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SOD1 deficiency: a novel syndrome distinct from amyotrophic lateral sclerosis

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Superoxide dismutase 1 (SOD1) is the principal cytoplasmic superoxide dismutase in humans and plays a major role in redox potential regulation. It catalyses the transformation of the superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide. Heterozygous variants in SOD1 are a common cause of familial amyotrophic lateral sclerosis. In this study we describe the homozygous truncating variant c.335dupG (p.C112Wfs*11) in *SOD1* that leads to total absence of enzyme activity. The resulting phenotype is severe and marked by progressive loss of motor abilities, tetraspasticity with predominance in the lower extremities, mild cerebellar atrophy, and hyperekplexia-like symptoms. Heterozygous carriers have a markedly reduced enzyme activity when compared to wild-type controls but show no overt neurologic phenotype. These results are in contrast with the previously proposed theory that a loss of function is the underlying mechanism in SOD1-related motor neuron disease and should be considered before application of previously proposed *SOD1* silencing as a treatment option for amyotrophic lateral sclerosis.

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Abbreviations: ALS = amyotrophic lateral sclerosis; SOD = superoxide dismutase

Introduction

Reactive oxygen species were traditionally considered to be detrimental to cell integrity and held responsible for a variety of damages to cellular structures, ultimately resulting either in premature cell death by apoptosis or in cancerogenesis (Lushchak, 2014). Over the course of years of intensive research, a more differentiated view on the role of reactive oxygen species both in health and disease was developed and continues to be refined (LeVine, 1992; Auten and Davis, 2009; Forman *et al.*, 2010; Fang, 2011; Szumiel, 2011).

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Among the enzymes regulating reactive oxygen species, superoxide dismutases (SODs) play an important role by facilitating the transformation of the superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2), which is then further processed by a variety of enzymes (Fridovich, 1997). Humans express three distinct SODs, namely SOD1–3. While SOD2 is localized within the mitochondria and SOD3 is located extracellularly, SOD1 is mainly found in the cytoplasm (Fukai and Ushio-Fukai, 2011). However, minor amounts are also localized in the mitochondrial intermembrane space and the nucleus of eukaryotic cells (Higgins *et al.*, 2002; Chung, 2017).

Previously, variants of *SOD1* have been implicated in the pathogenesis of familial amyotrophic lateral sclerosis (ALS), a debilitating neurological disorder characterized by the progressive degeneration of motor neurons. *SOD1* variants are causative for a significant proportion of familial ALS cases, ranging from about 13% in western to up to 30% in Asian populations (Kaur *et al.*, 2016; Zou *et al.*, 2017). While initially believed to exert mainly gain-of-function effects on the enzyme's activity, it has become clear that the described variants result in a variety of effects, some of which are suggestive of a prion-like pathomechanism (Vijayvergiya *et al.*, 2005) or mitochondrial dysfunction (Magrane *et al.*, 2009). These emerging new understandings of the pathomechanism of familial ALS and indeed also sporadic ALS (Alexander *et al.*, 2002) continue to give insights into the origins of this debilitating disorder. Interestingly, the overwhelming majority of *SOD1* variants associated with familial ALS show an autosomal-dominant inheritance pattern with homozygous variants being a rare exception (Andersen *et al.*, 1995, 1996, 1998). Heterozygous *SOD1* variants are therefore well established as a cause of familial ALS. These variants result in varying levels of SOD1 enzyme activity, ranging from severely reduced to levels above those observed in wild-type SOD1 (Borchelt *et al.*, 1994; Keskin *et al.*, 2017). Notably, the observed enzyme activity shows no association with clinical severity (Cleveland *et al.*, 1995).

There is a growing number of *Sod1*-deficient mouse models. While many were created using ALS-causing mutations observed in humans in order to study the associated phenotype (Dal Canto and Gurney, 1995; Tu *et al.*, 1996), complete knockouts have also been created. Interestingly, no motor neuron disease has been observed in these mice (Reaume *et al.*, 1996). However, they show extensive muscle involvement consisting of progressive motorneuronopathy with axonal denervation (Frey *et al.*, 2000; Hegedus *et al.*, 2007) resulting in secondary muscle pathology (Muller *et al.*, 2006). This phenotype is associated with increased oxidative stress secondary to mitochondrial dysfunction (Fischer *et al.*, 2012).

In addition to neuronal manifestations, these animals exhibit extraneuronal phenotypes with a shortened lifespan, hepatocellular carcinoma (Elchuri *et al.*, 2005) and altered hepatic energy metabolism (Wang *et al.*, 2012) as well as exhibiting endocrinological abnormalities (Matzuk *et al.*, 1998). For an extensive review of *Sod1*-deficient murine models, see Saccon *et al.* (2013).

In this study, we report on a homozygous loss-of-function *SOD1* variant identified in a patient with a debilitating neurological phenotype. The variant leads to SOD1 activity levels below measurable ranges and is associated with a phenotype marked by hyperekplexia, ataxia and muscular hypotonia in addition to severe psychomotor retardation.

Materials and methods

Subjects

Clinical evaluation was performed on the index patient as well as his parents. In addition, electrophysiological studies were performed on the index patient. All procedures were performed after consent of the patient's parents was obtained. Written consent for the publication of any photographs was obtained.

Neuroimaging and neuromuscular assessment

Cranial MRI was performed on the index patient at the ages of 2 and 6 years. Furthermore, electromyography and ultrasound of the right deltoid and left vastus lateralis muscle were performed at the age of 6 years.

Genetic analysis

DNA was prepared from EDTA blood samples using the QIAamp DNA Mini Kit (Qiagen). The coding and flanking intronic regions were enriched using in-solution hybridization technology and were sequenced using the Illumina HiSeq/NovaSeq system. Illumina bcl2fastq2 was used to demultiplex sequencing reads. Adapter removal was performed with Skewer. The trimmed reads were mapped to the human reference genome (hg19). Read duplicates that likely resulted from PCR amplification were removed. The remaining high-quality sequences were used to determine sequence variants (single nucleotide changes and small insertions/deletions). Variants were filtered and grouped into the following categories (Supplementary Table 1): *de novo* variants, homozygous variants, compound heterozygous, and hemizygous. Because of the family history and the absence of a manifest phenotype in the parents, an autosomal-recessive mode of inheritance was deemed most likely. *In silico* variant evaluation was carried out using the prediction software MutationTaster (Schwarz *et al.*, 2014) and Provean (Choi and Chan, 2015), as well as PolyPhen-2 (Adzhubei *et al.*, 2010). Identified variants were verified using traditional Sanger sequencing as described previously (Park *et al.*, 2015).

Superoxide dismutase functional assay

EDTA blood (10 ml) was mixed with 10 ml ACD-B (acid citrate-dextrose) and incubated for 2 h at room temperature to allow erythrocyte sedimentation. The supernatant was then centrifuged for 15 min at 1550 rpm. The resulting pellet was washed with a mixture of 0.8 ml 0.9% saline solution and 2.4 ml ultrapure water for 90 s, with 0.8 ml 3.6% saline solution added directly after the incubation period. This was followed by a further 10-min centrifugation at 1550 rpm. The supernatant was again discarded, and the pellet repeatedly washed until no erythrocytes were visible. After completion, the pellet was frozen until further processing.

SOD activity was measured using a spectrophotometric approach according to a previously published protocol (Spitz and Oberley, 1989, 2001).

Muscle biopsy

A muscle biopsy was taken from the index patient's vastus lateralis muscle at the age of 2 years. The biopsy was stained using Periodic acid–Schiff, NADH, ATPase pH 4.3 and 9.4, MADA, phosphofructokinase, and myo-phosphorylase staining. In addition, immunofluorescence for various skeletal muscle proteins and respiratory chain complex activity measurements were carried out.

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Results

Case report and radiography studies

The index patient is the third child of consanguineous parents (first degree cousins) of Afghan origin (Fig. 1). During pregnancy, polyhydramnios was noted. The patient was born at 39 weeks of pregnancy via emergency Caesarean section due to a maternal indication.

The family reported normal development until the age of 9 months when the patient was able to crawl. Shortly afterwards, progressive psychomotor decline marked by loss of motor abilities and progressive ataxia began.

Upon initial presentation at our tertiary care centre at the age of 6 years, he showed a significant combined developmental delay as he was not able to sit or stand unsupported. Additionally, he presented with dysmorphic features such as low set, posteriorly rotated ears (Fig. 2A) and overlapping toes (T2, 3 on the left and T3, 2 on the right) (Fig. 2B).

Clinical examination revealed an increased muscle tone of both upper and lower limbs with persistently bended arms in pronated position whereas muscular mass and distribution appeared normal. Corresponding to the spasticity he demonstrated hyper-reflexive brachioradial reflexes and more pronounced hyperreflexia in patellar reflexes together with enlarged reflex zones. Furthermore, we observed pyramidal path symptoms such as positive Babinski sign and bilateral exhaustible clonus of the feet (Supplementary Video 1). In addition, we stated pronounced symptomatic hyperekplexia with persistent glabellar tap sign as well as an incomplete Moro reflex consisting of the initial abduction, while the typical subsequent adduction reaction was lacking.

Truncal and proximal muscular hypotonia leading to the inability to sit or stand was noted. Therefore, it was not possible to examine for truncal ataxia. The patient showed no nystagmus or oculomotor dysfunction, but slight tremor of both hands and fingers upon movement. Cognitive functions were impaired; however, he was able to understand and/or implement simple correlations but not complexes requirements (e.g. directed pointing). Therefore, detailed assessment of these symptoms remains unanswered. Only non-verbal communication is possible.

Cranial MRI was performed at the age of 2 and again at 6 years. The initial MRI was inconclusive. At the age of 6 years, mild cerebellar atrophy with discreetly enlarged interfoliar spaces in the region of the anterior vermis was diagnosed (Fig. 3).

Extensive metabolic screening, including serum organic acids, serum acylcarnitine profile, urinary oligosaccharides and amino acids, purines/pyrimidines, and serum lactate analysis was performed but results were inconclusive.

Evaluation of antioxidant vitamins and trace elements revealed consistently low levels of blood manganese between 3.1 and 4 ng/ml (reference: 7–11 ng/ml) and zinc [672 µg/l (reference: 750–1400 µg/l)].

Heterozygous carriers identified within this family did not show any overt neurological phenotype. There were no cases of ALS reported in this family.

Genetic analysis

Array-based comparative genomic hybridization of the index patient was carried out without showing any abnormalities. Whole exome sequencing revealed that the patient was homozygous for the frameshift *SOD1* variant c.335dupG, resulting in a premature stop codon at position 112 of the resulting polypeptide (p.C112Wfs*11), thus terminating the polypeptide within GK2, i.e. the second β-sheet connection. Importantly, based on previous structural analysis, this disruption can be assumed to affect both a Cu binding site as well as an H_2O_2 liganding residue, which is believed to exert an important functional role by controlling the active site of the protein (Perry *et al.*, 2010). The parents as well as an older brother were heterozygous for the variant. Additional variants identified in the subjects are presented in the Supplementary material.

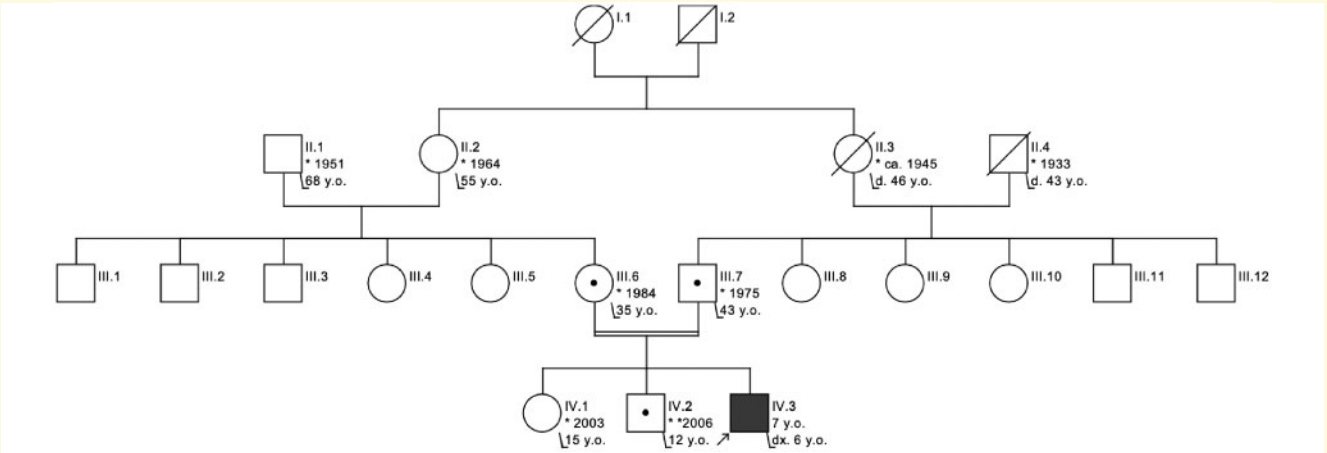

Figure 1 Pedigree of the index patient. His parents are first degree cousins. No other affected individuals were reported. Notably, there are no known cases of (familial) ALS in the kinship, while many of the reported individuals are at or above the mean age of onset observed in *SOD1*-related familial ALS.

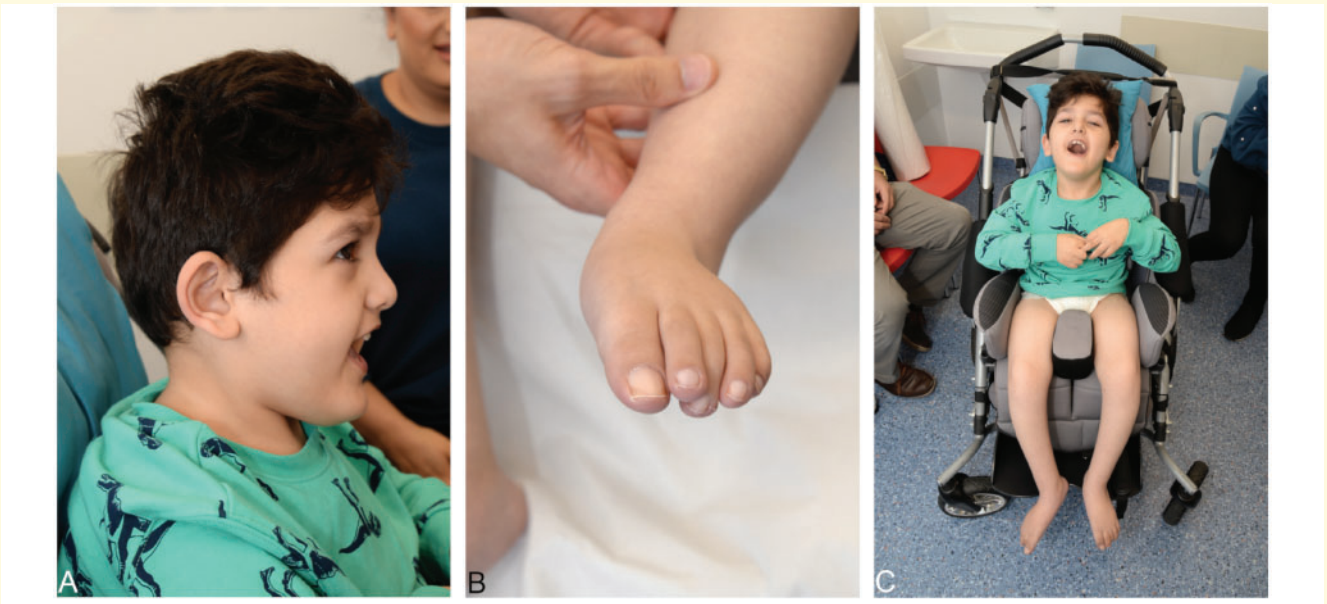

Figure 2 Phenotype of the index patient at 6 years of age. Dysmorphic features such as low set, posteriorly rotated ears and overlapping toes are present. A clinical examination demonstrating hyperekplexia-like symptoms is available online.

Superoxide dismutase functional assay

The activity of SOD1 of the index patient was below the detection level of 2 units/mg, whereas SOD2 activity was within normal ranges. Heterozygous carriers of the identified variant had an approximately halved SOD1 activity of 12 and 11 units/mg when compared to wild-type controls (55 and 51% of wild-type SOD1 activity, respectively).

In contrast, the activities measured in three healthy control subjects were all within reference ranges (Fig. 4).

Muscle biopsy and neurophysiological examination

The muscle biopsy obtained at the age of 2 years revealed a pathological finding with increased fibre size variability and atrophic fibres. The number of type 2 fibres was increased but no type grouping was present (Supplementary Fig. 1). Furthermore, focally decreased α-dystroglycan expression was observed, while the function of respiratory chain complexes I–IV was normal.

Muscle ultrasound examination showed multiple fasciculations of the right deltoid, right extensor digitorum,

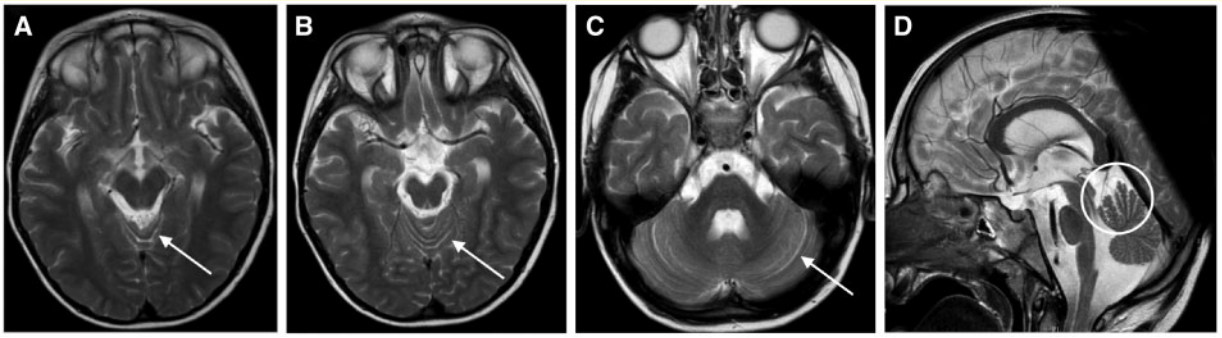

Figure 3 T_2-weighted cranial MRI of the index patient at the age of 6 years. In general, mild cerebellar atrophy is present. The cerebellar interfoliar spaces at the anterior vermis are discreetly enlarged (indicated by arrows) as are the cerebellar hemispheres in the lateral view (**D**).

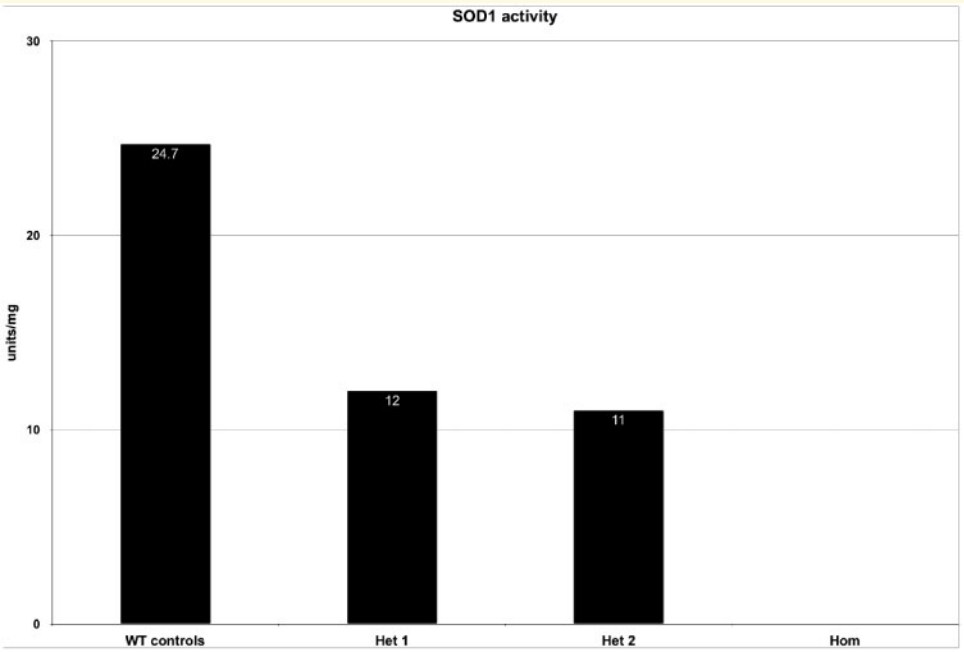

Figure 4 SOD1 activity measurement of the patient and heterozygous carriers compared to wild-type controls. Measurements were performed on isolated blood leucocytes. The index patient had no detectable SOD1 activity. Heterozygous carriers showed an approximately halved SOD1 activity of 12 and 11 units/mg protein, corresponding to 55% and 51% of the activity measured in wild-type (WT) controls, respectively.

and vastus medialis and tibialis anterior muscles on both sides.

Accordingly, multiple fasciculations of right deltoid and left vastus medialis muscle were identified using electromyography while no other pathologic spontaneous activity was seen. Besides these findings, nerve conduction studies and visual evoked potentials were normal.

Discussion

In this study, we describe a patient carrying a truncating homozygous *SOD1* variant resulting in the total absence of SOD1 activity. Human SOD1 deficiency has, to our knowledge, never been described before.

The link between *SOD1* variants and familial ALS has been firmly established. Indeed, research on SOD1-related ALS has been an important way of delineating the pathogenesis of this debilitating neurodegenerative disorder.

A vast number of variants in *SOD1* have been identified in familial ALS but despite extensive research, the exact pathomechanism by which these alterations cause familial ALS remains elusive. Recently, it has become clear that there are probably several ways in which *SOD1* variants exert their causative role in the disorder. It has been firmly

established that a vast number of variants leads to a gain-of-function of the gene product SOD1 (Bruijn *et al.*, 1998; Allen *et al.*, 2003). Furthermore, SOD1 aggregation (Jonsson *et al.*, 2008) and mitochondrial dysfunction (De Vos *et al.*, 2007) have been found to play a major role in the disorder.

The role of loss-of-function mutations is somewhat disputed. While this concept was initially believed to be the underlying cause of familial ALS (Deng *et al.*, 1993; Rosen *et al.*, 1993), mounting evidence against this notion has been derived from animal models (Ratovitski *et al.*, 1999; Boillee *et al.*, 2006). In addition, various human *SOD1* variants that retain the full dismutase enzyme activity have been described in familial ALS (Borchelt *et al.*, 1994; Hayward *et al.*, 2002). Therapeutic trials of antisense oligonucleotides in murine models carrying human *SOD1* variants support this notion (McCampbell *et al.*, 2018).

SOD1 loss-of-function is a new disease with recessive mode of inheritance and heterozygous carriers not affected by the disorder. Although the eldest investigated heterozygous carriers (35 and 44 years, respectively) could still develop symptoms of familial ALS, there have to be several older heterozygous carriers in earlier generations of the family without any symptoms of familial ALS (Fig. 1) (Bali *et al.*, 2017), showing that a reduction in enzyme activity—as in heterozygous carriers—does not lead to familial ALS whereas complete loss-of-function leads to a different disease.

The disorder observed in our patient differs significantly from motor neuron disease. While it shares some similarities, such as signs of muscular denervation observed indirectly in electromyography and muscle ultrasound, the vast majority of symptoms have not been described in ALS before. The age of onset in early childhood is atypical, as is the phenotype indicative of first motor neuron affection. The hyperekplexia-like presentation is a hallmark of the syndrome, which might facilitate diagnosis in the future.

The phenotype bears distinct similarities to the presentation of total murine *Sod1* knockouts (Matzuk *et al.*, 1998; Elchuri *et al.*, 2005; Wang *et al.*, 2012; Sakellariou *et al.*, 2018) in which similar features such as tremor, reduced muscle mass, and motor axonopathy (Shefner *et al.*, 1999) were noted.

The total absence of any measurable SOD1 activity suggests a major role of oxidative stress or dysregulation in the pathogenesis of the disease. This is in part supported by the finding of hypomanganesaemia in the index patient, as manganese is a known antioxidant, either as part of SOD2 or independently (Coassin *et al.*, 1992; Aguirre and Culotta, 2012).

Given the severe and debilitating nature of the disorder, the need for therapeutic options is evident. Based on SOD1's role in metabolism of reactive oxygen species, antioxidant therapy, e.g. by external supplementation, represents an intriguing approach. Indeed, the antioxidant *N*-acetylcystein was shown to have positive effects on anaemia and autoantibody generation in $Sod1^{-/-}$ mice (Iuchi *et al.*, 2007).

In addition to antioxidant compounds, SOD mimetics represent another promising therapeutic approach. These agents have been suggested as treatment options in the context of Parkinson's disease (Filograna *et al.*, 2016) and radiation damage (Anderson *et al.*, 2018), among others. In a phase 1b/2a trial in patients undergoing radiation therapy for oral carcinoma, the mimetic GC4419 showed acceptable safety (Anderson *et al.*, 2018), making it a candidate for application in SOD deficiency in the future.

The findings presented in this study not only shed light on the pathomechanism of SOD1-related ALS but are also of high relevance for the intensely investigated therapeutic strategy of *SOD1* silencing. Previous studies in various ALS disease models have established the general efficacy of silencing using antisense oligonucleotides, shRNA, miRNA, and other compounds (for a review see van Zundert and Brown, 2017). Due to the lack of ALS in SOD1 knockout mice, safety of silencing of both wild-type and mutant *SOD1* is assumed (van Zundert and Brown, 2017).

Given the severe neurological manifestation of SOD1 deficiency in our patient, the possibility of adverse effects following *SOD1* silencing must be considered. From a pathomechanistic point of view, silencing of the principal cytoplasmic superoxide dismutase might result in increased oxidative damage as well as altered redox signalling.

Findings from animal as well as early clinical studies did not indicate any adverse events attributable to SOD1 deficiency (Miller *et al.*, 2013; Thomsen *et al.*, 2014; Stoica *et al.*, 2016; Borel *et al.*, 2018). This can in part be explained by the incomplete representation of human (patho)physiology by animal models. Furthermore, currently published findings in humans made use of low doses of silencing agents not resulting in complete suppression of SOD1 activity (Miller *et al.*, 2013). Given the absence of an observable neurological phenotype in heterozygous carriers of the variant described in our study, these results do not exclude adverse events by *SOD1* silencing.

In conclusion, our data characterize the effects of a truncating mutation of *SOD1*, leading to total absence of SOD1 activity in the affected patient. The resulting phenotype is severe with tetraspasticity mainly affecting the upper limbs and hyperreflexia reflecting an affection of the first motor neuron. Antioxidant supplementation may represent a therapeutic approach, although further research is needed to characterize the effects of the variant on a deeper level. The results of this study call for a cautious approach to *SOD1* silencing as a therapeutic concept for ALS.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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