**Journal of Neurology**

**Congenital myasthenic syndrome with tubular aggregates caused by GFPT1 mutations**

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Abstract: Congenital myasthenic syndrome (CMS) is a clinically and genetically heterogeneous group of inherited disorders of the neuromuscular junction. A difficult to diagnose subgroup of CMS is characterised by proximal muscle weakness and fatigue while ocular and facial involvement is only minimal. DOK7 mutations have been identified as causing the disorder in about half of the cases. More recently, using classical positional cloning, we have identified mutations in a previously unrecognised CMS gene, GFPT1, in a series of DOK7-negative cases. However, detailed description of clinical features of GFPT1 patients has not been reported yet.

Here we describe the clinical picture of 24 limb-girdle CMS (LG-CMS) patients and pathological findings of 18 of them, all carrying GFPT1 mutations. Additional patients with CMS, but without tubular aggregates, and patients with non-fatigable weakness with tubular aggregates were also screened. In most patients with GFPT1 mutations, onset of the disease occurs in the first decade of life with characteristic limb-girdle weakness and fatigue. A common feature was beneficial and sustained response to acetylcholinesterase inhibitor treatment. Most of the patients who had a muscle biopsy showed tubular aggregates in myofibers. Analysis of endplate morphology in one of the patients revealed unspecific abnormalities. Our study delineates the phenotype of CMS associated with GFPT1 mutations and expands the understanding of neuromuscular junction disorders. As tubular aggregates in context of a neuromuscular transmission defect appear to be highly indicative, we suggest calling this condition congenital myasthenic syndrome with tubular aggregates (CMS-TA).

Response to Reviewers: The response to the reviewers’ comments will be uploaded as separate file.
Dear Roger,

Thank you very much for having our manuscript “Congenital myasthenic syndrome with tubular aggregates caused by GFPT1 mutations” (JOON-D-11-00756) reviewed by three experts. We were able to address all points that have been raised by the reviewers (please see details below). Corrections and changes to the manuscript are highlighted in yellow.

We found the reviewers’ suggestions very helpful and hope that the revised version of the manuscript is now fully acceptable for publication in the *Journal of Neurology*. Please let us know whether you require additional information. Thank you very much for your consideration.

Kind regards,

Hanns Lochmüller
Response to reviewers’ comments:

Reviewer #1:

We thank the reviewer for his excellent comments. However, we would prefer to keep tables 1 and 2 within the main body of the manuscript, as we feel that the information in the table is important for the reader.

Reviewer #2:

We thank the reviewer for his excellent comments. The following specific questions were asked:

1. The reporting of tubular aggregates in Table 1 is confusing, although Table 2 is clearer on this point. What is the difference between 'TA' and 'yes'. What does 'yes, on revision' mean. In particular, were the TA-negative biopsies 'revised'?

The column describing the presence of tubular aggregates in table 1 was changed for clarity in the revised manuscript. TA-negative biopsies were re-examined after the genetic diagnosis was made and GFPT1 mutations were found. In some biopsies, TAs were detected at re-examination which were not described at the first examination. This was added to the table legend of the revised manuscript.

2. It may be worthy of note that 2/3 families in which TAs were absent or inconclusive were the only ones with mutations p.M491T.

This is an interesting observation, indeed. One of the patients without TAs is homozygous for p.M491T and two siblings are heterozygous carriers of this mutation in combination with a frameshift mutation. One other family without TAs did not carry this mutation at all. At present we feel that the
number of families is too small for drawing any firm conclusions regarding genotype-phenotype correlation for the mutation p.M491T.

3. The 9 patients on whom SFEMG studies were made should be indicated in Table 1.

We indicated this in table 1 of the revised manuscript.

4. One patient with LGM and TAs was reported previously by some of the authors (Brain 2006). Was the status of GFPT1 investigated in that patient? If so, it would provide at least one patient in whom the structure and function of the NMJs had been investigated.

This patient was also included in the study by Palace et al. published in Brain, 2007. The GFPT1 gene was sequenced in this patient, but mutations were not identified.

5. One point not considered in the Discussion is the fact that while patients with DOK7 and GFPT1 mutations have weakness with a common and distinctive limb-girdle distribution, they differ markedly in their response to therapy (although this is referred to elsewhere in the paper). Do the authors have any insight into the possible implications of this difference?

It is currently not understood where this difference comes from. It might result from the fact that the NMJ structure is altered in DOK7 patients and this change cannot be reversed by esterase inhibitors. However, there is little information of NMJ structure in GFPT1 patients.
Reviewer #3:

We thank the reviewer for his excellent comments. The following specific questions were asked:

**Abstract:**
Please write gene names cursive, *DOK7* and *GFPT1*.

The italic formatting was lost when the abstract was pasted into the JOON online submission form. It was present in the original manuscript file, and is checked in the revised manuscript.

Is it possible to use abbreviations in the abstract like LG-CMS?

We now defined the abbreviation LG-CMS in the abstract of the revised manuscript as suggested.

A common feature is the sustained response to change the sentence this way, otherwise it seems to be confusing.

The sentence was corrected in the revised manuscript as requested.

At the end you introduce the "new name" for this subtype of CMS, but you will use it in cases with a mutation in *GFPT1*, won’t you? Then, please add this at the end of the abstract.

“CMS with tubular aggregates” refers only to cases with *GFPT1* mutations.

**Running title:**
*GFPT1* cursive, please check this for the whole text.

This was corrected as suggested, *GFPT1* has been italicised where we referred to the gene name.
Patients and methods:
Skip "there" in combination with table 3

The word “there” has been removed in the revised manuscript.

Results:
Clinical features:
p. 11: Please describe more in detail what you mean with delayed motor milestones, only independent walking?
These patients achieved independent walking at age 15-24 months. This is now explained in the text of the revised manuscript.

p. 12: They became non-ambulant? at which age? Can you describe where muscle atrophy could be observed?

Independent walking ability was lost permanently or for walking outdoors in all three patients around age 12 years. This is now stated in the revised manuscript.

We now give the distribution of reported muscle atrophy in families LGM1 and LGM5, the Lybian family LGM3 has been previously reported (ref. 13).

Neurophysiology:
Can you clarify how much patients were examined by the different neurophysiological methods and then, how much had pathological results and what kind of and where? This will be helpful for the reader.
Can you clarify what you mean with distal and proximal? Muscles, nerves??

We provide this additional information in the revised manuscript as requested. Table 1 of the revised manuscript shows the electrophysiological tests performed and their results in every patient. We refer to proximal and
distal muscles as explained in the text and table 1 of the revised manuscript. The patients who were negative for decrement on RNS when recording from distal muscles, are now reported in the text of the revised manuscript.

**Muscle pathology:**
*Add LGM to patient 14.3 (fig. 3)*

We corrected the figure legend as suggested.

**Can you give the number of muscle biopsies in which NADH stain and/or EM were done? Can you explain which investigation is more specific for TAs. Then the reader may better understand the interpretation of the results. Please, discuss this also in the discussion paragraph.**

EM more specific for identifying tubular aggregates; however – unlike the NADH staining - it is not done on routine basis on all muscle biopsies. We added a sentence to the discussion as suggested. Table 2 states whether EM has been performed for the respective patient.

**Response to therapy:**
*Would it be possible to add the dosage per KgBW in the first sentence?*
*Can you describe more in detail what was stabilized under the additional therapy with 3, 4-DAP, better than without?*

The dosage was given per kgBW by some of the referring physicians but not all of them. Moreover, the dosage was changed over time for many of the patients. We provide the information in the revised manuscript where it was available.

Concerning stabilization under additional therapy with 3,4-DAP a detailed explanation is given now in the text for patient LGM17.3. This means a
partial improvement in walking has been achieved and retained under this treatment.

References:
4. skip (  
5. change n to N  
8. change n to N  
10. Use the abbreviations: Am J Hum Genet  

We changed the references accordingly.

Discussion:
Do you use hip girdle and pelvic girdle for the same? If not please explain.

The terms hip girdle and pelvic girdle are used for the same muscles.

Tables:
Table 1: What do you mean with course?

“course” refers to whether the clinical symptoms were progressive or stable or improved with time.

RNS prox / dist? Symmetrical or not? Please explain, perhaps in the legend?

The clinical features were not reported to be asymmetrical in the patients which is now mentioned in the legend of table 1 in the revised manuscript.

Add all abbreviations in the legend.

The abbreviations were added to the table legend of the revised manuscript as requested.
Table 2: Clarify what means stainings? In a legend, perhaps. Others than NADH?

Table 2 only refers to NADH staining, and this was clarified in table 2 of the revised manuscript.

Table 2: What means EM not done for LGM 12.3? Was NADH done? Result?

Tubular aggregates were not investigated in patient LGM12.3 (see table 1). This has now been clarified in table 2 of the revised manuscript.
Congenital myasthenic syndrome with tubular aggregates caused by \textit{GFPT1} mutations

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Running title: **GFPT1** associated limb-girdle congenital myasthenia

**Abbreviations**: AChE = acetylcholinesterase; AChR = acetylcholine receptor; CK = creatine kinase; CMAP = compound muscle action potential; CMS = congenital myasthenic syndrome; 3,4-DAP = 3,4-Diaminopyridine; **DOK7** = downstream of kinase 7 gene; EM = electron microscopy; EMG = electromyography; LG-CMS = limb-girdle congenital myasthenic syndrome; NMJ = neuromuscular junction; RNS = repetitive nerve stimulation; SFEMG = single-fiber EMG; TA = tubular aggregates; **GFPT1/GFAT1** = glutamine-fructose-6-phosphate transaminase 1

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Disclosure:

The authors have nothing to disclose.

Author contributions:

The manuscript was written by VG, JSM, JS, AC and HL with the help of the coauthors. JS and MD performed genetic analysis of patients. AO, SM, and DB provided data from endplate studies. AO, CL, JC, CJM, AN, JJV, NM, JK, SN, AK, YN, BB, HN, CR, JPS, BS, BS, RH, TV, OKS, AN, AU, JP, DMS, FM, MGH and DB provided and evaluated clinical data and contributed patient materials to the study. KB, VS, DB, AA, HL and JSM supervised the study and acquired funding for the project.
Abstract

Congenital myasthenic syndrome (CMS) is a clinically and genetically heterogeneous group of inherited disorders of the neuromuscular junction. A difficult to diagnose subgroup of CMS is characterised by proximal muscle weakness and fatigue while ocular and facial involvement is only minimal. DOK7 mutations have been identified as causing the disorder in about half of the cases. More recently, using classical positional cloning, we have identified mutations in a previously unrecognised CMS gene, GFPT1, in a series of DOK7-negative cases. However, detailed description of clinical features of GFPT1 patients has not been reported yet.

Here we describe the clinical picture of 24 limb-girdle CMS (LG-CMS) patients and pathological findings of 18 of them, all carrying GFPT1 mutations. Additional patients with CMS, but without tubular aggregates, and patients with non-fatigable weakness with tubular aggregates were also screened. In most patients with GFPT1 mutations, onset of the disease occurs in the first decade of life with characteristic limb-girdle weakness and fatigue. A common feature was beneficial and sustained response to acetylcholinesterase inhibitor treatment. Most of the patients who had a muscle biopsy showed tubular aggregates in myofibers. Analysis of endplate morphology in one of the patients revealed unspecific abnormalities.

Our study delineates the phenotype of CMS associated with GFPT1 mutations and expands the understanding of neuromuscular junction disorders. As tubular aggregates in context of a neuromuscular transmission defect appear to be highly indicative, we suggest calling this condition congenital myasthenic syndrome with tubular aggregates (CMS-TA).
Introduction

Congenital myasthenic syndrome (CMS) is a rare and heterogeneous group of inherited muscle disorders caused by genetic defects that affect signal transmission at the neuromuscular junction (NMJ) [1, 2]. The clinical phenotype of CMS is fatigable weakness presenting usually from birth but later onset is also possible. To date, 14 different genes are known to cause CMS if mutated (http://neuromuscular.wustl.edu/synmg.html).

Limb-girdle congenital myasthenic syndrome (LG-CMS) is a previously recognised clinical entity [3] with prominent shoulder and pelvic girdle weakness and fatigue and minimal ocular and facial involvement. In 2006, mutations in the DOK7 gene were identified to cause a form of CMS with limb-girdle weakness where patients do not benefit from pyridostigmine treatment [4]. Subsequently, detailed clinical analysis of DOK7 patients revealed that many of them show external eye muscle involvement (often ptosis, less frequent ophthalmoplegia) contrary to the original concept of pure limb girdle weakness [4-9].

Recently, we have identified the underlying gene mutations in a second subset of CMS patients with prominent limb-girdle weakness responding well to esterase inhibitor therapy. We mapped the gene defect to the GFPT1 (glutamine-fructose-6-phosphate transaminase 1) gene on chromosome 2p13.3 [10]. GFPT1 is the key enzyme of the hexosamine pathway yielding the amino sugar UDP-N-acetylglucosamine, an essential substrate for protein glycosylation [11].

In this report, we describe the clinical features of 24 patients with GFPT1 mutations; muscle histopathology was available in 18 of these patients. This is the first detailed description of the phenotypic presentation of GFPT1 patients which should expedite diagnosis and treatment of LG-CMS in the future.
Patients and methods

All studies were carried out with informed consent of the patients or patients’ parents and approved by the institutional ethics review boards. Consent has been obtained for publishing any recognizable persons in photographs, videos, or other information.

Nine families are derived from the CMS patient cohort referred to the Friedrich-Baur-Institute in Munich, Germany, for genetic testing over the last fifteen years. Two families (LGM7 and 8) were recruited through the CMS service in Oxford, UK and reported previously in [7] (patients 1 and 3 in table 3). Two LGM families with tubular aggregates were previously reported in [12] (family LGM13) and [13] (family LGM3). GFPT1 mutations of all patients except family LGM17 have been reported in [10]. Family pedigrees are shown in [10], supplementary figure S2. Family LGM17 has not been reported previously.

Three further patients described with CMS in a limb-girdle distribution, tubular aggregates and benefit from esterase inhibitors (LGM4, 15 and 16 in [10] and [12]) and two patients reported in [7] did not carry GFPT1 mutations. GFPT1 mutations were also absent from a cohort of 52 unsolved cases with a wide range of different CMS phenotypes, but without tubular aggregates. We also screened a cohort of 4 patients with unexplained muscle weakness and tubular aggregates on biopsy, but without clear fatigability and without evidence of a neuromuscular transmission defect, for mutations in GFPT1, but did not detect any.

All pedigrees are compatible with autosomal recessive inheritance; all parents of the probands are reported to be healthy. Families LGM1-LGM4, LGM10 and LGM11 are consanguineous. The patients from families LGM1, LGM3, LGM5, LGM12, LGM13 and LGM17 are siblings. The age at examination varied between 7 and 63 years.
Tubular aggregates in muscle biopsies were present in 13 of 18 biopsies from the 14 families. Formal clinical assessment was performed in all patients as well as measurement of serum creatine kinase levels (CK) and titres of anti-acetylcholine receptor (anti-AChR) antibodies. Electromyography (EMG), nerve conduction studies (NCS), repetitive nerve stimulation (RNS) and single fibre EMG (SFEMG) were performed using standard techniques. Muscle biopsies were performed by open or needle technique. Electron microscopical analysis of one muscle biopsy specimen was performed after fixation in 2.5% glutaraldehyde, postfixation in OsO4 and embedding in resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate.
Results

The main clinical, electrophysiological and muscle biopsy features as well as the response to treatment of each patient and their GFPT1 genotypes are presented in Table 1.

Clinical features

The first symptoms were noted in the first decade of life in 21 out of 24 patients (range: 1st year of life – forties, median 6 years). Symptoms included difficulty in rising from a squatting position, climbing stairs, lifting the arms above the head, holding heavy objects and falls. All patients had normal motor milestones except patient LGM10.4, LGM12.3, LGM17.3 and LGM17.4 (delayed achievement of independent walking at age 15-24 months). Three patients manifested after the first decade of life: patient LGM9.3 experienced weakness on physical activity at age 13 years, patient LGM5.4 complained of shoulder girdle weakness in her forties, her brother (LGM5.3) had similar problems and presented at age 14 years.

On examination, weakness was more pronounced in the pelvic girdle muscles for most patients (fig 1F), but with the following exceptions. The shoulder girdle was initially involved in patient LGM5.3, LGM5.4 and in LGM3 family [13]. The pelvic and shoulder girdles were equally affected in patients LGM7.3 and LGM8.3, although weakness was first noted in the pelvic girdle in patient LGM8.3 that progressed to involve the shoulder girdle muscles six years later. Scapular winging and waddling gait were evident in half of the patients (fig 1E).
Only one patient had slight ptosis (patient LGM7.3) with all other patients exhibiting no significant ocular muscle involvement (fig 1A-C). Five patients exhibited mild facial weakness (LGM1.4, LGM1.5, LGM5.4, LGM7.3 and LGM8.3). Additionally, distal muscle weakness was noted in family LGM1 (long finger flexors and extensors as well as foot extensors), patients LGM5.3, LGM5.5, LGM7.3, LGM8.3 and LGM12.3. Neck muscles were weak in patients LGM7.3, LGM12.3, LGM12.4 and LGM5.4. One patient (LGM7.3) showed more generalised and severe muscle involvement (ptosis, facial, and neck weakness along with proximal and distal limb weakness). He also had subclinical involvement of the respiratory muscles and became non-ambulant within 4 years from presentation. Muscle atrophy was rarely observed, the two brothers from the Iranian family LGM1 were reported to have slight generalized muscle atrophy and the affectd members of family LGM5 – scapular winging. Mild proximal wasting was reported in family LGM3 [13].

The majority of patients reported prominent fluctuation of symptoms, both improvement and worsening over short periods of time (Table 1). Fixed muscle weakness was reported in six patients (patients LGM5.3, LGM5.4, LGM7.3, LGM11.3, LGM13.3 and LGM13.4). Diurnal fluctuations were reported in patients LGM2.4 and LGM6.4 while patient LGM14.3 experienced significant day-to-day fluctuations. Both heat and infections were noted to exacerbate neuromuscular weakness (LGM1, LGM2.4, LGM3 family and LGM8.2). Disease progression, e.g. reduced walking distance, was noted in the first two decades of life in most patients. Some patients experienced gradual worsening over decades. All patients retained independent walking abilities during the periods of observation except LGM7.3,
LGM8.3 and LGM17.4. It was lost permanently or for walking outdoors in all three patients around age 12 years.

**Laboratory tests and electrophysiology (Table 1)**

CK (creatine kinase) levels were normal or slightly elevated in most patients except in 3 individuals in whom the CK levels were moderately elevated (up to 8-12 times) (LGM6.4, LGM8.3 and LGM14.3). Anti-AChR antibodies were not detected in any of the patients.

When recording from distal muscles, RNS did not yield a decremental response in some patients (family LGM1, patients LGM5.4, LGM10.4, LGM11.3 and LGM17.4), but clear decrement was obtained from proximal muscles in all tested patients (Table 1). There was a single CMAP response to single nerve stimuli except in patient LGM10.4 with a double CMAP response tested twice on and off AChE inhibitors treatment. SFEMG showed abnormal jitter in all nine patients. Needle EMG performed in thirteen patients showed mild myopathic changes in proximal muscles with no spontaneous activity.

**Muscle pathological studies**

Most muscle biopsies showed unspecific or mild myopathic changes (summarised in Table 2). In addition, tubular aggregates (TAs) were identified in 11 families, best seen on NADH staining (Fig. 2). NADH staining and EM images of the TAs of affected individuals from families LGM3 and 4 have been published previously [12, 13]. The muscle pathology studies of patients LGM5.3, LGM5.5 and LGM12.4 revealed TAs but so small that they could be overlooked or misinterpreted as mitochondrial proliferation. In two patients (LGM10.4 and LGM11.3) we detected
inconclusive histological findings that did not fully match the criteria for TAs and were not further analysed under electron microscopy (Fig. 2D). Electron microscopy (EM) images of the tubular aggregates observed in patient LGM14.3 are shown in Fig. 2E and F. End plate morphology in patient 14.3 (Figure 3) revealed pronounced simplification of the postsynaptic membrane compared to the normal NMJ. There were numerous apparently normal synaptic vesicles in the axon terminals.

**Response to therapy**

Twenty of 22 treated patients responded well to AChE inhibitor treatment at a daily dosage of 80-540 mg (median 217 mg/day for patients aged 7-55 years), the remaining two patients (LGM5.4 and LGM12.4) did not receive treatment (Table 1). However, the benefit was not sustained in family LGM1 and LGM17. In family LGM1, although AChE inhibitors treatment led to significant improvement of the muscle weakness at a dosage of 2.8mg/kg/day in the older brother and 2.4mg/kg/day in the younger brother, treatment was later discontinued due to possible side effects (muscle twitching, depression and anxiety). The addition of 3,4-Diaminopyridine (3,4-DAP) in some patients was effective in improving or stabilizing the disease. In LGM 17, both siblings benefited from AChE in the first 18 months but seemed to relapse after that. Following an initial improvement with pyridostigmine by 12 years of age patient LGM17.3, started complaining of increased general fatigue, weakness in her hands and without medication she could barely walk for 2-3 minutes. Despite optimisation of pyridostigmine dose at 7.7mg/kg/day, she continued to deteriorate and by 13 years of age, the 28 feet walking time was 9 sec. By 14 years of age, she was started on 3,4-DAP at a dose of 50 mg/day. On a current optimised doses of combined pyridostigmine and 3,4-DAP her condition appeared to have stabilized. She resumed
walk for 10-15 minutes, her writing endurance improved and she could now reach for heavy objects.

**Additional features**

Retinal involvement was reported in two of the families. Both affected brothers from family LGM1 were diagnosed as having juvenile macular degeneration causing significant visual loss, more severe in the younger brother LGM1.5. No other family members showed clinical signs of retinal disease. Patient LGM7.3 exhibited an early squint and was subsequently diagnosed as having retinitis pigmentosa at the age of 5 years. None of the other families shows clinical signs of retinal disease, although ophthalmological studies were not performed in all patients. We were interested to note learning difficulties in patient LGM 17.3 and 4.
Discussion

We report on fourteen families with CMS due to mutations in the \textit{GFPT1} gene. The clinical phenotype associated with \textit{GFPT1} mutations seems to be distinct, and includes fatigable weakness of the shoulder and hip girdle muscles, normal eye movements, good response to esterase inhibitors, and evidence of tubular aggregates on muscle biopsy. Screening for \textit{GFPT1} mutations in 52 unsolved CMS cases with a wide range of different clinical phenotypes, but without tubular aggregates, was negative confirming that \textit{GFPT1} mutations are associated with a distinct and recognisable CMS phenotype. Tubular aggregates in muscle biopsies of patients with a proven neuromuscular transmission defect seem to be highly indicative of \textit{GFPT1} defects. Therefore, we would like to suggest naming this condition congenital myasthenic syndrome with tubular aggregates (CMS-TA). This follows the example of another clinically distinct form of CMS, congenital myasthenic syndrome with episodic apnea (CMS-EA), which is caused by mutations in \textit{CHAT} [14].

Notably, 5 patients with CMS and tubular aggregates (3 described in [12], 2 in [7]) did not have \textit{GFPT1} mutations. This may indicate that a small proportion of patients may carry cryptic mutations in \textit{GFPT1} not detectable by standard exon sequencing of genomic DNA, or may carry mutations in other, yet unknown genes. Moreover, patients with unexplained muscle weakness and tubular aggregates on muscle biopsy, but without evidence of a neuromuscular transmission defect, seem to be less likely to carry mutations in \textit{GFPT1}, and other causes of tubular aggregates such as periodic paralysis or chronic alcohol consumption need to be considered.
Previous reports on so-called limb girdle congenital myasthenia with and without tubular aggregates [7, 12, 15-22] described patients with a phenotype that may be compatible with the clinical phenotype observed in our GFPT1 patients. However, it was difficult to ascertain whether these patients belong to a single disease entity distinguishable from other forms of inherited neuromuscular junction defects. Genetic testing of these patients for GFPT1 can now be undertaken.

CMS with tubular aggregates caused by GFPT1 defects has clinical and pathological features that may help distinguishing it from other forms of CMS. This is particularly important as patients with an inherited neuromuscular transmission defect and predominant limb-girdle weakness seem to fall into two major categories. Half of the patients with CMS and prominent limb-girdle weakness carry mutations in the DOK7 gene [4, 6-8]. CMS caused by DOK7 mutations shows clear clinical and pathological differences from CMS with GFPT1 mutations: DOK7 patients may have ptosis, facial, bulbar and respiratory involvement and do not show sustained benefit from esterase inhibitor treatment, while muscle weakness may improve under ephedrine treatment [23, 24]. Muscle biopsies do not show tubular aggregates. Table 3 compares the main features of DOK7 and GFPT1 caused forms of CMS. Rarely, CMS patients with mutations in RAPSN or COLQ show a limb-girdle pattern of weakness. However, they were never found to have tubular aggregates on muscle biopsies [25-27].

The moderately elevated CK levels in three patients (LGM 6.4, LGM 8.3 and LGM 14.3) correspond to severe and fixed muscle weakness in just one of them (LGM 8.3) and clear dystrophic features are not found in their muscle biopsies. Myopathic
changes in needle EMG are found in all patients examined and do not correspond to
CK levels. Given the lack of clear fluctuations in eight of the patients, one has to
consider a wider differential diagnosis of primary muscle disorders like congenital
myopathies and muscular dystrophies. Indeed, many of our GFPT1 patients were
assigned a clinical diagnosis of a myopathy or muscular dystrophy prior to the
elucidation of a neuromuscular transmission defect. Evidence of decrement on repeat
nerve stimulation and the positive response to AChE inhibitors may be invaluable
clinical clues to help distinguish CMS with tubular aggregates from other muscle
disorders.

We were interested to note retinal involvement in two of our families (macular
degeneration in family LGM1 and retinitis pigmentosa in patient LGM7.3). It is
presently unclear whether retinal disease is associated with the GFPT1 mutations in
these families or whether the patients are affected by two genetically distinct
conditions.

The GFPT1 enzyme is expressed in both nerve and muscle tissue [28, 29]. Endplate
morphology analysis in patient LGM14.3 showed unspecific abnormalities and the
ultrastructural data we have available so far do not clarify if the origin of the
neurotransmission defect is primarily presynaptic or postsynaptic, or indeed a
combination of both pre- and postsynaptic abnormalities. Analysis of additional
patients, as well as deciphering the molecular consequences of impaired GFPT1
function or reduced GFPT1 levels in patients will be required to fully understand the
molecular pathogenesis of this disorder.
The origin and functional consequences of TAs have been investigated over the last 40 years. It is still unknown whether TAs are pathological structures or represent compensatory reactions to diverse pathogenic events such as periodic paralysis, dyskalaemia, intoxication, inflammatory myopathies, cramps and myalgias, myotonia congenita, familial myopathies, and several other myopathies of uncertain etiology [30, 31]. TAs are characterised as more or less densely packed aggregates of vesicular or tubular membranes of variable forms and sizes thought to derive from the sarcoplasmic reticulum (review in [15, 30]). TAs can be seen by light microscopy as dark inclusions in the NADH stain of muscle biopsies. A more specific way to identify TAs is by electron microscopy. This however is not done on a routine basis for all muscle biopsies. Caveolin-2(-/-)-deficient mice represent one animal model with TAs in muscle [32]. Currently, a molecular pathway linking TAs and the NMJ has not been established. Some families in our study (LGM10.4 and LGM11.3) share the same clinical features and harbour GFPT1 mutations but TAs were not conclusively detected. These are also some of the youngest patients in our study. This may hint to variable expression of TAs in different muscles or during life time of patients. Alternatively they may be an unspecific feature not directly related to the underlying pathomechanism. It will be interesting to see whether some of the patients described as unspecific myopathy with TAs carry mutations in GFPT1.

Our study confirms the phenotypic and genetic heterogeneity of CMS. In addition to DOK7–related CMS, GFPT1 associated CMS seems to be an important and distinct clinical and genetic entity associated with limb-girdle fatigable weakness, clear response to pyridostigmine, and frequent TAs on muscle biopsies. The identification
of GFPT1 as the predominant gene involved CMS-TA will allow better diagnosis, treatment and counselling of patients and their families.
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References


abnormalities associated with impaired neuromuscular transmission in a group of patients with 'limb-girdle myasthenia'. Brain 129, 2061-2076.


Figure Legends

Figure 1: Photographs of GFPT1-related CMS patients reported in this study. A, B. Patients LGM1.4 and LGM1.5. Note the absence of ptosis in the two brothers. C. Patient LGM10.4. Eye movements are not restricted. D. Patient LGM6.4. Note the absence of facial muscle weakness. E, F. Patient LGM11.3 shows weakness in the shoulder and pelvic girdle, and has difficulties rising from the floor and lifting the arms sideward.

Figure 2: NADH staining and range of tubular aggregates present in muscle biopsy sections of CMS patients with GFPT1 mutations.
A., B. Patient LGM1.4. Biopsy from the biceps brachii muscle showing variation in fiber size and increased internal nuclei. Some fibres show abundant TAs, other show tiny cytoplasmic vacuoles containing granular material. Image B shows a magnified view of the highlighted area in A.
C. Patient LGM9.3. Biopsy from the biceps brachii muscle, NADH reaction reveals TAs in a few fibres (arrows).
D. Patient LGM10.4. Significant subsarcolemmal accumulations of mitochondria and unevenness of staining, but unequivocal TAs were not seen in this patient’s biopsy.
E, F. Patient 14.3. Electron microscopy images of tubular aggregates beneath the sarcolemma. The tubules vary in diameter.
Figure 3: Ultrastructural findings at the neuromuscular junction (NMJ) in patient LGM14.3.

(a) Normal NMJ from a control demonstrating normal nerve terminal (N) and highly complex postsynaptic membrane folding with well formed secondary synaptic clefts (arrow head). Schwann cell processes (S) cover the nerve terminal, without extending into the normal primary synaptic cleft (arrow). The postsynaptic membrane (arrow heads) is well developed.

(b-f) NMJ of patient 14.3, deltoid muscle, three endplates were studied. The NMJ which is illustrated in (b) is shown at higher magnification (c) and (d). Additional NMJs are illustrated in (e) and (f).

Note pronounced simplification of the postsynaptic membrane compared to the normal NMJ. There are numerous apparently normal synaptic vesicles in the axon terminals.
Figure
<table>
<thead>
<tr>
<th>Clinical features</th>
<th>CK elevation x times</th>
<th>EMG</th>
<th>Muscle biopsy</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptom at onset / motor milestones</td>
<td>RNS: decrement at 3Hz %/muscle</td>
<td>Abnormal SFEMG Myopathic changes</td>
<td>Tubular aggregates</td>
<td>Response to AChE inhibitors</td>
</tr>
<tr>
<td>Symptom at onset / motor milestones</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Facial / bulbar / respiratory muscle weakness / other</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limb girdle weakness / fluctuations / course</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Therapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LGM1.4/m/Iran/yes/ p.D348Y homozygous</td>
<td>6yr/31</td>
<td>muscle weakness, fatigue, worse in summer/normal</td>
<td>yes/no/no/no/distal limb</td>
<td>yes/yes/improving after age 20</td>
</tr>
<tr>
<td>LGM1.5/m/Iran/yes/ p.D348Y homozygous</td>
<td>6yr/26</td>
<td>muscle weakness, fatigue, worse in summer/normal</td>
<td>yes/no/no/no/distal limb</td>
<td>yes/yes/improving after age 20</td>
</tr>
<tr>
<td>LGM2.4/f/Turkey/yes/ p.W240X homozygous</td>
<td>6yr/26</td>
<td>muscle weakness, fatigue and pain/normal</td>
<td>none</td>
<td>yes/yes/worsening</td>
</tr>
<tr>
<td>LGM3.3, LGM3.5, LGM3.6, LGM3.8, LGM3.9/2m,3f/Libya/yes/ p.R111C homozygous [13]</td>
<td>6yr/23-35</td>
<td>muscle weakness, fatigue and normal</td>
<td>none</td>
<td>yes, shoulder&gt;pelvic girdle/ND/ND</td>
</tr>
<tr>
<td>LGM5.3/m/Spain/no/ p.M492T and c.*22C&gt;A</td>
<td>14yr/55</td>
<td>weakness in the upper limbs/normal</td>
<td>no/no/no/distal limb</td>
<td>yes/no/ slight worsening</td>
</tr>
<tr>
<td>LGM5.4/f/Spain/no/ p.M492T and c.*22C&gt;A</td>
<td>forties/54</td>
<td>weakness in shoulder girdle/normal</td>
<td>no/no/no/neck muscles</td>
<td>yes/no/ slight worsening</td>
</tr>
<tr>
<td>LGM5.5/m/Spain/no/ p.M492T and c.*22C&gt;A</td>
<td>10yr/50</td>
<td>muscle weakness, falls/normal</td>
<td>no/no/no/distal limb</td>
<td>yes/yes/ slight worsening</td>
</tr>
<tr>
<td>LGM6.4/m/Germany/no/</td>
<td>5yr/16</td>
<td>muscle weakness/normal</td>
<td>none</td>
<td>yes/yes/ slight worsening</td>
</tr>
<tr>
<td>LGM</td>
<td>Sex</td>
<td>Age</td>
<td>Symptoms</td>
<td>Onset</td>
</tr>
<tr>
<td>-------</td>
<td>-----</td>
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<td>-------</td>
</tr>
<tr>
<td>LGM1</td>
<td>m</td>
<td>8yr</td>
<td>fatigue on walk/slight delay</td>
<td>yes/no/slight/slight neck and distal limb weakness</td>
</tr>
<tr>
<td>LGM2</td>
<td>m</td>
<td>6yr</td>
<td>repeated falls/normal</td>
<td>yes/no/no/ distal limb</td>
</tr>
<tr>
<td>LGM3</td>
<td>f</td>
<td>13yr</td>
<td>fatigue/normal</td>
<td>none</td>
</tr>
<tr>
<td>LGM4</td>
<td>f</td>
<td>1yr</td>
<td>muscle weakness, falls/normal</td>
<td>none</td>
</tr>
<tr>
<td>LGM5</td>
<td>m</td>
<td>7yr</td>
<td>muscle weakness/normal</td>
<td>none</td>
</tr>
<tr>
<td>LGM6</td>
<td>m</td>
<td>1yr</td>
<td>muscle weakness, falls/slight delay</td>
<td>no/no/no/neck and distal muscles</td>
</tr>
<tr>
<td>LGM7</td>
<td>f</td>
<td>1st decade/39</td>
<td>muscle weakness/normal</td>
<td>no/no/no/neck muscles</td>
</tr>
<tr>
<td>LGM8</td>
<td>m</td>
<td>10yr</td>
<td>muscle weakness/normal</td>
<td>none</td>
</tr>
<tr>
<td>LGM9</td>
<td>m</td>
<td>7yr</td>
<td>muscle weakness/normal</td>
<td>none</td>
</tr>
<tr>
<td>LGM10</td>
<td>m</td>
<td>1st decade/40</td>
<td>muscle weakness/normal</td>
<td>none</td>
</tr>
</tbody>
</table>
and p.R111C

| LGM17.3/f/Malta/no/ p.M491T and c.714_715insA | 8yr/9yr | frequent falls, fatigability when walking/ delayed | Learning difficulties | yes/yes/worsening | slightly | no/distal | ND/ND | no | positive at first, but worsening after 2 years of treatment. Improvement with addition of 3,4 DAP |
| LGM17.4/m/Malta/no/ p.M491T and c.714_715insA | 7yr/13 | difficulties in running, fatigability when walking/ delayed | Learning difficulties | yes/yes/worsening | slightly | ND | ND/ND | no | positive at first, but effect lost after 3 years of treatment despite adding 3,4 DAP |

Table 1: Clinical features of our patients with *GFPT1* mutations

ND: not done. CK: creatine kinase. EMG: electromyography. 3,4-DAP: 3,4-Diaminopyridine. RNS: repetitive nerve stimulation. SFEMG: single-fiber EMG. AChE: acetylcholinesterase.

yes, on revision: TA-negative biopsies were examined again after the genetic diagnosis was made and *GFPT1* mutations were found in those patients.

The clinical features were not reported to be asymmetrical.
<table>
<thead>
<tr>
<th>Patient</th>
<th>analysed muscle</th>
<th>Tubular aggregates (TAs)</th>
<th>additional findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGM1.4</td>
<td>biceps brachii</td>
<td>yes (NADH staining)</td>
<td>fiber size variation, type 1 fibre predominance, round or angular fibres</td>
</tr>
<tr>
<td>LGM2.4</td>
<td>vastus lateralis</td>
<td>yes, TAs exclusively in type 2 fibres</td>
<td>chronic myopathic changes</td>
</tr>
<tr>
<td>LGM3 [13]</td>
<td>biceps brachii</td>
<td>yes. Small subsarcolemmal aggregates (NADH staining), TAs in EM</td>
<td>-</td>
</tr>
<tr>
<td>LGM5.3</td>
<td>deltoid</td>
<td>yes. Subsarcolemmal enhancement (NADH staining), TAs in EM</td>
<td>mild myopathic changes, type 1 fibres predominance and ragged red-like fibres</td>
</tr>
<tr>
<td>LGM5.5</td>
<td>deltoid</td>
<td>yes. Subsarcolemmal enhancement (NADH staining), TAs in EM</td>
<td>unspecific myopathic changes, type 1 fibre predominance, ragged red-like fibres</td>
</tr>
<tr>
<td>LGM6.4</td>
<td>unknown</td>
<td>yes (NADH staining)</td>
<td>unspecific myopathic changes</td>
</tr>
<tr>
<td>LGM7.3</td>
<td>unknown</td>
<td>yes</td>
<td>muscle atrophy, multiple internal</td>
</tr>
<tr>
<td>LGM8.3</td>
<td>unknown</td>
<td>yes</td>
<td>nuclei, vacuoles, features consistent with denervation</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>-------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>LGM9.3</td>
<td>biceps brachii</td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td>LGM10.4</td>
<td>deltoid</td>
<td>not clear, suggestive of TAs (NADH staining)</td>
<td>uneven oxidative staining, accumulation of mitochondria</td>
</tr>
<tr>
<td>LGM11.3</td>
<td>deltoid</td>
<td>no, only one fibre with a possible inclusion (NADH staining)</td>
<td>-</td>
</tr>
<tr>
<td>LGM12.3</td>
<td>deltoid</td>
<td><strong>NADH staining and EM not done</strong></td>
<td>unspecific myopathic changes</td>
</tr>
<tr>
<td>LGM12.4</td>
<td>biceps brachii</td>
<td>yes. Enhancement and subsarcolemmal aggregates (NADH staining), TAs in EM</td>
<td>unspecific myopathic changes</td>
</tr>
<tr>
<td>LGM13.3</td>
<td>vastus lateralis [12]</td>
<td>yes, TAs in EM</td>
<td>-</td>
</tr>
<tr>
<td>LGM13.4</td>
<td>vastus lateralis [12]</td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td>LGM14.3</td>
<td>deltoid</td>
<td>yes, frequent fibres with TAs, TAs in EM</td>
<td>increased fiber size variability. Frequent fibres with internalized nuclei and</td>
</tr>
</tbody>
</table>
EM: electron microscopy.

Table 3: Comparison of main typical clinical features of CMS patients with *DOK7* and *GFPT1* mutations

<table>
<thead>
<tr>
<th>Disease onset:</th>
<th>CMS with <em>GFPT1</em> mutations</th>
<th>CMS with <em>DOK7</em> mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>average age at onset (range)</td>
<td>1st decade (birth – forties)</td>
<td>2nd year of life, sometimes late onset (birth – late twenties)</td>
</tr>
<tr>
<td>first symptoms</td>
<td>walking difficulties, weakness of shoulder or pelvic muscles</td>
<td>walking difficulties; ptosis, floppy tone and bulbar problems if onset at birth</td>
</tr>
<tr>
<td>delayed motor milestones</td>
<td>rare, 2/24 patients in our study</td>
<td>walking onset usually not delayed</td>
</tr>
</tbody>
</table>

**Pattern of muscle weakness:**

ocular muscles:
<table>
<thead>
<tr>
<th>Feature</th>
<th>Bilateral Ptosis</th>
<th>Ophthalmoparesis</th>
<th>Yes</th>
<th>Not Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Facial muscles</td>
<td>Not present</td>
<td>Not present</td>
<td>Yes</td>
<td>Not present</td>
</tr>
<tr>
<td>Bulbar muscles</td>
<td>Not affected</td>
<td>Not affected</td>
<td></td>
<td>Affected</td>
</tr>
<tr>
<td>Extremities, shoulder and pelvic muscles</td>
<td>Limb-girdle weakness pattern</td>
<td>Limb-girdle weakness pattern, waddling or sinuous gait</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory problems</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluctuation of weakness</td>
<td>Daytime-dependent, day to day fluctuations, fluctuations over longer periods</td>
<td>Day to day fluctuations, fluctuations over longer periods</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progression</td>
<td>Worsening in 11 patients, but independent walking ability retained in most</td>
<td>Progressive long-term deterioration leading to intermittent or permanent wheelchair use</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other features</td>
<td>Fixed weakness in some (8/24 patients)</td>
<td>Myopathic-like phenotype with permanent weakness, thinness of muscles, spinal deformities</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Muscle Pathology:**

| CK                                           | Elevated in 8/23 patients | Elevated in some |
| Tubular aggregates                           | Present in 13/18 biopsies | Not present |
| Endplate pathology                           | Unspecific changes (findings from only one patient) | Small simplified endplates, degenerating or highly simplified junctional folds, reinnervated, denervated and ectopic junctions |
| Other features                               | Fixed weakness in some (8/24 patients) | Myopathic-like phenotype with permanent weakness, thinness of muscles, spinal deformities |

**Therapeutic Options:**

| Response to esterase inhibitors              | Clearly positive in most | No effect or only short-term improvement, sometimes worsening |
| Successful long-term therapy with esterase inhibitors | Esterase inhibitors | Ephedrine, salbutamol |

**Mutation Analysis:**

| No obvious common mutation or mutation hotspot. No patient with complete loss of GFPT1 identified. | Common mutation 1124_1127dupTGCC present in the majority of patients, majority of patients have mutations in exon 7. No CMS patient with complete loss of Dok-7 identified |