Phenotypic and genetic spectrum of patients with limb-girdle muscular dystrophy type 2A from Serbia

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Limb-girdle muscular dystrophy (LGMD) type 2A (calpainopathy) is an autosomal recessive disease caused by mutation in the CAPN3 gene. The aim of this study was to examine genetic and phenotypic features of Serbian patients with calpainopathy. The study comprised 19 patients with genetically confirmed calpainopathy diagnosed at the Neurology Clinic, Clinical Center of Serbia and the Clinic for Neurology and Psychiatry for Children and Youth in Belgrade, Serbia during a ten-year period. Eighteen patients in this cohort had c.550delA mutation, with nine of them being homozygous. In majority of the patients, disease started in childhood or early adulthood. The disease affected shoulder girdle – upper arm and pelvic girdle – thigh muscles with similar frequency, with muscles of lower extremities being more severely impaired. Facial and bulbar muscles were spared. All patients in this cohort, except two, remained ambulant. None of the patients had cardiomyopathy, while 21% showed mild conduction defects. Respiratory function was mildly impaired in 21% of patients. Standard muscle histopathology showed myopathic and dystrophic pattern. In conclusion, the majority of Serbian LGMD2A patients have the same mutation and similar phenotype.

Key words: calpainopathy, c.550delA mutation, muscle magnetic resonance imaging, muscle histopathology

Introduction

Limb-girdle muscular dystrophy (LGMD) type 2A (calpainopathy) is an autosomal recessive disease caused by mutation in the CAPN3 gene (1). Calpain 3 is a skeletal muscle-specific isoform of the Ca2+-dependent non-lysosomal calpain cysteine protease, essential for normal muscle function (1). It plays a role in numerous functions in muscle cells, such as muscle regeneration, sarcoplasmic repair, cytoskeleton regulation and calcium homeostasis (2-5). Calpainopathy is the most common form of LGMD in majority of countries worldwide (6, 7).

Based on the distribution of muscle weakness and age at onset, three main phenotypes of LGMD2A have been identified: 1) pelvi-femoral phenotype is the most common one, with muscle wasting and weakness starting...
in pelvic girdle, later affecting shoulder girdle; 2) scapulo-humeral phenotype with affection of the shoulder girdle from the beginning; and 3) hyperCKemia, which may be considered as a presymptomatic stage of calpainopathy, most commonly exhibited in children and young adults (8-10).

Aim of this research was to analyze genetic and phenotypic features of Serbian patients with calpainopathy.

Methods

The study was approved by the Ethical Board of the Neurology Clinic, Clinical Center of Serbia. It comprises patients that have been genetically diagnosed with LGMD2A at the Neurology Clinic, Clinical Center of Serbia and the Clinic for Neurology and Psychiatry for Children and Youth in Belgrade, Serbia during a ten-year period from 2008-2017. These are the two largest neuromuscular centers diagnosing patients with LGMD from the whole country and the region. Genetic analysis was performed through the whole exome sequencing in 15 subjects (confirmed with Sanger), sequencing of the whole CAPN3 gene in one patient and analysis of four (exons 4, 6, 10 and 13) out of 24 coding exons in three subjects. Selection of these four exons was based on the previous genetic results of LGMD patients in the region and was done in order to lower the financial costs (11). Sequence variants were numbered according to the reference sequence NM_000070.3/ NP_000061.1.

Follow-up visits were regularly scheduled once or twice per year for all patients. Clinical assessment included a detailed medical and family history and neurological examination, performed by an experienced neurologist. First degree relatives of patients were assessed where possible. Muscle strength was scored using the Medical Research Council (MRC) scale (12), Electromyography (EMG) and nerve conduction studies were performed in 11 patients. Serum creatine kinase levels were measured in all patients at multiple time points during the course of the disease. Respiratory function was assessed by spirometry annually, while regular cardiac examinations included ECG each year and echocardiogram every second year. Muscle biopsies, usually of deltoid muscle, were performed in nine patients. Calpain 3 immunohistochemistry and immunoblot were not performed.

Muscle magnetic resonance imaging (MRI) was performed in four patients in axial and coronal planes of the lower limbs using the following sequences: T1-weighted (T1w), T2-weighted (T2w), proton-density weighted (PDw), and 3-point Dixon (13, 14). Images were assessed on an individual muscle basis and graded according to the five-point scale published by Mercuri et al. (15).

Methods of descriptive statistics were used: mean, standard deviation, median. Chi-square test, Mann-Whitney U test and Student t test were used for comparisons between two groups, as appropriate. Level of statistical significance was 0.05.

Results

We identified 19 LGMD2A patients from 18 families. This accounts for approximately 30% of all LGMD patients diagnosed in our two centers in observed period. Eighteen (95%) patients in this cohort had c.550delA (p.Thr184ArgfsTer36) mutation on at least one allele, with 9 (47%) patients being homozygous for this mutation (Tab. 1). Clinical presentation did not differ between c.550delA homozygous and heterozygous patients.

Majority of our patients were sporadic. Three of them had positive family history for LGMD2A (patients #6, #7 and #18). LGMD2A was diagnosed in patient #7 at the age of 12. Thus, his one-year older brother (#6) went through the full clinical examination – CK level was elevated with symptomatology showing mild wasting of the muscles in scapular region, contractures of the ankles, and inability to walk on heels.

We observed equal distribution regarding gender with eight (42%) patients being male (Tab. 2). The age at disease onset ranged between 7 to 40 years old (mean 16.4 ± 7.6, median 14.5 years). In one patient (#10) the disease started at the age of 40, while all of the others had disease onset between the age of 7 and 22. Almost half of the patients (42%) had multiple symptoms at the disease onset. In majority (74%) of the patients, the first symptom was proximal muscle weakness in lower limbs. Another common initial symptom was gait on tiptoes observed in 26% of patients. In two (11%) patients the disease started with proximal arm weakness. Two patients were asymptomatic, and they were accidentally diagnosed with hyperCKemia at age 9 and 11. Although patient #10 had late symptom onset, the disease showed more rapid progression, and the patient started to use a cane only after two years from the disease onset.

Average age at the last examination was 25.4 ± 10.4 years (median 26) (Tab. 2). Mean duration of disease was 11.6 ± 4.3 years (median 11.5). The facial and bulbar muscles still were not affected. Muscle atrophy and weakness was observed in pelvic girdle and thighs in 84% patients and the same number had shoulder girdle and proximal arm muscle wasting. Lower leg muscles were affected in 79% of patients and only one patient had distal muscle weakness in arms. Pseudohypertrophy of muscles was detected in ten (53%) patients, with seven of them exhibiting hypertrophy in calves, two in proximal muscles of lower limbs, and one in lower arms.
Table 1. Genetic findings in CAPN3 gene in investigated patients with LGMD2A.

| #  | Mutation in one allele       | Mutation in another allele       | Genetic method      |
|----|------------------------------|---------------------------------|---------------------|
| 01 | p.Thr184ArgfsTer36           | p.Glu566Lys                      | Gene sequencing     |
| 02 | p.Thr184ArgfsTer36           | p.Thr184ArgfsTer36              | Targeted exons sequencing |
| 03 | p.Thr184ArgfsTer36           | p.Arg440Trp                      | Whole exome sequencing |
| 04 | p.Thr184ArgfsTer36           | c.1194-9A > G                    | Whole exome sequencing |
| 05 | p.Thr184ArgfsTer36           | p.Thr184ArgfsTer36              | Whole exome sequencing |
| 06 | p.Thr184ArgfsTer36           | c.1746-20C > G                   | Whole exome sequencing |
| 07 | p.Thr184ArgfsTer36           | c.1746-20C > G                   | Whole exome sequencing |
| 08 | p.Thr184ArgfsTer36           | c.2380+1G > A                    | Whole exome sequencing |
| 09 | p.Thr184ArgfsTer36           | p.Thr184ArgfsTer36              | Whole exome sequencing |
| 10 | p.Thr184ArgfsTer36           | p.Thr417Met                      | Whole exome sequencing |
| 11 | p.Thr184ArgfsTer36           | p.Asp295LeufsTer57              | Whole exome sequencing |
| 12 | p.Thr184ArgfsTer36           | p.Thr184ArgfsTer36              | Whole exome sequencing |
| 13 | Deletion of 10362 kb (genomic coordinates 15:42676429-42686791) a | p.Gly441ValfsTer22              | Whole exome sequencing |
| 14 | p.Thr184ArgfsTer36           | p.Asn434LysfsTer37               | Whole exome sequencing |
| 15 | p.Thr184ArgfsTer36           | p.Thr184ArgfsTer36              | Whole exome sequencing |
| 16 | p.Thr184ArgfsTer36           | p.Thr184ArgfsTer36              | Whole exome sequencing |
| 17 | p.Thr184ArgfsTer36           | p.Thr184ArgfsTer36              | Whole exome sequencing |
| 18 | p.Thr184ArgfsTer36           | p.Thr184ArgfsTer36              | Targeted exons sequencing |
| 19 | p.Thr184ArgfsTer36           | p.Thr184ArgfsTer36              | Targeted exons sequencing |

a Genomic variants are based on build37/hg19.

Table 2. Sociodemographic, clinical and laboratory features of our patients with LGMD2A.

| #  | Sex | Age at onset | Symptoms at onset                                      | Creatin kinase increase | Age at the last visit | Ambulant at the last visit |
|----|-----|--------------|--------------------------------------------------------|-------------------------|-----------------------|---------------------------|
| 01 | M   | 20           | Difficulties climbing stairs and rising up from squatting | 10x                     | 30                    | Yes                       |
| 02 | F   | 9            | Increased CK                                           | 10-50x                  | 9                     | Yes                       |
| 03 | M   | 19           | Difficulties climbing stairs                           | 10x                     | 37                    | Yes                       |
| 04 | M   | 11           | Gait on tiptoes                                        | 10-50x                  | 21                    | Yes                       |
| 05 | F   | 11           | Gait on tiptoes, difficulties climbing stairs and rising up from squatting | 50x                     | 16                    | Yes                       |
| 06 | M   | 14           | Proximal arm weakness                                  | > 50x                   | 14                    | Yes                       |
| 07 | M   | 12           | Gait on tiptoes, elbow contractures                     | 50x                     | 15                    | Yes                       |
| 08 | M   | 22           | Difficulties climbing stairs and walking uphill         | > 5x                    | 35                    | Yes                       |
| 09 | F   | 14           | Gait on tiptoes, difficulties climbing stairs           | 10x                     | 26                    | Yes                       |
| 10 | F   | 40           | Wasting of proximal leg muscles, muscle pain            | > 50x                   | 51                    | Yes                       |
| 11 | M   | 7            | Weakness of proximal leg muscles                        | > 50x                   | 20                    | no (from age 19)           |
| 12 | M   | NA*          | Increased CK                                           | > 50x                   | 11                    | Yes                       |
| 13 | F   | 14           | Weakness of proximal leg muscles, difficulties walking on heels | 10x                     | 29                    | no (from age 27)           |
| 14 | F   | 13           | Gait on tiptoes, difficulties rising up from squatting  | 10x                     | 29                    | Yes                       |
| 15 | F   | 18           | Difficulties climbing stairs                           | 10-50x                  | 26                    | Yes                       |
| 16 | F   | 15           | Proximal arm and leg weakness                           | < 5x                    | 34                    | Yes                       |
| 17 | M   | 20           | Difficulties climbing stairs                           | 10x                     | 33                    | Yes                       |
| 18 | F   | 10           | Difficulties climbing stairs                           | 10-50x                  | 21                    | Yes                       |
| 19 | F   | 16           | Difficulties climbing stairs                           | 10-50x                  | 25                    | Yes                       |

M: male; F: female; NA: not applicable
Muscle strength was most severely reduced in the lower limbs, especially proximally with hip flexors and adductors being the most affected, and knee extensors relatively spared (Tab. 3). Dorsal flexors of foot and toes were significantly more affected than plantar ones. Shoulder abductors and adductors and elbow flexors were the most impaired upper limb muscle, with distal arm muscle strength being preserved in almost all patients. Scapular winging was present in 16 (84%) patients. Contractures of the ankles were present in all patients, with two of them as a result of surgery to prevent foot drop. Three (16%) patients had elbow contractures. All patients, except two, remained ambulant at the last examination. One patient started to use a wheelchair at the age of 19 after 12 years of disease duration, and another one at age of 27 after 13 years of having the disease. In the group of ambulant patients, two of them were using assisting walking device – one cane, and the other one four-wheel walker.

Serum CK levels during the course of the disease were elevated in all of the patients and were 4-80 times higher than upper border of reference values (normal value < 150IU/l). EMG was performed in 11/19 patients and it showed myopathic pattern in all patients with no spontaneous activity at rest. As for cardiology findings, none of the patients had cardiomyopathy. Electrocardiography showed incomplete right bundle branch block (RBBB) in one patient at age 11 when he was diagnosed with calpainopathy. Three other patients had mildly prolonged PQ interval observed at regular cardiologic check-ups between age 25 and 30. Neither of them had any cardiac complaints. Respiratory function was impaired in 4 (21%) patients who had restriction (FVC < 90%) with the lowest FVC being 72% in patient who had a long disease duration of 19 years. It is of interest that patient #2 had signs of mild restriction (FVC 85%) from the beginning of the disease.

Seven patients underwent biopsy of the deltoid muscle. One of them (#7) showed no pathological changes at age 15, three years after disease started. In all other patients, dystrophic pattern was observed, including fiber size variations (6/6), necrosis (4/6), and connective and/or fat tissue infiltration (6/6). Some patients had additional myopathic signs: split fibers (3/6) and internal nuclei (3/6). In four out of six patients, inflammation was observed: two of them had rare T lymphocytes and histiocytes, one rare eosinophiles and macrophages, and one significant eosinophilic infiltration (Fig. 1). Patient with significant eosinophilic infiltration was at age 12 at the time of the biopsy.

Muscle MRI of thighs and legs was performed in eight patients (Fig. 2). Uniform pattern of affection of the thigh was observed with gluteal muscles, posterior thigh compartment, hip adductors and iliopsoas muscles being severely affected. Adductors, semitendinosus and semi-

![Figure 1](Image)

**Figure 1.** Histopathological findings in patients with calpainopathy. A: normal muscle architecture in patient #7; B: fiber size variation, necrotic fiber with macrophage infiltration and split fiber (arrow) in patient #3; C,D: fiber size variation, internal nuclei, necrotic fibers with macrophage infiltration in patient #16; E: significant eosinophilic infiltrate in patient #12; F: connective and fat tissue infiltration in patient #15
### Table 3. Pattern of muscle involvement in our patients with LGMD2A.

| #  | Upper extremities – proximal muscles | Upper extremities – distal muscles | Lower extremities – proximal muscles | Lower extremities – distal muscles | As. | Scapular winging | Spine deformities | Cont. |
|----|-------------------------------------|-----------------------------------|--------------------------------------|----------------------------------|-----|-----------------|------------------|-------|
| 01 | S.Abd 4, S.Add 4, EF 3              | Normal                            | HF 4, HE 4, H.Abd 4, H.Add 2, KF 4, KE 3 | DF 4, DTF 4                      | -   | +               | Hyperlordosis    | Ankles |
| 02 | Normal                              | Normal                            | Normal                               | DF 4, DTF 4                      | -   | +               | None             | Ankles |
| 03 | S.Abd 4, S.Add 4, EF 3              | Normal                            | HF 1, HE 4, H.Abd 3, H.Add 2, KF 3, KE 4 | normal                          | +   | +               | Hyperlordosis    | Ankles |
| 04 | S.Abd 3, S.Add 4, EF 3, EE 4        | Normal                            | HF 2, HE 4, H.Abd 4, H.Add 3, KF 4, KE 4 | DF 4, DTF 4, PTF 4               | +   | +               | Hyperlordosis    | Ankles |
| 05 | S.Abd 4, EF 4                       | Normal                            | HF 3, HE 3, H.Abd 3, H.Add 3, KF 4, KE 4 | DF 3                             | -   | +               | Scoliosis         | Ankles |
| 06 | Normal                              | Normal                            | Normal                               | DF 4                             | -   | +               | None             | Ankles, elbows |
| 07 | S.Abd 4                            | Normal                            | HF 4, HE 4, H.Abd 4, H.Add 4          | DF 4                             | -   | +               | None             | Ankles, elbows |
| 08 | S.Abd 3, S.Add 2, EF 3, EE 3        | Normal                            | HF 1, HE 3, H.Abd 2, H.Add 2, KF 3, KE 3 | Normal                          | -   | +               | None             | Ankles |
| 09 | S.Abd 4, S.Add 3, EF 3, EE 4        | Normal                            | HF 2, HE 3, H.Abd 2, H.Add 2, KF 3, KE 3 | DF 4, PF 4                       | +   | +               | Hyperlordosis    | Ankles (surgery) |
| 10 | EF 3                                | Normal                            | HF 4, HE 3, H.Abd 3, H.Add 3, KF 4, KE 4 | Normal                          | -   | -               | Hyperlordosis    | Ankles |
| 11 | S.Abd 2, S.Add 3, EF 3, EE 3        | Normal                            | HF 2, HE 2, H.Abd 2, H.Add 2, KF 2, KE 2 | DF 2, PF 2, DTF 2, PTF 2         | -   | +               | Hyperlordosis    | Ankles |
| 12 | Normal                              | Normal                            | Normal                               | Normal                          | -   | -               | None             | Ankles |
| 13 | S.Abd 2, S.Add 2, EF 2, EE 2        | WE 4, FF 4, FE 4                  | HF 2, HE 2, H.Abd 2, H.Add 2, KF 3, KE 3 | DF 2, PF 4, DTF 2, PTF 4         | -   | +               | None             | Ankles, elbows |
| 14 | S.Abd 3, S.Add 3, EF 3, EE 4        | Normal                            | HF 2, HE 2, H.Abd 2, H.Add 2, KF 2, KE 2 | DF 3, PF 4, DTF 3, PTF 4         | +   | +               | Hyperlordosis    | Ankles (surgery) |
| 15 | S.Abd 4, S.Add 4, EF 4, EE 4        | Normal                            | HF 2, HE 3, H.Abd 4, H.Add 3, KF 3, KE 3 | DF 4, DTF 4                      | -   | +               | Scoliosis         | Ankles |
| 16 | S.Abd 2, S.Add 2, EF 3, EE 3        | Normal                            | HF 2, HE 2, H.Abd 2, H.Add 2, KF 3, KE 3 | DF 3, PF 4, DTF 3, PTF 4         | -   | +               | Scoliosis         | Ankles |
| 17 | S.Abd 3, S.Add 3, EF 3, EE 3        | Normal                            | HF 3, HE 2, H.Abd 2, H.Add 2, KF 2, KE 2 | DF 3, PF 4, DTF 3, PTF 4         | -   | -               | Hyperlordosis    | Ankles |
| 18 | S.Abd 3, S.Add 3, EF 3, EE 4        | Normal                            | HF 2, HE 2, H.Abd 2, H.Add 2, KF 3, KE 4 | DF 2, PF 3, DTF 2, PTF 3         | -   | +               | Hyperlordosis    | Ankles |
| 19 | S.Abd 4, S.Add 4, EF 4              | Normal                            | HF 2, HE 2, H.Abd 4, H.Add 3, KF 3, KE 4 | DF 4, PF 4, DTF 3, PTF 4         | +   | +               | Scoliosis         | Ankles |

AS: asymmetry in muscle strength; Cont.: contractures; DF: dorsal flexors; DTF: dorsal toe flexors; EE: elbow extensors; EF: elbow flexors; H.Abd: hip abductors; H.Add: hip adductors; HE: hip extensors; HF: hip flexors; KE: knee extensors; KF: knee flexors; PF: plantar flexors; PTF: plantar toe flexors; S.Abd: shoulder abductor; S.Add: shoulder adductor; ‘+’ present; ‘-’ absent; Muscle strength was graded according to the Medical Research Council Scale.
membranosus muscles were more affected than biceps femoris. It seems that sartorius (from the anterior compartment) and gracilis (from the medial compartment) were the most spared muscles of the thighs. In all patients the most commonly affected muscles of lower legs were of the posterior compartment, with medial head of the gastrocnemius and soleus being more affected than lateral head of the gastrocnemius. Anterior and especially deep posterior compartments were long preserved.

**Discussion**

Calpainopathy was diagnosed in one third of Serbian patients with LGMD and c.550delA was the most common mutation found in 95% of subjects (in half of them in homozygous state). Depending on the geographic region, LGMD2A represents approximately 30% of all LGMD (16, 17). The c.550delA mutation accounts for up to 75% of LGMD2A patients in Slavic countries, Turkey, Italy and Germany (18-23). It was marked as the Eastern Mediterranean founder mutation (18). One in 133 patients from Croatian general population carries c.550delA mutation (11).

In our patients, disease usually started from childhood to early adulthood (median 14.5 years) which is in accordance with previous data (18). First symptoms in our group were proximal muscle weakness in lower limbs, walking on tiptoes, and in some cases proximal arm weakness which is in line with the literature data (18). Due to the presence of proximal arm atrophy and weakness and scapular winging these patients may be misdiagnosed as facioscapulohumeral muscular dystrophy although the second disease can be differentiated because of the facial muscle weakness and asymmetric atrophy and weakness in upper limbs.

Two of our patients were diagnosed with LGMD2A due to incidentally discovered hyperCKemia. Asymptomatic hyperCKemia is usually seen in children or young adults with calpainopathy and it may persist for decades (18). Serum CK levels during the course of the disease were 4-80 times elevated in all of our patients and this is the most common and unvarying feature of the LGMD2A from early infancy (19, 24).

During the disease course, pelvic girdle/thighs and shoulder girdle/proximal arms were clinically affected with similar frequency although muscle weakness was more severe in lower limbs. MRI results in proximal leg muscles were in accordance with the clinical findings: hip extensors and adductors and knee flexors were the most affected, while knee extensors were relatively spared. This was clinically described even in the first papers on calpainopathy (25, 26). Similarly, Straub and colleagues described prominent involvement of the gluteal and posterior compartment of the thigh in LGMD2A (27). According to them, pathology seems to start in the adductor magnus muscle and spreads to the semitendinosus and thereafter to all the hamstring muscles which is similar to our findings. This typical and selective pattern may be of diagnostic significance (18).

Lower leg muscles were affected in around 80% of patients during the course of the illness. Dorsal flexors of foot and toes were clinically more affected than plantar. On the contrary, MRI results showed that the most commonly affected muscles of lower legs were of the posterior compartment which is in accordance with previous reports (27). Sparing of the tibialis posterior muscle was observed in our cohort and previously reported in the literature (27, 28).

Muscle pseudohypertrophy was detected in half of our patients, usually in calves. This has been previously
reported only as an occasional sign in LGMD2A, although 86% of Brazilian LGMD2A patients had this finding (18, 29). This may be of diagnostic importance since male patients with hyperCKemia and calf pseudohypertrophy may be misdiagnosed with dystrophinopathy. It was reported that up to 20% of LGMD patients and 7% of LGMD2A actually have dystrophinopathy (30, 31). Ankle contractures were present in all of the patients in our cohort, and three had elbow contractures. Literature data suggest that joint contractures are typical for LGMD2A and may be common even in early disease stages (18, 28).

After ten or more years of disease duration some of our patients became non-ambulant. Loss of ambulation in LGMD2A occurs about 10-30 years after the disease onset (32). Cardiomyopathy was not observed, and only minor cardiac conduction impairments were found in our cohort. Heart involvement is generally rare in LGMD2A, but there are some cases that had cardiomyopathy and cardiac arrest (33-37). Mild respiratory restriction was diagnosed in 21% of our LGMD2A patients, and in one patient it was present from the beginning of the disease. Reduced forced vital capacity is frequently reported in LGMD2A (38, 39) due to the diaphragm weakness, but usually after longer disease duration.

Standard muscle histopathology in our patients was nonspecific, showing a dystrophic (and myopathic) pattern. Nevertheless, biopsy may be of importance to exclude myofibrillar myopathies and myositis (18). Due to the progress in genetic methods, the American Academy of Neurology suggest muscle biopsy only if genetic testing is inconclusive (40). Some of our patients had inflammation in muscle samples, one of them had significant eosinophilic infiltration. This was previously described in young patients with LGMD2A (41-43). Some of these patients were previously treated with steroids for years without any effect (44). Krahn and colleagues reported that eosinophilic myositis is an early and transient feature of the calpainopathy since it was not found in older patients with LGMD2A (42, 43).

Conclusions

Almost all Serbian patients with calpainopathy had c.550delA mutation. In most of the patients, disease started in the childhood or early adulthood. The disease affected both shoulder girdle – upper arm and pelvic girdle – thigh muscles with similar frequency, although muscles of lower limbs are more severely impaired. None of the patients had cardiomyopathy, while 21% showed mild conduction defects. Respiratory function was slightly impaired in 21% of patients.

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Conflict of interest

The Authors declare to have no conflict of interest.

Ethical approval

All procedures performed in studies were in accordance with the ethical standards of the Ethical Board of the Neurology Clinic, Clinical Center of Serbia, and with the 1964 Helsinki declaration and its later amendments.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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