpredictable events.

Here, we assessed two biomarkers, neurofilament light chain (NF-L) and circulating cell free-mtDNA (ccf-mtDNA), in a cross-sectional study in MELAS patients, including both patients in the interictal period and during SLEs, and MELAS-Spectrum patients. Both biomarkers were significantly elevated in MELAS patients during SLEs, compared to the other patients. In addition, we found significant correlation between NF-L and m.3243 A > G blood heteroplasmy in MELAS patients, as well as between NF-L and clinical severity in the whole patients cohort.

Despite the limitations derived from the small sample size and the cross-sectional sample collection, our study confirms the value of NF-L and ccf-mtDNA as biomarkers efficiently hallmarking SLEs, highlighting their potential use to monitor the progression of MELAS.

1. Introduction

Primary mitochondrial disorders are heterogeneous conditions caused by pathogenic variants in genes encoded either by nuclear DNA or by the mitochondrial multi-copy genome (mitochondrial DNA, mtDNA) [1,2].

The m.3243 A > G variant in the *MT-TL1* gene is the most frequent [3] and one of the most phenotypically heterogeneous mtDNA single nucleotide variants causing mitochondrial disease. Large cohort studies have shown a remarkable phenotypic variability in patients carrying this variant: the most severe phenotype, mitochondrial myopathy, encephalopathy, lactic acidosis and recurrent stroke-like episodes (MELAS), affects only 10% of carriers of the variant, while the maternally inherited diabetes and deafness (MIDD) phenotype is far more common and represents one of the mildest phenotypes [4]. Other clinical features include myopathy, migraine, cardiomyopathy, gastrointestinal dysmotility, nephropathy, and retinopathy in various combinations [4], and stroke-like episodes (SLEs) can present throughout life in patients with m.3243 A > G variant [5,6]: indeed patients may initially have mild symptoms, such as MIDD, decades prior to their first SLE [7]. The

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variability of phenotypes between MELAS and MIDD, combining many clinical features of intermediate severity, has been defined as MELAS-Spectrum (MELAS-S) [8].

In addition to the m.3243 A > G variant, other less frequent pathogenic variants in the *MT-TL1* gene can also be associated with MELAS or MELAS-S [9]. A second large group of genetic defects leading to a MELAS-like disorder includes heteroplasmic variants affecting different mitochondrial encoded subunits of Complex I (i.e. ND1, ND3, ND5), which highlights how defective Complex I is central to this phenotype [9].

The heterogeneous disease course and lack of predictive and indicative biomarkers pose challenges both for clinicians and for patients, who live with the continual fear of disease progression [10]. In particular, the unpredictable occurrence of SLEs remains a challenge of paramount importance.

Neurofilaments are cytoskeletal components of neurons that are particularly abundant in axons. Their functions include the provision of structural support and maintaining the size, shape, and calibre of the axons [11]. Following central nervous system (CNS) axonal damage, neurofilament proteins released into cerebrospinal fluid (CSF) provide an indication of the extent of axonal damage and neuronal death. The neurofilament subtype most extensively studied in this context is neurofilament light chain (NF-L), and increased NF-L levels appear to reflect ongoing neuronal damage irrespective of the underlying pathology, making it an interesting biomarker for neuronal injury [12].

Similarly, the extracellular leakage of mtDNA following neuronal injury and death appears to be a hallmark of CNS degeneration [13]. Elevated circulating cell free-mtDNA (ccf-mtDNA) has been found in the plasma of MELAS patients in relation to SLEs or during the progression of neurodegeneration [14]. A similar finding has been observed in CSF [15], suggesting that mtDNA may be released from impaired and dying neuronal cells.

The aims of this study were: to investigate if circulating NF-L and ccf-mtDNA levels could differentiate MELAS patients from those without a history of SLEs, defined as MELAS-Spectrum (MELAS-S); to investigate the changes in these two biomarkers in MELAS patients during the acute phase (SLE ongoing) and, lastly, to explore the possible correlation between NF-L and ccf-mtDNA, and whether alterations in these biomarkers reflect a common mechanism of neurodegeneration.

2. Material and methods

2.1. Patients and samples

Samples (serum/plasma) were collected at Haukeland University Hospital (Norway) and at the IRCCS Istituto delle Scienze Neurologiche di Bologna (Italy). We analysed a total of 45 samples collected from a cohort of 37 patients harboring pathogenic variants m.3243 A > G (n = 31), m.3258 T > C (n = 1), m.3271 T > C (n = 2) and m.3279C > T (n = 1) in the *MT-TL1* gene, as well as the m.10191 T > C (n = 1) and the m.10197G > A (n = 1) variants in the *MT-ND3* gene.

We subdivided patients clinically into MELAS (n = 12) and MELAS-S (n = 25) groups, based on the presence or absence of SLEs in their clinical phenotype. Within the MELAS group, ten samples from four patients have been collected during SLEs (MELAS-SLE). Two different samples collected over five years from one MELAS-S patient were also available. Among the 31 patients harboring the m.3243 A > G variant, 14 have been previously assessed for ccf-mtDNA content [14], and their data have been incorporated into the present study.

Last, the difference in the number of samples analysed for the NF-L and ccf-mtDNA depends on the number of serum/plasma aliquots available for each patient. Similarly, for the correlation analyses, the number of samples relies on the availability of both biomarkers assessment in the same sample.

Genetic and demographic characteristics of the patients are reported in Table 1. A database containing the demographic characteristics and the clinical features of the patients is available in the Supplementary Material (*Clinical database.xlsx*). The Newcastle Mitochondrial Disease Adult Scale (NMDAS) was instrumental in assessing the disease burden [16]. Modified NMDAS scores (excluding item 10 on cognition) reported in the database were derived retrospectively from the clinical visit closest to blood sampling.

Table 1
Patients categorized into phenotypic groups.

Patient groups	Genetic defect (N)	Number of patients	Gender, Male (%)	Age (mean ± SD)
MELAS-S	m.3243 A > G (22) rare variants in *MT-TL1* (3)	25	12 (48%)	48 ± 14
MELAS	m.3243A > G (6) rare variants in *MT-TL1* (1) rare variants in *MT-ND3* (1)	8	3 (38%)	45 ± 15
MELAS-SLE	m.3243A > G (3) rare variants in *MT-ND3* (1)	4	2 (50%)	43 ± 15

Data are represented with ± standard deviation (SD) or percentage. N, number of patients.

2.2. Plasma and serum collection

Peripheral blood was collected in EDTA for plasma separation or in Serum Separation Tubes and centrifuged within 1 h from collection. Whole blood was centrifuged for 15 min at 3.000 ×g for plasma and serum separation. Plasma for ccf-mtDNA analysis was further centrifuged for 10 min at 15.000 ×g to avoid cell contamination. Aliquots were stored at −80 °C until processing.

2.3. NF-L analysis

The concentration of NF-L was determined in serum samples using the Simoa Assay (Uman Diagnostics, Quanterix), as previously reported [17].

2.4. Ccf-mtDNA analysis

Ccf-mtDNA was determined in plasma as previously reported [14]. Circulating cell free-DNA (ccf-DNA) was extracted from 200 µl of plasma samples using the NucleoSpin® Plasma kit (Machery & Nagel), following the manufacturer's instructions.

The ccf-mtDNA was quantified by droplet digital-PCR (dd-PCR, Bio-Rad) with a probe-based method, amplifying *MT-ND2*. The ccf-mtDNA was expressed as copies of the target gene on µl of template analysed (copies/µl template). Previously reported ccf-mtDNA values for 14 patients carrying the m.3243 A > G variant [14] were also included in the present study.

2.5. MtDNA heteroplasmy assessment in m.3243 A < G patients

For most patients carrying the m.3243 A > G variant, mtDNA heteroplasmy has been assessed on DNA extracted from blood mononuclear cells, by dd-PCR with a probe-based method that discriminates wild type and mutant alleles [14]. Results were normalized based on the age of patients using the online tool http://www.newcastle-mitochondria.com/m-3243ag-heteroplasmy-tool [18] and reported in the patients' database in the Supplementary Material.

2.6. Statistical analyses

The data were analysed using GraphPad Prism v.10.

Normal distribution of data was assessed by the Shapiro-Wilk test

and the Kolmogorov-Smirnov test. In case of non-normal distribution, nonparametric tests were used. Outliers have been identified using the ROUT method (Q = 1%). Significance for NF-L values was assessed by the Kruskal-Wallis test and the Dunn's multiple comparisons test (effect size expressed as $r = Z/\sqrt{N}$), whereas significance for ccf-mtDNA was calculated by One Way ANOVA and Tukey's multiple comparisons test (effect size expressed as Cohen's d). Correlations were assessed with Spearman's correlation analyses. Spearman's r is reported for the significant correlations as an indicator of the effect size.

3. Results

3.1. High NF-L and ccf-mtDNA levels hallmark ongoing SLEs

It has been previously shown that serum NF-L levels are increased in MELAS patients compared to healthy controls, especially in patients during SLEs [19]. Similarly, we have previously reported that, in contrast to other mitochondrial diseases, plasma ccf-mtDNA is elevated in m.3243 A > G patients compared to a control group of healthy individuals [14]. Here, we analysed both serum NF-L and plasma ccf-mtDNA comparing MELAS patients with those who have a clinical phenotype similar to MELAS but had never experienced SLEs, the MELAS-S, according to our definition. Moreover, we also analysed separately samples collected from MELAS patients with an ongoing SLE (MELAS-SLE).

The mean NF-L levels were comparable between MELAS-S and MELAS groups (Table 2, Fig. 1A). In MELAS-SLE patients, we found higher NF-L levels, which were significantly different from those of the other groups (Table 2, Fig. 1A). Similarly, while MELAS-S and MELAS patients showed similar ccf-mtDNA mean values, the MELAS-SLE patients had significantly higher ccf-mtDNA compared to the other groups (Table 2, Fig. 1B). An exploratory analysis of patients carrying rare mtDNA variants revealed a similar trend toward increased NF-L and ccf-mtDNA levels in the MELAS-SLE group (Supplementary Fig. 1). However, given the very limited number of samples, particularly within the MELAS-SLE subgroup, which includes samples from a single patient, these observations did not allow for meaningful statistical analyses and should be interpreted with caution.

Based on this first observation, we evaluated the potential relationship between these two biomarkers in the whole cohort and the different groups of patients. We failed to observe any significant correlation between NF-L and ccf-mtDNA in the whole cohort of patients (Fig. 1C), as well as in the different patient groups (Fig. 1D-F).

3.2. NF-L correlates with blood m.3243 A > G heteroplasmy in MELAS patients and with disease severity in the whole cohort of patients

It has been previously demonstrated in a large cohort of patients that age-adjusted blood m.3243 A > G heteroplasmy is highly correlated with disease burden and progression [18]. To evaluate whether NF-L and ccf-mtDNA values are related to the blood m.3243 A > G variant loads, we performed correlation analyses in 22 patients (16 MELAS-S, 3 MELAS, 3 MELAS-SLE) for NF-L (Fig. 2A-C) and for ccf-mtDNA (Fig. 2D-F). No significant correlations were observed in the whole patient cohort, as well as in MELAS-S (Fig. 2A-B and 2D-E). However, in MELAS patients, including both those in the interictal period and those sampled during SLE, a significant correlation was identified between NF-L and m.3243 A > G heteroplasmy (Fig. 2C) ($r = 0.84$, $p = 0.04$), whereas for ccf-mtDNA despite a similar trend we failed to observe a significant correlation (Fig. 2F).

Next, to investigate the possible relation between NF-L and ccf-mtDNA with the clinical severity, we assessed the correlation with the available NMDAS scores in 26 patients for NF-L (17 MELAS-S, 6 MELAS, 3 MELAS-SLE) (Fig. 3A-C) and 29 patients for ccf-mtDNA (19 MELAS-S, 7 MELAS, 3 MELAS-SLE) (Fig. 3D-F).

While for ccf-mtDNA we did not find any significant correlation (Fig. 3D-F), NF-L was significantly correlated with NMDAS score only when all patients were analysed together as a single cohort ($r = 0.48$, $p = 0.015$) (Fig. 3A).

Last, since plasma lactate is a key biomarker for MELAS, often elevated due to impaired mitochondrial oxidative metabolism and useful for supporting the clinical diagnosis, we evaluated its possible correlation with NF-L or ccf-mtDNA in 27 patients for NF-L (16 MELAS-S, 7 MELAS, 4 MELAS-SLE) and in 28 patients for ccf-mtDNA (17 MELAS-S, 7 MELAS, 4 MELAS-SLE). We failed to observe any significant correlation between NF-L and plasma lactic acid (Supplementary Fig. 2 A-C), whereas we found a significant negative correlation for ccf-mtDNA, exclusively for the MELAS-S patients ($r = -0.56$, $p = 0.02$) (Supplementary Fig. 2E).

4. Discussion

Our study highlights some observations of interest. Significantly higher levels of the two biomarkers NF-L and ccf-mtDNA were observed in MELAS patients when sampled during the recurrence of SLEs, as compared with patients during the interictal period and patients not suffering SLEs (Fig. 1A-B). The same trend was shown by the exploratory analysis of the subset of patients carrying rare pathogenic variants (Supplementary Fig. 1). Thus, our results suggest validating both biomarkers by following them along the evolution of SLEs, ultimately to be used as SLE hallmarks. While the higher levels of NF-L in MELAS-SLE were already noted [19], this finding is novel for ccf-mtDNA. Our results highlight the role of these biomarkers in relation to the possible neuronal injury and loss, which peaks during SLEs, as opposed to the presumably milder neurodegenerative activity of MELAS-S patients. Obviously, in this latter group of patients, individual cases can be equally as severe as in the MELAS group, but only a thorough longitudinal study may further highlight the usefulness of NF-L and ccf-mtDNA as prognostic biomarkers. Similarly, the predictive value of NF-L and ccf-mtDNA for the occurrence of SLEs also needs to be confirmed with a longitudinal study.

Dissecting further the results, we noted a different magnitude of NF-L and ccf-mtDNA elevation in samples taken during ongoing SLE, compared with those taken during the interictal period (fold changes 4.3 for NF-L and 2.6 for ccf-mtDNA) (Fig. 1A-B). This difference may reflect the fact that mtDNA release occurs not only in subacute neuronal cell death, such as during SLE, but also in neurons undergoing metabolic stress and not necessarily undergoing active cell death. Furthermore, while NF-L is assumed to be neuron-specific, ccf-mtDNA may be released by other tissues or organs, and this may mask specific neuronal

Table 2
Descriptive analysis for NF-L and ccf-mtDNA.

Biomarkers	Patient Groups (N)	Mean	SD	Median	95% CI of mean	Min-Max
NF-L	MELAS-S (24)	33.04	22.55	29.00	23.51–42.56	4–100
	MELAS (9)	42.41	31.97	30.00	17.84–66.99	15–96.70
	MELAS SLE (8)	181.4	108.7	165.5	90.54–272.2	34–397
Ccf-mtDNA	MELAS-S (23)	103.30	67.15	102.00	74.22–132.30	21.00–269.00
	MELAS (7)	82.00	31.27	85.00	53.08–110.90	28.00–115.00
	MELAS SLE (10)	209.80	170.20	136.50	88.06–331.50	27.00–581.00

N, number of samples; SD, standard deviation; CI confidence interval; Min minimum value; Max maximum value.

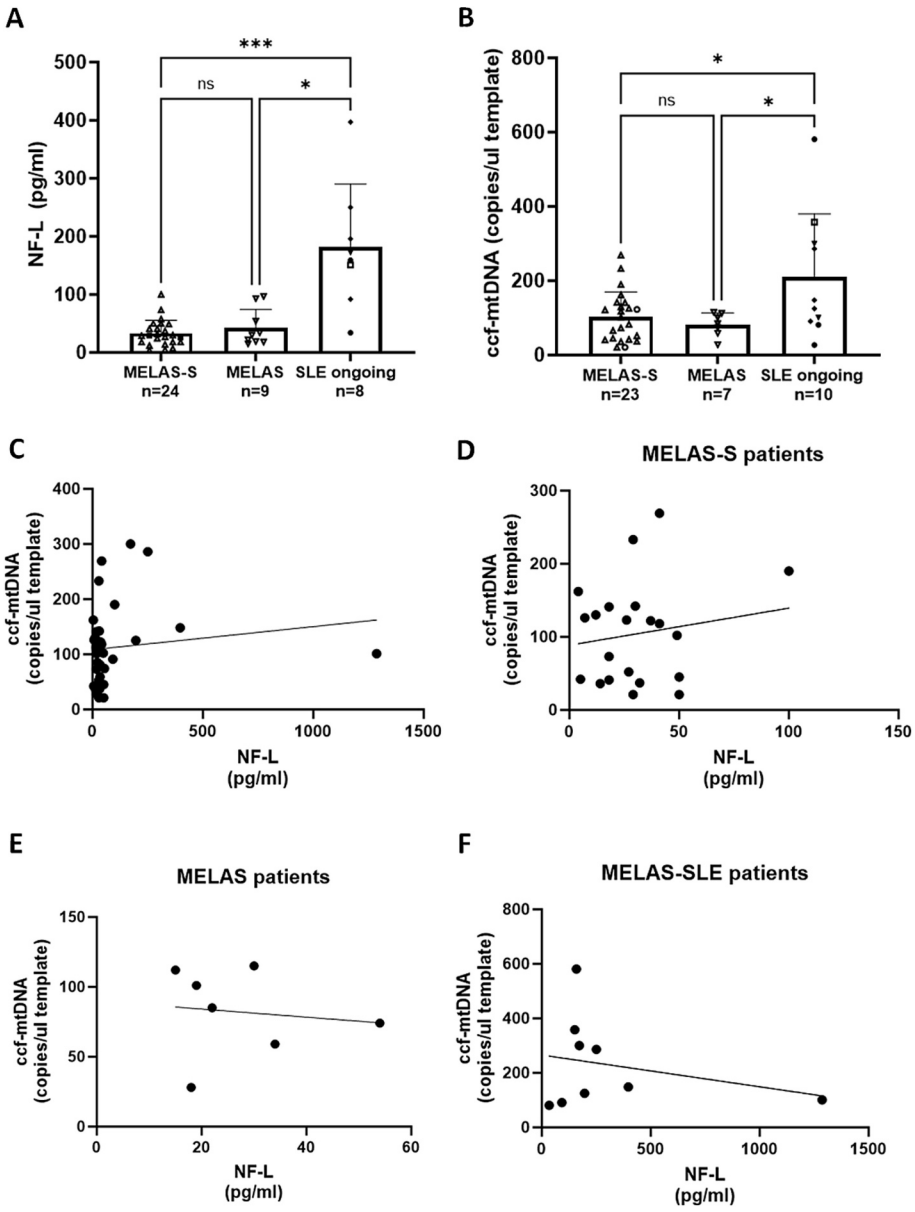

Fig. 1. Evaluation of NF-L and ccf-mtDNA in serum/plasma samples. (A) NF-L assessed in 23 MELAS-S (samples $n = 24$), 9 MELAS (samples $n = 9$) and 4 MELAS-SLE (samples $n = 8$) patients. One outlier was removed from the MELAS-SLE group. Samples derived from the same patient are indicated using the same symbol. Statistical significance was calculated using the Kruskal-Wallis test and the Dunn's test for multiple comparisons (*** $p = 0.0004$ $r = 0.68$, *$p = 0.0154$ r = 0.68). (B) Ccf-mtDNA assessed in 22 MELAS-S (samples $n = 23$), 7 MELAS (samples $n = 7$) and 4 MELAS-SLE (samples $n = 10$) patients. One outlier was removed from the MELAS group. Statistical significance was calculated using ANOVA and the Tukey's test for multiple comparisons (* $p = 0.02$ MELAS-S vs MELAS-SLE Cohen's d = −1.07, *$p = 0.0341$ MELAS vs MELAS-SLE Cohen's d = −1.29). (C—F) Spearman's correlation between NF-L and ccf-mtDNA in the whole cohort (33 patients, 35 samples, three outliers removed) (C), MELAS-S (20 patients, 21 samples) (D), MELAS (7 patients, 7 samples, one outlier removed) (E) and MELAS-SLE (4 patients, 9 samples) (F) patients. The number of samples for the correlation analyses relies on the availability of simultaneous assessment of NF-L and ccf-mtDNA and on the exclusion of outliers, when present.

involvement. Thus, we would suggest that NF-L is superior in monitoring neurodegeneration, while ccf-mtDNA is a more general biomarker of mitochondrial dysfunction derived from multiple tissues.

Another noticeable feature is the lack of correlation between the two biomarkers (Fig. 1C-F). There may be multiple reasons for this. First, the timing of the samples collected during SLEs might not be perfectly comparable, due to the retrospective nature of the study. Furthermore, following the previous consideration, the timing of release of NF-L and ccf-mtDNA might depend on different dynamics over time, and the possible supplementary contribution from other tissues to ccf-mtDNA could also impact this. In our experience, SLEs must be considered as part of a more global wave of bioenergetics imbalance, as frequently multiple markers of skeletal muscle dysfunction are also involved, like elevation of lactic acid, creatine phosphokinase, and eventually myoglobin, but with different timing and dynamics over SLE follow-up (Carelli and La Morgia, unpublished results). We have also previously reported a similar selective correlation of ccf-mtDNA with some biomarkers (alanine, lactic acid) and not with others (FGF21 and GDF15), despite all these biomarkers being increased in MELAS patients [14]. Finally, we must take into account the small number of MELAS/MELAS-SLE patients analysed, which might also justify the lack of correlation with basal levels of lactic acid in the plasma of this cohort. However, there is one exception for the MELAS-S group, in which we highlighted a negative correlation between ccf-mtDNA and basal lactic acid

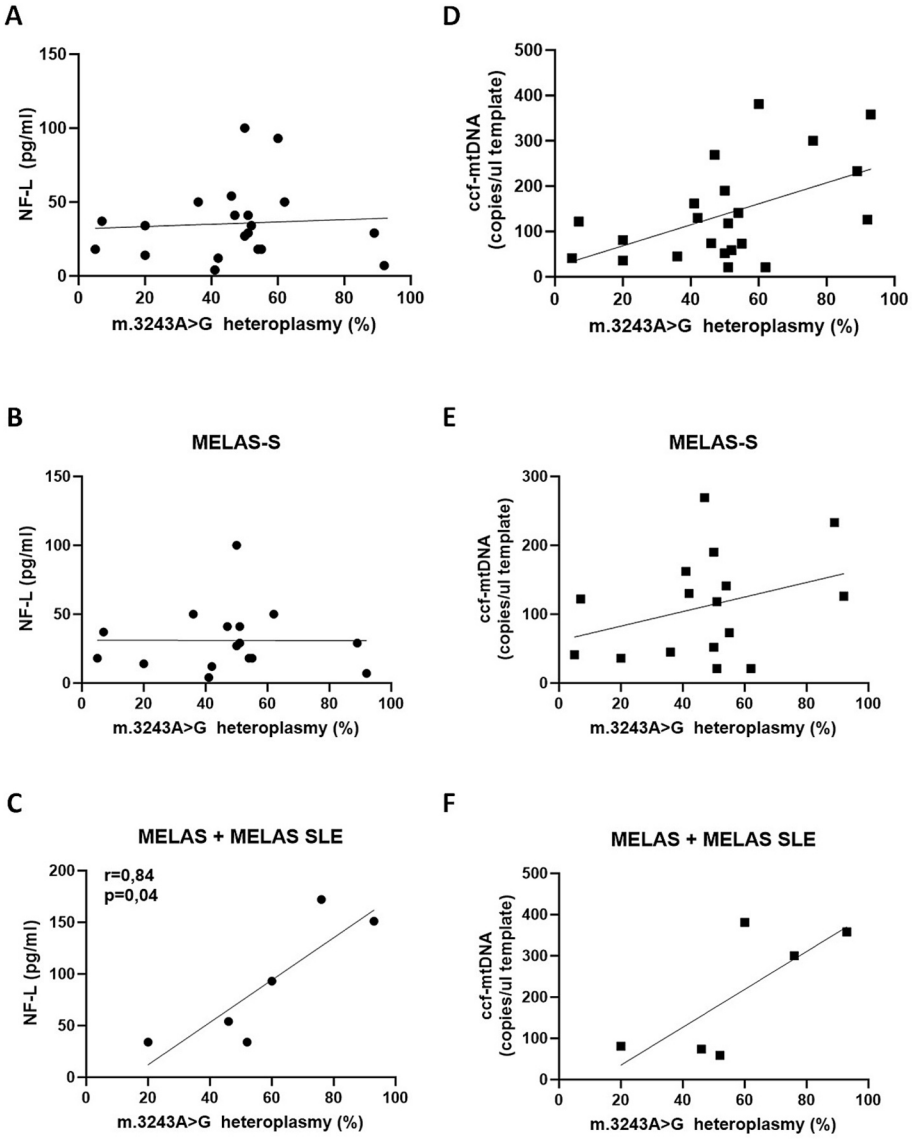

Fig. 2. Correlation analysis with the blood m.3243A > G heteroplasmy. (A-C) Spearman's correlation between serum NF-L levels and m.3243A > G heteroplasmy assessed in blood cells in the whole cohort (20 patients, 20 samples, two outliers removed) (A), in MELAS-S patients (16 patients, 16 samples) (B) and in MELAS patients (6 patients, 6 samples) (C), the latter including those in the interictal period and during SLEs. (D—F) Spearman's correlation between plasma ccf-mtDNA levels and m.3243A > G heteroplasmy assessed in blood cells in the whole cohort (22 patients, 22 samples) (D), in MELAS-S patients (16 patients, 16 samples) (E) and in MELAS patients (6 patients, 6 samples) (F), the latter including those in the interictal period and during SLEs. When statistically significant, Spearman r and *p* value are indicated in the graph. For each correlation analysis, the number of samples analysed relies on the simultaneous availability of NF-L/ccf-mtDNA and m.3243A > G heteroplasmy, as well as on the exclusion of outliers, when present.

(Supplementary Fig. 2), as was previously reported [14]. These considerations further advocate for the paramount importance of a well-designed longitudinal follow-up study of SLEs and of MELAS and MELAS-S patients in general, to truly capture their natural history and related biomarkers.

Another positive finding is the significant correlation observed between NF-L and both the NMDAS score and heteroplasmy levels, respectively in the whole cohort (Fig. 3A) and in the MELAS group, including the MELAS-SLE patients (Fig. 2C). While for ccf-mtDNA we failed to find significant correlations, a trend paralleling NF-L was observed for MELAS and MELAS-SLE patients (Fig. 2F). Lack of significance in this case is most probably due to the limited number of samples. NMDAS and blood heteroplasmy are indicative of clinical severity and may drive disease progression. Therefore, both biomarkers, if consolidated on longitudinal studies and larger cohorts, may serve as useful tools for monitoring disease progression in this patient population, both in clinical practice and as outcome measures in the context of clinical trials, since preventing disease progression, cerebral damage, and neurodegeneration should be the main goals of treatment in this group.

Circulating NF-L and ccf-mtDNA levels are elevated in MELAS patients compared to healthy individuals and in relation to SLEs [14,19]. Ccf-mtDNA may be regarded as a damage associated molecular pattern (DAMP), and in inflammatory neurological conditions, such as multiple sclerosis, both ccf-mtDNA and NF-L are increased during the most active inflammatory phases [20,21]. The role of inflammation in MELAS remains unclear and further studies on the pathogenesis of SLEs are needed. However, involvement of inflammation/innate immune system might suggest a role for anti-inflammatory or autoimmune therapy in the treatment of SLEs. The importance of the immune system in mitochondrial disease development was recently shown in a study highlighting a connection between the innate immune system and POLG, the mtDNA polymerase [22].

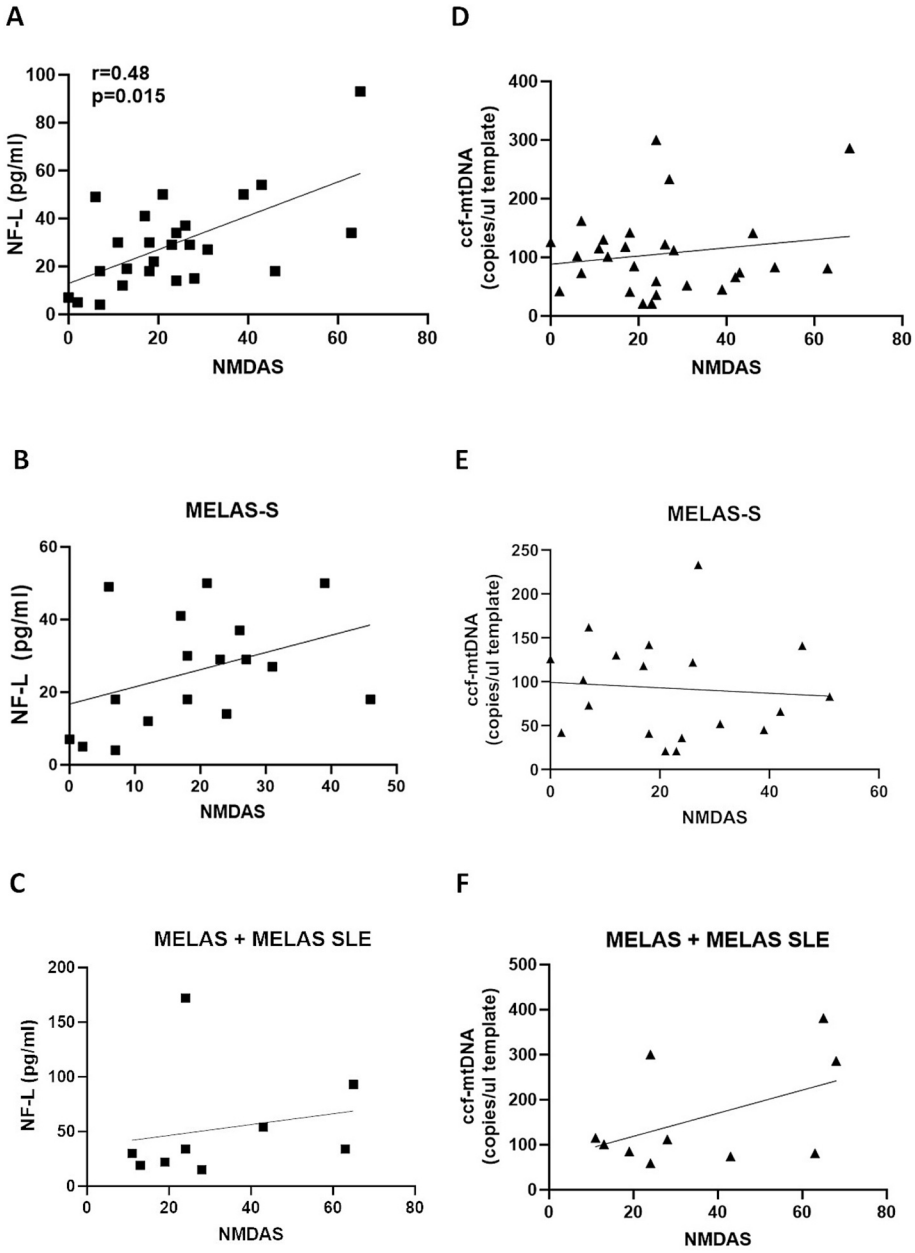

Fig. 3. Correlation analysis with the disease burden. (A-C) Spearman's correlation between serum NF-L levels and NMDAS scores in the whole cohort (25 patients, 25 samples, two outliers removed) (A), MELAS-S patients (17 patients, 17 samples) (B) and MELAS patients (9 patients, 9 samples, one outlier removed) (C), including the MELAS-SLE patients. (D—F) Spearman's correlation between plasma ccf-mtDNA levels and NMDAS scores in the whole cohort (28 patients, 28 samples, one outlier removed) (D), MELAS-S patients (19 patients, 19 samples) (E) and MELAS patients (10 patients, 10 samples) (F), including the MELAS-SLE patients. When statistically significant, Spearman r and p value are indicated in the graph. For each correlation analysis, the number of samples analysed relies on the availability of NF-L/ccf-mtDNA and NMDAS score, as well as on the exclusion of outliers, when present.

Our findings showing higher NF-L and ccf-mtDNA levels in MELAS-SLE compared to both MELAS patients in the interictal period and MELAS-S patients, hold promise on the possibility of combining biomarkers and making multivariate prediction models, e.g. to compute the risk of developing SLEs. Recently, four metrics (BMI, lactate levels, age-correlated blood mtDNA heteroplasmy levels, and sensorineural hearing loss) were used to quantify their cumulative impact on the risk of SLEs [6]. It is possible that the addition of NF-L and ccf-mtDNA could increase the predictive power of the model even further.

Our study has limitations. Despite an increased number of patients investigated compared to previous investigations [14], our study remains underpowered for more sophisticated stratifications and correlations. As already remarked, the cross-sectional nature of the study hampers full exploitation of these promising biomarkers, e.g. in describing the natural history of the disease or developing predictive algorithms, in particular for SLEs. Thus, a longitudinal design using a combined set of biomarkers is needed.

In conclusion, we provide consistent support for the combined use of NF-L and ccf-mtDNA as reliable biomarkers in MELAS patients to follow the disease course, to possibly predict the subacute events, and to be used as outcome measures in future clinical trials.

Clinical trial number

Not applicable.

CRediT authorship contribution statement

Alessandra Maresca: Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization, Writing – review & editing, Writing – original draft. **Monica Moresco:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Giulia Amore:** Methodology, Investigation, Data curation, Writing – review & editing. **Chiara La Morgia:** Methodology, Investigation, Data curation, Writing – review & editing. **Maria Lucia Valentino:** Methodology, Investigation, Data curation, Writing – review & editing. **Giada Capirossi:** Investigation, Writing – review & editing. **Giulia Sacchetti:** Investigation, Writing – review & editing. **Valerio Carelli:** Supervision, Funding acquisition, Conceptualization, Writing – review & editing, Writing – original draft. **Laurence A. Bindoff:** Conceptualization, Writing – review & editing. **Kristin N. Varhaug:** Investigation, Data curation, Conceptualization, Writing – review & editing, Writing – original draft. **Christian Vedeler:** Writing – review & editing.

Consent to participate

Subjects are enrolled and data is collected upon obtaining the written informed consent.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. The study was approved by the Norwegian Regional Committee for Medical Health Research Ethics (No:2019/479), and by the Institutional Ethical Board (Comitato Etico Interaziendale Bologna-Imola, CE-BI 13036).

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Declaration of competing interest

Dr. Monica Moresco, Dr. Giada Capirossi, Dr. Giulia Sacchetti, Dr. Maria Lucia Valentino and Dr. Christian Vedeler, Dr. Laurence A. Bindoff and Dr. Kristin N. Varhaug report no competing interests.

Dr. Alessandra Maresca: Speaker honoraria for meeting from First Class srl.

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Dr. Valerio Carelli: Advisory board and consultant for Chiesi Farmaceutici, GenSight Biologics, Pretzel Therapeutics; speaker honoraria and/or financial support for meetings from Chiesi Farmaceutici, GenSight Biologics, First Class srl. PI/SI for clinical trials sponsored by GenSight Biologics, Santhera Pharmaceuticals, Stoke therapeutics, Reneo, Stealth Biotherapeutics and OMEICOS therapeutics.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2026.109753.

Data availability

The authors confirm that the data supporting the findings of this study are available within the Supporting Information of the article.

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