Vitamin B1 deficiency leads to high oxidative stress and mtDNA depletion caused by SLC19A3 mutation in consanguineous family with Leigh syndrome

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Abstract
Leigh syndrome (LS) and Leigh-like spectrum are the most common infantile mitochondrial disorders characterized by heterogeneous neurologic and metabolic manifestations. Pathogenic variants in SLC carriers are frequently reported in LS given their important role in transporting various solutes across the blood–brain barrier. SLC19A3 (THTR2) is one of these carriers transporting vitamin-B1 (vitB1, thiamine) into the cell. Targeted NGS of nuclear genes involved in mitochondrial diseases was performed in a patient belonging to a consanguineous Tunisian family with LS and revealed a homozygous c.1264A > G (p.T422A) variant in SLC19A3. Molecular docking revealed that the p.T422A aa change is located at a key position interacting with vitB1 and causes conformational changes compromising vitB1 import. We further disclosed decreased plasma antioxidant activities of CAT, SOD and GSH enzymes, and a 42% decrease of the mtDNA copy number in patient blood.

Altogether, our results disclose that the c.1264A > G (p.T422A) variant in SLC19A3 affects vitB1 transport, induces a mtDNA depletion and reduces the expression level of oxidative stress enzymes, altogether contributing to the LS phenotype of the patient.

INTRODUCTION
Leigh syndrome (LS) was described for the first time in 1951 by Denis Leigh as a Subacute Necrotizing Encephalomyopathy (Leigh, 1951), and is defined today as a complex and incurable early onset pediatric mitochondrial disease which englobes several neurological manifestations (Stendel et al., 2020, Bakare et al, 2021). The common clinical features involve ataxia, hypotonia, developmental delay, seizures associated with dysphagia, failure to thrive, persistent vomiting, elevated serum or cerebrospinal fluid lactate levels, and abnormal ocular disturbances (Gerards et al, 2016; Chang et al, 2020; Ogawa et al., 2020; Stendel et al, 2020).

LS is genetically heterogeneous, resulting in a diverse mode inheritance including maternal or autosomal recessive transmissions, very rare X-linked and autosomal dominant inheritances, as well as de novo variants (Ruhoy and Saneto, 2014; Leigh et al., 2015; Lake et al., 2016; Bakare et al, 2021). Many years after LS first appeared, it was discovered that some patients had deficiency of pyruvate decarboxylase or pyruvate dehydrogenase complex (PDHc) (Evans, 1981; Devivo et al., 1979), and altered metabolism of thiamine (Worsley et al., 1965; Baertling et al., 2014; Maas et al, 2017). Thiamine or vitamin B1 (vitB1) is a water-soluble vitamin acting as a cofactor involved in energy metabolism and in the nucleic acids, antioxidants, lipids and neurotransmitters synthesis (Dhir, et al, 2019). There are many clinical manifestations of this vitamin deficiency among of them neurologic, respiratory, metabolic, and cardiovascular disorders which pose challenges for physicians. (Smith et al, 2021). Thiamine and biotin (vitamin B7) transports are performed by two membrane channels THTR1 and THTR2 encoded by SLC19A2 (OMIM 249270) and SLC19A3 (OMIM 607483) genes. SLC19A3 variants are responsible for biotin-thiamine responsive basal ganglia disease (BTRBGD), Leigh syndrome (LS), infantile spasms with lactic acidosis and Wernicke-like encephalopathy (Mayr el al, 2011; Gerards et al, 2013; Lake et al, 2016).
In some cases, \textit{SLC19A3} variants abrogate thiamine transport, resulting in vitB1 deficiency, and downstream alterations of many cellular pathways.

Here, we report a consanguineous Tunisian family including an affected individual with Leigh syndrome. NGSequencing of a panel of 281 nuclear genes encoding mitochondrial proteins was carried out in the index case and disclosed a homozygous pathogenic \textit{SLC19A3} variant (c.1264A > G; p.T422A). To gain insights on the pathogenicity of this variant, we performed the molecular docking of the \textit{SLC19A3} amino acid change and assessed the plasma oxidative stress and mtDNA copy number from the patient.

**PATIENT**

The present study has been approved by the institutional review board « Hadi Chaker » University hospital ethics committee* (Sfax, Tunisia). Written informed consent was obtained from the parents of the subject, in agreement with the Declaration of Helsinki.

He is a 2-year-old boy from a consanguineous family, born at 39 weeks of gestation following an uncomplicated pregnancy. His psychomotor development was normal.

At the age of 2 years, he developed an acute ataxia with impairment of consciousness. Brain MRI was characterized by bilateral symmetrical increased T2 and decreased T1 signal intensity at the basal ganglia, thalami, brainstem temporal-parieto-occipital cortex, and subcortical white matter and cerebellum (Fig. 1. A, B, C).

Clinical conditions spontaneously improved, but 2 months later he developed acute focal seizures. Both simple laboratory investigations (electrolytes, glucose, calcium, magnesium, phosphorus) and the cerebrospinal fluid analysis (CSF) were normal. His blood lactate level was elevated: 5.2 mmol/L (normal value < 2 mmol/L). Brain MRI examination showed bilateral abnormalities of signal intensity in the lenticular nucleus and in the bilateral periventricular white matter. He also showed abnormal high signal intensity in the bilateral periventricular white matter and the bilateral dentate nucleus (Fig. 1.D, E). The clinical and radiologic pattern was referred as Leigh’s syndrome or another type of mitochondrial encephalopathy. The boy died one month later at age of 2 and a half years, due to a refractory status epilepticus.

**CONTROLS**

Fifty healthy individuals from Tunisia with an average age of 2 years, were tested by Q-PCR for mtDNA depletion and deletions, and for the presence of an oxidative stress in the plasma.

**METHODS**

**Molecular analysis**
Library preparation and sequencing

Total DNA was extracted from peripheral blood using phenol chloroform standard procedures (Lewin, 1992). Custom NGS panel of 281 nuclear genes encoding mitochondrial proteins involved in the most frequent mitochondrial pathologies was used to screen for mutations in the index case. Library preparation for each sample was carried out using SureSelect Target Enrichment System for Sequencing on Ion Proton (Manuel Number G7530-90,005) as described as elsewhere (Felhi et al, 2020).

The selected variant was verified by Sanger sequencing. Exon 5 of the SLC19A3 gene was amplified using primer sets F: 5'- CCCTGTGGCCAATATGTTCT − 3'; R: 5'- TTGCTTGTTGTAAGGTTGAGAAA − 3'. PCR was performed with 50 ng of genomic DNA, with an initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 30 s, 60°C for 45 s and 72°C for 2 min, followed by a final extension at 72°C for 10 min. The amplified sequence was sequenced directly from purified PCR products using a BigDye Terminator Cycle Sequencing kit (version 3.1; Applied Biosystems; Thermo Fisher Scientific, Inc.) before analysis on an ABI 3130 automated DNA sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

MtDNA depletion and deletions:

The mtDNA copy number from blood was quantified by real-time quantitative polymerase chain reaction (qPCR) as described elsewhere (Felhi et al., 2020). MtDNA rearrangement and deletions were analyzed using eKLIPse software (Goudenege et al, 2019).

Molecular docking

Modeling of the 3D structure of wild type SLC19A3 protein was done with I-TASSER 5.1 (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). Structure-based function prediction was performed using the COACH method. The two models were optimized using the OPLS-AA force field until the gradient of 0.01 kcal/(Å.mol) was reached (Gschwend et al, 1996). The best-ranked docking pose of each chemical compound in the active site of SLC19A3 was obtained according to the scores and binding-energy value. The similarity between the best docking pose and experimental crystal pose was calculated using the root-mean square deviation (RMSD) (Lee et al, 2020). Receptor-ligand interactions were analyzed and drawn by using the Discovery Studio Visualizer (DSV) developed by Accelrys (BIOvIA, 2016).

Oxydative stress exploration

Protein quantification: Plasma protein contents were determined in alkaline medium to obtain blue color solutions assayed at 490 nm at 37°C as described by Lowry et al. (1951). Bovine serum albumin (BSA) was used as a standard.

TBARS-MDA Assay: As described by Draper and Hadley (1990), malondialdehyde (MDA) is measured with a spectrophotometer at 532 nm by using thiobarbituric acid (TBA), resulting in a TBA-MDA complex
(Rajneesh et al. 2008). Using the following equation, results were expressed as a percentage of inhibition for three replicates

\[
\% \text{ Inhibition} = \left( \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \right) \times 100
\]

**Enzymatic antioxidant activities (superoxide dismutase: SOD; Catalase: CAT):** The SOD and CAT activities were explored in the plasma of the patient and in five healthy age-matched individuals.

**SOD** activity was determined based on the photoreduction of the nitroblue tetrazolium (NBT) using the protocol described by Asada et al. (1974) at 37°C. Absorbance was recorded at 580 nm. Data were expressed as units/milligram of protein.

**Catalase (CAT) activity** was analyzed by the decomposition of hydrogen peroxide according to the method described by Aebi (1984) at 37°C. A decrease in absorbance due to H₂O₂ degradation was monitored at 240 nm for 1 min. Results were expressed as micromoles of H₂O₂ consumed/milligram of protein.

**Non-enzymatic antioxidant activity (glutathione: GSH)**

GSH reacted with 5,5 dithiodis-nitrobenzene (DTNB) forming thionitrobenzene (TNB) of yellow color measured at 412 nm at 37°C according to the method described by Ellman (1959). GSH contents were expressed as micromoles/milligram of protein.

**Statistical analyses**

*In vitro* and *in vivo* data were expressed as means standard deviation (SD). Statistical analyses were performed using SPSS 23.0 analysis software using the one-way analyses of variance (ANOVA) followed by the Fisher test (Stat View). The significance was accepted at p < 0.

**RESULTS**

In this report, we studied a consanguineous Tunisian family including an affected individual with clinical features suggestive of Leigh-like syndrome. Next-Generation Sequencing of a panel of 281 nuclear genes encoding mitochondrial proteins was performed in the indexed case (II.1) and variant filtering led to the identification of the homozygous variant c.1264A > G (p. T422A) in exon 5 of *SLC19A3*. Family segregation by Sanger sequencing disclosed the presence of the variant in the heterozygous state in the parents, and its absence in the healthy brothers (Fig. 1. II). This variant was predicted to be pathogenic according to the ACMG classification (PP3/PP5/PM2). Furthermore, analyses using I-Mutant and Mutpred softwares suggested a decrease in protein stability and an alteration of the transmembrane protein surface.

**Molecular docking of the mutated protein harboring the A422 amino-acid change.**
Since \textit{SLC19A3} encodes the vitB1 transporter, mutations in this channel are predicted to alter the passage of vitB1 through the cell membrane. Indeed, patients carrying the p.T422A substitution have been reported to have almost no transport activity (Sato et al, 2010).

First, we have analyzed the crystallized model of the SLC19A3 protein by I-TASSER. The visualization of the model built by Discovery Studio Visualizer disclosed a clear channel structure with parallel helices. The result showed that p.T422A change is located in a crucial position on an internal helix interacting with vitB1 (Fig. 2. A1, A2).

To analyze the effect of this variant on the passage of vitB1, we performed and compared the molecular docking between the wild-type and the mutated protein with vitB1. Results showed that the p.T422A leads to conformational changes of the whole protein structure and modifies the interactions with the substrate (Fig. 2. B1, B2). In the wild-type protein, vitB1 forms four hydrogen bonds with T422 amino acid, whereas the mutated A422 amino acid can establish only a single hydrogen bond with vitB1 (Fig. 2. C1, C2).

**Oxidative stress markers in patient plasma and healthy controls**

VitB1 deficiency alters the reactive oxygen species (ROS) balance at the cellular and mitochondrial levels. We evidenced that the plasma Malondialdehyde (MDA) level is significantly higher ($p < 0.01$) in the patient compared to control individuals (Fig. 3A), while plasma antioxidant activity associated to CAT, SOD and GSH enzymes were significantly lower ($p < 0.05$) in the patient compared to controls (Fig. 3B).

**MtDNA analysis**

We studied the consequences of the oxidative stress on mtDNA integrity by evaluating possible deletions or depletion. No mtDNA rearrangement or deletion was observed using the eKlipse software (data not showed), but the mtDNA copy number showed a 42% reduction in the patient compared to age-matched controls (Fig. 3.C)

**DISCUSSION**

In the present study, we performed molecular, biochemical and computational studies of samples issued from a Tunisian patient displaying a Leigh-like syndrome.

NGS sequencing of panel of nuclear genes encoding mitochondrial proteins revealed a known homozygous \textit{SLC19A3} variant c.1264A $>$ G (p.T422A), which is predicted to be pathogenic according to the ACMG classification and referenced as responsible for biotin-thiamine responsive basal ganglia disease (BTRBGD, OMIM# 607483). Previous functional studies confirmed its pathogenicity due to a down-regulation of the THTR2 thiamine transport (Vernau et al, 2014).

Molecular docking studies revealed that the affected amino acid is located on the channel surface and induces a mis-orientation of vitB1 in the mutated protein due to conformational changes induced by the
mutation and the decreased affinity of the protein for its ligand leading to an unfavorable environment for vitB1 transport.

VitB1 is a potent antioxidant, preventing harmful free radical damages and also and also inhibiting lipid peroxidation (Nga and Quang, 2019). Plasma analyses of our patient disclosed a significant increase in MDA, which corresponds to the primary marker of oxidative stress, reflecting abnormal lipid peroxidation. Furthermore, we found a significant decrease in antioxidant enzymes as the SOD and CAT, as well in the nonenzymatic antioxidant GPX. In a previous study carried on dogs, thiamine transport defects caused by SLC19A3 mutations increased oxidative stress (Vernau et al, 2014), which we confirm here in a human case.

Once in the cytosol, thiamine is converted into its active form by thiamine phosphorylase (TPP), generating a cofactor required for several enzymes involved in cell metabolism. Indeed, TPP serves as a cofactor for the pentose phosphate pathway enzyme and transketolase (TK) (Alfadhel et al, 2019). It produces reducing equivalents of NADPH that support fatty acid synthesis, antioxidant defenses, such as the glutathione peroxide-reductase system, and nucleotide biosynthesis, including ribose-5P, an essential precursor of nucleotide biosynthesis (Vernau et al, 2014). Thus, the deficiency in NADPH and ribose-5P can explain the increase in oxidative stress and the decrease in mtDNA copy number in the patient. The lower mtDNA copy number observed is supported by previous studies in dogs and seals (Vernau et al, 2014; Croft et al, 2013), leading to an imbalance of nucleotide pools between the cytosol and mitochondria (Fig. 4). Furthermore, various problems in neurotransmission could produce in lack of thiamine, most reported the glutamatergic and GABAergic systems, resulting in a toxic neuroexcitatory state that contributes to the neurologic impairment in all described patients (Butterworth, 1989; Todd et al, 1999; De Freitas-Silva et al, 2010).

SLC19A3 deficiency was initially reported in patients from Saudi Arabia as biotin-responsive basal ganglia disease (Ozand et al, 1998). These patients presented in childhood with a subacute encephalopathy and symmetrical lesions in the basal ganglia, particularly the caudate nucleus and putamen. Today, the spectrum of SLC19A3 variants has expanded to neurologic mitochondrial syndromes, with the most common being LS (Gerards et al, 2013; Ortigoza-Escobar et al., 2014; Ortigoza-Escobar et al., 2016). The p.T422A variant is a recurrent pathogenic mutation in the Arab region, which was identified with a founder effect in the Saudi Arabian population for BTRBGD (Alfadhel et al, 2013) and was recently reported in a Tunisian LS patient (Hechmi et al, 2022). Supplementation of thiamine and biotin can be effective in treating patients harboring deficiency in thiamine transporter genes such as SLC19A3 and SLC25A19, since it can increase intracellular thiamine levels. Thus, this disease is potentially treatable, but only if the genetic diagnosis is provided early at the onset of the disease. Indeed, among patients who received vitB1 treatment within 1 month after the disease onset, 73.3% of them displayed a good recovery, becoming either symptom-free or with mild deficits, while this proportion decreased to 35.0% with patients that received a delayed therapy (Wang et al, 2021). Individualized doses according to the variant impact on vitB1 deficiency should be recommended for patients. Indeed, patients with mutations that impair vitB1 passage should be treated differently as those with mutations that
completely block the passage. Unfortunately, our patient did not benefit from thiamine and biotin supplementation, because the genetic diagnosis was obtained too late. He was admitted to the hospital pediatric service at the age of 18 months for ADEM (Acute Disseminated Encephalomyelitis and Encephalitis). The brain MRI performed at the age of 2 years showed basal ganglia and white matter involvement suggesting a Leigh / Leigh like syndrome diagnosis and he passed away after few months.

**Conclusion**

In summary, we reported a patient from a consanguineous Tunisian family with a clinical presentation of LS, who harbored the founding Arab pathogenic SLC19A3 variant p.T422A affecting vitB1 transport. Further modelization of the mutation impacts and biochemical analyses confirmed the pathogenicity of the variant and its involvement in LS.

**Declarations**

**Acknowledgments**

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**Conflicts of interest/Competing interests**

The authors declare that they have no conflicts of interest or financial relationships relevant to this article to disclose. The authors alone are responsible for the content and writing of the paper. No financial or non-financial benefits have been received or will be received from any party related directly or indirectly to the subject of this article.

**Availability of data and material (data transparency)**

All data are available.

**Code availability**

Not applicable.

**Authors' contributions**
Felhi R, Charif M, Lenaers G and Fakhfakh F had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Sfaihi L, Kamoun TH: Clinical investigations. Fakher F: molecular docking. Aoiadni N: oxidative stress. Analysis or interpretation of data: Felhi R, and Fakhfakh F. Draft of the manuscript: Felhi F, Charif M, Lenaers L and Fakhfakh F.

**Ethics approval**

The study was conducted in accordance with the principles stated in the Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects, Helsinki, Finland, 1964, and as amended in Fortaleza, Brazil, 2013. The study design was approved by the committee on research ethics of the University of Sfax, Tunisia.

**Consent to participate**

Informed consent for publication of this study was obtained from all patients and/or families involved.

**Consent for publication**

We certify that all contributors have read and approved the submission to this journal and that there is no financial or commercial involvement, or other conflict of interest by any author.

**References**


**Figures**

**Figure 1**

(I): Brain MRI of the patient at the age of 2 years: Bilateral and symmetrical decreased signal intensity in T1-weighted images (A) with increased signal intensity in axial T2-weighted images (B, C) in basal
ganglia, thalamus, splenium of corpus callosum, midbrain, temporal-parieto-occipital cortex, and subcortical white matter and cerebellum. Diffusion weighted imaging: b1000 images (D, E) showing extensive areas of restricted diffusion (hyperintensity) in cerebral white and gray matter structures (splenium of corpus callosum, basal ganglia, thalamus cortex, cerebellum, dorsal pons, and midbrain).

(II) Pedigree of the family with the segregation of the c.1264A>G variant in SLC19A3.
Figure 2

Molecular modeling of SLC19A3 transporter and the docking of wild type (T422) and mutated model (A422). (A1) modeling structure of the SLC19A3 channel into the phospholipidic membrane. (A2): location of the affected amino acid within the channel, (B1). (C) molecular docking showing the distribution and location of the amino acids interacting with vitB1 in the wild type (B1) and the mutated model (B2), (C) binding of the wild type A422 (C1) and mutated T422 (C2) amino acid to vitB1.

Figure 3

(A): Dosage of lipid peroxidation (MDA), (B) Dosage of antioxidant activities: SOD, CAT and GSH in patient’s plasma compared to control plasmas (C) Quantification of mtDNA copy number in patient and controls.
Figure 4

Impact of \textit{SLC19A3} mutation on the metabolism related to VitB1/thiamine: Impact on mtDNA content and oxydative stress state