Mitochondrial DNA copy number and D-loop region methylation in carriers of amyotrophic lateral sclerosis gene mutations

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Abstract

Aims: To investigate mitochondrial DNA (mtDNA) copy number and D-loop region methylation in carriers of *SOD1*, *TARDBP*, *FUS,* and *C9orf72* mutations.

Methods: Investigations were performed in blood DNA from 114 individuals, including amyotrophic lateral sclerosis (ALS) patients, pre-symptomatic carriers and non-carrier family members.

Results: Increased mtDNA copy number (P = 0.0001) was observed in ALS patients, and particularly in those with *SOD1* or *C9orf72* mutations. *SOD1* mutation carriers showed also a significant decrease in D-loop methylation levels (P = 0.003). An inverse correlation between D-loop methylation levels and the mtDNA copy number (P = 0.0005) was observed.

Conclusions: De-methylation of the D-loop region could represent a compensatory mechanism for mtDNA up-regulation in carriers of ALS-linked *SOD1* mutations.

Keywords: Amyotrophic Lateral Sclerosis; epigenetics; mitochondrial DNA methylation; D-loop mitochondrial region; mtDNA copy number; SOD1.

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by progressive death of upper and lower motor neurons with progressive weakness and atrophy of voluntary skeletal muscles, ultimately leading to patient death within 2 to 5 years after symptoms onset. Unfortunately no cure for ALS is available, and treatments only slow the progression of the symptoms [1]. The biological processes that lead to neuronal death are not yet completely understood, but numerous pathological mechanisms have been linked to ALS, including oxidative stress, mitochondrial dysfunction, impaired axonal transport, protein aggregation, excitotoxicity, and neuroinflammation. Particularly, mitochondrial dysfunction has been observed in early disease stages either in animal models or in the central nervous system and peripheral blood of ALS patients [2,3].

The majority of ALS is sporadic (defined as SALS), and only 5–10% of the cases are inherited (familial ALS, FALS). Four major genes account for most of FALS and for a small percentage of SALS, namely *SOD1* (12% of FALS and about 1% of SALS), *TARDBP* (5% of FALS and less than 1% of SALS), *FUS* (4% of FALS and less than 1% of SALS) and *C9orf72* (40% of FALS and about 7% of SALS). A few other genes play a significant role in inherited ALS forms, each accounting for less than 1% of the cases [4]. In addition to these highly penetrant genes, genome-wide screening has identified more than 100 low penetrance ALS loci, suggesting a polygenic inheritance model and a strong contribution of environmental factors in disease pathogenesis [4,5].

Many non-genetic factors have been linked to ALS risk, such as advanced age, male gender and environmental factors. ALS-linked environmental factors include infections, intense physical activity, exposure to organophosphates, and exposure to heavy metals such as lead, mercury, cadmium and selenium [6,7].

Epigenetic mechanisms regulate gene expression levels without altering the DNA sequence, and are influenced by environmental factors [6]. Many investigators observed epigenetic changes in

ALS tissues, including global changes in DNA methylation leading to an altered expression of dozens of genes, and likely contributing to disease onset and progression [8-11]. Despite that epigenetic modifications of the nuclear DNA have been well documented in ALS tissues [9], little is still known concerning the epigenetic modifications of the mitochondrial DNA (mtDNA). However, mitochondrial impairment is a feature of neurodegeneration, and some investigators have suggested that epigenetic modifications of the mtDNA, the so-called "mitoepigenetics," might occur in Alzheimer's disease (AD), Parkinson's disease (PD), ALS, and other neurodegenerative conditions, but evidence is limited to a few studies [8,12-14]. Particularly, increased levels of the DNA methyltransferase 3A (DNMT3A), one of the enzymes that catalyse DNA methylation, were observed post-mortem in nuclear and mitochondrial fractions of human ALS motor cortex [8], while increased methylation of the mitochondrial gene coding for the 16S rRNA was detected in spinal cord neurons and skeletal muscle myofibrils of ALS transgenic mice [12]. Furthermore, aberrant DNA methylation of the mitochondrial displacement-loop region (D-loop) was observed in brain regions of individuals in preclinical stages of AD, in AD mice, in PD brains, as well as in peripheral blood of AD patients [13,14]. The D-loop region is critical for mtDNA replication and transcription, and recent studies in colorectal cancer cells revealed that an increased methylation of this region was linked to a decreased copy number of the mtDNA [15].

In order to further address mtDNA methylation in ALS, we investigated D-loop methylation levels and mtDNA copy number in blood DNA of ALS individuals. Particularly, we collected ALS patients with mutations in *SOD1, TARDBP, FUS* or *C9orf72* genes, and their relatives, including carriers of the mutations in a pre-symptomatic stage and non-carrier family members. We then searched for differences in mtDNA D-loop methylation and copy number among groups.

Materials and methods

Subjects and genetic screening

Blood samples have been collected from 54 ALS patients with mutations in one of the major ALS genes (*SOD1*, *FUS*, *TARDBP* and *C9orf72*), and from 60 of their relatives, including pre-symptomatic carriers and non-carrier family members (Table 1). Particularly, we collected 29 ALS patients with *SOD1* gene mutations, three with mutations in *TARDBP*, four with mutations in *FUS*, and 18 with a *C9orf72* expansion. Details on the carried mutations are provided (Supplementary Material). A total of 28 asymptomatic carriers were available from those families, as well as 32 healthy non-carrier family members (Table 1). All included patients fulfilled the El Escorial criteria [16] for probable or definite ALS and have been diagnosed by expert neurologists. All the study participants were offered genetic testing and counselling, and all signed the informed consent approved by the Ethics Committee of the Niguarda Hospital (ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy). Genomic DNA extraction was performed using the QIAmp DNA blood Mini Kit (Qiagen, Milan, Italy, Catalog N◦ 51106) following the manufacturer's protocol. The search of *SOD1*, *TARDBP*, *FUS* and *C9orf72* gene mutations was performed as described elsewhere [17-19], and details are provided in Supplementary material.

D-loop methylation analysis

Methylation of the D-loop region was assessed by means of methylation sensitive-high resolution melting (MS-HRM) technique as reported elsewhere [14]. Briefly, 200 ng of DNA from each sample were treated with sodium bisulfite in order to convert all unmethylated cytosines into uracil using the EpiTect Bisulfite Kit (Qiagen, Milan, Italy, Catalog N° 59104). A sample of completely unmethylated genomic DNA (Amplified human genomic DNA, completely unmethylated, Qiagen, Cat No./ID: 59568) was used as control assay to check the bisulfite conversion efficiency that resulted to be of 99% in average. In order to avoid potential batch effects,

each kit was used to treat simultaneously the same number of ALS and healthy samples, and samples derived from different bisulfite treatments were analyzed independently on separate occasions to verify the inter-assay variability, observing a very good reproducibility. Furthermore, in order to ensure that the circular structure of the mtDNA did not affect the bisulfite conversion efficiency, the mtDNA was linearized by restriction digestion using *PvuII* (ThermoFischer Scientific, Catalog N° ER0631). *PvuII* specifically recognize CAGCTG and cuts at the position of GC. There is only one *PvuII* restriction site in mtDNA at nt 2650~nt 2655 (GenBank: J01415.2). Digestion was performed incubating DNA with *PvuII* at 37 °C overnight in a 20 µl reaction mix containing nuclease-free water, 0.5 µg of DNA, 2 µl of 10X Buffer G and 1 µl of *PvuII*. We next assessed the comparison of methylation data between *PvuII* digested (linearized) and undigested (circular) mtDNA, observing similar results for the D-loop methylation levels (Supplementary Material).

We used the following forward 5'-GGAGTTTTTTATGTATTTGGTATTTT-3' and reverse 5'-ACAAACATTCAATTATTATTATTATATCCT-3' primers to amplify a D-loop region of 222-bp which comprises the nucleotides 35-256 (GenBank: J01415.2) and includes 10 CpG sites. The MS-HRM analyses were performed using a CFX96 Real-Time PCR detection system (Bio-Rad) with the following protocol: 1 cycle of 95°C for 12 min, 50 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for 30 s, followed by an HRM step of 95 °C for 10 s and 50 °C for 1 min, 65 °C for 15 s, and continuous acquisition to 95 °C at one acquisition per 0.2 °C. PCR was performed in a final volume of 10 µl, containing 5 µl of master mix (Qiagen, Milan, Italy, Catalog N° 59445), 10 pmol of each primer and 10 ng of bisulfite modified DNA template. Each reaction was performed in duplicate. Fully methylated and unmethylated DNA (EpiTect methylated and unmethylated human control DNA, bisulfite converted, Qiagen, Milan, Italy, Catalog N◦59695) was mixed to obtain the following ratios of methylation: 0%, 12.5%, 25%, 50%, 75%, 100%. Standard DNA samples with known methylation ratios were included in each assay in order to generate standard curves to be used for the deduction of the methylation ratio of each sample. In order to obtain single methylation

percentage values from MS-HRM assays, rather than a range, we applied an interpolation method developed and described in our laboratory [20].

D-loop sequencing

In order to verify that MS-HRM results were not altered by the presence of mutations that could have affected the melting temperature of the PCR products of the analysed D-loop region, Sanger sequencing of samples showing 0% methylation and of methylated samples was performed. One microliter of a tenfold dilution of PCR product was added to a 20 µl reaction containing 3.2 pmol of the D-loop amplifying forward primer, 1 µl of BigDye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA, Catalog N. 4336791) and 4 µl of 5x Sequencing Buffer (Applied Biosystems, Catalog N° 4336697). A cycle sequencing modified protocol was followed: 1 cycle of 96°C for 1 min, 30 cycles of 96 °C for 10 s and 48 °C for 4 min. Reactions were purified with Mag-Bind SeqDTR (Omega Bio-Tek, Georgia, USA, Catalog N. M1300-05) following company protocol and run on ABI PRISM 310 Sequencing Analyzer (Applied Biosystems) with Data Collection Software v 3.1, using POP4 polymer and the "*KB_310_POP4_BDTv1_36Rapid*" mobility file. Data analysis was performed with the Sequencing analysis Software v. 5.1 (Applied Biosystems). Sequencing of the analyzed D-loop region did not show the presence of genetic variants that could interfere with the observed methylation levels.

Mitochondrial DNA copy number

MtDNA copy number was performed as reported elsewhere [21]. Briefly, 10 ng of total cellular DNA was used as input for quantitative PCR (qPCR). Primers amplifying a nuclear DNA region (hemoglobin subunit beta) and a mtDNA region (chrM:3313-3322) were taken from literature [22]. qPCR reactions were performed with a C1000^{TM} Thermal Cycler equipped with a CFX 96^{TM} Real-Time System (Bio-Rad, Milan) with the following conditions: 15 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 45 sec at 55°C and 45 sec at 72°C. qPCR was performed in a final volume

of 10 µl, containing 5 µl of master mix (Qiagen, Milan, Italy, Catalog N° 59445), 10 pmol of each primer and 1 µl (10 ng) of DNA template. Each reaction was performed in triplicate. Threshold cycle (*Ct*) values were obtained with the Bio-Rad CFX Manager Software (Bio-Rad, Milan). To determine the mtDNA content relative to nDNA, the following equations were used:

1) ΔCt = nDNA *Ct* – mtDNA *Ct*

2) Relative mtDNA content = $2 \times 2^{\Delta Ct}$

Statistical analyses

All data were tested for normality using the Shapiro-Wilk test. Since both D-loop methylation levels and mtDNA copy number were not normally distributed, natural logarithm transformation was done before analysis. Differences in D-loop methylation levels and copy number among ALS patients, asymptomatic carriers and non-carrier family members were compared by one-way multifactorial analysis of variance (ANOVA) correcting for age at sampling and gender. Multifactorial ANOVA was also used to compare D-loop methylation levels and copy number among different mutant genes (i.e. non-carriers of mutations, *SOD1* carriers, *TARDBP* carriers, *FUS* carriers and *C9orf72* expansion carriers), and to compare mean mtDNA copy number levels between samples with unmethylated D-loop and those showing D-loop methylation. Linear regression analysis was performed to search for correlation between D-loop methylation levels and mtDNA copy number. Statistical analyses were performed with STATGRAPHICS 5.1 plus software package for Windows. Because we investigated four different genes, Bonferroni's correction for multiple comparisons was applied and we considered statistically significant only *P*-values <0.05/4 = 0.0125 when comparing the four classes of gene mutations with respect to non-carriers of mutations in those genes.

Results

Figure 1A shows differences in mtDNA copy number among non-carriers of mutations, asymptomatic carriers and ALS patients. The mtDNA copy number was higher in ALS patients respect to non-carriers of mutations (*P*=0.0001), and asymptomatic carriers showed and intermediate copy number between the two other groups (Figure 1A). Multifactorial ANOVA revealed that age at sampling and gender had no significant effect on mtDNA copy number in the whole cohort of subjects (*P*=0.43 and 0.59 respectively). Figure 1B shows D-loop methylation differences among non-carriers of mutations, asymptomatic carriers and ALS patients. D-loop methylation showed a tendency to be lower in ALS patients than in non-carriers of mutations (*P*=0.06). Asymptomatic carriers showed an intermediate methylation level between ALS patients and non-carrier family members (Figure 1B). Multifactorial ANOVA revealed that age at sampling and gender had no significant effect on D-loop methylation levels in the whole cohort of subjects (*P*=0.58 and 0.23 respectively).

The comparison of mtDNA copy number between samples with unmethylated D-loop (0%) and those showing D-loop methylation revealed a significantly higher copy number in the first group (*P*=0.004), Figure 2A. In addition, we observed an inverse correlation between D-loop methylation levels and mtDNA copy number (r= -0.32; *P* = 0.0005) (Figure 2B).

When data were analysed according to the mutant gene, we observed that both *SOD1* mutation carriers (*P* = 0.0001) and *C9orf72* expansion carriers (*P* = 0.002) showed higher mtDNA copy numbers than non-carriers of ALS-linked mutations (Figure 3A). However, only carriers of *SOD1* mutations showed significantly lower methylation levels of the mtDNA D-loop region (*P* = 0.003) (Figure 3B).

Because both *SOD1* and *C9orf72* mutation carriers showed higher mtDNA copy number, but only *SOD1* mutation carriers showed a reduced methylation of the mtDNA D-loop region, we next

stratified carriers into asymptomatic carriers and ALS patients (Figure 4). Figure 4A shows that, concerning *SOD1* carriers, ALS patients showed a significant increase in mtDNA copy number with respect to both non-carriers of mutations (P=0.0001) and to asymptomatic *SOD1* mutant carriers (P=0.0011). ALS carriers of a *C9orf72* expansion showed a significantly higher mtDNA copy number than non-carriers (P=0.0078), but no significant difference was observed between symptomatic and asymptomatic *C9orf72* expansion carriers (Figure 4A). Concerning D-loop methylation levels, both symptomatic and asymptomatic *SOD1* carriers showed lower methylation levels than non-carriers of mutations (P=0.01), whilst *C9orf72* expansion carriers, either asymptomatic or ALS affected, showed similar D-loop methylation levels than the control group (non-carriers of ALS-linked mutations) (Figure 4B). No significant difference between symptomatic and asymptomatic carriers of both *TARDBP* and *FUS* mutations was observed in terms of both mtDNA copy number and D-loop methylation levels (not shown).

Discussion

In the current study we investigated mtDNA copy number and the methylation levels of the mitochondrial D-loop region in blood DNA of individuals belonging to families in which segregate ALS-linked mutations. Overall, we observed a significant increase in mtDNA copy number and a tendency toward decreased D-loop methylation levels in ALS patients respect to non-carriers of ALS-linked mutations, whilst the asymptomatic/pre-symptomatic carriers showed intermediate levels of both biomarkers. Furthermore, we observed that the mtDNA copy number was higher in individuals with a de-methylated D-loop region respect to those showing D-loop methylation, and an inverse correlation between D-loop methylation and mtDNA copy number was detected. However, when individuals were stratified according to the mutant gene, both carriers of *SOD1* and *C9orf72* mutations showed significantly higher levels of mtDNA copy number than non-carriers of ALS-linked mutations, but only *SOD1* mutation carriers showed a significant reduction of D-loop methylation levels. Interestingly, concerning *SOD1* carriers, the increase in mtDNA copy number was significant only in ALS patients, but the decrease of D-loop methylation levels was already detectable in the presymptomatic carriers. By contrast, *C9orf72* expansion carriers showed an increased mtDNA copy number than non-carriers of ALS-linked mutations, but nor asymptomatic carriers, neither ALS patients, showed a decrease of D-loop methylation levels. *TARDBP* and *FUS* carriers showed no significant increase in mtDNA copy number and no significant decrease of D-loop methylation than non carriers of ALS-linked mutations, but data should be considered preliminary as we only had few subjects with those mutations.

In recent years several research has been carried out in the context of aberrant epigenetic mechanisms in ALS pathogenesis, global and gene-specific methylation changes were detected in blood and neuronal DNA from ALS patients by either genome-wide or candidate gene approaches, but most of those studies focused on nuclear DNA methylation changes [10,11,23-31]. However, although mitochondria play a central role in ALS pathogenesis, very little is still known about a

possible contribution of aberrant mtDNA epigenetic mechanisms, and the available evidence includes changes of mitochondrial DNMT protein levels in human ALS motor cortex and in both spinal cord neurons and skeletal muscle myofibrils of transgenic ALS mice at pre-symptomatic and early disease stages [8,12].

Recent studies suggest that the mtDNA is largely hypomethylated, but the mtDNA D-loop region shows inter-individual differences in methylation levels that have been linked to different human conditions, including neurodegeneration [13,14]. D-loop is a non-coding region of the mtDNA of about 1.1 kb, critical for both mitochondrial replication and transcription [32]. Several authors reported that D-loop methylation is inversely correlated with mtDNA copy number in different human tissues, including placenta, peripheral blood, colorectal cancer specimens and retinal endothelial cells [15,33-36], suggesting that the methylation levels of this mitochondrial region could have a role in mtDNA regulatory mechanisms. Indeed, we observed a significant inverse correlation between D-loop methylation levels and mtDNA copy number, strengthening previous evidence that these mechanisms are related to each other [15,33-36]. Studies in colorectal cancer samples also suggested a link between the methylation status of the D-loop region and the expression levels of mtDNA genes. Particularly, hypo-methylation of the D-loop region was associated with an increased mtDNA copy number and with increased expression of the nicotinamide adenine dinucleotide subunit 2 (*ND-2*) gene [34]. Unfortunately, we do not have available RNA samples to search for correlation between D-loop methylation and mtDNA gene expression levels.

Concerning neuronal function and degeneration, regional differences in D-loop methylation were observed in human post-mortem brain tissues [37], decreased D-loop methylation levels were observed post-mortem in the *substantia-nigra* of PD patients [13] and in blood DNA of AD patients [14]. Furthermore, a dynamic pattern of methylation of this region with disease progression was observed in the entorhinal cortex of patients with AD-related pathology and in brain regions of AD mice [13].

In the present study we observed that the mtDNA copy number is increased in the blood DNA of ALS patients. This observation strengthens previous evidences of mitochondrial impairment detectable in peripheral blood of ALS patients [38-40] as well as studies linking mitochondrial impairment to ALS pathogenesis [41-44]. Some authors detected mitochondrial dysfunction in muscular biopsies from ALS patients with sporadic ALS [41,42], and recent findings of mutations in the *CHCHD10* gene, that encodes a mitochondrial protein located in the intermembrane space, suggest a pivotal role for mitochondrial dysfunction in some ALS families or sporadic cases [43,44]. Concerning the peripheral tissues, Ehinger and collaborators observed an increase of mitochondrial content in platelets isolated from peripheral blood of ALS patients respect to healthy controls, and suggested that this event was a compensatory phenomenon for the observed reduction of complex IV-activity of the electron transport chain [40]. Moreover, we observed that the increased mtDNA copy number is significantly evident in both *SOD1* and *C9orf72* patients. Interestingly, Masser and coworkers showed an age-related increase in absolute mtDNA copy number in the skeletal muscle of *Sod1*$^{-/-}$ mice [45], whilst others observed an increase of mitochondrial DNA content and mass in human fibroblasts with the *C9orf72* expansion mutation [46]. Collectively, present and previous data suggest that both *SOD1* and *C9orf72* impairment result in increased mtDNA content, likely as a compensatory mechanism of mitochondrial dysfunction.

However, only *SOD1* mutant carriers showed a significantly lower methylation of the D-loop region. Quite recently it has been demonstrated that different ALS-linked genes can have diverse effects on mitochondrial dynamics and function; for example, it has been shown that mutations in *TARDBP*, *C9orf72* and *SOD1* genes impair mitochondria in a different manner [46,47]. Particularly, both *TARDBP* and *C9orf72* mutations altered mitochondrial morphology in cultured fibroblasts exposed to oxidative conditions, but an increase of mtDNA content and mass was observed only in mutant *C9orf72* cells, whilst *TARDBP* mutant cells were not able to activate mitochondrial proliferation to counteract mitochondrial dysfunctions [46]. Conversely, no morphological mitochondrial changes were observed in mutant *SOD1* fibroblasts when exposed to similar

oxidative conditions, but cells showed an increase in oxygen consumption indicating an increased reliance on mitochondrial function [47,48]. Indeed, studies in ALS transgenic mice have shown that the increase in oxidative stress in *Sod1*$^{-/-}$ mice is not associated with increased heteroplasmy or mtDNA deletions [49], but rather with increased mtDNA copy number as an attempt to restore mitochondrial function [45]. SOD1 is one of the major antioxidant enzymes and plays a pivotal role in the regulation of reactive oxygen species (ROS) homeostasis of the cells. In fact the *SOD1* gene encodes for a protein that catalyses the inactivation of superoxide (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2), thus providing an important antioxidant defence in nearly all cells [50]. Abnormal mitochondrial dynamics are early events in skeletal muscles of *Sod1* mutant mice, already detectable before disease onset [51]. Studies in ALS mice, however, include different models such as *Sod1* knockout mice and mice that carry human ALS-linked *SOD1* mutations, such as the G93A one, the first lacking enzymatic activity and showing only a relatively mild phenotype, and the latter over-expressing mutant proteins with a toxic gain of function and better resembling the ALS phenotype [52,53], likely explaining the differences in mitochondrial behaviour between knockout and ALS-linked mutants. However, by analyzing published data of ALS patients, Saccon and coworkers found a marked reduction of SOD1 enzyme activity arising from several of the known *SOD1* mutations, suggesting that not only a toxic gain of function of the mutant allele, but also the resulting reduced activity can play a role in the ALS phenotype [54]. The question of whether SOD1 loss of function is implicated in ALS pathogenesis is still debated, however both transgenic and knockout mice showed evidence of mitochondrial dysregulation [45,51].

The present observation of a significant decrease in D-loop region methylation levels in both pre-symptomatic and symptomatic *SOD1* mutant carriers suggests that the hypomethylation of this region could represent a compensatory mechanism to oxidative stress, leading to an increased mtDNA copy number. We however collected samples with different *SOD1* mutations, likely leading to different enzyme activities and variable disease phenotype [54]. Our cohort is relatively

small to investigate if the different *SOD1* mutations impair mtDNA methylation and copy number in a different manner, and further studies in larger cohorts are required to clarify this issue.

It is also clear from previous studies that several mechanisms could be responsible for an mitochondrial accumulation in ALS tissues, but it is still questioned if they are functional mitochondria leading to an increased expression of mitochondrial genes [55-57]. Mitophagy (mitochondrial autophagy) is an essential mechanism that selectively eliminates damaged mitochondria, and there is accumulating evidence of impaired mitophagy in neurodegeneration, resulting in the accumulation of dysfunctional mitochondria [55]. Several ALS-linked mutations have been shown to impair autophagy [56], and there is also evidence of feedback mechanisms regulating mitochondrial biogenesis and turnover that, when mitophagy is impaired, could instigate mitochondrial biogenesis [57]. Unfortunately we do not have RNA samples to clarify if the observed increase in mtDNA copy number results or not in increased mtDNA gene expression in our cohort, and also this issue requires to be addressed in future studies.

Conclusion

In conclusion, the present study showed increased mtDNA copy number in peripheral blood DNA from ALS patients, and particularly in those with *SOD1* mutations and *C9orf72* expansions. We also observed an inverse correlation between D-loop methylation levels and the mtDNA copy number. However, only *SOD1* mutations accounted for a significant decrease in D-loop methylation levels, suggesting that it could represent a compensatory mechanism for mtDNA up-regulation peculiar to *SOD1* mutant carriers to couple with increased oxidative stress. We must acknowledge that most of the ALS patients included in the study were carriers of *SOD1* or *C9orf72* mutations; therefore, additional studies in individuals with other ALS-linked mutant genes, and particularly in *FUS* or *TARDBP*, are required to confirm that the observed epigenetic dysregulation is confined to *SOD1* mutants. Present results also strengthen previous evidence of a dysregulated D-loop methylation in neurodegenerative diseases.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Summary points

- We investigated mtDNA copy number and the methylation levels of the mitochondrial D-loop region in blood DNA of individuals belonging to families in which segregate mutations in the major ALS genes, namely *SOD1*, *TARDBP*, *FUS* and *C9orf72*.
- A significant increase in mtDNA copy number was detected in ALS patients respect to non-carriers of ALS-linked mutations. The asymptomatic carriers showed intermediate levels of mtDNA copy number.
- We observed that mtDNA copy number was higher in individuals with a de-methylated D-loop region respect to those showing D-loop methylation, and an inverse correlation between D-loop methylation levels and mtDNA copy number was detected.
- Analyzing the data in relation to the presence of the different mutant genes, patients with *SOD1* mutation or *C9orf72* expansion showed higher mtDNA copy numbers than non-carriers, but only carriers of *SOD1* mutations, either pre-symptomatic or affected by ALS, showed significantly lower methylation levels of the mtDNA D-loop region.
- De-methylation of the D-loop region could represent a compensatory mechanism for mtDNA up-regulation to counteract oxidative stress in carriers of ALS-linked *SOD1* mutations.

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***Interesting paper that demonstrated an increase in global DNA methylation levels in peripheral blood of ALS patients.**

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Table 1. Demographic characteristics of the study population

	ALS patients (n = 54)	Asymptomatic carriers (n = 28)	Non-carriers (n = 32)
Age (mean±S.D.)	56.4±12.3	45.0±16.6	55.3±13.6
Gender F/M	27/27	17/11	16/16
Mutation in *SOD1*	29	13	-
Mutation in *TARDBP*	3	4	-
Mutation in *FUS*	4	5	-
Mutation in *C9orf72*	18	6	-

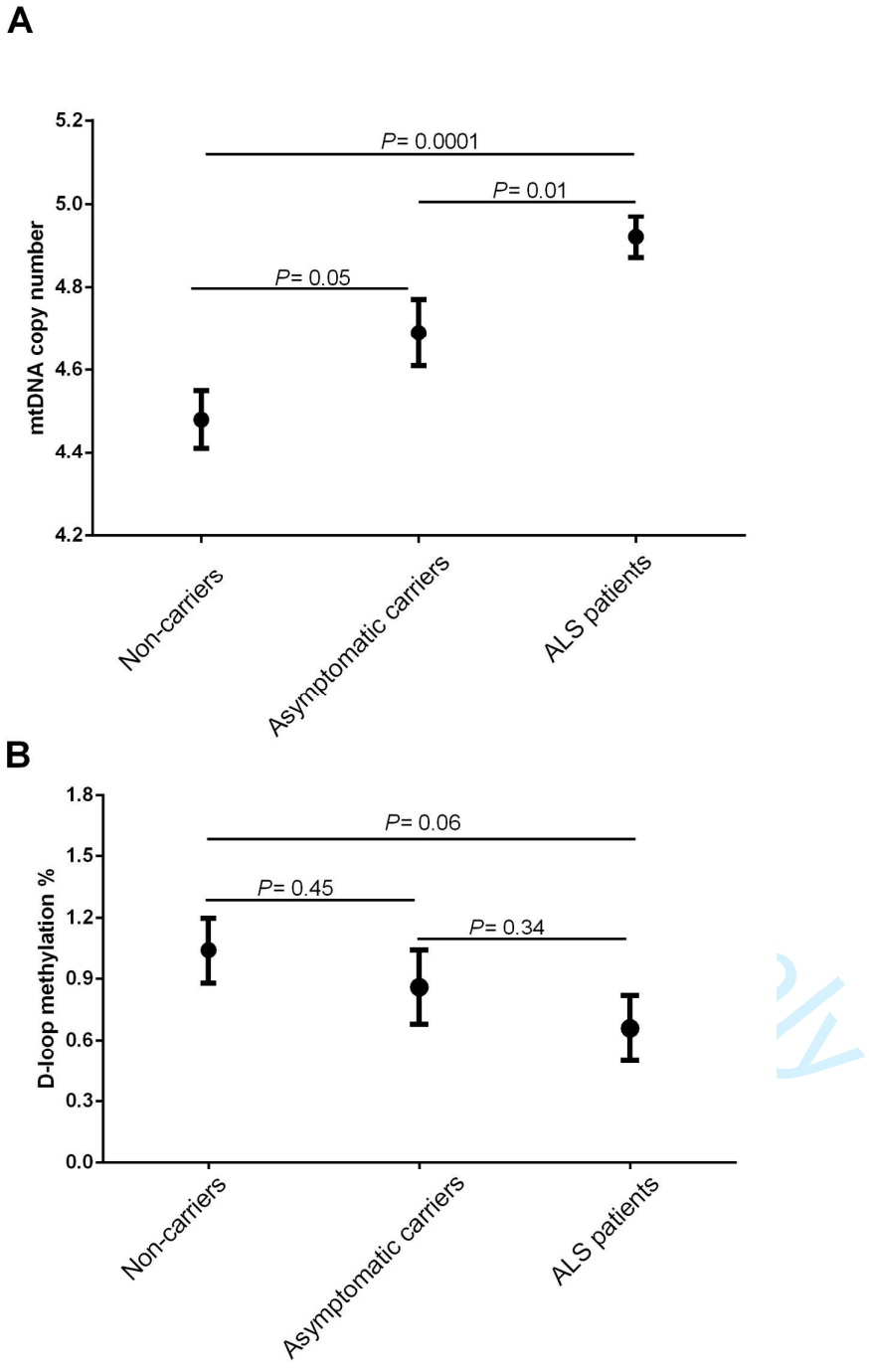


Figure 1. (A) mtDNA copy number in non-carriers of mutations, in asymptomatic carriers and in ALS patients. *P*-value obtained by means of multifactorial analysis of variance adjusted for age at sampling and gender. (B) D-loop methylation levels in non-carriers of mutations, asymptomatic carriers and ALS patients. *P*-value obtained by means of multifactorial analysis of variance adjusted for age at sampling and gender. MtDNA copy number and D-loop methylation levels are expressed as log-transformed data.

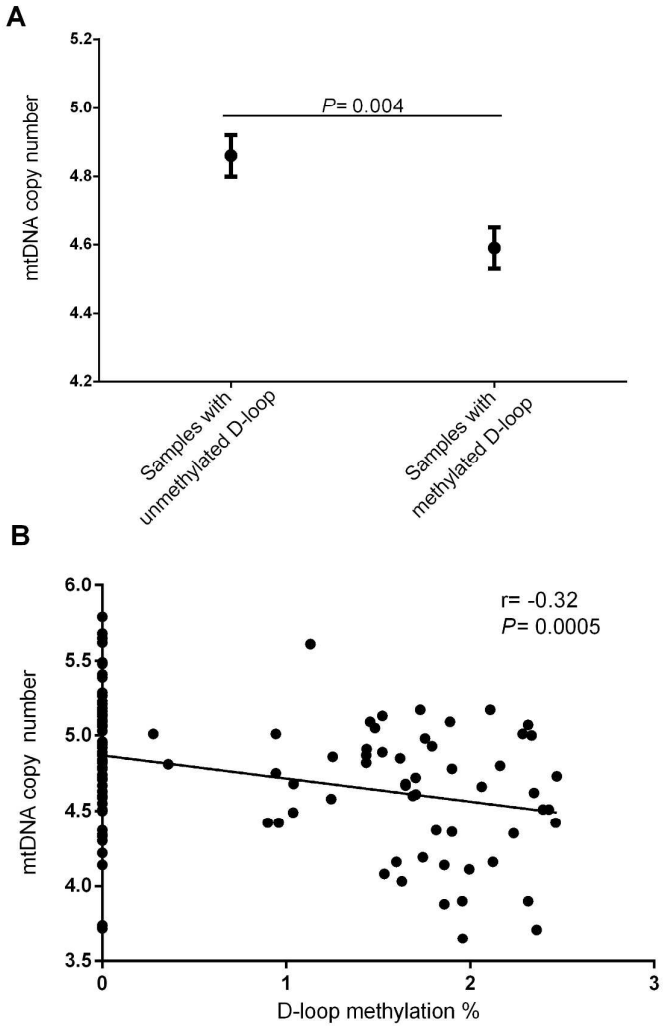


Figure 2. (A) Comparison of mtDNA copy number between samples with unmethylated D-loop and those with methylated D-loop. *P*-value obtained by means of multifactorial analysis of variance adjusted for age at sampling and gender. (B) Correlation between mtDNA copy number and D-loop methylation. MtDNA copy number and D-loop methylation levels are expressed as log-transformed data.

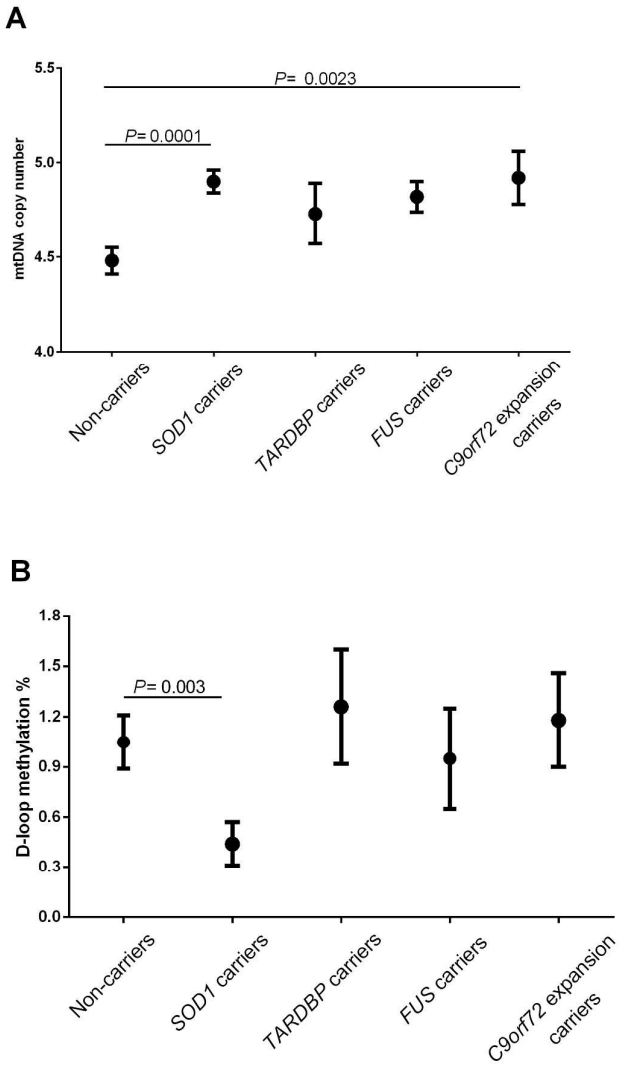


Figure 3. (A) MtDNA copy number in non-carriers of mutations, and in carriers of *SOD1*, *TARDBP*, *FUS*, and *C9orf72* mutations. *P*-value obtained by means of multifactorial analysis of variance adjusted for age at sampling and gender. (B) D-loop methylation levels in non-carriers of mutations, and carriers of *SOD1*, *TARDBP*, *FUS*, and *C9orf72* mutations. *P*-value obtained by means of multifactorial analysis of variance adjusted for age at sampling and gender. MtDNA copy number and D-loop methylation levels are expressed as log-transformed data. Only *P*-values that survived correction for multiple comparisons are shown.

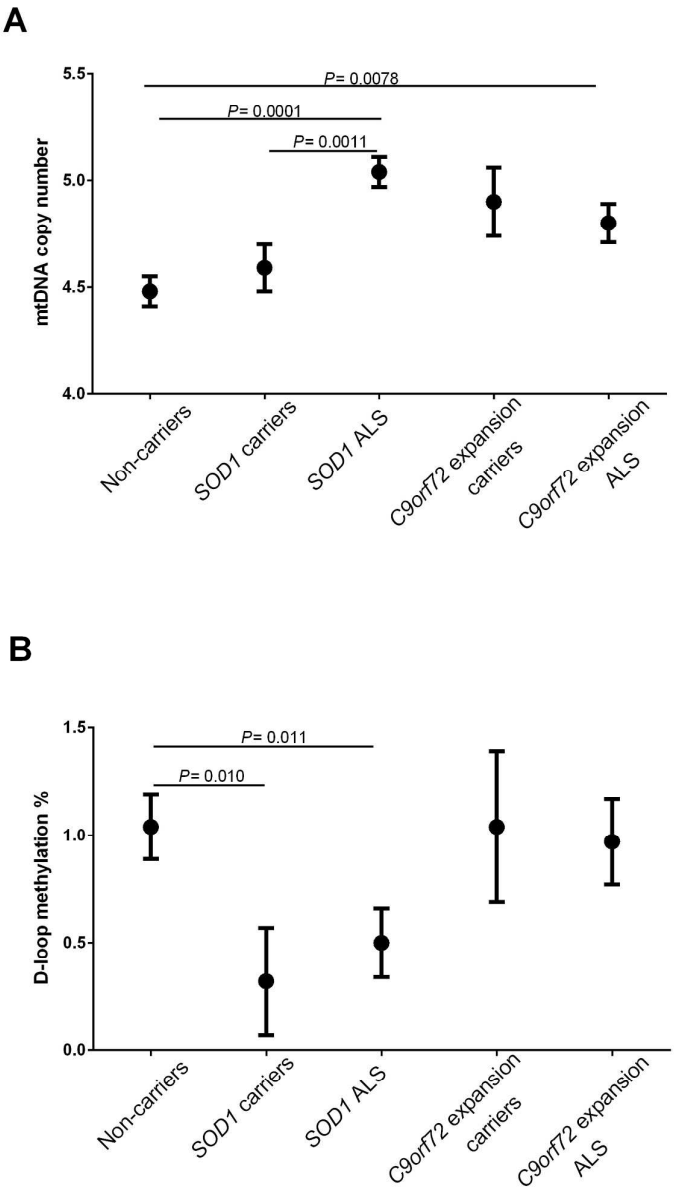


Figure 4. (A) MtDNA copy number in non-carriers of mutations, and in both pre-symptomatic and affected carriers of *SOD1* and *C9orf72* mutations. *P*-value obtained by means of multifactorial analysis of variance adjusted for age at sampling and gender. (B) D-loop methylation levels in non-carriers of mutations, and in both pre-symptomatic and affected carriers of *SOD1* and *C9orf72* mutations. *P*-value obtained by means of multifactorial analysis of variance adjusted for age at sampling and gender. MtDNA copy number and D-loop methylation levels are expressed as log-transformed data. Only *P*-values that survived correction for multiple comparisons are shown.

Mitochondrial DNA copy number and D-loop region methylation in carriers of amyotrophic lateral sclerosis gene mutations

Supplementary material

Details on the carried mutations.

In the current manuscript we collected peripheral blood from 54 ALS patients and 28 asymptomatic carriers with mutations in one of the major ALS genes (*SOD1*, *FUS*, *TARDBP* and *C9orf72*). In Supplementary table 1 are reported the specific mutations carried by ALS patients and asymptomatic carriers.

Supplemetary Table 1. Mutations of the major ALS genes detected in ALS patients and in asymptomatic carriers enrolled in the study, and number of carriers of each mutation.

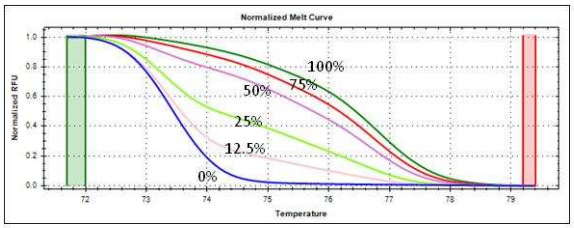
	ALS patients (total n = 54)	Asymptomatic carriers (total n = 28)
Mutations in *SOD1* (total n= 42)		
p.Gly93Asp	n= 6	n= 9
p.Gly72Ser	n= 1	n= 2
p.Asn65Ser	n= 3	n= 1
p.Gly130_Glu133del	n= 1	n= 1
p.Leu144Phe	n= 3	-
p.Leu84Phe	n= 2	-
p.Glu134del	n= 2	-
p.Asp90Ala	n= 3	-
p.His80Arg	n= 1	-
p.Thr137Ala	n= 1	-
p.Ala4Val	n= 1	-
p.Ser59Arg	n= 1	-
p.Gln153Arg	n= 1	-
p.Val5Met	n= 1	-
p.Ile150Thr	n= 1	-
p.Glu121Gly	n= 1	-
Mutations in *TARDBP* (total n=7)		
p.Gly294Val	n= 1	-
p.Ala382Thr	n= 1	n= 3
p.Met359Val	n= 1	n= 1
Mutations in *FUS* (total n=9)		
p.Gly245Val	n= 1	n= 1
p.Arg521Cys	n= 2	n= 4
p.Arg522Gly	n= 1	-
Expansion in *C9orf72* (total n= 24)		
Presence of the GGGGCC hexanucleotide expansion	n= 18	n= 6

Genetic screening for *SOD1*, *TARBP*, *FUS* and *C9orf72 mutations*

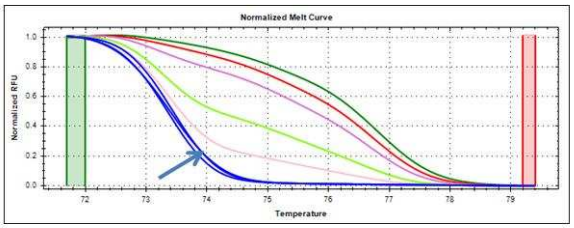
Exons 1-5 of *SOD1*, exon 6 of *TARDBP*, and exons 5, 6, 13, 14, 15 of *FUS* were analyzed by direct sequencing as previously described elsewhere (17, 18, 19). The analysis was performed on an automated 3730 DNA analyzer (Applied Biosystems, Milan, Italy) using the BigDye Terminator Cycle Sequencing Kit version 1.1 or 3.1 (Applied Biosystems, Milan, Italy), depending on PCR products size.

The repeat-primed PCR (RP-PCR) assay was used to identify the presence of the GGGGCC hexanucleotide expansion in *C9orf72* gene as described in the original report (18). ALS-associated pathological expansions in *C9orf72* have been defined as a repeat number greater than 30 and the typical saw-tooth pattern with a 6-bp periodicity in RP-PCR. Fragment-length analysis of non-expanded alleles was also performed through an independent PCR with labeled primers. All PCR fragments were analyzed on an ABI 3730 Genetic Analyzer (Applied Biosystems, Milan, Italy) and data were examined by using the GeneMapper software v.4.0 (Applied Biosystems, Milan, Italy). All positive results of the genetic test were communicated in the presence of a multidisciplinary team including geneticist, neurologist, and psychologist.

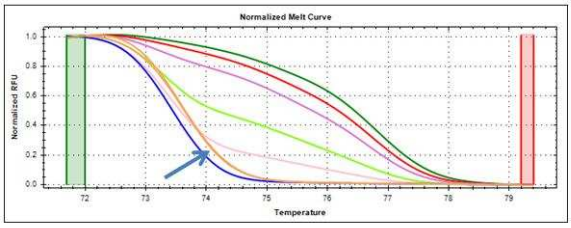
Supplementary Figure 1. Comparison of D-loop methylation levels between circular and linear mtDNA



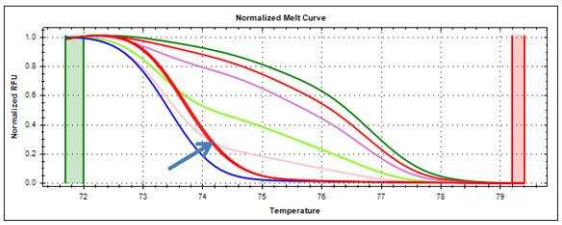
Standard DNA



Sample showing about 0% of methylation



Sample showing about 4% of methylation



Sample showing about 8% of methylation

In order to verify if results obtained after linearization were comparable or not to those obtained with a circular mtDNA structure, we compared MS-HRM curves of samples with different percentages of D-loop methylation levels either digested with *PvuII* to obtain a linear structure, or not digested (circular DNA) before the bisulfite treatment. The figure shows the MS-HRM curves of three samples that showed 0%, about 4% and about 8% of D-Loop methylation, respectively. For each sample we run both digested and non digested DNA. It is evident that pre-digestion with *PvuII* did not affect MS-HRM curves, and samples showed very similar levels of methylation in digested and non-digested DNA as indicated by the overlapping of their melting curves. We can therefore conclude that pre-digestion does not limit the in vivo relevance of the results.