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Identifying novel *AGRN* variants in congenital myasthenic syndrome: insights from three Iranian families

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ABSTRACT

Congenital myasthenic syndromes (CMS) are rare inherited disorders characterized by defective neuromuscular transmission, with the *AGRN* gene recognized as a notable yet infrequent cause of CMS. This study aims to delineate the clinical and molecular characteristics of three Iranian patients diagnosed with *AGRN*-related CMS. Whole exome sequencing (WES) was performed, leading to the identification of three novel variants in the *AGRN* gene. One patient had a homozygous indel variant, another patient had a missense mutation in a homozygous state, and a third patient had two missense variants that were inherited in a compound heterozygous manner. Each patient exhibited distinct clinical presentations ranging from progressive weakness, fatigue, to muscle atrophy and this heterogeneous clinical presentation contributed to diagnostic delays. Electrophysiological studies confirmed neuromuscular junction disorder through significant decremental responses after repetitive nerve stimulation. Treatment outcomes varied, demonstrating the complexity of therapeutic efficacy among patients with agrin mutations. These findings underscore the phenotypic diversity associated with *AGRN* mutations and emphasize the challenges in the diagnosis and management of CMS. This research enhances understanding of the clinical and molecular landscape of *AGRN*-related CMS in Iranian patients and highlights the importance of tailored therapeutic strategies.

1. Introduction

Congenital myasthenic syndromes are a group of inherited disorders caused by genetic mutations that result in defective signal transmission at the neuromuscular junction (NMJ) [1]. CMS are classified as rare disorders, with prevalence estimates reported in the literature ranging from 1.8 per million in the general population to 22.2 per million in pediatric populations [2,3].Early-onset fatigable muscle weakness is the defining clinical feature of CMSs. Furthermore, the severity of symptoms and the age of onset exhibit considerable variability across different genetic subtypes [4].

The rapid advancements in next-generation sequencing (NGS) technology over the past decade have led to a continuous increase in the identification of molecular defects, with over 35 monogenic disease genes associated with presynaptic, synaptic, and postsynaptic components of the NMJ documented to date [1,5]. Currently, mutations that affect the development and maintenance of the NMJ are classified into a distinct category of CMSs and are ranked as the second most common cause of the disease, following defects in acetylcholine receptors (AChRs). The genes associated with this condition include *RAPSN, DOK7, LRP4, MUSK*, and *AGRN* [6]. Agrin, produced by the *AGRN* gene (MIM #103,320), is a cell-specific heparan sulfate proteoglycan generated through alternative splicing. Agrin, which is derived from motoneurons, is secreted from nerve terminals into the synaptic cleft, facilitating the clustering and synthesis of postsynaptic AChRs by activating the postsynaptic LRP4-MuSK-Dok-7 complex [5,7]. To date, several cases with a mutation in this gene have been documented [8]. In this study, we aimed to delineate the clinical and molecular spectrum of *AGRN*- related CMS in three unrelated Iranian patients.

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2. Method and material

2.1. Subjects and whole exome sequencing

Three unrelated Iranian patients diagnosed with CMS were clinically and genetically analyzed at the Neuromuscular Research Center, affiliated with Tehran University of Medical Sciences (TUMS) in Iran. The pedigrees (family 1 and 2) indicated autosomal recessive inheritance patterns, with the two affected individuals being born to healthy first-degree consanguineous parents, or occurring sporadically in family 3 (Fig. 1). We followed the guidelines outlined in the Declaration of Helsinki and informed consent was obtained from the patients or their guardians. Blood samples were collected from the patients, their parents, and any available family members for molecular analysis. The genomic DNA extracted from the proband's blood underwent whole exome sequencing (WES), and the methodology and analysis of the SNVs (single nucleotide variant) and CNVs (copy number variation) have been detailed in our previous studies [9–11]. To validate the identified genetic variants and for co-segregation analysis, Sanger sequencing was performed using ABI Big Dye terminator chemistry along with an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA).

2.2. Structural modeling of the p.Arg140Trp variant on the AGRN protein

We utilized the Missense3D database (https://missense3d.bc.ic.ac.uk/), a web-based tool to predict the potential structural effects of p. Arg140Trp novel missense variant.

3. Results

The proband from family 1 (1-III1, Fig. 1A) was a 19-year-old female, born to consanguineous parents. She first presented at the age of 12 with weakness in her lower extremities. Her condition progressed slowly; initially, she was unable to run, and over time, she experienced difficulty climbing stairs. In the past year, she developed problems raising her arms. She reported experiencing fatigue but did not have significant fluctuations in her symptoms. Upon examination, she had a high-arched palate, with normal cognitive function and speech. There was no evidence of ptosis, ophthalmoparesis, or facial weakness. Weakness was observed in bilateral shoulder abduction and finger abduction (grade 4/5), while strength in other upper extremity muscles was normal. Conversely, her lower limb muscles exhibited bilateral symmetric weakness (grade 4/5 in hip flexion, knee flexion, and knee extension; grade 3/5 in ankle dorsiflexion; and grade 4/5 in ankle plantar flexion). Gowers' sign was positive, and her calf muscles appeared atrophic. Axial muscle strength and tone were normal, although mild lumbar scoliosis was noted. Deep tendon reflexes (DTRs) were normal. She had a waddling gait and could not perform heel or toe walking. Pulmonary function tests revealed a reduced forced vital capacity (FVC) at 70 % of predicted values. Electrophysiological studies revealed normal routine nerve conduction studies (NCS), but the repetitive nerve stimulation (RNS) test demonstrated significant decrement, and electromyography (EMG)showed low amplitude, and short-duration motor unit potentials. Treatment with pyridostigmine at a dose of 60 mg three times per day led to improvement in fatigue and muscle weakness. After a few months, she mentioned some deterioration and salbutamol 2 mg three times a day was added with significant improvement in muscle force (all muscles were 5/5 except for shoulder abduction and hip flexion which were 4+).

Whole exome sequencing identified a homozygous indel variant in exon 26 of *AGRN*: NM_198,576.4:c.4584_4586del, p. Gln1528_Arg1529delinsHis (Fig. 2). This variant has not been previously reported in the literature and has been classified as a variant of uncertain significance (VUS) according to the criteria established by the American College of Medical Genetics and Genomics (ACMG). This classification is based on the application of criteria PM2 and PM4. The PM4 rule is particularly significant as it indicates that the variant is associated with a change in protein length that arises from in-frame deletions or insertions occurring in a non-repetitive region of the gene, or as a result of a stop-loss variant.

Furthermore, if we incorporate the strong evidence provided by rule PP4, which relates to the alignment of the variant with the clinical phenotype, alongside rule PP1, which assesses co-segregation of the variant within affected families, the classification of this variant can be upgraded to likely pathogenic [12].

Proband of the second family (2-III1, Fig. 1B) was a 38-year-old female who also had consanguineous parents and presented with difficulty running and a tendency to fall during childhood. Her condition had a stepwise progressive course. She experienced proximal weakness in her lower extremities starting at age 20, and weakness in her proximal upper extremities at age 27, following the births of her first and second child, respectively. Her symptoms worsened over the last three years, accompanied by complaints of dyspnea at rest. She reported intermittent leg cramps. On examination, she had no signs of ptosis, ophthalmoparesis, or facial weakness. Examination of her upper extremities showed symmetric weakness in several muscle groups (grade 4/5 in shoulder abduction and elbow flexion; grade 3/5 in thumb abduction; grade 4/5 in finger abduction), while other muscle groups remained strong. In the

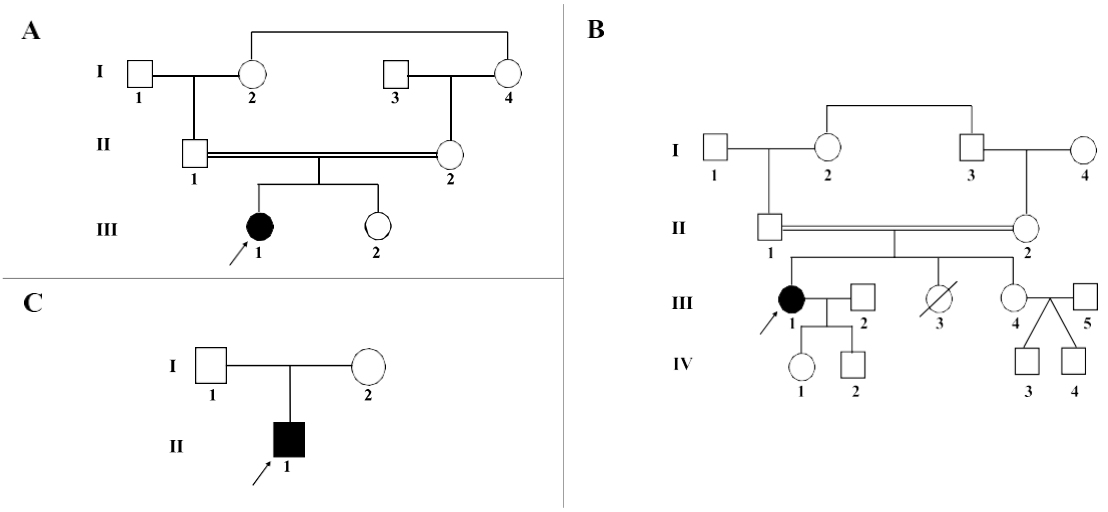

Fig. 1. (A) Family 1, (B) Family 2 and (C) Family 3 pedigrees. The arrow denotes the proband. Blank circles and squares: normal individuals; Dark circle and squares: CMS- affected individuals.

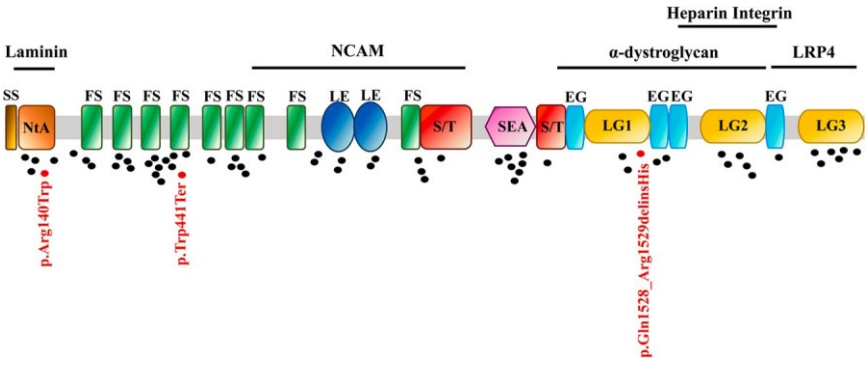

Fig. 2. Domains of agrin protein according to UniProtKB are drawn to scale. Previously reported variants (black dots) and variants identified in the current study (red dots) according to NM_198,576.4 (RefSeq) are indicated. SS, secretion signal peptide; FS, follistatin-like domain; LE, laminin EGF-like domain; S/T, serine/threonine-rich domain; SEA, a sperm protein, enterokinase, and agrin domain; EG, EGF-like domain; and LG, laminin G-like domain. Horizontal bars indicate positions where the indicated binding partners bind.

lower extremities, her knee extension was weak bilaterally, but other muscle groups showed normal strength. Gowers' sign was positive. DTRs were normal. Electrocardiogram (ECG) and echocardiogram results were normal, and pulmonary function tests indicated an FVC of 92 % of predicted values. Serum CPK levels were recorded at 342 U/L (normal range < 200 IU/L [13]) on one occasion but were normal on subsequent tests. At the age of 26, she underwent a muscle biopsy, which showed no significant abnormality. Electrophysiological studies revealed normal routine NCS, but the RNS test demonstrated significant decrement, and EMG showed low amplitude, short duration motor unit potentials. Treatment with pyridostigmine (60 mg three times per day) resulted in a worsening of her symptoms, leading her to discontinue the drug after one month. Subsequently, she was treated with salbutamol (3mg three times per day), resulting in a marked improvement in muscle strength, and after 6 months of treatment, her muscle force became normal on manual muscle testing. Follow-up spirometry showed an FVC of 93 % of predicted, although she still reported mild dyspnea.

WES unraveled that she carries a homozygous missense variant, c.418C>T, p.Arg140Trp, in exon 2 of *AGRN* gene (Fig. 2). This variant is a novel variant of uncertain significance (VUS) according to the PM2 criterion outlined by the ACMG. However, the classification of this variant can be reassessed with the incorporation of additional ACMG evidence-based criteria, specifically PP4 and PP1. When considering these two additional criteria—strong evidence of clinical correlation (PP4) and co-segregation within families (PP1)—the classification of this variant shifts from VUS to likely pathogenic.

After introducing the protein data bank (PDB) file of the AGRN protein, the probable pathogenic effects of the p.Arg140Trp variant were analyzed. Missense3D data indicated that this substitution leads to an expansion of the cavity volume of the protein, suggesting that this mutation may potentially cause structural damage (Fig. 3).

The proband of family 3 (1-II1, Fig. 1C) was a 33-year-old male who presented with proximal weakness and gait difficulties in early childhood. The course of his disease was relatively stable, although he reported exertional dyspnea since childhood. He reported postprandial nausea and vomiting, with a diagnostic workup revealing distal esophageal stenosis. On examination, he displayed bifacial weakness and mild bilateral ptosis without ophthalmoparesis. Mild postural hand tremors were observed. In the upper extremities, bilateral finger and thumb abduction were weak (grade 4/5), while strength in other muscle groups remained normal. In the lower extremities, there was mild symmetric weakness (grade 4+/5) in hip flexion, knee flexion, but ankle dorsiflexion was 4-, with other muscle groups showing normal strength. Gowers' sign was positive. Pulmonary function tests revealed reduced FVC at 68 % of predicted values. Electrophysiological studies showed normal routine NCS and normal needle EMG, with the RNS test demonstrating significant decrement, compatible with a neuromuscular junction disorder. Treatment with pyridostigmine (60 mg three times per day) led to a marked improvement in his symptoms. However, after nine months of treatment, he reported some worsening of his condition. The dosage of pyridostigmine was increased to 60 mg four times per day, and salbutamol (2 mg per day) was added to his regimen, resulting in significant improvement. After 8 months of dual therapy, he was examined and muscle force was normal.

WES revealed that he harbored the c.418C>T (p.Arg140Trp) and c.1323G>*A* (p.Trp441Ter) variants in the *AGRN* gene in a compound heterozygous state (Fig. 2). Both variants are novel, with one classified as a variant of uncertain significance (VUS) and the other as pathogenic, based on the ACMG criteria.

The identified variants in three families were confirmed by Sanger sequencing and completely co-segregated with the phenotypes in the related family members.

4. Discussion

In our cohort study of congenital myasthenic syndrome patients (CMS) in Iran, we identified mutations in several notable genes, including *CHRNE, COLQ*, various glycosylation pathway genes (such as *GFPT1, GMPPB*, and *ALG2*), as well as *DOK7, RAPSN*, and *AGRN*. While some of these cases have been documented in the literature (Karimi et al. [14]; Hesami et al. [15]; Ziaadini et al. [16]; Ghasemi et al. [17]), others are still under review. Previous studies report that mutations in the *AGRN* gene account for approximately 0.84 % of all CMS cases [6,18]. This study specifically aims to highlight CMS cases associated with mutations in the *AGRN* gene, where we identified three novel variants in three unrelated Iranian patients.

All our patients had normal muscle tone at birth and achieved typical early motor milestones, but symptoms emerged in childhood after a period of normal development. The clinical presentation primarily involved limb weakness with minimal cranial muscle involvement, consistent with previous studies such as Finsterer et al. (2019) [19]. All patients exhibited significant distal weakness, varying in severity and distribution. Distal weakness may also be evident in some other congenital myasthenic syndromes including *MYO9A, SYT2* and *DOK7*-related CMSs [5]. Additionally, two patients had notably low forced vital capacity (FVC) levels during their initial visits, aligning with the effects of gene mutations (e.g., *AGRN, LRP4, MUSK, DOK7*) associated with bulbar and respiratory involvement [20].

Moreover, our findings underscore the diverse phenotypes associated with *AGRN*-related CMS, with symptom onset ranging from birth to early adulthood. This variability is echoed in existing literature, where cases have presented with a wide clinical spectrum, including limb-girdle weakness and fatigue, as reported by Wang et al. (2020) [18] and Ohkawara et al. (2020) [8]. It is noteworthy that similar to our

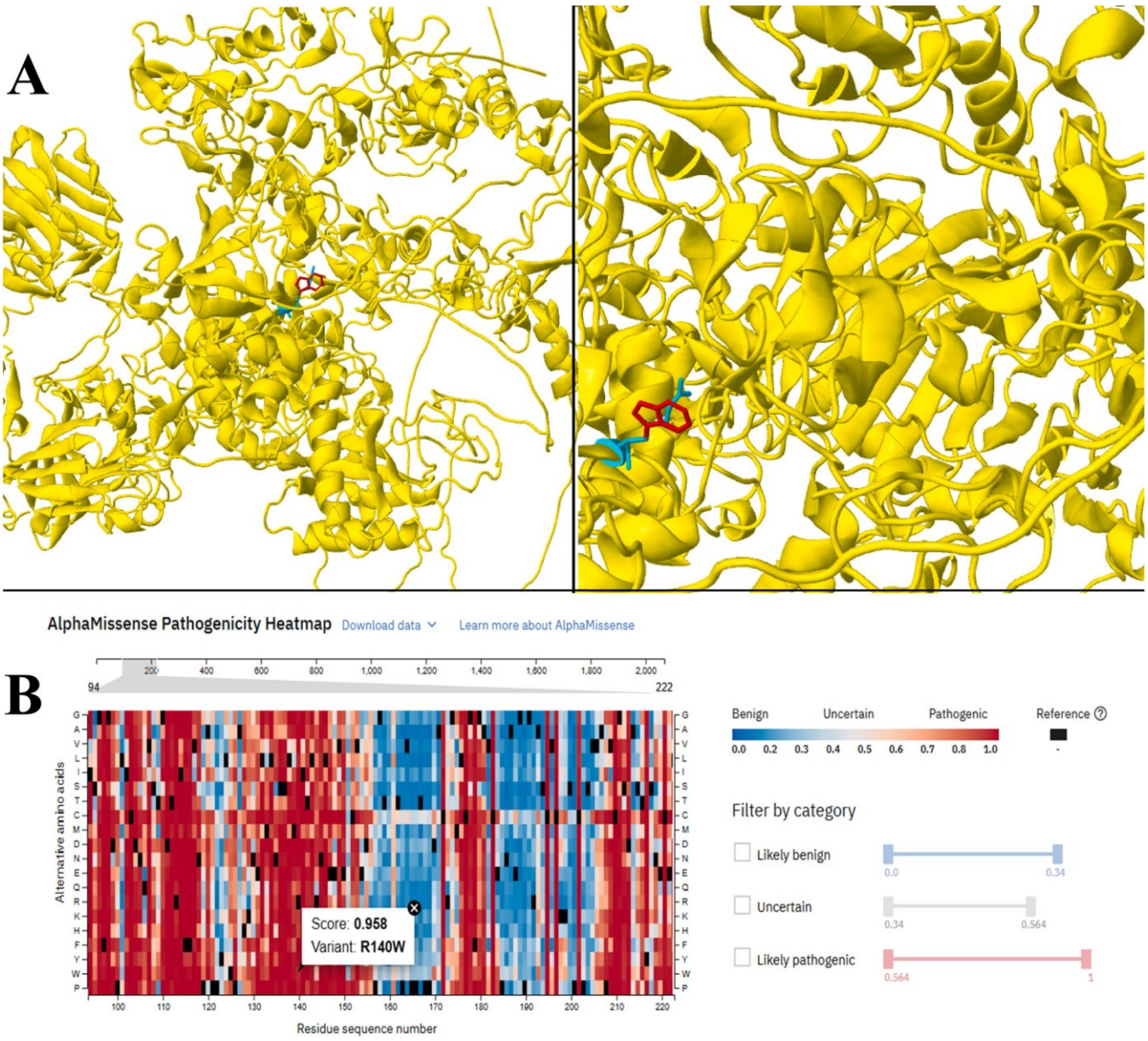

Fig. 3. (A) Structural modeling of the p.Arg140Trp variant's impact on the AGRN protein. This modeling was conducted using the Missense-3D database. The Blue and red colors represent the normal (Arg140) and mutant (Trp140) amino acids associated with the p.Arg140Trp variant, respectively. (B) The AlphaMissense Pathogenicity Heatmap which shows the *AGRN*:p.Arg140Trp variant (https://alphafold.com/).

findings, previous studies have identified significant diagnostic delays for CMS patients, often leading to misdiagnoses that include muscular dystrophy or spinal muscular atrophy, as highlighted in a study by Kao et al. (2018) [21].

One patient (P3) exhibited non-fatigable ptosis and weakness of eye closure. Limb weakness in our patients was relatively fixed, with no evidence of objective fatigability. Repetitive nerve stimulation (RNS) tests typically revealed significant decremental responses in patients with *AGRN*-associated CMS, and our cases confirmed this pattern; decrement ranged from 18.5 % to 57.6 % in the abductor pollicis brevis muscle across all patients (Table 1). Notably, the most prominent decrement response was seen in abductor pollicis brevis muscle in all of our three patients. Additionally, two patients (P1 and P2) displayed myopathic pattern on needle EMG. The combination of a myopathic EMG pattern and decremental response to RNS may serve as a specific indicator of neuromuscular junction disorders, including various subtypes of congenital myasthenic syndrome [22], recommending RNS testing for such patients in the appropriate clinical context. Previous reports suggest a high rate of acute disease exacerbation and subsequent ICU admission in patients with agrin-associated CMS [23]; however, none of our patients experienced severe exacerbation requiring hospital admission. One patient (P2) reported symptom worsening post-delivery.

While *AGRN*-related CMS generally shows a favorable response to adrenergic agonists like salbutamol, responses to pyridostigmine can vary significantly. Two of our patients initially responded well to pyridostigmine; however, both later required a combination of pyridostigmine and salbutamol, while another patient responded exclusively to salbutamol. Reports suggest that the specific type of mutation may influence treatment efficacy [19,24]. Notably, one of our patients experienced deterioration with pyridostigmine, mirroring findings in CMS related to *COLQ* and *DOK7* genes, as well as in patients with slow-channel syndrome [25]. This observation may provide insights into the underlying genetic variant associated with the CMS phenotype.

A recent Phase 1b trial is currently underway to evaluate the efficacy

Table 1
Summary of molecular and clinical features of three reported cases.

	Patient 1-III1	Patient 2-III1	Patient 3-II1
Variant of *AGRN*: NM_198,576.4	c.4584_4586del; p. Gln1528_Arg1529delinsHis	c.418C->T; p.Arg140Trp	c.418C->T;p. Arg140Trp c.1323G->*A*; p.Trp441Ter
Zygosity	Homozygous	Homozygous	Compound heterozygous
Age of onset (years)	12	early childhood	early childhood
Presenting symptoms	Lower extremity weakness	Difficulty in running	Gait difficulty
Ptosis	–	–	
Ophthalmoparesis	–	–	–
Facial weakness	–	–	
Axial weakness	–	–	–
Proximal limb weakness			
Distal limb weakness			
FVC at the first visit (percent of predict)	70	92	68
Decremental response to RNS (Percentage of decrement)	**(18.5)**	**(57.6)**	**(25.8)**
Response to pyridostigmine		–	
Response to salbutamol	++		
Other findings on examination	High-arched palate, mild scoliosis	–	–

of ARGX-119, a humanized monoclonal antibody that activates Muscle-Specific Kinase (MuSK). Given its potential to stabilize and enhance neuromuscular junction function—which is compromised in agrin-associated CMS—ARGX-119 may offer potential therapeutic benefits for this subgroup of CMS patients [26].

The *AGRN* gene, located at chromosome 1p36.33, codes for a protein named agrin [27]. The agrin protein is vital for the organization of the neuromuscular junction (NMJ) and plays a critical role in muscle function. It consists of three globular C-terminal laminin G (LG) domains, an N-terminal (NtA) domain, and follistatin-like domains (Fig. 2). The NtA domain is crucial for binding to the basal lamina, while the C-terminal LG3 domain is essential for the aggregation of acetylcholine receptors (AChRs) and other associated molecules at the NMJ. Additionally, the LG1 and LG2 domains interact with α-dystroglycan, a multimeric transmembrane protein complex that contributes to the structural integrity of the muscle cell membrane [6].

The **p.Gln1528_Arg1529delinsHis** variant in P1-III1, located in the LG1 domain of agrin, disrupts its structural integrity and binding capability, potentially destabilizing the postsynaptic apparatus and impairing neuromuscular junction (NMJ) organization (Fig. 2). This variant, along with the previously reported **p.Ala1506Thr** mutation, compromises agrin's role in AChR clustering and synaptic stability, leading to similar clinical manifestations. Both mutations result in marked decrements in repetitive nerve stimulation, indicating disrupted synaptic transmission. These findings highlight shared pathophysiological pathways in congenital myasthenic syndromes linked to *AGRN* mutations [8,18].

The **p.Arg140Trp** variant in P2-III1, located in the NtA domain of agrin, impairs its binding to laminin, crucial for AChR clustering. Similar to the **p.Arg43Ala** mutation, this variant disrupts neuromuscular transmission, as evidenced by significant decrement in RNS. Both mutations cause childhood-onset symptoms, progressive proximal weakness, and ambulation difficulties. However, the p.Arg140Trp patient presented symptoms later with a more stepwise progression, suggesting variability in clinical severity. These findings underscore shared mechanisms but distinct phenotypic expressions in agrin-related congenital myasthenic syndromes [18,28,29].

Arginine, a positively charged hydrophilic amino acid, is replaced by Tryptophan, a neutral and highly hydrophobic residue, in the p. Arg140Trp variant. This substitution also involves a significant size difference, with Arginine weighing 174 Da and Tryptophan, the largest amino acid, weighing 204 Da. We propose that this change likely alters the properties of the AGRN protein, contributing to the CMS phenotype (Fig. 2 and 3A). The p.Arg140Trp variant has been classified as pathogenic, supported by the Alphamissense algorithm, which assigns it a pathogenicity score of 0.958 (Fig. 3B). Alphamissense, part of the Alphafold platform, predicts the impact of missense mutations on protein function using structural and functional data. The high score suggests this mutation significantly disrupts protein function, leading to disease (Fig. 3).

The proband from Family 3 carried both the **p.Arg140Trp** and **p. Trp441Ter** variants. The latter variant, located in the fourth fasciclin (FS) domain, introduces a premature stop codon, resulting in the truncation of the agrin protein. This truncation leads to the loss of critical structural and functional components essential for maintaining an effective synaptic architecture and AChR clustering at the NMJ (Fig. 2) [30]. The co-occurrence of these variants in a compound heterozygous state further emphasizes the potential pathogenicity associated with disrupted agrin function (Fig. 2) [30].

In conclusion, our findings emphasize the complexity of CMS presentations, which can lead to diagnostic delays due to nonspecific symptoms. The novel variants identified, particularly in critical domains of the agrin protein, suggest potential mechanisms by which these alterations disrupt neuromuscular junction function. This study underscores the urgent need for tailored diagnostic and therapeutic strategies to improve outcomes for patients with *AGRN*-related CMS, particularly within the Iranian population. Future studies should focus on elucidating the functional implications of these variants to refine treatment protocols.

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Statements and declarations

Ethical publication statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Abbreviations

- **AChR**: Acetylcholine Receptor
- **ACMG**: American College of Medical Genetics and Genomics
- **CMS**: Congenital Myasthenic Syndrome
- **CNV**: Copy Number Variation
- **CPK**: Creatine Phosphokinase
- **DTR**: Deep Tendon Reflex
- **ECG**: Electrocardiogram
- **EMG**: Electromyography
- **FVC**: Forced Vital Capacity
- **NCS**: Nerve Conduction Studies

- **NGS**: Next Generation Sequencing
- **NMJ**: Neuromuscular Junction
- **RNS**: Repetitive Nerve Stimulation
- **SNV**: Single Nucleotide Variation
- **WES**: Whole Exome Sequencing
- **VUS**: Variant of Uncertain Significance

CRediT authorship contribution statement

Hadi Gharebaghian: Writing – original draft, Visualization, Investigation, Formal analysis. **Aida Ghasemi:** Writing – original draft, Methodology, Formal analysis. **Omid Hesami:** Writing – review & editing, Visualization. **Shahriar Nafissi:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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