Reduced mtDNA Copy Number in the Prefrontal Cortex of C9ORF72 Patients

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Abstract
Hexanucleotide repeat expansion in C9ORF72 gene is the most common genetic cause of amyotrophic lateral sclerosis and frontotemporal dementia (C9ALS/FTD). Loss of C9ORF72 protein function and a toxic gain-of-function directly by the RNA or RAN translation have been proposed as triggering pathological mechanisms, along with the accumulation of TDP-43 protein. In addition, mitochondrial defects have been described to be a major driver of disease initiation. Mitochondrial DNA copy number has been proposed as a useful biomarker of mitochondrial dysfunction. The aim of our study was to determine the presence of mtDNA copy number alterations in C9ALS/FTD patients. Therefore, we assessed mtDNA copy number in postmortem prefrontal cortex from 18 C9ORF72 brain donors and 9 controls using digital droplet PCR. A statistically significant decrease of 50% was obtained when comparing C9ORF72 samples and controls. This decrease was independent of age and sex. The reduction of mtDNA copy number was found to be higher in patients’ samples presenting abundant TDP-43 protein inclusions. A growing number of studies demonstrated the influence of mtDNA copy number reduction on neurodegeneration. Our results provide new insights into the role of mitochondrial dysfunction in the pathogenesis of C9ALS/FTD.

Keywords mtDNA copy number · C9ALS/FTD · Neurodegeneration · C9ORF72 · ddPCR

Introduction
In 2011, abnormal expansion of a GGGGCC (G4C2) hexanucleotid repeat in a non-coding region of the C9ORF72 gene was identified as the most common genetic cause of familial and sporadic forms of both amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (C9ALS/FTD) [1, 2].

Although the mechanisms of disease of C9ALS/FTD remain unknown, three pathological mechanisms of C9ORF72 gene mutation have been described. First, loss of function of the C9ORF72 protein through haploinsufficiency [1–3]. Second, a toxic RNA gain-of-function suggested by the accumulation of sense and antisense RNA transcripts containing the G4C2 repeat within nuclear foci [4]. Third, gain-of-function by repeat-associated non-ATG (RAN) initiated translation of dipeptide-repeat protein (DPR) from the sense and antisense strand [reviewed in 5]. Five potential DPRs, called “poly(GA),” “poly(GP),” “poly(GR),” “poly(PA),” and “poly(PR),” can be produced by RAN translation [6]. Although the role of DPR in the development of the disease remains uncertain, aggregation of poly(GR) has
recently been observed in the brain of patients and associated with neurodegeneration [7, 8]. Apart from DPRs, post-mortem examinations of C9ALS/FTD brain tissue exhibit cellular inclusions containing transactive response DNA-binding protein 43 kDa (TDP-43). The topographical distribution of this pathology usually determines the phenotype of the disease, i.e., ALS vs FTLD, and correlates with the degree of degenerative changes [9]. Of these features, DPR pathology and nuclear RNA foci are unique and highly specific to C9ALS/FTD. In addition to these three molecular mechanisms, many more downstream cellular pathways have been described to be affected in C9ALS/FTD, including nucleo-cytoplasmic transport, RNA processing, function of nucleolus, formation of membrane-less organelles, translation and ubiquitin proteasome system alterations, and DNA damage repair pathways impairment [5, 10]. Despite all efforts, nowadays it is still unclear which molecular events initiate the disease.

Mitochondrial dysfunction is known to be a hallmark of a broad spectrum of diseases, including neurodegenerative disorders, and variation in the mitochondrial DNA (mtDNA) copy number is used as a biomarker of mitochondrial dysfunction. In fact, it is known that mtDNA levels oscillate in response to changes in the environment surrounding the cell [11], and they have been tested as a non-invasive biomarker for various diseases such as Parkinson’s disease and Alzheimer’s disease [12, 13]. There are several studies supporting a role for mitochondrial dysfunction in the pathogenesis of C9ALS/FTD. It has been shown that poly(GR) in C9ORF72 neurons compromise mitochondrial function and cause DNA damage in part by increasing oxidative stress [14, 15]. Importantly, these in vitro studies revealed that when reducing the levels of poly(GR) or the induced oxidative stress, disease phenotypes were partially suppressed or reversed [14, 15]. Overall, these studies pointed out that mitochondrial defects are a major driver of disease initiation in C9ALS/FTD. In an attempt to provide more insights into the role of mitochondrial dysfunction in C9ALS/FTD, we assessed the mtDNA copy number in human postmortem samples of the prefrontal cortex of C9ORF72 patients in order to decipher its role in the pathogenesis of the disease.

Material and Methods

Subjects

Deep frozen postmortem prefrontal cortex was obtained from 18 C9ALS/FTD brain donors (8 males and 10 females) and 9 controls (5 males and 4 females) with no clinical evidence or neuropathological finding of ALS/FTD or other main neurodegenerative diseases but mild changes commonly observed in the aging brain. All donors were of Caucasian origin. The disease duration, defined as the time between symptoms onset and death, in the C9ALS/FTD group ranged from 1 to 21 years. Table 1 summarizes clinical, molecular, and neuropathological findings of subjects recruited in the study.

No significant difference was observed in sex between both groups (p = 0.6). However, significant differences were detected in terms of C4/C2 repeat length and age. While all C9ALS/FTD samples exhibited a G4C2 repeat expansion greater than 145 repeats, control samples had normal G4C2 repeat size (≤23 G4C2). The mean age ± standard deviation (SD) in years was 79 ± 11 for C9ALS/FTD patients (range 55–88) and 67 ± 9 for controls (range 58–90) (p = 0.004). Therefore, age was taken into account as a co-variable for statistical correction.

The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona. Brain samples were obtained from the Hospital Clinic-IDIBAPS Brain Bank under approved protocols of the Ethical Committee (Hospital Clinic Barcelona, Spain).

Brain Sample Preparation

Total mitochondrial and genomic DNA was extracted for each sample using a method that preserves the in vivo ratio between them as previously described [16]. Briefly, the frozen brain tissue was rapidly homogenized in 1 ml of 100ST-DNA, RNA, and protein solubilization buffer (#DCQ100ST, DireCtQuant, Spain) per 10 mg of brain tissue using Dounce homogenizer with 25 μm clearance. The samples were incubated at 90°C for 3 min and centrifuged at 10,000 rcf for 10 min. The clear supernatant was transferred to a fresh tube and used to measure the absolute mtDNA copy number and nuclear genomes by digital droplet PCR (ddPCR).

Neuropathological Examination

Immunohistochemistry was performed on formalin clear supernatant was transferred to a fresh tube anti-C9RANT (Novus Biologicals, Centennial, CO, USA; dilution 1:1,000) and anti-phosphorylated TDP, Centennial, (Cosmo Bio, Tokyo, Japan; dilution 1:20,000). The DAKO EnVision® detection kit (Dako, Glostrup, Denmark) was used for visualization of antibody reactions and nuclei were counterstained with hematoxylin. The frequency of TDP-43 aggregates was scored following a semiquantitative analysis in the frontal cortex. A 4-point grading scale (0.5 = isolated; 1 = sparse; 2 = moderate, and 3 = abundant) was used to evaluate the severity of TDP-43 immunoreactivity. No attempt was made to score cytoplasmic and neuritic lesions separately, but most cases with a high TDP-43 score had dystrophic neurites in addition to neuronal cytoplasmic inclusions.
| ID sample   | Sex | Age at disease onset (years) | Age at death (years) | C9orf72 (GGGGCC)n | TDP-43 inclusions in prefrontal cortex | Clinical diagnosis                      | Overall neuropathological diagnosis      | NIAAA neuropathological criteria for AD |
|------------|-----|-----------------------------|----------------------|-------------------|----------------------------------------|----------------------------------------|------------------------------------------|----------------------------------------|
| Control_1  | Male| -                           | 78                   | 2,2               | 0                                      | Control                                | Minimal incidental LBP (Braak 1)        | A1B1C0                                 |
| Control_2  | Male| -                           | 83                   | 2,7               | 0                                      | Behavioral disorder                    | Incipient AgD (Saito I)                 | A1B0C0                                 |
| Control_3  | Female| -                         | 81                   | 2,8               | 0                                      | Control                                | Minimal limbic TDP43                    | A3B1C2                                 |
| Control_4  | Male| -                           | 64                   | 2,6               | 0                                      | Control                                | Minimal incidental LBP (olfactory bulb only) | A2B1C1                                 |
| Control_5  | Female| -                         | 90                   | 2,2               | 0                                      | Control, acute brainstem hemorrhage    | Intermediate AD + acute brainstem hemorrhage + mild limbic TDP43 | A2B2C2                                 |
| Control_6  | Male| -                           | 58                   | 2,13              | 0                                      | Control                                | Incipient AgD (Saito I) + chronic metabolic encephalopathy | A0B0C0                                 |
| Control_7  | Female| -                         | 88                   | 2,4               | 0                                      | Control                                | Moderate AgD (Saito II)                 | A2B1C2                                 |
| Control_8  | Female| -                         | 83                   | 5,10              | 0                                      | Control                                | ARTAG + moderate AgD (Saito II) + minimal incidental LBP (Braak 1) | A0B1C0                                 |
| Control_9  | Male| -                           | 86                   | 2,2               | 0                                      | Control                                | ARTAG + moderate AgD (Saito II) + minimal incidental LBP (Braak 1) | A2B1C1                                 |
| C90RF72_1  | Male| 51                          | 61                   | >145              | 2                                      | FTD                                    | FTLD-TDP                               | A0B2C0                                 |
| C90RF72_2  | Female| 65                        | 66                   | >145              | 2                                      | FTD/ALS                                | FTLD-TDP + ALS                         | A0B2C0                                 |
| C90RF72_3  | Female| 68                        | 71                   | >145              | 1                                      | FTD/ALS                                | FTLD-TDP + ALS                         | A1B2C0                                 |
| C90RF72_4  | Female| 63                        | 69                   | >145              | 3                                      | FTD/ALS                                | FTLD-TDP + ALS                         | A1B0C0                                 |
| C90RF72_5  | Male| 61                          | 66                   | >145              | 3                                      | FTD                                    | FTLD-TDP                               | A0B1C0                                 |
| C90RF72_6  | Female| 52                        | 55                   | >145              | 3                                      | FTD                                    | FTLD-TDP                               | A0B1C0                                 |
| C90RF72_7  | Male| 73                          | 80                   | >145              | 1                                      | LBD                                    | FTLD-TDP + LBP (limbic)                | A0B2C0                                 |
| C90RF72_8  | Male| 55                          | 69                   | >145              | 2                                      | FTD                                    | FTLD-TDP + HS                          | A0B0C0                                 |
| C90RF72_9  | Female| 53                        | 57                   | >145              | 0.5                                     | ALS                                    | ALS-TDP + incipient AgD (Saito I)       | A0B0C0                                 |
| C90RF72_10 | Female| 65                        | 75                   | >145              | 0.5                                     | FTD                                    | FTLD-TDP                               | A1B2C1                                 |
| C90RF72_11 | Female| 55                        | 58                   | >145              | 3                                      | FTD/ALS                                | FTLD-TDP + ALS                         | A1B2C0                                 |
| C90RF72_12 | Male| 72                          | 73                   | >145              | 2                                      | AD vs LBD                              | FTLD-TDP                               | A0B2C0                                 |
| C90RF72_13 | Female| 67                        | 69                   | >145              | 0.5                                     | LBD vs CBS                             | FTLD-TDP + ALS + intermediate AD       | A3B2C2                                 |
| C90RF72_14 | Female| 56                        | 57                   | >145              | 3                                      | FTD/ALS                                | FTLD-TDP                               | A1B1C0                                 |
| C90RF72_15 | Male| 52                          | 55                   | >145              | 3                                      | ALS/FTD                                | ALS + FTLD-TDP                         | A0B1C0                                 |
| C90RF72_16 | Male| 75                          | 80                   | >145              | 1                                      | DLB                                    | LBP (limbic) + FTLD-TDP                | A1B1C0                                 |
| C90RF72_17 | Male| 65                          | 69                   | >145              | 2                                      | FTD                                    | FTLD-TDP + ALS                         | A0B1C0                                 |
| C90RF72_18 | Female| 35                        | 61                   | >145              | 3                                      | FTD                                    | FTLD-TDP + LBP (limbic) + incipient AgD (Saito I) | A2B1C1                                 |

AD Alzheimer’s disease, AgD argyrophilic grain pathology, ALS amyotrophic lateral sclerosis, CBS Cortico basal syndrome, FTD frontotemporal dementia, FTLD-TDP frontotemporal lobar degeneration with TDP-43 immunoreactive pathology, HS hippocampal sclerosis, LBD Lewy body dementia, LBP Lewy body pathology, NIAAA National Institute of Aging-Alzheimer’s Association [37]
**mtDNA Copy Number Assessment by Digital Droplet PCR**

Digital droplet PCR (ddPCR) technology was chosen to measure the absolute number of mtDNA copies per diploid genome as previously described [16]. Briefly, mtDNA copy number was measured using QX200 Droplet Digital PCR System following the manufacturer’s recommendations (Bio-Rad Laboratories, Inc.). In order to normalize mtDNA copies relative to nuclear DNA, two genes, TBP-1 and TEFM, were used as reference genes.

MtDNA was amplified directly in human brain solubilized samples with the 5 multiplexed directly in human brain solubilizable DNA Digital PCR System from primers to amplify a 92 bp fragment from the human mitochondrial sequence. The amount of genomic DNA was measured in multiplex fluorescent intensity assay using two single-copy genes located in different chromosomes thus controlling unlikely event of copy number variation of one of the single-copy genes. TEFM-1 was amplified with 50k event of copy number variation of one of the singlem following single event gTBP-1 amplified with 50k event of copy number variation of one of the singlem follow The ddPCR experiments were performed using EvaGreen based assay following the manufacturer’s recommendations (Bio-Rad Laboratories).

The presence or absence of amplification per droplet was evaluated using a QX200 Droplet Reader and analyzed using the QuantaSoft Analysis Pro (Bio-Rad Laboratories) software. An assumption that a diploid genome contains 2 copies of each single-copy gene TBP-1/TEFM was made. mtDNA copy number was calculated by dividing the number of mtDNA copies detected by number of diploid genomes (measured by TBP-1/TEFM).

**Statistical Analysis**

Results were expressed as mean ± standard error means (SEM). For comparisons of the means, the statistical significance of the differences was examined using general linear model univariate analysis, the parametric independent t-test, or the non-parametric Mann–Whitney U and Kruskal–Wallis tests. Correlations were studied using Pearson’s correlations. All statistical analyses were performed by the SPSS program, version 25 (SPSS, Inc. Chicago, IL, USA). Significance was accepted for p-value < 0.05.

**Results**

We studied the mtDNA copy number in postmortem prefrontal cortex obtained from 18 C9ALS/FTD patients compared to 9 control samples. The amount of mtDNA copy number was analyzed by ddPCR and results are shown as the mtDNA copy number per diploid genome (Fig. 1). A statistically significant decrease was observed when comparing C9ALS/FTD samples (3923 mtDNA copies/diploid genome ± 566) with controls (7364 mtDNA copies/diploid genome ± 909) (p = 0.003) (Fig. 1). Moreover, taking into account the age of individuals as a co-variable, the reduction in the mtDNA copy number in C9ORF72 prefrontal cortex was statistically significant (linear model univariate analysis C9ALS/FTD vs controls with age as a covariate, marginal means for C9ALS/FTD: 3818 ± 648 and controls: 7574 ± 968; p = 0.006). We further performed Pearson’s correlation analysis to determine the association between mtDNA copy number and age in C9ALS/FTD patients and controls. There was no correlation between mtDNA copy number and age (R² = 0.05, p = 0.26).

Having shown a significant 50% decrease in the mtDNA copy number in prefrontal cortex of C9ALS/FTD patients, we investigated whether this decline was correlated with the abundance of DPR inclusions or TDP-43 aggregates. Neuropathological examination evidenced DPR inclusions and TDP-43 aggregates in all frontal cortices of C9ALS/FTD patients. While no differences could be determined regarding the abundance of DPR inclusions since all samples exhibited high abundance of these inclusions across all cortical layers, the semiquantitative analysis of TDP-43 aggregates showed differences in its frequency (Fig. 2). The correlation analysis with mtDNA copy number evidenced that C9ALS/FTD patients with isolated or sparse TDP-43 inclusions (n = 6) showed a reduction of 35% in the mtDNA copy number compared to controls (Fig. 3 and Table 2). This reduction was more evident in the group of C9ALS/FTD patients with moderate/severe inclusions (n = 12), in which a mtDNA copy number reduction of 63% was obtained when comparing to the control group and of 42% when comparing to C9ALS/FTD patients with isolated or sparse TDP-43 inclusions (Fig. 3 and Table 2).

Statistical analysis identified only significant differences when comparing mtDNA copy number between controls.
and C9ALS/FTD patients with moderate/abundant TDP-43 inclusions ($p = 0.009$) (Fig. 3 and Table 2). These results suggest that C9ALS/FTD patients with isolated/sparse TDP-43 inclusions in prefrontal cortex brain region might represent an intermediate state in terms of neuronal cell degeneration or death.

Finally, we further analyzed whether there was a relationship between mtDNA copy number and clinical manifestation of C9ORF72 expansion patients. Patients were divided into three groups based on clinical manifestations: C9ALS, C9FTD, and C9ALS/FTD. Although no statistically significant differences were obtained, those patients diagnosed as

![Image](https://example.com/image.png)
C9ALS/FTD showed the lowest mtDNA copy number value (Fig. 4).

Discussion

Mitochondria are subcellular organelles which have multiple important roles, including ATP production, Ca\(^{2+}\) homeostasis, and reactive oxygen species (ROS) production [reviewed in 17]. Each mitochondrion contains multiple copies of mtDNA that encode proteins involved in oxidative phosphorylation. Mitochondrial DNA integrity is necessary for preserving cellular energetics, and in fact, several quality control systems neutralize processes that lead to mitochondrial dysfunction [18]. Loss of mtDNA integrity by altered mtDNA copy number and increased mutations is implicated in cellular dysfunction with aging and disease [19]. A growing number of studies demonstrated the influence of mtDNA copy number reduction on neurodegeneration [e.g., 20–22].

Although the exact pathogenetic mechanism of C9ALS/FTD is still elusive, several evidences suggest that damaged mitochondrial likely plays a fundamental role [23–25]. The relevance of DPR pathology in causing C9ALS/FTD has also been widely discussed and it is currently believed that its production is a key molecular event downstream of C9ORF72 repeat expansion [24, 26–28]. In C9ORF72 cellular and animal models, it has been reported that DPR proteins have a toxic effect in an accumulative and age-dependent manner, compromising several cellular pathways such as mitochondrial function and causing DNA damage by increasing oxidative stress [7, 8, 14, 15, 29]. It has been suggested that DPR protein aggregation precedes and instigates TDP-43 pathology [30, 31].

This study investigated the changes in mtDNA copy number in prefrontal cortex of patients with C9ALS/FTD. Additionally, the study evaluated the relationship between mtDNA copy number and the clinical phenotype. For these purposes, we used a ddPCR technique since it is a highly sensitive and specific method for absolute quantification of mtDNA copy number and the ratio of mtDNA per cell. Results showed a statistically significant mtDNA copy number reduction of 50% in C9ALS/FTD patients compared to controls \((p = 0.006)\). However, the analyzed tissues contain a mixture of several cell types of the brain, making it difficult to attribute this reduction to a particular one. Therefore, our results might reflect an average number attributable to all cell types. Nevertheless, since neurons rely more on oxidative phosphorylation to meet their energy requirements than the oligodendrocytes, astrocytes, and microglia [32], on the one hand, and the other, abnormal TDP-43 protein aggregates observed in our C9ORF72 expansion cases were predominantly neuronal [33], we speculate that the decreased mtDNA copy number herein reported may have a greater effect on neurons. All our samples showed marked DPR pathology whereas the frequency of TDP-43 aggregate was different among the C9ALS/FTD samples analyzed. Even the sample size was relatively small, we found a positive correlation between the abundance of TDP-43 inclusions and the mtDNA copy number decrease, meaning that those patients presenting with moderate or abundant TDP-43 inclusions showed an even more pronounced mtDNA copy number reduction than those with isolated or sparse inclusions (Fig. 3). The relationship between mitochondrial dysfunction and TDP-43 proteinopathy has been previously documented. Alterations in mitochondrial dynamics, trafficking, and mitophagy have been reported in association with TDP-43 pathology [reviewed in 34]. To our knowledge, this is the first study that shows a reduction of mtDNA copy number in human C9ALS/FTD postmortem brain samples that correlates with the abundance of TDP-43 inclusions.
Our results might reflect a late stage of mitochondrial impairment and suggest that TDP-43 inclusion formation contributes in an accumulative manner reducing the mtDNA content and finally further compromising survival or disease progression. However, our data do not exclude that other pathologies, like DPRs, also contribute to these changes.

The C9ORF72 expansion is associated with clinical heterogeneity, with affected patients displaying symptoms of either ALS or FTD, or mixed features of both. Although several factors have been analyzed, such as the repeat expansion length and the distribution and amount of TDP-43 and DPR protein aggregation, results are inconclusive and the molecular basis for this clinical variation is still unknown [31]. Reports on clinical and neuropathological correlation with DPR protein pathology have been conflicting. While some authors found no relationship between specific DPR species and clinicopathologic subtypes of C9ORF72-related disease [35], others describe either a correlation with DPR distribution [36] or neurodegeneration [8]. In our experience, the distribution of TDP-43, and not DPR pathology, determines the clinical phenotype (unpublished data). In this line, although our results need validation in a larger series of cases, they showed a positive tendency correlation between the mtDNA copy number and the clinical phenotype. mtDNA copy number in the prefrontal cortex of C9ORF72 patients was lower in cases with ALS/FTD than in cases with ALS and FTD (Fig. 4). Thus, although interpreted with caution, these results suggest that an impaired mitochondrial function could be more pronounced in C9ORF72 patients with ALS/FTD phenotype.

Nowadays, mtDNA copy number has been described as an attractive non-invasive biomarker for various neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease, and FXTAS [16]. In order to promote its usage for disease biomarker, it would be necessary to characterize mtDNA copy number in living symptomatic and asymptomatic C9ORF72 expansion carriers using minimally invasive approaches such as from peripheral blood.

Conclusions

Together, our results are in agreement with similar reductions in mtDNA copy number reported in a number of other age-related neurological disorders, where mitochondrial dysfunction is also a pathogenic hallmark. We have observed a significant reduction of mtDNA copy number in C9ALS/FTD patients, particularly associated with the amount of TDP-43 pathology. This novel finding might help to unravel the role of mitochondrial dysfunction in the pathogenesis of C9ALS/FTD. Overall, these data suggest that the maintenance of mitochondrial genome copy number may play a crucial role in replacing damaged mitochondrial complexes to preserve cellular viability under stress conditions. The increased understanding of C9ALS/FTD pathogenesis might help to unravel therapeutic targets that can be applied at early stages of the disease.

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Author Contribution Maria Isabel Alvarez-Mora: formulation and evolution of overarching research goals. Preparation and presentation of the published work

Petar Podlesniy: development and design of methodology

Teresa Riazuelo and Nuria Serra: conducting research and investigation process

Laura Molina: provision of study materials and preparation of the published work

Ellen Gelpi: preparation and presentation of the published work and critical review.

Laia Rodriguez-Revenge: formulation and evolution of overarching research goals. Preparation and presentation of the published work

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Availability of Data and Material The datasets of the current study are available upon request with no restriction.

Code Availability Not applicable.

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Laia Rodriguez-Revenge: formulation and evolution of overarching research goals. Preparation and presentation of the published work

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Availability of Data and Material The datasets of the current study are available upon request with no restriction.

Code Availability Not applicable.

Declarations

Ethics Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona. Brain samples were obtained from the Hospital Clinic-IDIBAPS Brain Bank under approved protocols of the Ethical Committee (Hospital Clinic Barcelona, Spain).

Consent for Publication Not applicable.

Consent to Participate Not applicable.

Conflict of Interest The authors declare no competing interests.

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