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Prevalence and genetic subtypes of congenital myasthenic syndromes in the pediatric population of Slovenia

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ABSTRACT

Aim: Congenital myasthenic syndromes (CMS) are rare, genetically and phenotypically diverse disorders of neuromuscular transmission. Data on prevalence among children are scarce. Whole exome sequencing facilitated discovery of novel CMS mutations and enabled targeted treatment. Our aim was to identify the prevalence, genetic subtypes and clinical characteristics of CMS in pediatric population of Slovenia.
Methods: In this observational, national, cross-sectional study, medical records were retrospectively reviewed. Children with genetically confirmed CMS, referred over a 19 – year period (2000–2018) to the University Medical Centre, Ljubljana, Slovenia, were included in the study. Genetic and phenotypic characteristics were collected and prevalence of CMS in children was calculated.
Results: Eight children with a confirmed genetic mutation in 5 different genes (CHRNE, CHRND, RAPSN, CHAT, MUSK) causative of the CMS were identified. Calculated prevalence of genetically confirmed CMS was 22.2 cases per 1.000.000 children at the end of 2018.
Interpretation: The prevalence of genetically confirmed CMS in Slovenian children at the end of 2018 exceeds previously reported prevalence by more than two-fold, which suggests that prevalence in the literature is likely to be underestimated. Two extremely rarely detected mutations in MUSK and CHRND gene were detected and patient's clinical descriptions add important information on genotype-phenotype correlation.

1. Introduction

Congenital myasthenic syndromes (CMS) are rare, genetically and phenotypically diverse genetic disorders of neuromuscular transmission. The disease usually presents during the first and second year of life with fluctuating weakness, fatigability and exercise intolerance, typically involving ocular, bulbar, and limb muscles [1,2]. Episodic apneas in neonates, which can be the presenting symptom in certain subtypes, can result in brief resolved unexplained events (BRUE) [3].

Up to the last decade, typical clinical picture, positive family history, abnormal neuro-physiology tests, namely repetitive 3 Hz nerve stimulation (RNS) or single fiber electromyography (SF-EMG) and negative ACh and Musk antibodies have been hallmarks of the diagnosis [4]. In the last few years, next generation sequencing (NGS) and whole exome sequencing have become important and more readily available methods of confirming the diagnosis and the genetic subtype. The genetic landscape of CMS has been expanding fast in the past few years. In 2012, a total of 14 different genes known to cause CMS were reported and to date over 30 genes have been implicated [5].

Correct diagnosis is especially important because of treatment possibilities for certain subtypes [6–8]. Drugs with a positive effects for a specific CMS subtype may worsen other forms of CMS, depending on the underlying genetic defect. That is why the genetic subtyping of CMS is key to rational and optimized pharmacological treatment [2,9–11].

The aim of this study was to identify the prevalence and the genetic subtypes of childhood CMS in Slovenia. Knowledge on the

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most common genetic subtypes and corresponding phenotypes in our region could assist the physicians with establishing correct diagnosis and management of patients with CMS locally and globally.

2. Methods

In this retrospective, national, cross sectional study, all pediatric patients with genetically confirmed diagnosis of CMS obtained by December 31, 2018, and referred over a 19 − year period (2000−2018) to the Department of Child, Adolescent and Developmental Neurology, University Medical Centre, Ljubljana, Slovenia, were included. Considering ours is the only tertiary centre for child neurology and the only specialized pediatric centre for treatment of children with neuromuscular diseases in Slovenia, all children with neuromuscular diseases in the country are referred to our centre. We are also curating the national Registry of Slovenian Children with Neuromuscular Diseases and collaborating with the European Treat-NMD Neuromuscular Network. We therefore assume to cover all identified pediatric patients with CMS in our country.

We have checked our electronic health record system and paper medical archives, searching for patients with any of the diagnosis according to the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD-10) of G70.0* (Myasthenia gravis and other myoneural disorders) or G70.2.* (Congenital and developmental myasthenia). Only patients with genetically confirmed CMS were included in the study.

Genetic analyses prior to 2016 have been performed in foreign laboratories (Neuromuscular Research Laboratory, Mayo Clinic, Rochester and Centogene Laboratory, Rostock). Since 2016, genetic testing was performed at the Clinical institute of medical genetics and Centre for Medical Genetics, University Children's Hospital Ljubljana of the University Medical Centre Ljubljana and was based on either targeted sequencing of 4.813 genes associated with monogenic conditions (using the Illumina TruSight One panel) or whole exome sequencing (using the Illumina Nextera Coding Exome v1.2). The library preparation was performed according to manufacturer's recommendations and sequencing was performed on the Illumina sequencing platforms (Illumina MiSeq and Illumina NextSeq 550). An in-house pipeline in accordance with GATK best practice guidelines was employed for data analysis and annotation [12]. The interpretation of data was focused on the panel of 55 genes, associated with various forms of congenital myasthenia and conditions presenting with. The interpretation of sequence variants was based on ACMG/AMP standards and guidelines [13].

The population of children (age less than 19 years) in Slovenia at the end of 2018 was obtained from the Statistical Office of the Republic of Slovenia. Prevalence of genetically confirmed CMS in children at the end of 2018 was calculated.

Patients' clinical data, such as age at diagnosis, type of genetic mutation, electrophysiological studies, therapy and outcome were considered from medical data and clinical neurological examination which performed in 2019.

3. Results

3.1. Prevalence

We have identified 8 children (3 males, 5 females) with a confirmed genetic CMS who were born between January 1, 2000 and December 31, 2018. All 8 children were included in the study and none of the identified children died in the study period.

The population of children (age less than 19 years) in Slovenia at the end of 2018 was obtained from the Statistical Office of the Republic of Slovenia and was 360.161. All eight identified children included in the study were still under 19 years of age at that time. Therefore, the estimated prevalence of genetically confirmed CMS in children of Slovenia was 22.2 cases per million.

3.2. Genetic subtypes of CMS

The 8 children with a CMS in our cohort came from 6 unrelated families; among them were two pairs of siblings with an identical genetic mutation. In all other families, no other relatives with CMS or other neuromuscular disease were present. All patients were of Slovene nationality and Caucasian origin, although 1 family (2 children, siblings) had Albanian ancestors. None of the patients were born in consanguineous marriages.

Since 2016, 10 patients were tested for CMS at the Clinical institute of medical genetics, University Medical Centre Ljubljana. The diagnosis of CMS was confirmed in four of them, mutations in genes connected to myopathies were detected in two patients (not included in this study), while in four remaining patients genetic testing was negative. Genetic mutations in other patients in our cohort were diagnosed in laboratories abroad, for which we do not have additional data on CMS-negative tests (Fig. 1).

Regarding the genetic subtypes of CMS in our patients (see Table 1), 4 patients had a primary acetylcholine receptor (AChR) defect caused by a mutation in one of the subunit genes (3 CHRNE and 1 CHRND), three patients had a defect in the end-plate potential development and maintenance caused by a RAPSN (two patients) or MUSK (one patient) mutation and one had a choline acetyltransferase (ChAT) deficiency due to a mutation in the CHAT gene.

Nine different mutations (Table 1) were identified in our cohort and two of them have not previously been reported in association with disease in humans. Novel mutations were found in patients 2 and 8, namely in MUSK and CHRNE genes. Patient 2 was compound heterozygous for two missense variants in MUSK gene (c.1724T > C and c.2365G > A). While the c.1724T > C variant has previously been reported as pathogenic, the novel c.2365G > A variant in MUSK gene is absent from the control populations of the gnomAD project and a majority in-silico algorithms predicted its damaging effect on protein function. According to ACMG guidelines, we classify the novel c.2365G > A variant as likely pathogenic (criteria PM2, PM3, PP3, PP4).

The novel c.3G > A CHRNE gene variant was observed in homozygous state in patient 8 and is predicted to disrupt the initiation codon of the CHRNE sequence and thus leading to loss of function of the gene. Additionally, the variant is also absent from

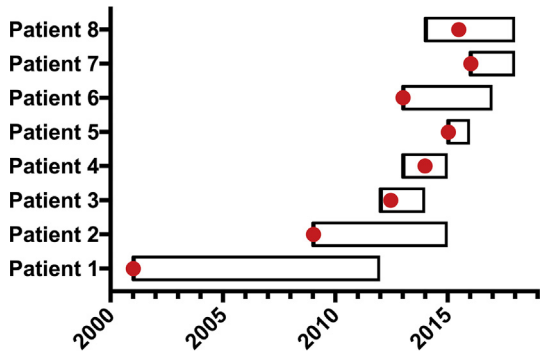

Fig. 1. Patients' year of birth (left side of the bar), time of first clinical signs (red dot), and year of genetic diagnosis (right side of the bar). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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Table 1
Genetic characteristics of CMS subtypes in Slovenian cohort of patients.

Patient No.	Family No.	Gene	Coding transcript change	Predicted protein change	ACMG classification	Genotype
1	1	CHRND	c.481G > A	p.(δD140 N)	Likely pathogenic	Compound heterozygous
			c.821-2A > C		Pathogenic	
2	2	MUSK	c.1724T > C	p.(Ile575Thr)	Pathogenic	Compound heterozygous
			c.2365G > A	p.(Gly789Ser)	Likely pathogenic	
3	3	CHRNE	c.1327delG	p.(Glu443Lysfs*64)	Pathogenic	Homozygous
4	3	CHRNE	c.1327delG	p.(Glu443Lysfs*64)	Pathogenic	Homozygous
5	4	CHAT	c.1249G > A	p.(Gly417Arg)	Likely pathogenic	Homozygous
6	5	RAPSN	c.264C > A	p.(Asn88Lys)	Pathogenic	Compound heterozygous
			c.490C > T	p.(Arg164Cys)	Pathogenic	
7	5	RAPSN	c.264C > A	p.(Asn88Lys)	Pathogenic	Compound heterozygous
			c.490C > T	p.(Arg164Cys)	Pathogenic	
8	6	CHRNE	c.3G > A	p.(Met1?)	Pathogenic	Homozygous

control populations of the gnomAD project. According to the ACMG guidelines, the variant is considered to be pathogenic (criteria PVS1, PM2, PP4). Segregation analyses in unaffected parents showed that they are both heterozygous carriers of this variant, which is compatible with recessive inheritance for CHRNE-associated disease.

After 10 years of genetic testing, compound heterozygous mutations in CHRND gene were finally identified in patient 1 and detailed pathophysiologic consequences were thoroughly described by Shen et al., in 2016 [14].

Half of our patients were homozygotes for the reported mutations, but 1 pair of siblings (patients number 6 and 7) and patients 1 and 2 were compound heterozygous.

3.3. Phenotypes of CMS

Main clinical characteristics, response to pharmacological treatment, dosage of drugs and EMG results according to the underlying defects are more extensively presented in Table 2.

All patients, except the three with CHRNE mutation, presented in the neonatal period. In patients with mutations in the CHRNE gene, the clinical picture manifested in the first 16 months of life (3–16 months). Time to genetic diagnosis in these patients was from 1 to 11 years (median 2.2 years). Five patients were treated with ACEI before the confirmed genetic diagnosis patients (patient 1/CHRND; patient 2/MUSK; patient 3/CHRNE; patient 5/CHAT; patient 8/CHRNE) based on clinical and electrophysiological findings. After the genetic diagnosis was confirmed, we have initiated the treatment in 3 patients (patient 4/CHRNE; patient 6/RAPSN; patient 7/RAPSN) and modified treatment according to the genetic subtype in 1 patient (patient 2/MUSK).

Moderate to severe generalized hypotonia and muscle weakness were the first presenting signs in all patients. Mild to severe ptosis was reported in all but one patient (patient 7) with the RAPSN mutation. Mild contractures of several fingers were noted in both sisters with RAPSN mutations, which were the main indication for NGS CMS panel testing.

Ophthalmoplegia was detected only in patient 1 with CHRND mutation, but bulbar weakness, manifesting as feeding problems (difficulties in sucking, chewing or swallowing), was observed in half of our cohort (patients 1/CHRND; patient 2/MUSK; patient 5/CHAT; patient 6/RAPSN).

Severe, life-threatening respiratory distress that required invasive ventilation with tracheostomy was present in three patients (patients 1/CHRND, patient 2/MUSK, patient 5/CHAT). Nevertheless, all the patients were prone to recurrent respiratory tract infections due to respiratory muscles weakness except one (patient 4/CHRNE).

Pyridostigmine in the dosage of 3–12 mg/kg daily was the first line treatment in all the patients, but was ineffective in the patient with MUSK mutation. This particular patient partially reacted to 3,4-Diaminopyridine (3,4-DAP) treatment. 3,4-DAP was added to pyridostigmine in three other patients with pronounced clinical picture (patients 1/CHRND; patient 3/CHRNE; patient 6/RAPSN) and had a beneficial effect in these patients.

3.3.1. Electrophysiology

Electrophysiological studies (motor and sensory nerve conduction studies, repetitive nerve stimulation and/or single fiber electromyography (SF-EMG) were performed in seven of eight patients. The results were consistent with a neuromuscular junction disease in only three patients, but only after the first year of life, in one of them the investigation was performed in sedation; in others the EMG did not reveal findings, consistent with the diagnosis of CMS. In general, the key to diagnosis was clinical picture combined with genetic testing.

4. Discussion

Despite the fact that CMS is considered to be a very rare disease, the possibility of effective treatment adds to the importance of early diagnosis of CMS.

Data on prevalence of CMS are scarce, the studies were mostly conducted in non-European regions [9,15–18]. In Europe, the reported prevalence was between 1.8 cases per million total population in Spain to 9.2 per million children in UK [15,17]. Our study revealed the prevalence of 22.2 cases per million children, which exceeds previous reported prevalence by more than two fold. Increase in the incidence of genetically confirmed CMS cases in the last few years is mostly due to easily accessible and advanced genetic testing. However, this prevalence most likely still underestimates the real prevalence of the disease in Slovenia. There is a high possibility, that more cases would have been identified in the studied period, had the genetic testing been more available earlier on and some children with CMS born in the recent years might have not been identified yet. In our centre, the vast majority of patients with a clinical suspicion of a neuromuscular disease, even with normal EMG results, are genetically tested, using mainly NGS methods. Thus, even the patients with less marked and less specific signs for CMS have been identified, as for example the siblings with RAPSN mutation in our cohort. Higher prevalence of CMS in pediatric population of Slovenia might also be connected to healthcare organization, due to a centralized care, as all patients with a suspected neuromuscular diseases are usually referred to our tertiary centre. Another possibility would be that the genetic background of Slovene children is different from other studied populations. More likely, the lack of data makes reliable estimation of a prevalence in Europe hard and could explain big diversity of previously published

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Table 2
Clinical characteristics of children with CMS. 3,4-DAP: 3,4-Diaminopyridine; EMG: electromyography; SF-EMG: single fiber electromyography; RNS-EMG: repetitive nerve stimulation electromyography.

Patient No.	Sex	Mutated Gene	Age of Clinical Onset	Age at Genetic Diagnosis	Neurological Symptoms	Respiratory Symptoms	Therapy/ Response to Therapy	EMG
1	F	CHRND	neonatal	11 y	Birth asphyxia, severe generalized muscle weakness and ptosis, feeding difficulties (need for gastrostomy), lordosis and scoliosis	Hypoventilation, invasive home ventilation (tracheostomy from 6th year on) Recurrent severe respiratory distress during respiratory tract infections.	Pyridostigmine 2,5 mg/kg; 5x/d 3,4-DAP 0,15 mg/kg; QID Pyridostigmine from 1st month on	Unsuccessful in the 1st year RNS-EMG in the 2nd year characteristic of ChAT CMS
2	M	MUSK	neonatal	5 y	Mild birth asphyxia, severe ptosis, moderate to severe generalized hypotonia, feeding difficulties, lumbal lordosis	Inspiratory stridor, recurrent severe respiratory distress, hypoventilation. Transient invasive home ventilation (tracheostomy: 1–29 mo).	Pyridostigmine and salbutamol ineffective 3,4-DAP 0,25 mg/kg; QID Pyridostigmine: 16–27 mo	RNS-EMG in the 1st year unspecific. SF-EMG (20 mo): moderate abnormalities of NMJ disease consistent with CMS.
3	F	CHRNE	3 mo	2 y	Ptosis, generalized hypotonia, week cry, week cough, muscle weakness	Recurrent respiratory tract infections	Pyridostigmine 2 mg/kg; 5x/d from 10th mo on, 3,4-DAP 0,05 mg; QID from 19th mo on	SF-EMG at 13th mo (sedation): severe abnormalities of NMJ consistent with postsynaptic type
4	F	CHRNE	1 y	2 y	Mild ptosis, generalized hypotonia	None	Pyridostigmine 1,25 mg/kg; QID After genetic dg was made	/
5	M	CHAT	neonatal	1 y	Moderate birth asphyxia with convulsions, severe generalized hypotonia, mild contractures at birth, feeding difficulties (need for gastrostomy)	Respiratory arrest in neonatal period, invasive home ventilation (tracheostomy from 1st mo on), recurrent respiratory tract infections	Pyridostigmine 2,5 mg/kg; 5x/d Pyridostigmine from 7th month on.	RNS-EMG in the 1st year normal and uncharacteristic for NMJ disease
6	F	RAPSN	neonatal	5 y	Moderate generalized hypotonia, mild ptosis, contractures of fingers, craniosynostosis of metopic suture, feeding difficulties, muscle weakness, fatigability	Recurrent respiratory distress during respiratory tract infections	Pyridostigmine 1,7 mg/kg; QID 3,4-DAP 0,2 mg/kg; TDI After genetic dg was made	SF-EMG: no abnormalities
7	F	RAPSN	neonatal	1.5 y	Mild asphyxia at birth, discrete contractures of fingers, moderate generalized hypotonia, fatigability	Recurrent respiratory distress during respiratory tract infections	Pyridostigmine 2 mg/kg; QID After genetic dg was made	SF-EMG: no abnormalities
8	M	CHRNE	16 mo	4 y	Intermittent ptosis, torticollis, muscle weakness, fatigability	Pneumonia 1x	Pyridostigmine 1 mg/kg; TID or QID from 3rd year	RNS-EMG: negative.

data [15,17]. In any case, our data reiterates the proclaimed fact that CMS is often a missed diagnosis and prevalence in the literature is likely to be underestimated [10,11]. More effective diagnostic strategies and disease awareness among healthcare professionals are therefore of major importance for identification of CMS patients.

As for the genetic CMS subtypes, the nicotinic AChR defects are reported to be the prevailing mutations, followed by defects in endplate potential development and maintenance [2,5,19]. In Slovenian children the genetic mutations have a similar distribution, since half of the CMS patients are diagnosed with AChR mutations. CHRNE gene mutations account for 37% of CMS patients altogether, which is comparable to data reported by Parr et al. from UK children and slightly higher than detected in the Spanish population [15,17]. The C.1327delG (p.Glu443Lysfs*64) CHRNE recessive mutation, which is linked to south-eastern European or/and Roma ancestors [10], was the most prevalent form. The detected RAPSN mutation in siblings (patients 6,7) contributed to 25% of the cohort compared to 15.0–17.5% in big CMS cohorts at Mayo Clinic (n = 356) and Spain (n = 64) [2,5,15]. The genotype in our patients happened to be a compound heterozygote mutation, with one of the allele being the most common c.264C > A (p.Asn88Lys) mutation previously reported in European countries. Our results are therefore consistent with the Spanish study, where c.264C > A (p.Asn88Lys) mutation was detected either as in a compound heterozygote or homozygote state in all of the patients with RAPSN CMS [15]. Two rarely reported genes were causative of CMS in two of eight patients, namely CHRND and MUSK mutations.

Genotype-phenotype correlations in our cohort were similar to those found in previous reports, namely RAPSN and CHRNE mutations carrying a relatively benign and stable clinical course, compared to patients carrying CHAT, CHRND and MUSK mutations who are prone to episodic apnea in early period [3,20,21]. Tracheostomy due to episodic apnea and/or severe respiratory distress was

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in our cohort performed in the first months of life in patients with CHAT and MUSK mutation and at the age of six years, in the patient with CHRND mutation. CHAT mutation has been known as the most common cause of episodic apnea and respiratory distress in CMS, but correlation with MUSK mutation has not been so obvious [3,16]. According to recent publication by Finsterer, MUSK mutations are very rare and only 9 patients were reported so far, among which respiratory insufficiency and nocturnal apnea was observed in one patient only. Referring to the same article, CHRND mutations are even less prevalent, with 5 patients reported up till now [21]. Detailed clinical description is available for two of those, with feeding difficulties, moderate generalized weakness, and recurrent episodes of respiratory insufficiency provoked by infections as a hallmark of disease – these symptoms were also present in our patient with CHRND mutation, as well as scoliosis and lordosis. Brief clinical description of patient 1, with emphasis on biochemical characteristics of mutant AChR protein, was already previously published in collaboration with Neuromuscular Research Laboratory, Mayo Clinic, where genetic diagnosis of CMS was confirmed and mutations in CHRND gene were identified [14].

One of the important advantages of a genetic diagnosis in CMS is the ability of treatment optimization according to underlying genetic mutation [22]. In general acetyl cholinesterase inhibitors (ACEI) such as pyridostigmine are the most commonly used therapeutic agents, but additional use of the potassium channel blocker (3,4-DAP), AChR open channel blockers (fluoxetine or quinidine) and the β_2-adrenergic receptor agonists (ephedrine and salbutamol) can be effective as well [2,9–11]. Efficacy of pyridostigmine was confirmed in our cohort as well, except in patient 2 with MUSK mutation, where insufficiency was expected. Pyridostigmine has been reported to even aggravate the symptoms in MUSK CMS, which was not the case in our patient. However, reports on clinical benefit of 3,4-DAP and salbutamol in these patients promoted a trial in our patient as well, who profited from 3,4-DAP treatment [9,21].

Historically, a typical clinical picture, negative AChR and MUSK antibodies and abnormal neuro-physiology tests indicating a NMJ disease were key to the diagnosis of CMS. Repetitive 3 Hz stimulation test showing decremental response or abnormal jitter and blocking on single-fiber electromyography are usually typical findings in patients with CMS. In our cohort electrophysiologic tests were conclusive of CMS in only three patients but not before the age of one year. In other patients showed no abnormalities or the investigation was technically unsuccessful and in one was not performed at all. The reason for such low gain from neurophysiological tests in our population could be the technical complexity of this test in the youngest children and lack of experienced investigators. However, electrophysiological tests can be normal in CMS patients, particularly in RAPSN mutations [20]. Time to the diagnosis of CMS has evidently shortened and incidence of CMS increased after the availability of genetic diagnostic tests increased. According to our results, genetic testing seems an important and often crucial step in any case of clinical suspicion of CMS, despite negative electrophysiological results.

To conclude, this study revealed a more than two-fold higher prevalence of genetically confirmed CMS in children in Slovenia, compared to the previous reports from other European and non-European regions. This underlines the importance of disease awareness among healthcare professionals and reliable diagnostic tests, particularly genetic testing, in order to diagnose CMS patients as soon as possible, considering the availability of effective symptomatic treatment. The most prevalent genetic subtypes in Slovenian children were identified, which provide valuable information for directing genetic tests in our population. Two rarely reported genes were causative for CMS in two patients, namely CHRND and MUSK mutations. The clinical descriptions of these patients add important information on genotype-phenotype correlations in those otherwise sporadically recorded genes. Due to the rarity of CMS, multicentric studies with large cohorts would be beneficial in the future in order to further enlighten genotype-phenotype correlations of CMS in children.

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