

Molecular and functional imaging biomarkers in myotonic dystrophy

Doctoral (Ph.D.) thesis

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Pécs, 2021

Table of contents

Abbreviations.....	3
1.Introduction.....	4
2. Myotonic Dystrophy.....	4
2.1. Genetics and pathogenesis	5
2.2. Phenotypes	6
3. Elevated FGF-21 in myotonic dystrophy type 1 and mitochondrial diseases.....	8
3.1. Biomarkers in myotonic dystrophy.....	8
3.2. Aims	9
3.3. Materials and methods	9
3.4. Results	11
3.5. Discussion.....	13
4. Cortical involvement during myotonia in myotonic dystrophy: an fMRI study	14
4.1. Literature review.....	14
4.2. Aims.....	15
4.3. Materials and methods	15
4.4.Results	18
4.5. Discussion.....	21
5.Summary of novel findings.....	23
6.References.....	24
7.Publications.....	26
<i>Acknowledgments.....</i>	<i>29</i>

Abbreviations

BMI	body mass index
BOLD	blood oxygen level dependent
dACC	dorsal anterior cingulate cortex
DM1	myotonic dystrophy type 1
DM2	myotonic dystrophy type 2
DMPK	myotonic dystrophy protein kinase
ECG	electrocardiogram
ELISA	enzyme-linked immunosorbent assay
EMG	electromyography
FGF 21	human fibroblast growth factor 21
FSHD	facioscapulohumeral muscular dystrophy
HOMA-IR	Homeostasis Model Assessment - Insulin Resistance
ICD	implantable cardioverter defibrillator
JMDRS	Japanese Mitochondrial Disease Rating Scale
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes
MRC	Medical Research Council
NT-proBNP	NT-proB-type natriuretic peptide
PEO	progressive external ophthalmoplegia
SMA	supplementary motor area
STR	short tandem repeat

1. Introduction

Muscular dystrophies are hereditary diseases causing progressive degeneration of the skeletal muscle, but neural structures remain spared. This group of diseases is characterized by symmetrical muscle weakness and atrophy, the pattern of muscle wasting makes possible to recognise the type of the disease. In this modern era, the diagnosis is based not only on the clinical characteristics, but also on the underlying molecular genetic defects and consequent pathogenesis. Thus, muscular dystrophies are classified on the basis of their molecular background. Recent research enabled to look forward to future genetic and molecular therapies.

The most common adult muscular dystrophy is myotonic dystrophy, followed by facioscapulohumeral muscular dystrophy (FSHD) [1, 2]. The diagnosis of muscular dystrophies, beside the clinical presentation, relies on muscle biopsy and molecular genetic testing. In recent decades, several research groups made efforts to find easily detectable and adequate biomarkers in muscle diseases. One of those emerging biomarkers is fibroblast growth factor-21 (FGF-21), a regulator of lipid and glucose metabolism. Based on several studies it is a possible biomarker for mitochondrial and other muscle diseases. Based on this hypothesis, we studied the serum levels of FGF-21 in several muscle diseases (mitochondrial myopathy, DM, FSHD), which is discussed in the first part of my thesis.

In the second part of our work, we turned to functional magnetic resonance imaging (fMRI), which allows examining the brain areas activated during myotonia, the most characteristic symptom of DM1. Our findings can reveal the potential of fMRI as a biomarker of the disease.

2. Myotonic Dystrophy

The prevalence of the disease varies in different regions of the world. Most studies in Europe found 10-20/100.000 cases [3-7], but this is probably underestimated because many patients are not recognized. This is also indicated by a screening programme in New York State, where the prevalence of myotonic dystrophy was estimated to 48/100.000 [8].

The disease is characterized by progressive muscle weakness, atrophy and myotonia. Myotonia is the temporary inability of a muscle to relax after exertion.

This phenomenon most often presents during shaking hands, speech or jaw opening.

There are two subtypes of the disease: myotonic dystrophy type 1 (DM1) was described in the 19th century as the classical Steinert's dystrophy, while type 2 (DM2) was first discussed in 1994. In DM2, muscle involvement is predominantly proximal, while other symptoms are similar but milder than in DM1. Currently there are no clear data on its worldwide prevalence, but in Europe it appears to be similar to that of DM1 [9].

In addition to muscle symptoms, myotonic dystrophy is characterized by multisystemic involvement (eyes, brain, cardiovascular system, endocrine system, etc.).

2.1. Genetics and pathogenesis

Myotonic dystrophy shows great variability in terms of severity of the symptoms, course, and onset of the disease. Multisystemic involvement and clinical heterogeneity are caused by a nucleotide repeat expansion of variable length. In DM1 a CTG repeat expansion occurs in the unstable "DM-critical region" of the myotonic dystrophy protein kinase (DMPK) or myotonin gene at the 19q13.3 locus [10]. In healthy people the number of repeats is between 5-37, up to 50 repeats (pre-mutated allele) the disease is asymptomatic. Above 50 repeats, allele penetration is complete, with disease manifesting in almost all cases. In the mild form of DM1, the number of CTG repeats are found to be 50-150, in more severe cases 150-1000, and in the congenital form longer than 2000. The severity of the disease and the time of onset of the first symptoms are related to the length of expansion [11].

In DM2, a cytosine-cytosine-thymine-guanine (CCTG) tetranucleotide expansion occurs at the 3q21.3 locus, in the ZNF9 or CNBP gene. The number of repeats is approximately 26 in healthy people and about 5,000 in DM2 patients.

In both subtypes of the disease, the expansion occurs in the 3' non-translated region of the respective mRNAs causing sequestration and functional impairment of different cellular proteins, mostly splicing factors. The difference between the subtypes of the disease might be caused by the different spatial position of the DMPK and CNBP genes and the different nearby genes. An important factor may be that the function of MBNL1 splicing regulator protein is impaired in both types, however, in DM1 it is associated with overexpression of CUGBP1, another

regulator of alternative splicing, which further enhances defective splicing [12]. In summary, spliceopathy is an essential molecular feature of both types of DM.

2.2. Phenotypes

2.2.1. Congenital myotonic dystrophy

- the most common cause of neonatal respiratory failure [2]
- the newborn has facial diplegia, is hypotonic („floppy infant”), needs ventilation, is unable to breastfeed, has thin ribs and high diaphragm
- mental and motor development is delayed
- cerebral atrophy and dilated ventricles are common
- as a child he will be able to walk, but bowel incontinence is common [11]
- diagnostic tests: genetics, muscle biopsy, sometimes EMG.

2.2.2. Childhood/juvenile myotonic dystrophy

- the first symptoms typically appear before the age of 10: cognitive and behavioural problems, attention deficit, lower intelligence [13]
- later symptoms similar to adult DM1 develop
- the incidence of arrhythmias in adolescence is 15-20%, and are exacerbated by sports and physical activity [14].

2.2.3. Adult-onset myotonic dystrophy

Clinical aspects

- muscle involvement: facial muscles, sternocleidomastoid, distal limb muscles [15], worsens with age, gait instability is common [16]. Myotonia is also present in the facial muscles, hands and forearm. Grip myotonia can improve with repeated contractions - 'warm-up phenomenon'.
- multisystemic involvement:
 - cardiovascular system: conduction disturbances and tachyarrhythmias, cardiac care is of paramount importance from the diagnosis of the disease [17]
 - central nervous system: both DM1 and DM2 are characterized by mild cognitive deficit, white matter lesions and cerebral atrophy are common [18]

- cataract: posterior subcapsular cataract occurring earlier than 55 years of age and its familial incidence may also indicate myotonic dystrophy [1, 19]
- endocrine disorders: thyroid, pancreas, hypothalamus and gonads are affected. Diabetes is not more common in DM1, but insulin resistance can be caused by abnormal splicing of insulin receptor mRNA [20]
- respiratory failure: characteristic primarily of congenital DM, but general anaesthesia can often be associated with respiratory and cardiovascular complications in adults [21-23] .

Diagnostic workup

- based on familial history and clinical aspects
- final diagnosis: genetic testing, but EMG and muscle biopsy are also important
- imaging: Magnetic Resonance (MR) of central nervous system abnormalities and monitoring of atrophy in skeletal muscles

Patient care, therapeutic options

- genetic counselling, prevention of complications caused by multi-organ involvement
- musculoskeletal rehabilitation
- Studies were performed with small molecules bound to CUG repeats, for post-transcriptional gene silencing, and for genome editing. Their use in vivo is still pending [24-27].
- myotonia: Na-channel blockers (e.g., mexiletine, procainamide, propafenone, carbamazepine, etc.), but these agents may impair muscle strength in addition to improving myotonia [28].
- prevention of cardiac complications (ECG monitoring, pacemaker or ICD implantation) [29]
- treatment of sleep apnoea
- consideration of the risk of anaesthesia
- patients' life expectancy in the childhood and congenital forms of DM1 is clearly reduced, and is also lower than the average in adult-onset DM1. For DM2, life expectancy is generally the same as in the healthy population [30-33].

3. Elevated FGF-21 in myotonic dystrophy type 1 and mitochondrial diseases

3.1. Biomarkers in myotonic dystrophy

A remarkable proportion of therapeutic attempts in myotonic dystrophy target RNA treatment. Thus, the detection of mRNA formed during defective alternative splicing before and after treatment may be an important future biomarker of the therapeutic response. The utility of circulating serum/plasma miRNAs as biomarkers is also promising, however, although miRNAs may be expressed tissue-specifically, there may be overlaps, and the same molecule may be produced in multiple tissues. Considering the multisystemic involvement of DM1, the isolation of miRNA secreted by different tissues causes difficulties [34]. Serum and plasma concentrations of several subtypes of miRNAs (miR-1, miR-133a, miR-133b, miR-206 [35] and miR-133a, -133b and -206 [36]) correlated with the degree of muscle weakness according to a number of studies [35, 36]. In addition to the number of further studies required to determine the specificity of miRNAs, a possible cost-effective and time-efficient protocol for the quantitative measurement of miRNAs is not yet available. For DM1, a biomarker panel could be useful to assess several secondary complications that may develop in patients [35, 37].

Regarding cardiac complications, NT-proBNP and copeptin has been shown to be possible independent predictors of atrial fibrillation in DM1 patients [38].

3.1.2. FGF-21 as a biomarker

FGF-21 (human fibroblast growth factor 21) is a 181-amino acid protein belonging to the human FGF superfamily. The proteins in the family play diverse roles in endocrine, paracrine, and intracellular processes. FGF21 is expressed by liver cells, adipose tissue, thymus, and skeletal muscle. FGF-21 plays a central role in glucose and lipid metabolism and adaptation to starvation [39]. Elevated levels of FGF-21 in skeletal muscle have also been found in an animal model of mitochondrial disease [40]. FGF-21 levels have also been shown to be elevated in human mitochondrial diseases involving muscle [41]. Several recent studies have demonstrated elevated FGF-21 levels in patients with mitochondrial disease compared with other neuromuscular diseases and healthy controls [42, 43]. These results suggest that mitochondrial dysfunction induces a starvation-like state, and

that, like liver and brown adipose tissue, diseased skeletal muscle is capable of secreting FGF-21 [40, 44] .

3.2. Aims

- to determinate the serum concentrations of FGF-21 in several neuromuscular diseases (mitochondrial myopathy, myotonic dystrophy, FSHD) and healthy controls
- comparison and correlation analysis of the obtained values with several clinical parameters

3.3. Materials and methods

3.3.1. Patients

We enrolled 30 patients diagnosed with mitochondrial disease, 16 patients with dystrophy myotonica (DM1), 5 patients with facioscapulohumeral muscle dystrophy (FSHD), and 20 healthy controls. The study was approved by the local ethics committee (approval number: 4581/2012). Patients signed a consent form. There was no significant disease in the history of healthy controls, their BMI was in the normal range, and they were not undergoing medical treatment or medication. Patients and controls with an abnormal BMI (<17 or> 30) were excluded from the study. In addition to clinical examinations, the patients' diagnosis was confirmed by genetic testing.

Mitochondrial patients included 5 patients with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes), 15 with PEO (progressive external ophthalmoplegia), one of whom was diagnosed with Kearns-Sayre syndrome, and 10 with mitochondrial myopathy. The diagnosis of

	Control gr.	Mitochondrial patients			DM1	FSHD
		MELAS	PEO	Myopathy		
N (male/fem.)	20 (9/11)	5 (3/2)	15 (9/6)	10 (3/7)	16 (7/9)	5 (2/3)
Age (yrs)	45.9±16.1	51.2±23.8	59.6±15.3	57.7±9.6	50.9±12.6	41±12.3
First sy. (yrs)	NA	41.2±21.6	39.2±23.1	47.1±14	35.1±15.9	30.4±14.6
BMI (kg/m ²)	26.0±5.3	23.7±6.5	26.3±6.0	27.1±8.1	27.1±5.4	21.7±2.6
Diab. M.	0/20	0/5	3/15	2/10	3/16	0/5

Table 1. Demographics of enrolled healthy controls and patients. Age, years; N, number of subjects (male / female); First sy, first symptoms of the disease (age); Diab. M., Number of diabetic subjects; NA - not applicable

mitochondrial disease was made according to the criteria published in 2002 [45]. Patient's demographics are summarized in Table 1.

3.3.2. Methods

All patients underwent detailed general and neurological examinations. Blood was collected at the same time each time (8:00, on an empty stomach), and serum was isolated and stored at -80°C until enzyme-linked immunosorbent assay (ELISA) was done.

HOMA-IR (Homeostasis Model Assessment - Insulin Resistance) index was calculated for the detection of insulin resistance in myotonic dystrophy and mitochondrial patients [46].

Summarized MRC (Medical Research Council) score was used to quantify muscle weakness. This was calculated from bilateral evaluations of the muscle strength of neck flexors, extensors, and 15 limb muscles.

Systemic involvement was assessed using the Japanese Mitochondrial Disease Rating Scale (JMDRS) [47]. In our study, we also added a score for evaluation of the eye movement abnormalities.

All patients underwent muscle biopsy the histopathological examination was performed by a neuropathologist according to international standards.

Genetic testing of mitochondrial patients was performed mostly using a muscle sample, in some cases using DNA isolated from blood. Diagnosis of FSHD and DM1 was also confirmed by genetic testing.

Duplicate determinations of serum FGF-21 were performed with a commercially available ELISA kit (Quantikin ELISA kit, R&D Systems Inc). Serum FGF-21 concentration (pg/ml) was calculated according to the manufacturer's instructions. The ELISA test was performed on a blinded manner by E.L. and M.C.

Statistical analysis was performed with the use of SPSS v19. (IBM Co., Armonk, New York) software. Clinical data were compared using nonparametric tests (Mann-Whitney U test and analysis of variance). Person correlation was used to compare FGF-21 levels and clinical data. We used a significance level set to 0.05.

3.4. Results

FGF-21 levels were determined from 71 samples, of which 20 were healthy controls and 51 patients with the mentioned muscle diseases. The BMI of the controls and patients ranged from 18 to 30. Patients with BMI below 17 and above 30 were excluded, since we found that serum FGF-21 levels in controls were highly correlated with BMI ($r=0.75$, $p=0.0005$). No significant correlation was found between BMI and FGF-21 levels in any of the patient groups.

Statistical analysis showed that JMDRS was significantly higher in PEO patients than in patients with mitochondrial myopathy, DM1, and FSHD, but there was no significant difference compared with patients with MELAS (figure 1/A). Patients' CK levels ranged from the normal range (up to 200 U / l) to moderately elevated, with no significant difference between the study groups. Serum lactate levels were higher in mitochondrial patients compared to DM1 patients, especially in the PEO group. (Figure 1/B). Serum FGF-21 levels showed similar values in healthy controls, MELAS, and patients with mitochondrial myopathy, and were slightly elevated in FSHD.

FGF-21 levels were significantly higher in PEO (589.5 ± 496.4 (mean \pm SD), range 5.2-1705.7) and DM1 (429 ± 328.5 (mean \pm SD), 36.5- range 1130.9) than in healthy controls (138 ± 173.3 (mean \pm SD), range 0-510) (figure 1/C). The HOMA-IR index was significantly higher in DM1 patients than in the mitochondrial group (6.13 ± 4.86 vs. 1.8 ± 1.2 , (mean \pm SD) $p = 0.012$). The results of muscle biopsy examinations are summarized in Table 2.

Correlation analysis showed an association of lactate levels and FGF-21 ($r = 0.69$, $p = 0.0008$) and RBF ratios ($r = 0.44$, $p = 0.03$) in the mitochondrial group.

The subgroup analysis revealed an even stronger correlation between the level of lactate and the ratio of RBFs in the PEO group. In addition, FGF-21 levels also correlated with JMDRS scores (Fig. 2).

There was no significant difference in serum FGF-21 levels between the genders and it was not related to the age of the patients. No association was found between FGF-21 and muscle weakness or CK values. HOMA-IR did not correlate with any of the clinical or biochemical parameters in the studied groups.

	Control	Mitochondrial patients			DM1	FSHD
		MELAS	PEO	Myopathy		
Diff. mit. acc.	NA	2.8±1.3	2.9±1.3	2.9±1.6	0.8±2	0
RBF (SDH)	NA	7.4±11.9*	10.8±13.9*	6.2±15.7*	0.27±0.57	0.03±0.05
COX- fibers	NA	3.2±4.5	16.6±16.5*	1.0±0.7	0.5±0.7	0.03±0.05
COX+RBF	NA	8.2±13.3*	3.3±2.5	15.7±25.4*	0	0

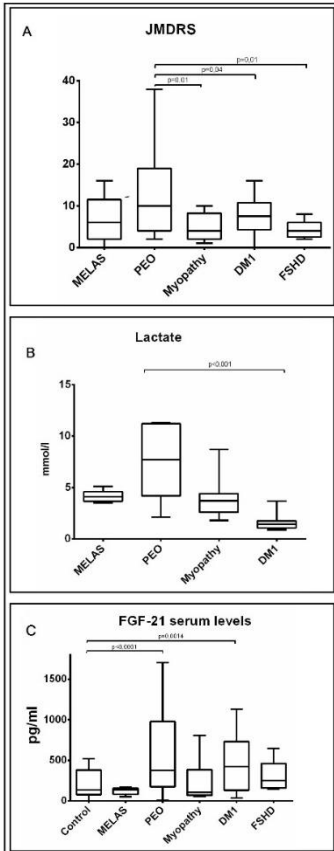


Table 2. Mitochondrial abnormalities in muscle biopsy specimens. Data are presented as mean ± standard deviation. * significant difference ($p < 0.05$). Diff. mit. acc., diffuse mitochondrial accumulation; NA, not applicable; MELAS, mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes; PEO, progressive external ophthalmoplegia; DM1 dystrophy myotonica type 1; RBF, ragged blue fibers; SDH, succinate dehydrogenase; COX, cytochrome-1 oxidase.

Figure 1. JMDRS, Lactate, and FGF values in healthy controls and patients enrolled in the study. JMDRS (A) and serum lactate levels (B) were highest in the PEO group. Serum fibroblast growth factor 21 (FGF-21) levels (C) were significantly higher in PEO and DM1 patients compared to healthy controls. The box-plot diagram shows the mean (with 25th and 75th percentiles) and the whiskers the min-max values. Significance levels are indicated.

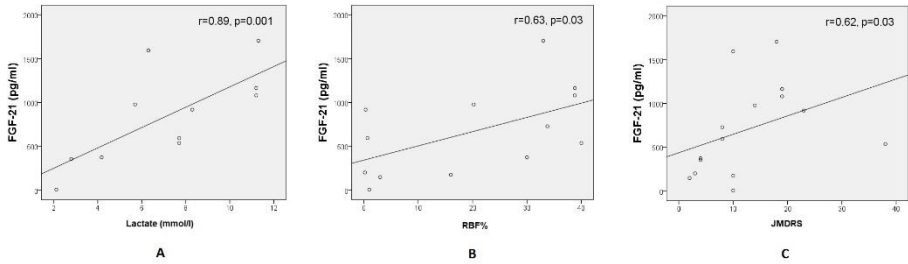


Figure 2. Correlation of serum FGF-21 levels with biochemical, histological, and clinical findings in PEO patients. Serum FGF-21 levels correlated strongly with serum lactate levels (A), the ratio of RRF fibers in muscle biopsy specimens (B), and JMDRS (C).

3.5. Discussion

- serum levels of FGF-21 were elevated in mitochondrial patients: it was almost normal in MELAS and mitochondrial myopathy, while it was significantly elevated in PEO. Normal FGF-21 levels in isolated myopathy may be explained by milder muscle involvement, exclusion of overweight patients, and a high incidence of diabetes mellitus [41].
- FGF-21 levels correlated with the proportion of RBF fibers, one of the main histological indicators of mitochondrial dysfunction.
- Our results suggest that FGF-21 is a much more sensitive marker of mitochondrial disease than CK or lactate levels [48].
- In line with histological characteristics, normal FGF-21 levels were found in FSHD patients, and the incidence of signs of mitochondrial lesions and dysfunction was low.
- One of our most important findings is the elevated FGF-21 level observed in DM1 patients, which may be explained by insulin resistance, supported by the fact that insulin resistance (elevated HOMA-IR) was significantly more common in DM1 than in mitochondrial patients. Another possible explanation is the development of mitochondrial dysfunction in DM1 patients, which contributes to FGF-21 production, as indicated by histological abnormalities (Table 2).
- In DM1 patients, we could not show a significant association between FGF-21 levels and the severity of mitochondrial involvement or clinical symptoms, so it is possible that insulin resistance has a greater effect on FGF-21 levels than mitochondrial dysfunction.

- Serum levels of FGF-21 are associated with mitochondrial dysfunction; however, mitochondrial syndromes and subgroups differ in terms of serum levels of FGF-21.
- Further studies are needed to analyse the correlation between phenotype, severity of muscle involvement, and FGF-21. It is important to consider insulin resistance when assessing FGF-21 levels in neuromuscular diseases.
- In the future, we plan to determine serum FGF-21 levels in a larger group of patients with myotonic dystrophy as well as in several muscle diseases. Correlation studies with other clinical parameters (lipid metabolism abnormalities, proBNP, serum microRNA concentrations) are also planned.

4. Cortical involvement during myotonia in myotonic dystrophy: an fMRI study

4.1. Literature review

The most characteristic symptom of myotonic dystrophy is the involuntary maintained muscle contraction, which clinically manifests as the difficulty in relaxing the hand muscles – grip myotonia. Myotonia is thought to be caused by a disorder of a muscle chloride channel [49]. Myotonia temporarily ameliorates after exercise – this is the warm up phenomenon [50], however, the background of this phenomenon is yet not known.

Myotonic dystrophy is a multisystem disease that also affects the central nervous system. A wide range of brain lesions have been detected in DM1, primarily white matter lesions and atrophy have been observed [51, 52]. An earlier MRI study of finger-thumb opposition showed significantly higher BOLD (blood oxygen level dependent) activation in several brain areas (e.g., sensorimotor cortex, parietal lobe, thalamus, premotor area, insula, supplemental motor area) in DM1 patients than in healthy controls. These findings have been attributed to compensatory mechanisms such as reorganization and redistribution of functional networks that may result from the accelerated aging process, a characteristic of the disease [53]. Another review found decreased glucose uptake and cerebral perfusion in the frontal, temporal, and parietal lobes [54]. Considering that

primarily the neuromuscular abnormalities have been studied in DM1, the extent and background of brain involvement during myotonia is not yet known.

4.2.Aims

- We hypothesized that compensatory mechanisms are activated in the brain during myotonia, but it cannot be ruled out that the brain is at least partially involved in the maintenance of myotonia.
- We aimed to map the brain areas activated during myotonia using fMRI.
- We intended to answer whether there is a significant difference in the BOLD signal in brain areas between myotonic and non-myotonic DM1 patients during the performance of a „grip task”
- Based on our findings and literature data we aimed to assess neural mechanisms underlying myotonia.

4.3.Materials and methods

4.3.1. Patients

16 patients (9 women, 7 men, aged 31–60 years) diagnosed with DM1 were enrolled in the study. Exclusion criteria were a history of alcohol abuse, drug abuse, cognitive deficit (Mini mental state test less than 25/30 points), and any other reason when an MRI scan could not have been performed. All patients were right-handed, based on “Edinburgh Handedness Inventory Test” [55]. The clinical characteristics of the patients are summarized in Table 3.

Patients were divided into two groups: In the DM / Myotonia + group, myotonia was clinically detectable during complete hand grip, while in the DM /

	Gender (M/F)	Age (Yrs)	Disease duration (Yrs)	Myotonia onset (Yrs)	Sum-MRC
DM/Myotonia⁺(1) (n=8)	5/3	48,33±8,33 (31-60)	15,22± 10.37 (5-39)	33± 9,84 (18-44)	459.0± 40,34 (383-516)
DM/Myotonia⁻(2) (n=8)	4/4	47,29± 8,14 (39-58)	22,14± 5,46 (15-29)	24,86±6,12 (17-34)	454,7±34,61 (387-492)
All patients (n=16)	9/7	47,88± 7,99 (31-60)	18,25± 9,04 (5-39)	29,44±9,16 (17-44)	457,1± ±36,77 (383-516)

Table 3. Clinical characteristics of patients. (1) DM1 patients with grip myotonia (2) DM1 patients without grip myotonia. Data are shown as mean ± standard deviation (min-max values). SUM-MRC: sum of the muscle strength measurements (scale 1-10) performed on 24 muscles. There was no statistically significant difference between the two groups (Mann-Whitney U test, $p > 0.05$).

Myotonia - group, no myotonia was observed during the examination (EMG confirmed subclinical myotonia). Based on this, 8-8 patients were enrolled in both groups.

4.3.2. Imaging

Structural and functional imaging was performed. High-resolution T1- and T2-weighted images were taken to assess structural changes (see below for details). Functional imaging consisted of three phases according to the protocol outlined in Figure 3.

Prewarm-up examination

The following consecutive blocks were repeated six times (the first block was introduced by a rest period of 30 seconds):

1. Grip block: Patients performed grip in left hand. This block was introduced by the word ‘grip’ and lasted for 10s.
2. Post-grip block (‘myotonia’ condition in DM/myotonia+ group, ‘myotonia absent’ condition in DM/myotonia group): This block was introduced by the word ‘release’; however, DM/ myotonia+ patients were not expected to be able to immediately release their grips; instead of that, myotonia phenomenon was expected to occur. Patients were told not to force relaxation, but let myotonia

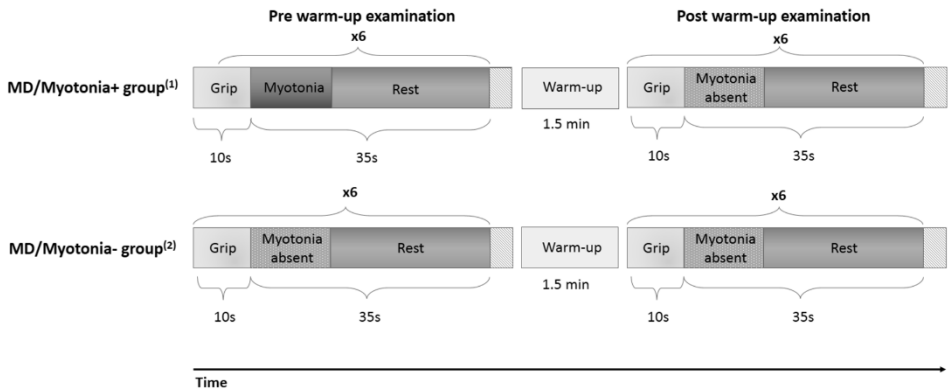


Figure 3. Arrangement of pre- and post-warm-up examinations. (1) Myotonic dystrophy patients with apparent grip myotonia; (2) myotonic dystrophy patients without apparent grip myotonia. Grip: left-hand grip; warm-up: repeated opening and closing of left hand as long as needed for maximal possible attenuation.

spontaneously pass. Duration of the triggered myotonia was considered as time until complete hand relaxation and was recorded at each block by examiner.

3. Rest (baseline) block: Time left between grip blocks lasted 35s; that is, rest block lasted 35s minus post-grip block duration.

To avoid disturbing effects of visual processing and linked functions, patients were told to keep eyes closed during the entire acquisition.

Post-warm-up examination

The post-warm-up examination was executed in the same way as the pre-warm-up examination; however, due to the preceding ‘warm-up’ exercise, no myotonia was expected to occur (‘myotonia absent’ conditions in both DM/myotonia+ and DM/myotonia- groups).

Passive hand movement task

Passive hand movement task started with a 30s rest period, then included five repeated blocks of passive hand movement (investigator performed opening and closing movement on the patient’s left-hand fingers while patient was told not to counteract) and resting, both lasting 30s.

Image acquisition parameters

Imaging was performed on a 3T Magnetom TIM Trio human whole body MRI scanner (Siemens AG, Erlangen, Germany) with a standard 12-channel head coil.

The T1-weighted high-resolution measurement was performed using a three-dimensional (3D) MPRAGE sequence. Functional images were obtained using a standard 2D spin echo EPI sequence; interleaved slice order was applied to avoid crosstalk between contiguous slices. A total of 150 volumes were acquired in 300s. Subjects were positioned supine in the scanner.

Data analysis

Preprocessing and statistical analysis were performed using FMRI Expert Analysis Tool (FEAT) 5.98 version.

In the DM / Myotonia+ group, the timing of the “myotonia” and “rest” phase was adjusted to the duration of the observed myotonia. Myotonia durations recorded in DM/Myotonia+ group were used not only for pre-warm-up studies but also for

examination of post-warm-up myotonia absent condition of DM/Myotonia- group for a direct comparison of activations.

In addition to the “grip” and „rest”, the“ myotonia absent ”state was used for the DM/Myotonia- group studies to make the data comparable to those recorded in the DM/Myotonia+ group. The “myotonia absent” state lasted for 5s, as this was the average duration of myotonia in the DM/Myotonia+ group.

The following groupwise comparisons were made:

A: Prewarm-up examination conditions vs postwarm-up examination conditions (intrasubject analysis, i.e., prewarm-up grip vs post-warm-up grip condition; prewarm-up myotonia vs postwarm-up myotonia absent condition). Extraction-based contrast maps were calculated using paired t-test design. The comparisons of pre- and post-warm-up examination conditions were performed in the DM/myotonia- group as well to reveal if repetition or fatigue in itself had an effect on BOLD response.

B: Prewarm-up examination conditions of DM/myotonia+ group vs prewarm-up conditions of DM/myotonia group (intersubject analysis, i.e., DM/myotonia+ group grip vs DM/myotonia group grip condition; DM/myotonia+ group myotonia vs. DM/myotonia- group myotonia absent condition). Extraction-based contrast maps were calculated using unpaired t-test design.

Passive hand movement tasks were compared between myotonia and control groups using extraction-based contrast maps with unpaired t-test design.

4.4.Results

Structural imaging

Mild, non-specific abnormalities were found in patients, such as atrophy, ventricular dilatation, white matter lesions, and wider Virchow-Robin spaces. There was no lesion interfering with functional imaging.

Functional imaging

All patients in the DM/Myotonia+ group had at least 3 seconds (mean 5 seconds, max. 12 seconds) of myotonia after handgrip prior to warm-up. No myotonia longer than 1 second was recorded when testing handgrip after warm-up. In the DM/Myotonia- group, no myotonia occurred at any of the complete hand grips.

DM/Myotonia+ group

Significantly ($P < 0.05, Z > 2.3$) higher BOLD signal was found during the pre-warm-up examination in myotonia condition than during the post-warm-up myotonia absent condition, in the supplementary motor area (SMA) and in the dorsal anterior cingulate cortex (dACC) (see Fig. 4, Table 4.). No significant difference was found between pre- and post-warm up „grip” phases.

DM/Myotonia- group

No significant difference in BOLD signal was found between pre- and post-warm-up conditions in DM/Myotonia- group. Significantly ($P < 0.05, Z > 2.3$) higher BOLD signal was found in the DM/myotonia+ group in myotonia condition than in the DM/myotonia- group myotonia absent condition, in the SMA and in the dorsal anterior cingulate cortex (dACC) (see Fig 5. and Table 5.). No significant difference was found between grip condition BOLD signals.

Passive hand movement task

No significant difference in BOLD signal during passive hand movement task was found between DM/myotonia+ and DM/myotonia- groups.

Cluster	Voxel #	P	Max. Z	Z max. coordinates (MNI152) ¹			Atlas label ²
				X (mm)	Y (mm)	Z (mm)	
1	314	0.00681	4.29	6	-10	62	49 % SMA ³
2	224	0.0452	5.09	2	-2	30	65% ACC ⁴

Table 4. Cluster details of voxels of significantly higher blood oxygen level dependent signal in prewarm-up myotonia condition (Cluster 1) than in post-warm-up myotonia absent condition (Cluster 2). SMA, supplementary motor area; ACC, Anterior cingulate cortex. ¹Coordinates in mm, MNI152 standard space. ²Atlas label according to the Harvard-Oxford Cortical Structural Atlas.

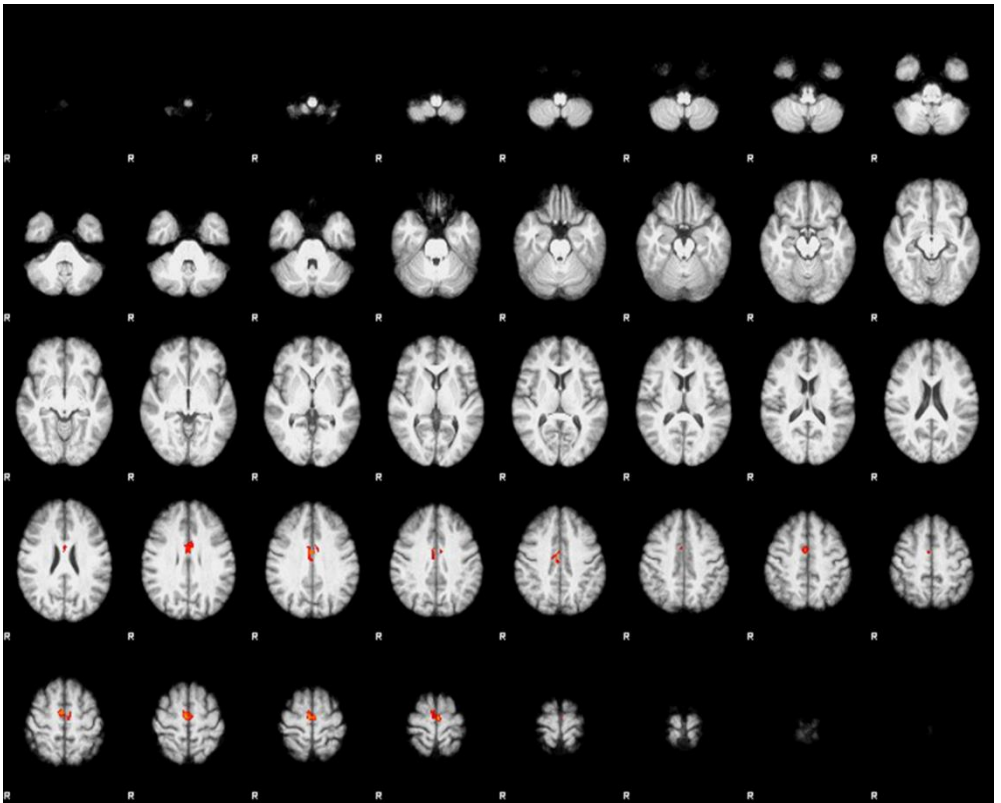


Figure 4. Comparison of prewarm-up myotonia condition and post-warm-up myotonia absent condition in the DM/myotonia+group. Red-yellow clusters (overlaid on MNI 152 standard brain) represent voxels of significantly ($P < 0.05$, $Z > 2.3$) higher blood oxygen level-dependent signal at myotonia condition compared to myotonia absent condition. Clusters are located in the supplementary motor area (SMA) and the dorsal anterior cingulate cortex (dACC). Cluster details are listed in Table 4.

Cluster	Voxel #	P	Max. Z	Z max coordinates (MNI152) ¹			Atlas label ²
				X (mm)	Y (mm)	Z (mm)	
1	682	0.00014	3,79	0	12	34	81 % ACC
2	516	0.003	2,91	-4	-12	62	50% SMA

Table 5. Cluster details of voxels of significantly higher blood oxygen level-dependent signal in DM/myotonia+ group myotonia condition (Cluster1) than in DM/myotonia- group myotonia absent condition (Cluster 2). ACC, Anterior cingulate cortex; SMA, supplementary motor area.¹Coordinates in mm, MNI152 standard space. ²Atlas label according to the Harvard-Oxford Cortical Structural Atlas.

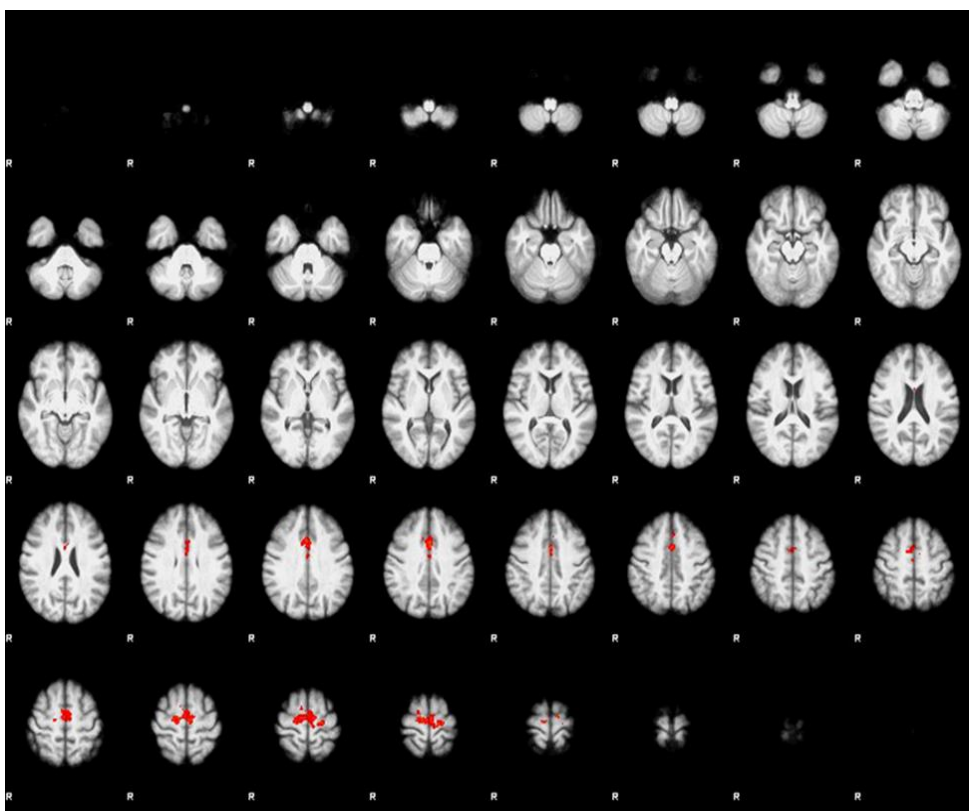


Figure 5. Comparison of DM/myotonia+ group prewarm-up myotonia condition and DM/myotonia- group prewarm-up myotonia absent condition. Red-yellow clusters (overlaid on MNI 152 standard brain) represent the significantly ($P < 0.05$, $Z > 2.3$) higher blood oxygen level-dependent signal rate voxels in the DM/myotonia+ group myotonia condition compared to DM/myotonia- group myotonia absent condition. Clusters are located in the supplementary motor area (SMA) and the dorsal anterior cingulate cortex (dACC). Cluster details are listed in Table 5.

4.5. Discussion

- To our knowledge, this is the first study exploring brain involvement during grip myotonia using fMRI.
- First, based on the warm up phenomenon, then based on the two, myotonia+ and myotonia- groups we were able to examine separately the BOLD activations during myotonia. Significantly higher BOLD signals were found in the supplementary motor area (SMA) and dorsal anterior cingulate cortex (dACC).
- primary motor areas did not show activation during myotonia so the brain functions are unlikely to be involved in the development of myotonia phenomenon
- myotonia is likely to lead to compensatory cortical activation, these areas are also thought to be involved in normal muscle relaxation
- Activations in the medial frontal cortex during myotonia may theoretically be caused by different motor control mechanisms as movement initiation/preparation, 'active' motor termination, but may be part of conflict-related mechanisms as well.
- Voluntary muscle relaxation has been associated with SMA in several studies [56-59], suggesting that the higher BOLD signal observed in SMA during myotonia indicates a similar involuntary inhibitory function.
- the anterior cingulate cortex (dACC) is also located in the motor control area of the medial frontal cortex and is responsible for the activation of cognitive functions related to movement. In myotonia, based on former studies this area is likely to be activated in connection with error reporting/failed execution [60-63].
- the absence of significant BOLD signal difference between groups and sessions during passive hand movement task underscores that the dACC and SMA activations are myotonia related and are not a result of other possible motor or sensory mechanism alteration caused by DM1.
- We showed that myotonia is related to cortical activations in high-order motor control areas. This cortical involvement is most likely to represent action of inhibitory circuits intending motor termination.
- Our results may add important information to the field of motor area research and may help understanding the cortical contribution to other peripheral and central disorders affecting motor system such as Parkinson's disease, dystonias, or apraxias.

- We plan further investigations of muscle MRI and fMRI with a larger group of DM1 patients as well as patients having congenital myotonia.

5. Summary of novel findings

In summary, the most important results of my clinical research during my doctoral work were:

- Serum FGF-21 levels were determined in several muscle diseases. This was most frequently elevated in mitochondrial patients, primarily PEO, but almost normal FGF-21 levels were measured in MELAS and isolated myopathy patients. This was likely caused by the mild muscle involvement, exclusion of overweight patients, or association of FGF-21 levels with diabetes mellitus.
- Our results confirm that FGF-21 levels are associated with the severity of mitochondrial damage in patients with myopathy; however, there was no significant association between FGF-21 levels and the degree of muscle weakness.
- Our results confirmed the association between FGF-21 and lactate levels.
- Primary and secondary mitochondrial dysfunction in muscle tissue can lead to FGF-21 production.
- An important result of our study was that we measured elevated FGF-21 levels in DM1 patients. One explanation for this may be the well-known insulin resistance in the disease, and another possible explanation may be the likely mitochondrial dysfunction in DM1 patients.
- Our results show that in neuromuscular diseases, it is important to consider insulin resistance when assessing FGF-21 levels.
- In our fMRI study, we showed that primary motor regions are not involved in the development of myotonia, but this muscle-derived phenomenon leads to secondary, possibly compensatory cortical activation.
- Prolonged muscle relaxation allowed fMRI mapping of areas that are likely to be involved in normal muscle relaxation as well. These are the medial frontal cortex, the anterior cingulate cortex, and the supplemental motor area. The first two are responsible for motor control mechanisms, but may also be part of “error detection” mechanisms. Supplementary motor area may cause inhibition leading to suspension of motor response.

- Simultaneous activation in the dorsal-anterior cingulate cortex and the supplementary motor area suggests that inhibited muscle relaxation elicits a secondary response in the areas responsible for motor control.
- Our results may provide important information for the study of motor areas and the understanding of cortical responses in some particular central and peripheral diseases.

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7. Publications

Peer-reviewed articles related to the thesis:

Lovadi, E ; Csereklyei, M ; Merkli, H ; Fülöp, K ; Sebők, A ; Karcagi, V ; Komoly, S ; Pál, E Elevated FGF 21 in myotonic dystrophy type 1 and mitochondrial diseases MUSCLE & NERVE 55 : 4 pp. 564-569. , 6 p. (2017)

Speciality of the journal: Neurology (clinical), ranking: Q2, IF (2017): 2,496.

Tóth, A; Lovadi, E*; Komoly, S; Schwarcz, A; Orsi, G; Perlaki, G; Bogner, P; Sebők, A; Kovács, N; Pál, E** et al. Cortical involvement during myotonia in myotonic dystrophy: an fMRI study. *Acta Neurologica Scandinavica* 132: 1 pp. 65-72., 8 p. (2015)

*shared first authors **shared last authors;

Speciality of the journal : Neurology, ranking: Q2., IF (2015): 2,559.

Cumulative impact factor related to the thesis: 5,055

Cumulative impact factor: 5,404

Book chapter related to the thesis:

Pal E, Lovadi E, Gati I, Merkli H, Komoly S. Muscle biopsy: Diagnostic value in suspected myopathies. In: Washington, HS; Castillo, Jimenez CE (editor) *Myopathies: : New Research* Hauppauge (NY), USA : Nova Science Publishers (2012) pp. 57-74., 18 p.

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Lovadi E, Pál E, Sebők Á, Bajkó Z Methodology of histometry measurements in Dystrophia Myotonica. University of Medicine and Pharmacy Tg. Mures , Students Academic conference, Tg Mures, 27-30.03.2011.

Nusser N, Sebők Á, Lovadi E Rehabilitation of myotonic dystrophy patients at the hospital in Harkány. Hungarian Society of Medical Rehabilitation and Physical Medicine XXXIII. Travelling Assembly, Szolnok, 4-6 September 2014.

Scientific posters related to the thesis:

Lovadi E, Nusser N, Sebők Á, Pál E, Complex care and rehabilitation in Myotonic Dystrophy. XXXV Travelling Assembly of the Hungarian Society of Neuroscientists, Debrecen, 22-24 November 2012, 3rd prize.

Lovadi E, Sebők Á, Nusser N, Komoly S, Pál E Complex rehabilitation in myotonic dystrophy. XXXVI Conference of the Hungarian Society of Neurologists, Eger, 20-22 October 2016.

Lovadi E, Nagy Sz, Sebők Á, Komoly S, Pál E Quantitative MRI of the muscle atrophy in patients with myotonic dystrophy1. XXXVI Conference of the Hungarian Society of Neurologists, Eger, 20-22 October 2016

E. Pál, E. Lovadi, M. Cserekyei, Á. Sebők, S. Komoly FGF21: A biomarker of neuromuscular diseases. — WCN 2013, No: 2471, Topic: 7 — Neuromuscular disorders, Wien, Austria, 21-26 September 2013..

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Speciality of the journal: clinical and experimental medicine, ranking: Q3, IF (2016):0,349

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Scientific posters not related to the thesis:

Lovadi E, Gyimesi Cs, Janszky J, Sebők Á, Illés Zs The "Great Imitator": Neurosyphilis diagnosed after non-convulsive status epilepticus. XXXV Travelling Assembly of the Hungarian Society of Neuroscientists, Debrecen, 22-24 November 2012,

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Rozgonyi R, Lovadi E, Csécei P, Faludi B The importance of sleep tests in thalamus and brainstem stroke. The XIII Conference of the Hungarian Stroke Society and X Conference and of the Hungarian Neurosonological Society, Győr, 5-7 October 2017

Acknowledgments

I would like to express my thanks and gratitude to those who have helped my scientific and clinical work over the last ten years, with their advice and often with their encouragement.

I had the opportunity to meet Professor Sámuel Komoly in Targu Mures in 2010, and he was immediately open and helped me to spend my Erasmus study exchange trip at the Department of Neurology in Pécs. The foundations and new acquaintances made at that time led to the writing of this thesis.

My first uncertain steps were assisted by Dr. Endre Pál and Dr. Ágnes Sebők with their guidance, competence and trust in advance, which has not ran out over the years. Professors Sámuel Komoly and József Janszky, as well as the whole staff of the Department of Neurology in Pécs, supported my clinical and scientific work all along. I would like to express my gratitude and appreciation for them.

Without the support, faith and trust of my family, without the help of my husband Tibor, this thesis and the scientific work on which it was based would also not have been possible.

Finally yet importantly, I would like to thank our patients, who have often travelled hundreds of kilometres to take part in our studies and who have relied on our research team throughout. We all have learned a lot from their words and attitudes.

'In history, coincidences can open strange doors to the future. And when a door like this opens, you have to step in.'

Voltaire