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MITOCHONDRIAL ABERRATION AND THEIR CONNECTION TO NOTCH 3 GENE MUTATIONS IN CADASIL PATIENTS

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ABSTRACT

CADASIL patients with mutations in the *NOTCH3* nuclear gene has shown ultrastructural and biochemical abnormalities of the mitochondria in their skeletal muscle biopsies suggesting that Notch signaling pathway may directly or indirectly influences the mitochondrial respiration. In this study five CADASIL patients belonging to five unrelated pedigrees were investigated to detect any mitochondrial abnormalities in their circulating leucocytes which may help to eliminate the need for invasive muscle biopsies. Entire mitochondrial coding region was sequenced in both directions for these CADASIL patients; also their mitochondrial respiratory activity (MRA) in circulating leukocytes was assessed and relative mtDNA content was quantified. The results showed that in comparison with ethnicity matching healthy controls (n=159), mean number of mtDNA sequence variants both synonymous and nonsynonymous in CADASIL patients was not statistically significant (p=0.378). No novel pathologic mitochondrial DNA (mtDNA) mutation(s) were found in the patients after excluding haplogroup specific single nucleotide polymorphisms (SNPs). No significant difference was seen in relative mtDNA content between patients and controls (p=<0.32). Mean MRA depicted a significant decline of 22% in CADASIL patients when compared with ethnicity and demographically matching controls (p=< 0.001). In conclusion, this study enforces that hypothesis that Notch3 gene mutation are linked to mitochondrial abnormalities and both act in a synergetic manner to develop CADASIL. The exact mechanism is worth investigating further and may shed light on how neuro-signaling interact with mitochondrial energetic to develop neurological diseases.

INTRODUCTION

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is more common that initially thought and is caused by mutations in the *NOTCH3* gene, which encodes for the transmembrane receptor *Notch3* [1]. The disease belongs to a family of disorders called the Leukodystrophies. The most common clinical manifestations are migraine headaches and transient ischemic attacks or strokes, which usually occur between 40 and 50 years of age, although MRI is able to detect signs of the disease years prior to clinical manifestation of disease. The mean age of onset of ischemic episodes is approximately 46 years (range 30–70) and the ischemic strokes typically occur in the absence of traditional cardiovascular risk factors. Recurrent silent strokes, which is a common feature of CADASIL, leads to cognitive decline and overt subcortical dementia. When muscle and nerve biopsies from CADASIL patients were examined under electron microscope, enlarged mitochondria with needle-like calcium precipitates were observed [2]. Another study reported mitochondrial impairments in a CADASIL patient with frameshift deletion in the *Notch3* gene where muscle biopsy and brain magnetic resonance spectroscopic imaging revealed ultrastructural mitochondrial abnormalities and increased parenchymal brain lactate respectively [3].

Finnila and others reported a patient having NOTCH3 mutation and a mitochondrial mutation (5650 G>A) in the tRNA (Ala) gene with less abundance of mitochondria and structural impairment of mitochondrial network in fibroblasts and myoblasts obtained from a patient; it can be assumed that co-occurrence of these two mutations is not coincidental and NOTCH3 mutation may be a mutation sin mtDNA [4].

77 CADASIL patients and 192 matching controls were sequenced for the whole mtDNA coding region revealing higher number of polymorphisms among patients when compared to the controls [5]. Muscle biopsy of a Spanish CADSAIL patient and histochemical analysis of his affected family members, showed significant decrease in the activity of complex I and presence of ragged-red fibers with abnormal cytochrome c oxidase staining respectively [6]. Dotti et al. did not to detect any common pathogenic mtDNA mutations in DNA sample extracted from the skeletal muscles of a CADASIL patient [3].

Since mitochondrial dysfunctions can be detected in circulating leukocytes of patients with various mitochondrial disorders [7-10] in this study we examined different mitochondrial parameters in leukocytes of patients with CADASIL with and without *NOTCH3* mutation to study its importance as a noninvasive procedure in detecting mitochondrial abnormalities.
Following parameters are included in this study: i) sequencing the entire mtDNA coding region, ii) comprehensive pathogenicity evaluation of detected mtDNA sequence variants, iii) measurements of relative mtDNA content and iv) assessing mitochondrial respiration activity.

MATERIALS AND METHODS

Patients and Controls

A brief Inclusion Criteria form (see below) for the disease was mailed to all members of the Pan Arab Union of Neurological Sciences (PAUNS) requesting them to identify cases and participants for this study. Families were included when an index case had both a history of transient ischemic attacks (TIA) or subcortical stroke of unidentified etiology, positive family history of stroke with early death or dementia compatible with autosomal dominant traits, and a cranial MRI scan showing diffuse or focal microangiopathic white matter abnormalities. Five patients, from five unrelated families with pure Arabic ethnic backgrounds fulfilled the inclusion criteria.

Family (A) and (B) were from Saudi Arabia, Family (C) was from Kuwait, Family (D) was from the Sudan and finally, Family (E) was from Yemen (see Table). Blood donors from king AbdulAziz University Hospital (KAUH) with matching ethnicity and demographics were recruited as healthy control subjects (n= 159). The subjects reported no symptomatic metabolic, genetic, ocular or neurological disorders on an extensive questionnaire about family history, past medical problems and current health.

Clinical Assessment

Detailed assessment of various risk factors and clinical evaluations was done for all subjects, their siblings and other available family members (Figure 1). All previous and current imaging results were collected. In one family (Family B) MRI brain scans were performed in all sib ships of the index case. The proband in Family B was diagnosed as possible CNS angiitis, so brain and meningeal biopsy was performed and showed small vessels angiopathy with multiple osmiophilic, granular electron-dense material (GOM) without atherosclerosis or amyloid deposition. Detailed clinical evaluations of these patients has been previously described [11].

Sample collection and DNA extraction

After obtaining required consent from all participating individuals 10ml of blood were collected in EDTA tubes. PUREGENE DNA isolation kit from Gentra Systems (Minneapolis, USA) was used for DNA extraction from whole blood samples of all CADASIL patients.

Isolation of lymphocytes from peripheral blood and preparation of cell suspension

Phosphate buffered saline (PBS) was mixed with 5ml blood sample at a ratio of 1:1 within 1 hr of extraction and slowly layered onto a 4.5 ml Ficoll-Hypaque separating solution contained in 15 ml screw cap tube. The tubes were centrifuged for 20 min at 1000xg to isolate the lymphocyte-containing layer which was collected using a sterile pipette. After adding the lymphocyte mix it was further diluted in 10 ml PBS and centrifuged for 10 min at 660xg. 5 ml of hypotonic PBS lysing buffer was added after discarding the supernatant and the pellet was gently suspended in it for about 45 seconds. 5 ml of 2 x NaCl solutions was added to the mixture and then gently mixed by pipetting. The suspension was further centrifuged at 600xg for 10 min and the supernatant is discarded. The pellet is then suspended in RPMI-1640 medium (Gibco, Invitrogen Corporation) supplemented with L-glutamine. The optical density (OD 660) of the lymphocyte suspension was adjusted to 0.20, which is equivalent to a cell density of approximately $5x10^5$ cells/ml. Using this protocol, cell viability assessed by 0.2% trypan blue was 96 ± 2%. These cells were used for mitochondrial respiration testing.

Mutation analysis of the NOTCH3 gene

DNA from patients and their families was amplified using primers designed to amplify the 33 exons, including the intron-exon boundaries, of the *NOTCH3* gene. The same amplification primers were also used for sequencing. Sequencing was carried out using the Dynamic Terminator Reagent Sequencing kit [Amersham Pharamcia, US]. The samples were then run on the DNA analyzer [MegaBACE 1000 Capillary system; Molecular Dynamics, Amersham Pharmacia Biotech]. Data from the analyzer were analyzed using the Chromas-Pro version

1.34 (Technelysium, Pty, Ltd, Australia). We also investigated the frequency of each detected mutation in our normal controls. In this study, we excluded known proven silent-polymorphisms found in patients and controls from reporting and further analysis.

DNA Amplification and Sequencing

The entire coding region of the mitochondrial genome was amplified in all patients and controls in 24 separate polymerase chain reactions (PCRs) using single set cycling conditions as detailed elsewhere [12]. Primers were used to amplify the entire coding region of the mitochondrial genome except the D-loop [13]. PCRs were run under the following PCR conditions: 20 ng of each DNA sample in a 50 ml PCR reaction mixture containing 200 mM dNTP, 0.2 mM of each primer-pair, 1 unit of TaqDNA polymerase, 50 mM KCL, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.3).

Polymerase chain reaction was performed for 35 cycles and 55°C annealing temperature in a GeneAmp 9700 PCR system (Perkin-Elmer, Foster City, CA). PCR-Primers were designed to avoid amplifying mtDNA-like sequences in the nuclear genome. Each successfully amplified fragment was directly sequenced using the same primers used for amplifications and the BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Samples were run on the ABI prism 3100 sequencer (Applied Biosystems). A similar sequencing protocol was followed for the nuclear genes.

Sequence Analysis of the Mitochondrial DNA Coding Region

The full mtDNA genome was sequenced except for the D-loop. Sequencing results were compared to the corrected Cambridge reference sequence [13]. All fragments were sequenced in both forward and reverse directions at least twice for confirmation of any detected variant. All sequence variants from both CADASIL patients and controls were compared to the Mitomap database [14], the Human Mitochondrial Genome Database, GenBank, and Medline listed publications. Reported homoplasmic synonymous or non-synonymous (NS; resulting in amino acid change) polymorphisms used predominantly for haplogroup analysis were excluded from further consideration [15].

Prediction of Pathogenicity

Pathologic characteristics of each NS sequence change, in both CADASIL patients and controls, were estimated according to a combination of: standard pathogenicity criteria [16]; an evaluation of interspecies conservation using the PolyPhen database (http://genetics.bwh.harvard.edu/pph/), and the Mamit-tRNA website (http://mamit-trna.u-strasbg.fr/index.html). The following was performed when necessary: assessment of the possible impact of an amino acid substitution on three-dimensional protein structure using the Protean program, part of the LASERGENE V.6 software (DNASTAR, Inc. Madison, WI, USA), which predicts and displays secondary structural characteristics; assessment of the possible effect of the mtDNA change on protein function using PolyPhen [17] and the SIFT (Sorting Intolerant From Tolerant) program (http://blocks.fhcrc.org/sift/SIFT.html), which predicts whether protein substitutions are tolerated [18].

Therefore, a NS nucleotide change was considered potentially pathologic, if it met all of the following criteria, when applicable: 1) it was not found in at least 100 controls of matching ethnicity; 2) it changed a moderately or highly conserved amino acid; 3) Protean predicted an alteration of protein structure; 4) it was assessed as possibly or probably pathologic by PolyPhen; 5) it was not established as one of the haplogroup-specific SNPs and 6) It was not reported as an established polymorphism in MITOMAP database (last updated August 2007), the Human Mitochondrial Genome Database (http://www.genpat.uu.se/mtDB; last updated August 2007), GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html; last updated August 2007), and Medline listed publications.

Determination of Relative Mitochondrial DNA Content

A competitive multiplex PCR was carried out with two simultaneous primer sets as described previously [19], a technique that has been applied successfully to a variety of tissues [20, 21], including blood of patients with LHON [22] and a number of other optic neuropathies [23-25]. One primer-pair was designed to amplify a 450 bp fragment of the ND1 mitochondrial gene (forward primer sequence 5'-ACA TAC CCA TGG CCA ACC TC-3' and reverse primer sequence 5'-AAT GAT GGC TAG GGT GAC TT-3') and the other pair to amplify a 315 bp fragment of the ß-actin nuclear gene (forward primer sequence 5'-ATG TTT GAG ACC TTC AAC AC-3' and reverse primer sequence 5'-CAT CTC TTG CAC GAA GTC GA-3'), which served as an internal control.

Control and patient PCRs were run simultaneously under the following PCR conditions: 20 ng of each DNA sample in a 50 ml PCR reaction mixture containing 200 mM dNTP, 0.2 mM of each of the ND1 primer-pair, 0.6 mM of each of the ß-actin primer-pair, 1 unit of Taq DNA polymerase, 50 mM KCL, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl (pH 8.3). Polymerase chain reaction was performed for 25 cycles in a GeneAmp 9700 PCR system (Perkin-Elmer). One ml of SYBR® Green I stain was added to the reaction mixture in the last cycle to label the PCR products. PCR products were separated on 1% agarose gel at 100 V for 1 h, and intensity of the two bands was quantified by the use of gel imager (Typhoon 9410; GE Amersham Biosciences, Schenectady, NY).

The ratio of ND1 to ß-actin was determined for each patient and control by dividing the fluorescence intensity of the ND1 band by the intensity of the ß-actin band.

Measurement of Mitochondrial Respiratory Activity

Resazurin is a redox-active blue dye that becomes pink and highly fluorescent when reduced. It competes with oxygen for electrons in a standard preparation of circulating lymphocytes, and change in fluorescence (corrected for background and protein concentration) reflects respiration. Lymphocytes from patients and controls were incubated with 6 μM Resazurin without and with mitochondrial inhibition by amiodarone 200 μM, and the fluorescence intensity resulting from Resazurin reduction was monitored spectrofluorimetrically over time.

Mitochondrial Respiratory Activity (MRA) was calculated as the difference between uninhibited and inhibited measurements at 240 minutes, taken in triplicate, averaged, and normalized for protein concentration and background activity, as described previously [10]. Mitochondrial metabolic activity has been assessed using Resazurin in synaptosomes from spinal cord-injured animals [26] and neonatal rat cerebellum [27] and in isolated yeast mitochondria [28]. The current technique has been validated in systemic mitochondrial disorders [10] including LHON-like optic neuropathies [23].

Figure 1. Family Pedigrees of five Arab families with CADASIL

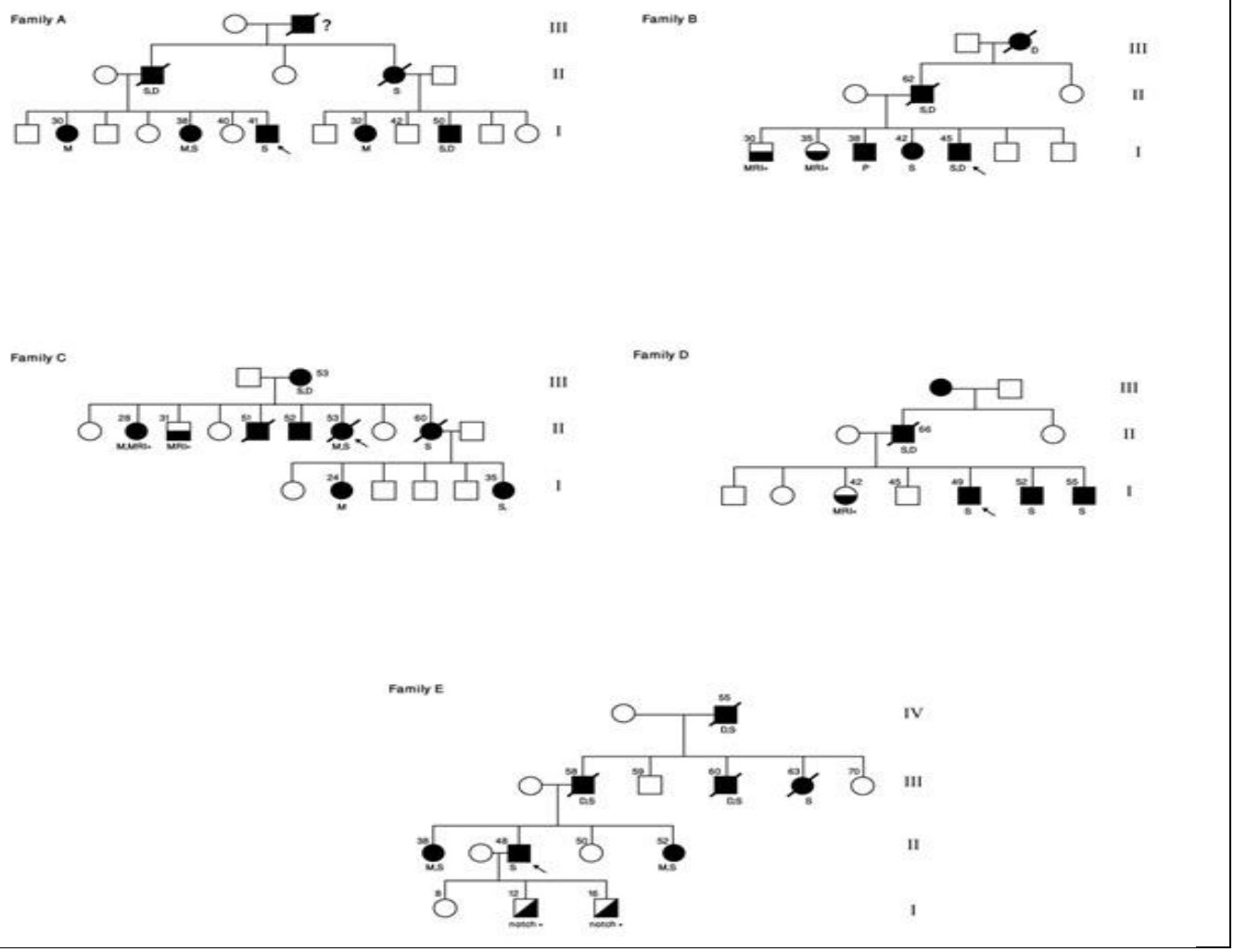

Table 1. Non-synonymous mtDNA sequence changes detected in CADASIL patients

Nucleotide substitution	Amino Acid substitution	Base substitution type	Location	Hetero-plasmy (%)	Interspecies conservation	PolyPhen prediction	Pathogenicity prediction
3851 C>G	A182G	Transversion	TM domain of ND1 gene	N/A	Moderate	Benign	Non-pathologic
14113 T>C	F593L	Transition	TM domain of ND5 gene	N/A	Low	Benign	Non-pathologic

14171 A>G	I168T	Transition	TM domain of ND6 gene	N/A	Low	Benign	Non-pathologic
14966 A>C	N74H	Transversion	Outside the functional domain of	N/A	High	Benign	Non-pathologic
15048 G>C	G101A	Transversion	Outside the functional domain of	N/A	Moderate	Benign	Non-pathologic

Base substitution type – Transversion = A mutation in which a purine/pyrimidine replaces a pyrimidine/purine base pair or vice versa [G:C > T:A or C:G, or A:T > T:A or C:G]; Transition = A mutation in which a purine/pyrimidine base pair is replaced with a base pair in the same purine/pyrimidine relationship [A:T > G:C or C:G > T:A]. Interspecies conservation was assessed using PolyPhen (http://genetics.bwh.harvard.edu/pph/), which determines interspecies conservation for an altered amino acid by performing alignment with all available amino acid sequences for other species. Pathogenicity prediction = A sequence variant was considered potentially pathogenic if it satisfied all the condition detailed in methods. None of above listed non-synonymous sequence changes were found in ethnicity-matched.

Table 2. Summary of the *Notch3* mutations and mitochondrial abnormalities detected in CADASIL patients

Family	Ethnicity	*Notch3* mutation detected	NS[+] mtDNA sequence changes	Relative mtDNA content[•]	MRA [#]
A	Saudi	c.406 C>T	14113	1.25	17.80
B	Saudi	ND	3851	1.20	17.30
C	Kuwait	c.406 C>T	14171	1.22	15.80
D	Sudan	ND	15048	1.19	16.20
E	Yemen	c.475 C>T	14966	1.15	13.20

ND= not detected, no mutation detected after sequencing the full *Notch3* gene

[+] All non-synonymous (NS) mtDNA sequence changes reported above were not detected in 159 matching healthy controls.

[•] Relative mtDNA content: the optimum value of the ratio to distinguish between CADASIL patients and controls was 1.21, see methods.

[#] MRA= Mitochondrial Respiratory Activity.

RESULTS

Clinical findings and *NOTCH3* mutation detected in these CADSIL patients have been described previously [11, 29] in details. After sequencing the whole *NOCTH3* gene, we detected pathogenic mutation [c.406C>T (p.Arg110>Cys)] in exon 3 of the *NOTCH3* gene in the probands of Families A and C. In Family E, we detected another reported pathogenic mutation [c.475C>T (p.Arg133>Cys)] in exon 4 of the gene. While, In Families B and D, we could not detect any reported or potentially pathogenic sequence change after sequencing the full *NOTCH3* gene [29].

Here, we sequenced the full mtDNA coding region in five CADASIL patients and 159 ethnicity matching controls. The mean number of sequence changes, relative to the Cambridge reference sequence, detected in CADASIL patients (n= 5) was 18 sd 0.707 and among healthy controls (n=159) was 17.4 sd 1.51. The difference between the two groups was not statically significant (p=0.378).

Table 1 details the five non-synonymous mtDNA sequence changes found in CADASIL patients after excluding all synonymous mtDNA changes, established non-synonymous polymorphisms, and non-synonymous mtDNA sequence changes relevant primarily to haplogroup designation. None of these non-synonymous mtDNA sequence changes were novel (not previously reported) and all were not present in ethnicity-matching healthy controls (n= 159). Three of the five non-synonymous sequence variants were transversions and the remaining two were transitions. All were homoplasmic and applying the pathogenicity prediction criteria detailed in the methods, none of these non-synonymous sequence changes were potentially pathologic.

Table 2 details the *NOTCH3* mutation in each patient, the nonsynonymous mtDNA sequence changes, relative mtDNA content and MRA. Four CADSIL patients had *NOTCH3* mutation and one patient from the Sudan had none after sequencing the full *NOTCH3* gene. None of the CADSIL patients had any potentially pathological mtDNA sequence changes or a change in the relative mtDNA content. Relative mtDNA content was not statistically different (p=<0.32) between CADASIL patients (mean 1.20; sd 0.04) and simultaneous controls (mean 1.25; sd 0.185). However, the mean MRA in CADASIL patients (16.1±1.6; sd 0.80) was significantly (p=0.021) lower than

healthy matching controls (21.3±0.2; sd 1.79). All CADASIL patients had MRA lower than the mean value for the age, sex and ethnicity matching controls.

DISCUSSION

In our previous study we have described five Arab patients with clinical phenotype typical of CADASIL belonging to five unrelated pedigrees. Four out of five patients were found to be positive for *NOTCH3* mutations suggesting that clinical phenotype and genotype of CADASIL is similar for Arabs and other ethnic groups [11]. Various recent studies has shown that there is a considerable occurrence of mitochondrial abnormalities in CADASIL patients, to further study this we investigated certain mitochondrial features in leukocytes of five CADASIL pedigrees.

Parameters included in this study were sequencing the entire mitochondrial coding region, measuring relative mtDNA content and assessing mitochondrial respiratory activity (MRA). CADASIL patients compared to healthy controls have earlier been reported with higher number of mtDNA-polymorphisms and three pathogenic mtDNA mutations were detected in three separate CADASIL pedigrees [5]. In our study we did not find any difference in frequency of mtDNA mutations among our patients and controls; also no pathogenic mtDNA mutation was detected in any of our subjects. Different haplogroup frequencies between CADASIL patients and controls could be a possible factor in differences between mtDNA-polymorphisms found in our study and as described in above stated previous study [5].

Their findings cannot be taken as an overall picture as only 3/77 (3.9%) of their CADASIL pedigrees had pathogenic mtDNA mutations and in that way our results support their outcome. According to previous studies including our own it can be hypothesized that *NOTCH3* contributes to mtDNA maintenance and mutations in *NOTCH3* may increase the frequency of mutations in mtDNA [4-6] although it might not be compelling enough. We further investigate mitochondrial abnormalities in mitochondrial respiration or relative mtDNA which might be induced by NOTCH3 mutation we measured mitochondrial respiration in leukocytes of CADASIL patients and compared that to age, sex and ethnicity matching controls. Considerable decline in mitochondrial respiration (22%) was detected in CADASIL patients when compared to the controls which might be indicative of oxidative stress status. Mitochondrial respiration malfunction in absence of mtDNA mutations is not surprising as we have previously detected Complex I respiratory defect in LHON plus dystonia patients with no mtDNA mutation [10].

Actual mechanism responsible for decline in mitochondrial respiration of our CADASIL patients is not evidently clear although there might be possibility that *NOTCH3* gene mutation may induce premature apoptosis eventually reducing mitochondrial respiration as recent evidence suggests Notch proteins may be involved in apoptosis regulation [30]. In conclusion, mitochondrial respiration measurement may be a useful parameter to assess when evaluating CADASIL patients for mitochondrial abnormalities. Also, mitochondrial respiration deficiencies may play a potential role in the pathogenesis of CADASIL.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

STATEMENT OF HUMAN AND ANIMAL RIGHTS

All procedures performed in human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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