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Highlights

• This report provides three novel pathogenic variants in *SLC5A7* causing CMS type 20. • CMS type 20 is remarkably variable in clinical phenotype, severity and prognosis. • Additional treatment with β 2-adrenergic agonists may be useful in some patients.

Q1



Presynaptic congenital myasthenic syndrome due to three novel mutations in *SLC5A7* encoding the sodium-dependant high-affinity choline transporter

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Abstract

SLC5A7 encodes the presynaptic sodium-dependant high-affinity choline transporter 1 (CHT), which uptakes choline to the presynaptic nerve terminal following the breakdown of acetylcholine by the acetylcholinesterase within the synaptic cleft. We report 5 patients from three consanguineous families with congenital myasthenic syndrome type 20 caused by novel mutations in *SLC5A7*. The individuals from family 1 and 2 were homozygous for c.320G>A; (p.Arg107His) and c.886G>A (p.Ala296Thr), respectively, and their phenotype was characterised by recurrent apnoeic attacks early after birth and learning and speech difficulties in childhood. Individuals from family 3 were homozygous for c.1240T>A (p.Tyr414Asn) and suffered from more severe central and peripheral manifestations with lack of spontaneous movements and respiratory drive and overall minimal response to external stimuli. All individuals tested showed neurophysiological defects compatible with impaired neuromuscular transmission. Combined treatment with cholinesterase inhibitors and β 2-adrenergic agonists was beneficial in patients from family 1 and 2. Affected individuals from family 3 died from complications directly related to their underlying genetic condition. This report provides three novel pathogenic variants in *SLC5A7* and highlights the variability in the clinical phenotype, severity and prognosis of this syndrome.

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1. Introduction

Congenital myasthenic syndromes (CMS) are a heterogeneous group of inherited neuromuscular disorders caused by mutations in genes encoding proteins that are essential for neuromuscular transmission. There are currently more than 30 subtypes of CMS and they can be classified according to the location of the encoded protein into presynaptic, synaptic and postsynaptic disorders [1]. Until

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¹ The Oxford Clinical Whole Genome Sequencing consortium (a full list of consortium members is available in the supplementary data).

recently, most of the CMS described were postsynaptic in origin with a few being located to the synaptic space or the presynaptic terminal. However, the number of presynaptic CMS has now expanded with abnormalities identified in the synthesis and recycling of ACh [2,3], the exocytosis of synaptic vesicles [4,5] and axonal transport [6]. Many of the patients within the presynaptic group present with episodic apnoeas but also with severe central deficits derived from the additional expression of the encoded proteins within the central nervous system [7]. This expands the boundaries of the classic definition of CMS from being confined to a neuromuscular junction deficit to a component within multisystemic disorders.

SLC5A7 encodes the presynaptic HC-3 sodium-dependant high-affinity choline transporter 1 (CHT), which uptakes choline (Ch) into the presynaptic terminal following the breakdown of acetylcholine (ACh) at the synaptic cleft by the acetylcholinesterase [8]. CHT consists of 580 amino acids and is predicted to organise into 13 transmembrane domains. No crystal structure has been solved to date although CHT is thought to form a homo-oligomer in the cell surface [9]. CHT is essential for life as shown by *SLC5A7*^{-/-} mice that become immobile, breath irregularly and die within an hour of being born [10]. Their NMJs are characterised by increased axonal sprouting and decreased acetylcholinesterase (AChE) activity and they exhibit a time-dependant loss of spontaneous and evoked responses. By contrast, *SLC5A7*^{+/-} cholinergic terminals seem to compensate for significant reductions in CHT protein levels although they have increased sensitivity to stress [10].

Diseases associated with *SLC5A7* mutations include autosomal dominant distal hereditary motor neuronopathy type VIIA (dHMN VIIA, MIM: 158850) and autosomal recessive CMS type 20 (MIM: 617143). The first one has been reported in association with truncating mutations while missense changes have been found to underlie CMS. Both conditions are considered very rare disorders with only a few cases reported. Choline uptake is reduced in mutant CHTs but it is unclear how different types of mutations give rise to the different diseases. One study suggested that CHTs bearing missense mutations are prevented from reaching the NMJ due to loss of export into axonal processes, while those with truncating mutations are not “filtered” causing dominant-negative interference from oligomerisation with the WT molecule [11]. Peripheral neuropathy is not typically associated with CMS except for mutations in *SYT2* that cause an autosomal-dominant form of Lambert-Eaton myasthenic syndrome and non-progressive motor neuropathy [4].

We present five patients from three different kinships underlying novel *SLC5A7* missense mutations. This report expands the genetic spectrum of CMS type 20 and emphasizes the variability in the clinical phenotype and prognosis.

2. Methods

Consent for DNA analysis and publication of clinical and genetic data was obtained with ethical approval from OXREC

B: 04.OXB.017 and Oxfordshire REC C 09/H0606/74. Genomic DNA was isolated from the patient and parents peripheral blood by standard methods.

Whole genome sequencing (WGS) was performed by OxClinWGS with 150bp paired-end format (Illumina HiSeq 2500 System) and reads were mapped to reference genome hs37d5 using Stampy v1.0.23. Variants were called with Platypus v0.8.1 using default settings (except minFlank=0) and filtered with Ingenuity Va (Qiagen) using several confidence filters. Only rare variants with population allele frequency of 0.2% or less (1/500 chromosomes) or 0.5% in WGS500 and requirement for homozygosity were considered given the reported consanguinity.

Whole exome sequencing (WES) was carried out using SureSelect Focused Exome enrichment technology (Agilent, Santa Clara, USA) and Illumina NextSeq (2 × 100bp paired end reads) for sequencing. Variants with allele frequency <0.001 (equivalent to 0.1%) in gnomAD exomes database (2.0.1 v2) were prioritized by identifying homozygous changes that are predicted to be either loss of function according to the human sequence ontology, or are missense variants which are predicted to be damaging by at least 3 out of 6 in silico tools from the dbNSFP v3.0 functional prediction voting.

In silico tools for the interpretation of genetic variants included the SIFT algorithm [12], PolyPhen-2 [13], Mutation Taster [14], Mutation Assessor [15], FATHMM [16], and fathmm-MLK [17]. Furthermore, we used ensemble scores (MetaSVM and MetaLR) from dbNSFP v3.0, which are based on 10 component scores and the maximum frequency observed in the 1000 genomes populations to provide additional information [18]. Putative pathogenic variants were confirmed by Sanger sequencing and segregation analyses were performed when available.

3. Results

3.1. Genetic analysis

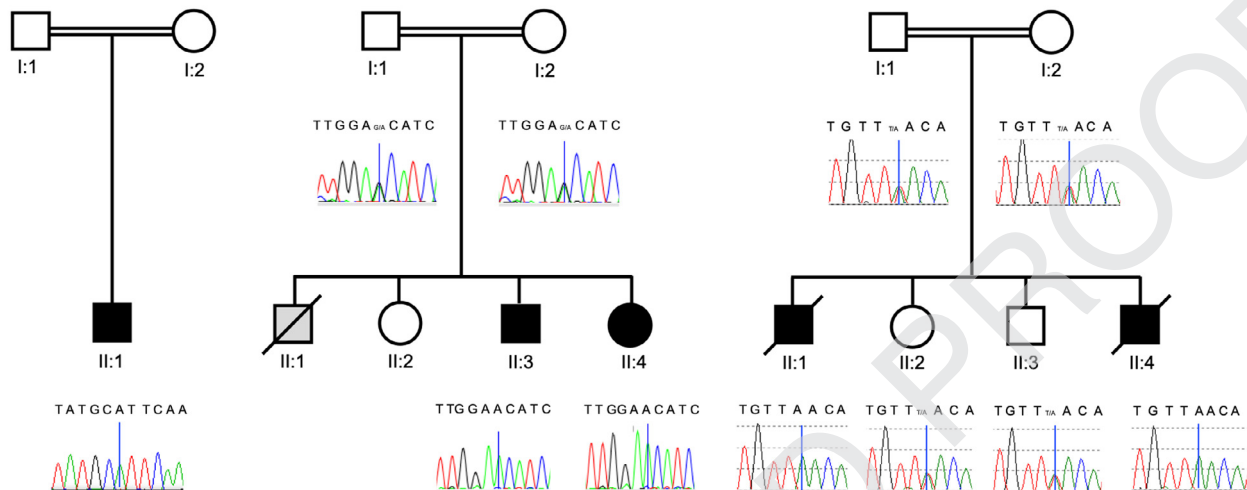
WGS analysis for the consanguineous singleton case showed that ~10% of genome was made up of regions of homozygosity (ROH), consistent with a high degree of consanguinity. Using a simple recessive inheritance model, more than twenty rare homozygous variants were identified (supplementary data) from which the variant NM_021815.4:c.320G>A; p.Arg107His in *SLC5A7* (Fig. 1A) was observed in 28/28 reads and stood out on account of a high CADD (Combined Annotation dependant Depletion) score of 30, rarity and to the gene’s known role in disease. The variant, located within a 25.6 Mb region of autozygosity, corresponds to a transition in exon 4, and results in the substitution of arginine to histidine within the cytoplasmic region (Fig. 2A). It is predicted to be damaging by 6/6 in silico tools and it was not listed in the ExAC (Exome Aggregation Consortium) database, but was present in the Genome Aggregation Database [(gnomAD, Cambridge, MA

A

c.320G>A (p.Arg107His)

c.886G>A (p.Ala296Thr)

c.1240T>A (p.Tyr414Asn)



B



Fig. 1. Segregation and conservation analysis of *SLC5A7* variants. (A) Segregation analysis of *SLC5A7* variants in the families reported. Pedigree symbols are shaded according to the presence of clinical symptoms. Individual II:1 from family 2 is shaded grey due to lack of genetic confirmation. (B) Conservation analysis showed that the Arg107, Ala296 and Tyr414 residues are conserved across species. The protein alignments were performed using the Clustal Omega sequence alignment program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

(URL: <http://gnomad.broadinstitute.org>) (June 2019)) [19] on just 2/252,394 chromosomes on heterozygosis.

WES identified a novel *SLC5A7* variant in homozygosis in family 2 corresponding to a transition in exon 7, leading to the substitution of Alanine to Threonine (NM_021815.4:c.886G>A; p.Ala296Thr) within the extracellular space (Fig. 2A). The variant was not listed in the Genome Aggregation Database [(gnomAD, Cambridge, MA (URL: <http://gnomad.broadinstitute.org>) (June 2019))] and was not present in a database containing >13,000 Arab exomes (May 2020), courtesy of Prof FS Alkuraya - King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia. In silico analysis classified the variant as deleterious by 3/6 individual tools and by the ensemble scores (MetaSVM and MetaLR) from dbNSFP v3.0. Additional candidate variants are not available for this pedigree.

In family 3, WES data showed that ~9% of the patient's genome was made up of ROH of above 5Mb. Six variants were identified after filtering (supplementary data), from which the *SLC5A7* variant (NM_021815.4:c.1240T>A;

p.Tyr414Asn) was highlighted for clinical attention by being located within a ROH of 26.2 Mb and predicted to be damaging by 6/6 in silico tools. The variant, corresponding to a transversion in exon 9 within the transmembrane region (Fig. 2A), was absent from the gnomAD exomes database and affected a gene highly likely to be intolerant to damaging recessive variation according to ExAC 0.3 functional gene constrains scores (pRec=0.897).

No variants within other known presynaptic CMS causative genes were identified in the three families. Sanger sequencing demonstrated segregation of *SLC5A7* variants within families 2 and 3 (Fig. 1A). Comparative analysis showed that Arg107, Ala296 and Tyr414 residues are conserved across species (Fig. 1B).

3.2. Clinical features

3.2.1. Family 1 (homozygous for p.Arg107His)

Individual II:1 was born prematurely at 33 weeks of gestational age (weight 2.02 kg) from first-degree consanguineous parents of Kuwaiti origin. He presented soon

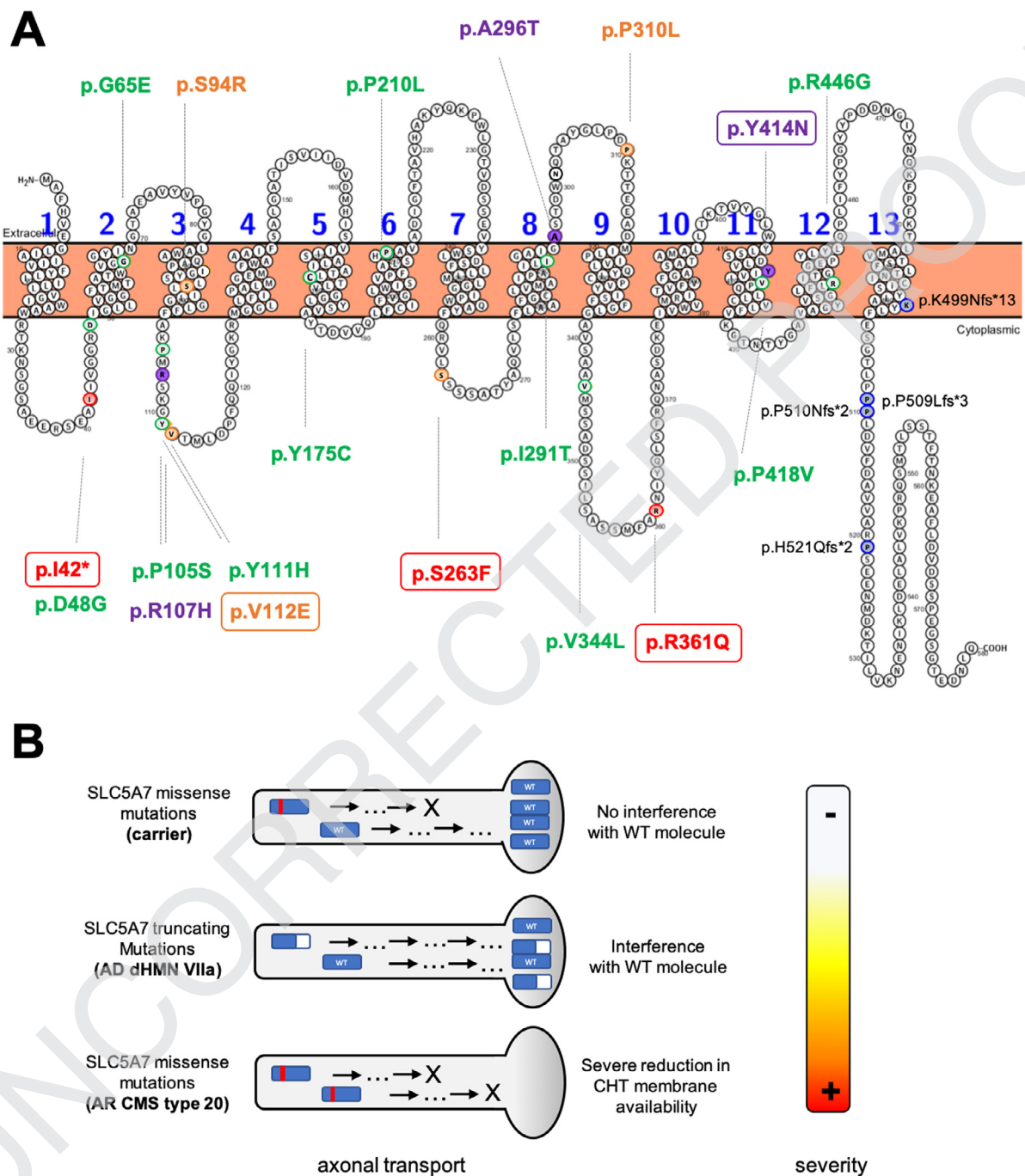


Fig. 2. Schematic representation of the choline transporter, localisation of *SLC5A7* mutations reported to date and proposed molecular mechanisms. **A.** The choline transporter is a multi-pass protein consisting of 580 amino acids that are predicted to organise in 13 transmembrane domains. Mutations causing dHMN VIIa (blue) are typically located towards the C-terminus domain [27]. By contrast, the CMS causative mutations are present along the remaining length of the protein. The CMS mutations are shown colour-coded according to the associated clinical features as CMS with episodic apnoeas (green), severe generalised phenotype (orange) and antenatal onset phenotype (red). The novel *SLC5A7* mutations are displayed in purple. Mutations associated with lethality are displayed inside boxes. This cartoon was generated using Protter® [33]. **B.** The mechanism proposed by Wang et al. suggests that truncated forms of CHT reach the NMJ causing a dominant-negative interference resulting from oligomerisation with the WT molecule. By contrast, missense mutations may be filtered from reaching the NMJ, which could be an explanation for the lack of effect in asymptomatic carriers where the WT allele ensures physiological CHT levels and the profound severity of those carrying biallelic mutations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

after birth with hypotonia and episodic apneas triggered by intercurrent infections, some of which required ventilation, and seizures. During the first year of life, motor development was delayed and generalised hypotonia, moderate ptosis and mild ophthalmoparesis were noted. Treatment with pyridostigmine (5.5 mg/kg/day) from age 12 months was beneficial with clear improvement in gross motor development over the course of two months. In the last examination, at 14 months, he was able to stand holding on and tried to pull himself up. Then oral salbutamol 200 mcg daily was added with subjective improvement, but no formal evaluation has been conducted yet.

3.2.2. Family 2 (homozygous for p.Ala296Thr)

Individual II:3 was born at 36 weeks of gestational age from first-degree consanguineous Emirati parents with low birth weight (1.7 kg) due to intrauterine growth retardation. He suffered from multiple severe apnoeic episodes from the first month of life requiring ventilation and occasionally cardiopulmonary resuscitation. Triggers included crying, high temperature and hot weather. This resulted in prolonged hospital admission to intensive care for approximately 6 months. Treatment with pyridostigmine was initiated age 5 months and increased up to 4.5 mg/kg/day in 6 divided doses. There was no without apparent beneficial effect on the frequency of apnoeic episodes, the last of them being recorded at 3 years old. He also had transient history of early dysphagia but subsequent problems with eating and swallowing were not reported. He had delayed motor developmental milestones (sitting at 9 months and walking at 2 years of age) and subsequent difficulties with rising from the floor, fatigue with walking and frequent falls due to proximal weakness and coordination difficulties. Treatment with salbutamol 6 mg daily was added at the age of 7 years with a positive effect on his muscle strength in the form of improved ability to rise from the floor and increased walking tolerance up to 20 min. At the age of 8 years, there was no evidence of facial or limb weakness and only mild ptosis and intermittent squint were present, but he had significant learning and behavioural difficulties and problems with hand manipulation and fine motor skills.

Individual II:4 was born at 37 weeks of gestational age following a normal pregnancy. She was noted to have bilateral ptosis, generalised hypotonia and feeding difficulties from birth that were more noticeable at 2 months. Treatment with pyridostigmine (6 mg/kg/day) and salbutamol 1.5 mg daily was initiated at two and a half months old with improvement of dysphagia. However, during infancy, she was found to have a weak cry and required thickened liquids although artificial feeding was not needed. Her motor milestones were delayed (walking at 16 months). Unlike her sibling, she had a single apnoeic episode to date at 20 months old, which did not require ventilation. At the age of 2 years, she had mild ptosis and ophthalmoparesis with normal muscle power elsewhere on examination. By contrast, the phenotype was driven by global delay but mainly significant speech and language difficulties.

Individual II:1 from family 2 died at 10 months of age with similar manifestations to his siblings although no formal genetic or clinical diagnosis was made at that time.

3.2.3. Family 3 (homozygous for p.Tyr414Asn)

Individual II:4 was born at 38 weeks of gestational age from first-degree consanguineous parents of Pakistani origin by elective caesarean section following an uneventful pregnancy. He had a poor respiratory effort from birth that required intubation and mechanical ventilation. He also had poor suck requiring artificial feeding from birth. He was started on treatment with pyridostigmine (7.2 mg/kg/day) on day five of life with no response. He had a short trial of 3,4-Diaminopyridine, which was coincidental with epileptic seizures and then was discontinued. Additional treatment with salbutamol 900 mcg daily at the age of 2 months was not helpful. He required continuous ventilatory support and did not manage any time off the ventilator. He also suffered from recurrent respiratory infections. On examination, there was a lack of spontaneous respiratory effort and minimal spontaneous movements only in his upper limbs. Muscle tone was overall reduced in axial and limb muscles. At the age of 3 months, he was unable to follow and fix and lacked social smile. He also developed epileptic seizures consisting of episodes of generalized rhythmic twitching of arms and legs associated with desaturation and increased heart rate for which he was started on regular phenobarbitone. Following genetic diagnosis, limitation of patient care was advised following the guidelines from the Royal College of Paediatrics and Child Health [20].

Individual II:1 suffered from severe generalised and bulbar weakness from birth. He was long-term ventilated via tracheostomy due to lack of respiratory drive and permanently fed via gastrostomy. He remained non-communicative and had very limited cognitive and motor development with no real awareness of surroundings. Autonomic dysfunction was also noted with low core temperatures down to 32 °C. He underwent a trial of pyridostigmine during childhood without significant benefit. Over the years he suffered from multiple admissions to hospital due to a combination of respiratory problems and recurrent infections. He also suffered from severe gastrointestinal complications including gut perforation. He eventually died at the age of 15 years although there were no clear changes in his clinical status from birth. Genetic diagnosis was achieved by exome sequencing after his death and prior to the birth of his younger sibling (Individual II:4).

3.3. Investigations

3.3.1. Neurophysiological studies

Abnormalities consistent with impaired neuromuscular transmission were noted in all patients undergoing neurophysiology. Individual II:1 from family 1 showed significant decrement (>10%) to 3 Hz repetitive nerve stimulation (RNS) from *abductor pollicis brevis*. Individual II:3 from family 2 showed significant decrement to 3 Hz RNS

from the *abductor digiti minimi* and the *abductor pollicis brevis* with 30% and 12% reduction in CMAP amplitudes, respectively. Stimulated potential analysis using concentric needle electrodes (SPACE) from right *orbicularis oculi* also showed marked increased jitter (MCD 93.5 μ s) and blocking in 24 out of 39 pairs. No incremental response or repetitive CMAPs were observed and nerve conduction studies were reported as normal. SPACE from *orbicularis oculi* in Individual II:4 from family 2 showed increased MCD of 70.3 μ s and blocking in 5 out of 30 pairs. Individual II:4 from family 3 had a marked decremental response of 68% to 3 Hz repetitive nerve stimulation from *abductor hallucis*.

3.3.2. Other investigations

A number of tests were requested to evaluate the potential contribution of the central nervous system to the clinical manifestations. Individual II:3 from family 2 showed predominantly frontal discharges in EEG studies performed during infancy and his recurrent apnoeic episodes were originally thought to be epileptic in origin. Individual II:4 from family 3 showed bilateral slow-wave posterior activity with frequent sharp waves but no clear epileptiform discharges were observed during the recording period. Individual II:1 from family 1 was diagnosed initially with epilepsy, but we do not have records of the EEG studies.

Individual II:4 from family 3 underwent brain MRI at 7 weeks old that showed abnormal white matter signal throughout the brain, more evident in the frontal lobes indicative of increased water content/ relative lack of myelin.

4. Discussion

We describe the clinical and complementary features of three families with presynaptic CMS due to previously unreported missense mutations in *SLC5A7*. We emphasise the clinical heterogeneity of patients with CMS type 20 and report our experience with the use of β 2-adrenergic agonists in this syndrome.

CMS type 20 is extremely rare with only 14 patients reported to date [2,11,21,22]. Most cases lie within the severe spectrum of the congenital myasthenic syndromes although there seems to be at least 3 different associated phenotypes. First, a neonatal phenotype characterised by recurrent apnoeic episodes from birth associated with a variable degree of generalised hypotonia, bulbar weakness and oculofacial symptoms [2]. Patients within this category suffer from life-threatening respiratory episodes early in life that are often misdiagnosed as epileptic seizures. The clinical course seems to improve over time with decreased frequency of respiratory events and positive response to pharmacological treatment. However, some patients also exhibit cognitive deficits and behavioural problems, which become more apparent later in life if the neuromuscular symptoms improve. This is likely related to the multisystemic expression of the choline transporter within major cholinergic projections of the central nervous system [23] although hypoxic brain damage may also contribute. CMS associated with episodic

apnoeas include ChAT [24], Rapsyn [25] and fast channel syndromes [26] although the list is expanding with more recently described presynaptic syndromes [7].

Secondly, there is a more severe and generalised global phenotype characterised by marked hypotonia, lack of respiratory drive and spontaneous movements and severe bulbar weakness [11,21]. Patients within this category also present with severe central deficits and minimal response to external stimuli. The overall prognosis is typically poor with minimal response to cholinesterase inhibitors and continuous need for artificial feeding and mechanical ventilation. Finally, a severe antenatal (lethal) phenotype has also been reported with hydramnios, generalised hypotonia, severe respiratory distress, arthrogryposis and multiple malformations including brain atrophy [2,22].

Affected individuals from family 1 and 2 would classify as neonatal onset phenotype with episodic apnoeas although Individual II:4 had a milder disease course with a single episode and no artificial feeding requirements. This may be related to the early start of combined treatment with pyridostigmine and salbutamol although the existence of a milder phenotype non-previously reported is also possible. By contrast, affected individuals from family 3 are representative of the more severe and generalised phenotype with lack of respiratory drive and spontaneous movements from birth. It remains unclear what drives the differences in the clinical phenotype of patients with CMS type 20. The missense mutations reported to date are distributed along the length of the protein and no specific hot spots or phenotype-genotype correlations have been identified (Fig. 2A). Mutations causing dHMN VIIa (MIM: 158850) are typically located towards the C-terminus domain [27] and are thought to have a dominant-negative effect [11] (Fig. 2B). Evaluation of the choline transporter activity in heterologous cells using 3 H-choline transport assays has shown the deleterious effect of missense variants on choline uptake [2,11]. Although not formally proven, it is possible that the clinical severity relates to the activity of the mutant transporter, which may also be influenced by its trafficking insertion into the presynaptic membrane [11]. For instance, the functional activity of CHT – p.Ser263Phe was similar to cells expressing an empty vector [22] in keeping with a severe antenatal phenotype. Alternatively, modifier factors for the expressivity of *SLC5A7* mutations could also contribute to the phenotype, especially in consanguineous families with loss of heterozygosity regions.

We believe that the novel missense variants reported in this study are pathogenic based on the clinical phenotype and the presence of robust neuromuscular transmission abnormalities on neurophysiological testing. Moreover, the three missense variants all substitute for an amino acid residue that is highly conserved during evolution and are reported as deleterious by 6/6 silico prediction tools except for p.Ala296Thr, which is predicted to be pathogenic by 3/6. This difference could be related in part to the location of the residue within the extracellular space, but also to the fact that prediction scores from SIFT, Mutation Assessor and Polyphen2 tend to cluster together and be highly correlated [28] as they may

use the same training data or similar methods to elaborate a prediction. However, we are reassured by the deleterious prediction from the ensemble scores (MetaSVM and MetaLR) from dbNSFP v3.0, which are known to outperform all their component scores [28], and more importantly, by its absence from a database containing >13,000 Arab exomes.

The response to pharmacological treatment in patients with CMS type 20 seems to be phenotype-dependant. In keeping with the patients currently reported, individuals presenting with episodic apnoeas seem to improve on cholinesterase inhibitors while those with the severe global phenotype did not show any effect [2]. Since choline uptake serves as a limiting step for sustained ACh synthesis [8], it is plausible that cholinesterase inhibitors may only be beneficial as long as the mutant CHTs hold some residual activity. Studies in *SLC5A7*^{+/-} transgenic mice showed increased sensitivity to stresses that interfere with ACh turnover [10]. This would suggest a potential benefit from additional doses of cholinesterase inhibitors during infections, hot weather or other precipitating factors of sudden apnoeic attacks. It is not known whether 3,4-diaminopyridine is helpful in this subtype of CMS. Individual II:4 from family 3 developed epileptic seizures while on this drug, which highlights the need for using it with caution especially if central manifestations are present. Affected individuals from family 1 and 2 had additional benefit from treatment with salbutamol, a β 2-adrenergic receptor agonist. This was also reported in a patient homozygous for the p.P210L substitution on combined treatment with pyridostigmine and ephedrine [11]. β 2-adrenergic agonists have shown to enhance neuromuscular transmission by improving the structure of the postsynaptic membrane [29–31]. Since β 2-adrenergic receptor agonists are affordable drugs with a good safety profile, we would recommend assessing their effect on muscle strength if muscle weakness remains on treatment with cholinesterase inhibitors. Finally, the future development of novel compounds that positively modulate CHT transport enhancing cholinergic signalling could be beneficial in the most severe cases [32].

In conclusion, we present three families with novel mutations in *SLC5A7* and different clinical phenotypes. CMS should be suspected in infants with apparent life-threatening events and in newborns with severe hypotonia and lack of respiratory drive and spontaneous movements. Pharmacological treatment can be beneficial in patients with respiratory apnoeas while those with the more severe global phenotype are typically non-responsive.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2020.10.006.

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