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CLINICAL AND GENETIC STUDY OF HEREDITARY CEREBELLAR DEGENERATIONS IN BULGARIA

THESIS SUMMARY

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"Doctor of Science"

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Abbreviations

AA- Aminoacids
AFP- Alpha fetoprotein
ALS- Amyotrophic lateral sclerosis
ATP- Adenosin triphosphate
BsEP- Brain stem evoked potentials
CP- Cerebral palsy
CT- Computer tomography
CTX- Cerebrotendinous xantomatosis
CV- Conduction velocity
DNA- Deoxyribonucleic acid
DOPA- Dopamine
DRPLA- Dentatorubral-pallidoluysian atrophy
EF- Ejection fraction
EEG- Electroencephalography
EMG- Electromyography
ERG- Electroretinography
GTCS- Generelized tonic clonic seizures
HCA- Hereditary cerebellat ataxias
HJ- Hip joint
MEP- Motor evoked potentials
MJD- Machado Joseph disease
MRI- Magnetic resonance imaging
MS- Multiple sclerosis
MSA- Multisystem atrophy
mRNA- messanger ribonucleic acid
ncRNA- non coding ribonucleic acid
NCS- Nerve conduction studies
NGS- Next generation seqencing
NPA- Niemann Pick type A disease
NPB- Niemann Pick type B disease
NPC- Niemann Pick type C disease
NPD- Neuropsychological development
OCT- Optical coherence tomography
PET- Positron emission tomography
RNA- Ribonucleic acid
SCA- Spinocerebellar ataxia
SSEP- Somatosensory evoked potentials
TR- Tendon reflexes
HDL- High density lipoproteins
VEP- Visual evoked potentials
WES- Whole exome sequencing
WGS- Whole genome sequencing

Introducton

Hereditary cerebellar degenerations represent a large group of clinically and genetically heterogeneous diseases (Manto M et al. 2020). In some forms, there is an isolated cerebellar involvement, while in others additional extracerebellar manifestations are present, such as retinal degeneration, extrapyramidal movement disorders, pyramidal manifestations, cortical involvement (seizures, cognitive and behavioral disorders), peripheral neuropathy, and involvement of other organs and systems.

During the last 20 years, with the advances of molecular-genetic diagnostic methods, the knowledge about hereditary cerebellar ataxias (HCA), encompassing the underlying gene defects and the pathogenetic mechanisms, which would also be the basis for future therapeutics, has significantly expanded.

From a clinical point of view, new classifications were introduced. Given the overlapping phenotypes of HCA caused by mutations in different genes and the wide phenotypic spectrum due to defects in the same gene, clinical and paraclinical studies prove to be insufficient to clarify the exact diagnosis.

The main goal of the present study is to evaluate the HCA present in Bulgaria, as well as to determine the genotype-phenotype correlations for these forms. The importance of this thesis is determined by the lack of modern studies on the epidemiology and genotype-phenotype correlations of HCA in Bulgaria, consistent with the progress of molecular genetic diagnostics.

1. Aim and tasks

1.1 Aims of the study:

1. To assess the prevalence of different forms of hereditary cerebellar degenerations in Bulgaria

2. To study the genotype-phenotype correlations in the diagnosed forms of hereditary cerebellar degenerations in Bulgaria

1.2 Tasks:

To achieve the above-mentioned goals, the following tasks have been set:

1. Collection of a cohort of patients with cerebellar ataxias. This cohort is subclassified into two parts: sporadic cases and familial cases

2. Genealogical study of affected families with cerebellar ataxias and their classification depending on the pattern of inheritance as:

- Autosomal-dominant
- Autosomal-recessive
- X-linked
- Mitochondrial

2. Performing a DNA analysis and determining the gene defect (specific gene and type of mutation).
3. Determining the distribution of the different forms within the country and among the different ethnic groups.
4. Establishing a registry of patients with HCA
5. Detailed clinical phenotyping using the following methods:
   • Clinical, incl. specific ataxia scales
   • Neurophysiological
   • Neuropsychological (specific batteries of neuropsychological tests, tailored to expected cognitive impairments)
   • Neuroimaging methods
6. Referral of patients with cognitive, behavioral and mental disorders for appropriate therapy and neurorehabilitation.
7. Monitoring the effect of treatment in curable hereditary diseases with cerebellar ataxia
8. Referral of patients and their relatives to genetic counseling and prenatal diagnosis, in order to prevent new cases of the hereditary celebellar degenerations.

2. Material and methods:

2.1. A total of 162 patients belonging to 95 pedigrees were evaluated. They are classified as follows:

• 113 are cases with family history, corresponding to:

o AD or pseudodominant type of inheritance in 63.

o AR type of inheritance in 50.

• 49 are sporadic cases.

2.2. Clinical phenotyping:

All patients underwent a complete clinical phenotyping, including the following studies:

- Medical history, incl. family with a genealogical analysis
- Neurological examination, incl. scales for assessing the severity of ataxia SARA (Schmitz-Hubsch T et al. 2006), and spasticity SPRS (Schule R et al. 2006).
- Somatic examination
- Neuro-ophthalmological examination (visual acuity, perimetry, oculomotor, fundus evaluation, if necessary, OCT)
- Neurophysiologic tests:
  - Nerve conduction studies (NCS) with study of n. peroneus, n. tibialis, n. medianus, n. ulnaris, n. suralis, n. peroneus superficialis
  - Electromyography (EMG)
  - NCS and EMG in the patients were performed on a Dantec–Keypoint portable electromyograph (Natus, Copenhagen, Denmark).

  - Sudoscan
  - Evoked potentials - VEP, BsEP
  - Electroencephalography (EEG)
- Standard blood laboratory tests, incl. alpha-fetoprotein and lysosphingolipids
- In patients with mutations in the *POLR3A* gene, basal levels of hormones from the gonadal axis and levels after stimulation with gonadotropin releasing hormone and clomiphene citrate were additionally examined to assess the presence of hypogonadotropic hypogonadism.
- In patients with Niemann Pick B and C disease, abdominal echography was also performed in order to assess the degree of organomegaly.
- Neuropsychological assessments are presented in table 1.
  In patients with a low education from the Roma ethnic group, the results were compared to age and sex matched healthy controls from the same community.

Table 1. Neuropsychological tests used in patients with HCA.

Evaluation of the cognitive functions	**Neuropsychological assessments**
Evaluation of the general cognitive functioning and intelligence	Mini-Mental State Examination (MMSE)
	Wechsler intelligence scale for children
	Raven progressive matrices
Memory ❖ Verbal	Rey Auditory Verbal Learning Test (RAVLT)
	Test with 10 words
	Digit span- forwards
Executive functions	Trial making test (TMT)
	Stroop test
	Digit span - backwards Phonemic Verbal fluency
Verbal fluency	Semantic verbal fluency

- In patients with mutations in the *POLR3A* gene, in addition, orthopantomography was performed to assess the presence of hypodontia

- Magnetic resonance imaging of the brain and spinal cord - 3 Tesla MRI unit (Siemens Verio).

2.3. Genetic studies:

Blood was collected from the patients and their unaffected relatives, with subsequent isolation of DNA.

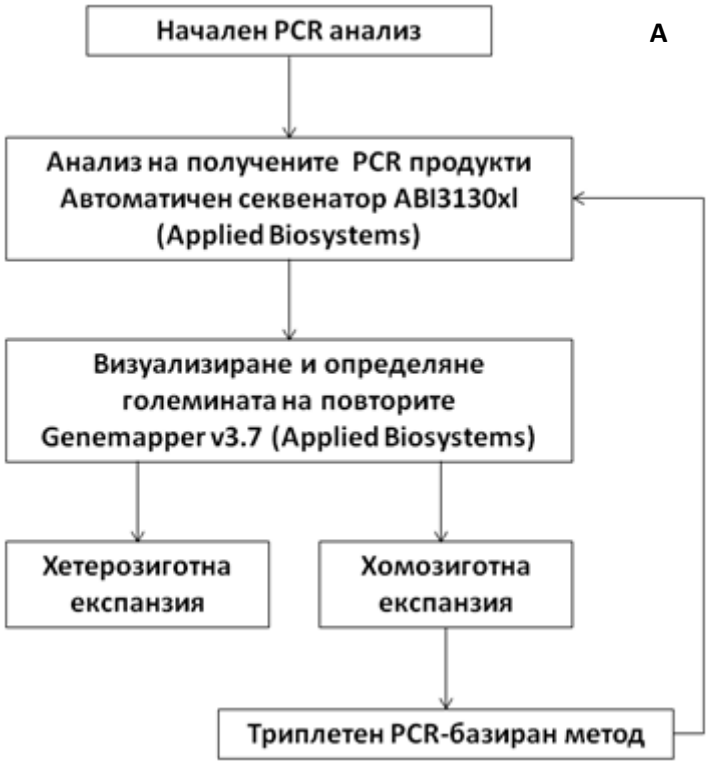
The genetic studies of these patients were performed at the National Genetic Laboratory, MU-Sofia, Medical Diagnostic Laboratory "Genika"-Sofia, Molecular Genetics Laboratory, Western Australian Institut for Medical Research and Center for Medical Research, The University of Western Australia, Perth, Australia and Molecular Neurogenomics Group, VIB Department of Molecular Genetics, Antwerp, Belgium.

2.3.1. Screening for dynamic repeats in NMA-related genes.

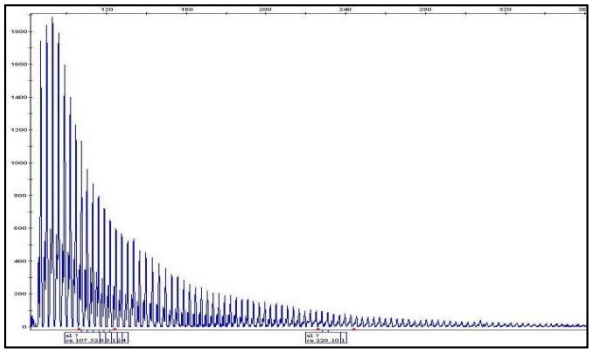
The predominant type of mutations leading to AD forms of HCA are trinucleotide expansions located in the coding part of genes in SCA type 1, 2, 3, 6, 7, 17. Another category of nucleotide expansions are located outside the protein-coding sequence of the corresponding genes, characteristic for SCA8, 10 and 12. The most common defects leading to AR-SCA are GAA expansions in the first intron of the *FXN* gene in Friedreich's ataxia (FRDA), (Gasser T et al. 2010).

Using the dynamic repeat screening system presented in Figure 1, 45 new patients with history of AD inheritance or sporadic cases, lacking prior genetic analysis were screened. Primary PCR-analysis of trinucleotide repeats in loci CCA1, 2, 3, 6, 7, 8, 10, 12, 17 was performed in all of them. Patients in whom a homozygous state was reported for any of the studied loci were included in screening to rule out expansion at this locus by TP-PCR analysis.

А


Начален PCR анализ
Анализ на получените PCR продукти
Автоматичен секвенатор ABI3130xl
(Applied Biosystems)
Визуализиране и определяне
големината на повторите
Genemapper v3.7 (Applied Biosystems)
Хетерозиготна
експанзия
Хомозиготна
експанзия
Триплетен PCR-базиран метод

Б



В

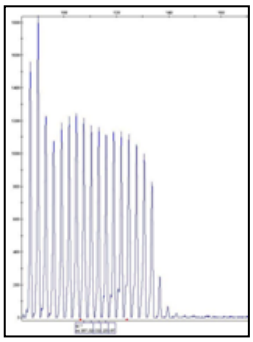


Figure 1. Analysis of dynamic nucleotide expansions in known SCA-related genes. Systematic course of analysis (A). Electrophorogram of the triplet PCR in a patient with an expansion (A) and in a normal control (B). The x-axis shows the size of the fragments in bp, while the y-axis shows the fluorescence intensity. The assessment of the pathogenicity of the expansions in each patient was made according to the number of established repeats in the respective locus in the different SCA forms, according to literature data (table 2), (Gasser T et al., 2010).

Table 2. Normal ranges of dynamic nucleotide repeats in the most common forms of spinocerebellar ataxia.

Disorder	Gene/protein	Type of trinucleotide expansion	Length of the expansion:	
			Normal allel	Expansion
SCA 1	*ATXN1*/ ataxin-1	CAG	27–36	39–82
SCA 2	*ATXN2*/ ataxin -2	CAG	15–32	33–64
SCA 3	*ATXN3*/ ataxin -3	CAG	12–43	52–86
SCA 6	*CACNA1*/ calcium channel	CAG	8–14	20–33
SCA 7	*ATXN7*/ ataxin -7	CAG	7–35	38–200
SCA 8	*KLHL-1*/Kelch-similar protein 1	CAG/CTG		107–128
SCA 10	*ATXN10*/ ataxin-10	ATTCT		1000–4000
SCA 12	*PPP2R2B*/ phosphatase	CAG		66–78
SCA 17	*TBP*/TATA binding protein	CAG	29–42	50–63
FRDA	*FXN*/frataxin	GAA		90-1300

2.3.2. Exome sequencing

In patients without pethological expansions in the studied loci for SCA and Friedreich's ataxia, the analysis continued with exome sequencing to search for point mutations, small deletions/insertions as the cause of the disease. In this regard, the following activities were carried out:

- Whole Genome Sequencing (WES) data obtained in the first stage
- Exome sequencing in the newly registered families in which dynamic repeat expansions were not found in the studied loci for SCA and Friedreich disease.
- Analysis of exome sequencing data in newly registered families.

A group of patients, in whom the detection of mutations in already known genes associated with various forms of HCA was expected based on clinical data, were referred for exome sequencing of a panel of 4813 genes associated with known diseases.

Whole exome sequencing to search for new and rare forms of HCA.

In order to unravel novel and rare forms of HCA, the present study used the method of whole exome sequencing (WES), which consists in high-throughput parallel sequencing of all protein-coding regions in the genome (exome) of a given individual. BGI Tech Solutions' exome sequencing includes DNA library preparation and targeted enrichment of the human exome with the SureSelect Human All Exon v5 kit (Agilent), parallel sequencing of the prepared libraries using the Illumina HiSeq 2000 platform, and primary bioinformatics analysis of the data.

2.3.2.1. Analysis of WES data obtained from the primary bioinformatic analysis.

The obtained data were processed using the GenomeComb or VarSeq® software packages, and the analysis included:

- Alignment and mapping of the reads to the reference human genome version hg19.
- Removal of duplicate reads, indel realignment and base quality score recalibration (BQSR)
- Identification of variants in the reference genome
- Annotation using public databases for population frequency, position in the relevant protein and mRNA, predictions for pathogenicity, evolutionary conservatism, etc.
- Prioritization of the identified variants by filtering initially by gene panels, and subsequently in the remaining genes in the exome depending on the mode of inheritance of the disease in the family.

In patients belonging to isolated populations or inbred communities, the method of homozygous mapping based on exome sequencing data (HOMWES) was applied with standard analysis parameters described by Kancheva D. et al., 2016.

2.3.2.2. Confirmatory analyzes for the association of the identified candidate mutations with the clinical phenotype in the family.

Candidate pathogenic variants identified by exome sequencing were confirmed and their co-segregation with the disease in the particular family was verified by classical Sanger sequencing. The functional effect of the candidate mutations was evaluated in silico by using different prediction programs calculating the possible phenotypic effect of the variant. SIFT (http://sift.jcvi.org/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), Mutation Taster (http://www .mutationtaster.org/), Meta-SNP (http://snps.biofold.org/meta-snp/), Condel (http://bg.upf.edu/fannsdb/), for variants potentially leading to changes in splicing - Automated Splice Site And Exon Definition Analyzes (http://splice.uwo.ca/), NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/), Human splicing finder (http:// www.umd.be/HSF3/HSF.html).

2.3.2.3. Cell culture study of fibroblasts cultured at 37 C under humidified 95% air/5% CO2 in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies) containing glucose (4.5 g l 1), 2 mM glutamine, penicillin (100U ml 1), streptomycin (100 mg ml 1) and 10% fetal bovine serum (FBS). Fibroblasts were grown in the presence and absence of 100 ng/ml cycloheximide (CHX) for 24 h.

2.3.2.4. Isolation of RNA from fibroblasts and whole blood using the PAXgene Blood RNA system to determine functional effect of mutations.

Additional evidence in favor of the pathogenicity of the candidate variants was collected in the databases and in the literature by the following indicators:

- Expression of the affected gene in the central nervous system

- Phenotypic expression of mutations in the given gene in animal models
- Participation of the affected gene to a cellular cascade with a known role in the development of the relevant brain structures and/or in the pathogenesis of the disease

Localization of the amino acid residue affected by the mutation in an important structural or functional domain of the protein, or near another mutation with the same clinical phenotype in the 3D structure of the protein.

A registry of patients with HCA was created, including data on the clinical phenotype, traite of inheritance, presence of consanguinity in the family, ethnic origin, geographical area, availability of biological materials for research.

2.3.3. For the purpose of mutational screening of diseases with high prevalence in a given region, such as AT, DNA was isolated from died blood spots from the neonatal screening. Filter blanks were used for neonatal screening purposes and were provided by the National Genetics Laboratory. The main advantage of this type of samples is the ability to store them at room temperature without harming the quality of the material, as well as their easy transportation.

Principle:

The heterozygosity screening was performed by direct PCR amplification on dry blood spots from Guthrie cards. The samples were provided by the National Genetic Laboratory, Sofia used for phenylketonuria screening.

For testing the carrier status regarding the mutation in the target genes, they were amplified, followed by direct Sanger sequencing (BigDye Terminator cycle sequencing kit, Applied Biosystems, Foster City, CA, USA). Primers were de- signed to specifically amplify the coding part of the exon and exon-intron boundaries (available upon request).

Theoretically calculated hybridization temperatures were used to optimize a protocol for amplification of target regions. After a series of optimizations, a protocol was developed for the optimal components of the amplification reaction.

2.4. Statistical analysis

Data were entered and processed with the statistical package IBM SPSS Statistics 19.0. $p < 0.05$ was chosen as the level of significance at which the null hypothesis is rejected.

In the statistical analysis, the following are applied:

• Descriptive analysis – the frequency distribution of the examined signs, broken down by research groups, is presented in tabular form.

• Non-parametric Mann-Whitney test – to test hypotheses of difference between two independent samples

3. Results

3.1. Autosomal dominanat ataxias

In the current study the following forma were described:

- SCA1
- SCA2
- SCA6
- SCA7

- SCA14

3.1.1. SCA1

The study encompassed 21 patients belonging to 17 pedigrees. Data, regarding age at onset and age of loss of independent gait was collected from the probands for the remaining members of their pedigrees from previous generations.

The mean age at onset was 41.2 years (SD 7.2), with a variation between 27 and 56 years. The initial clinical symptoms in all of the affected were ataxia of stance and gait. The frequency of the clinical symtoms in our group are presented on figure 2.

Ataxia of stance and gait, intention tremor, saccadic eye movements, gaze evoked nystagmus and dysarthria were present in all 21 patients. SARA varies between 6 and 24 in the represented patients. In 3 patients, independent ambulation was impossible. It was lost 10, 11, 14 years after the clinical onset. In 8/21 (38.0%) a limitation of the vertical eye movements was obsetved. Pyramidal involvement, without weakness, but with brisk tendon reflexes, more pronounced for the lower limbs, combined with pathological reflexes from the groups of Babinski and Rosolimo were found in 15/21 (71.4%), while pseudobulbar involvement with dysphagia and oral automatisms in 3/ 21 (9.5%). Spastically increased muscle tone in the lower limbs was present in 5/21 (23.8%). Peripheral nerve involvement with distal hypesthesia for superfecial sensation was present in 12/21 (57.1%), NCS was consistant with peripheral axonal damage of the sensory fibers in 12/21 (57.1%) patients, while in 5/21 (23.8%) involvement of the sensory and motor fibers was observed. In these 5 patients the complaints have been present for more than 5 years. Neuropsychological assessment was consistant with mild cognitive impairment in 18/21 (85.7%) with predominant involvemen of the executive functions.

Brain MRI showed evidence of cerebellar atrophy in all patients, while in 12/21 (57.1%) additional atrophy of the brainstem structures was found. In 5/21 patients (23.8%), in whom the history of the disease was over 7 years from the beginning to the current examination, cortical atrophy was also present (figure 3).

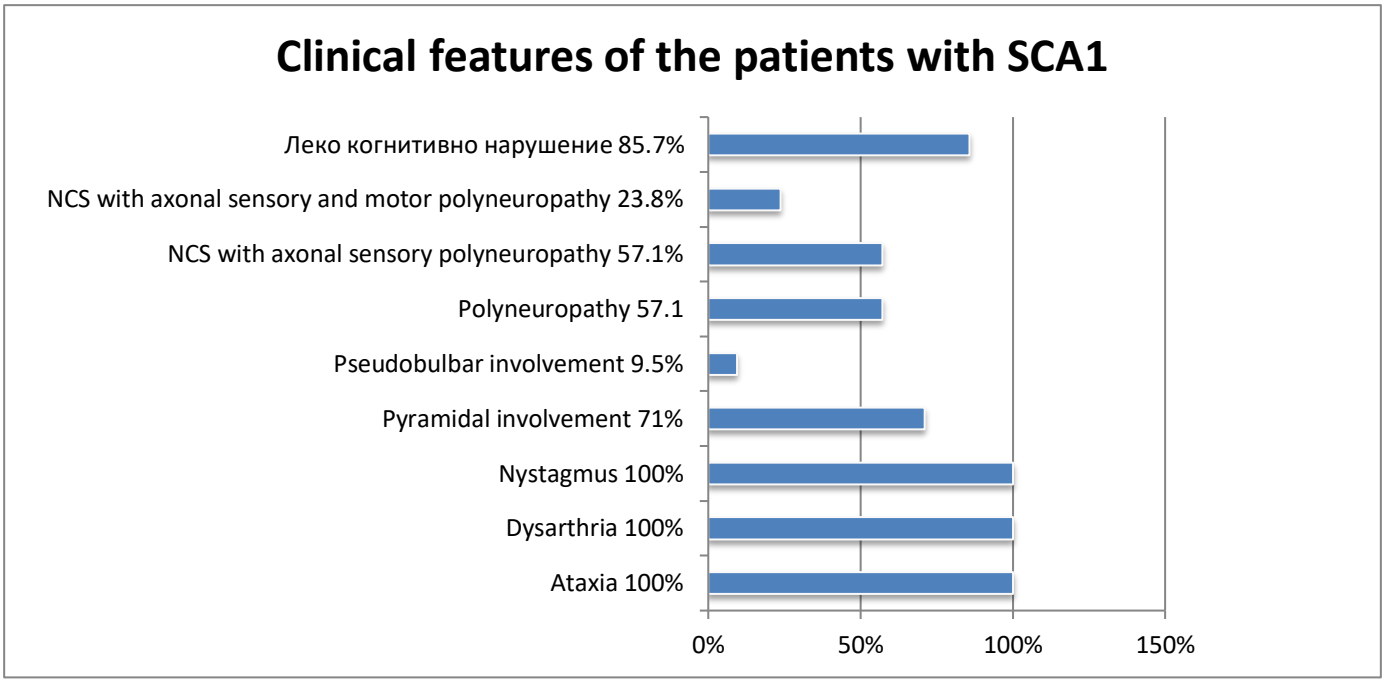


Figure 2. Clinical features of the patients with SCA.

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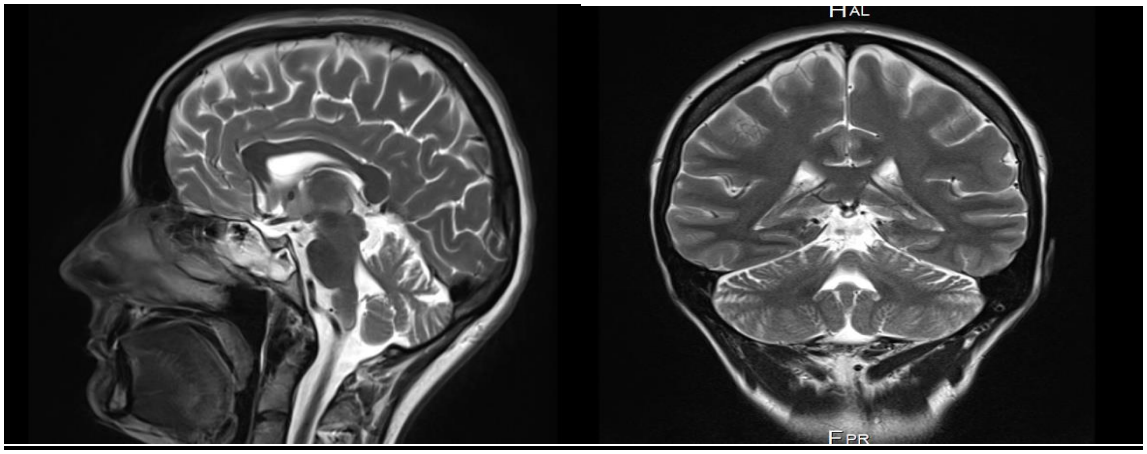


Figure 3. MRI, showing mild cerebellar atrophy combined with atrophy of the medulla oblongata and cervical spine in a 32-year-old female patient with a 2-year history of complaints and an expansion of 50 CAG in the *ATXN1* gene. A SAGITAL T2. B Coronal T2.

In the evaluated group, CAG repeats in the *ATXN*1 gene varied between 40 and 54 with a normal values below 39.

In three of the pedigrees where there was evidence of the onset of complaints in at least three consecutive generations, anticipation was found with an earlier onset between 2 and 10 years for each subsequent generation (Figure 4).

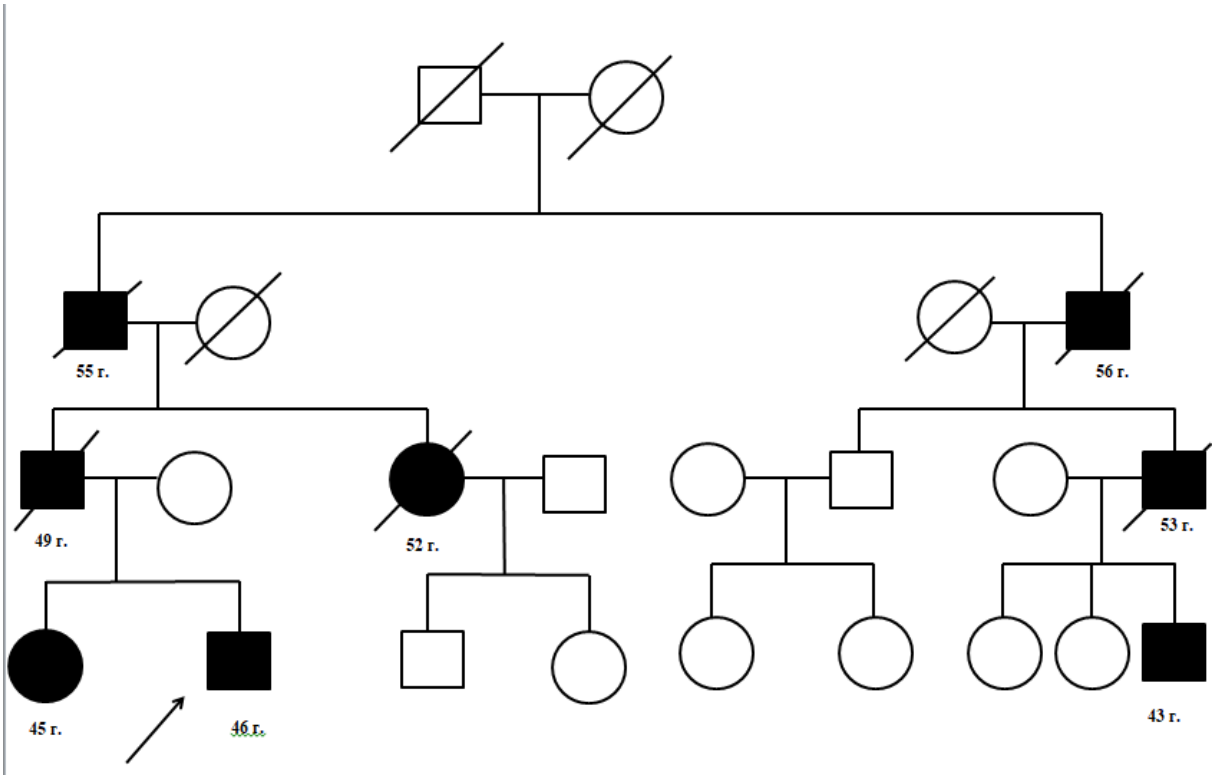


Figure 4. Pedigree of patients with genetically verified SCA. The age at onset is indicated for those with clinical symptoms. Antisipation is observed in the subsequent generations.

Discussion:

SCA 1 is the first genetically verified AD ataxia (Orr HT et al. 1993), caused by dynamic mutations in the *ATXN1* gene. The age at onset is highly variable between 4 and 74 years, which correlates with the size of the CAG-expansion (Di Donato S et al. 2012). The normal range of CAG repeats within the gene is below 39, and in patients with SCA 1 described so far, the number varies between 39 and 83 (Schols et al. 2004). The is the most frequent AD form of SCA in Bulgaria, followed by SCA2.

In our cohort, the number of CAG repeats in the *AXN1* gene varied between 40 and 54, with a mean age at onset of 41.2 years (SD 7.2). Despite the wide variation in the clinical onset (between 27 and 56 years), among the Bulgarian patients there is no case with childhood onset, which is likely due to of the smaller expansions. The typical earlier-onset anticipation was found in all pedigrees in which there was data for the clinical onset of those affected in three consecutive generations, which was related to the instability of the CAG repeats during meiosis (Orr HT et al. 1993).

Ataxia, present in all patients, is most often associated with pyramidal features, and in ¼ of our patients there is also spastically increased tone in the lower limbs, which necessitates the differential diagnosis with a complicated form of HSP (Di Donato S et al. 2012).

Oculomotor disorders due to cerebellar and extracerebellar disturbances are typical, with saccadic eye movements and nystagmus being an early manifestation present in all our patients. In those with a longer progression, a limitation of vertical eye movements is also observed (Klockgether T et al. 1998). Rare features described in other reports, such as optic

atrophy, choreic and dystonic hyperkinesias, were not found in our group (Di Donato S et al. 2012).

Cognitive impairment was observed in 85.7% of our patients, but it was within the range of mild cognitive impairment with predominant executive dysfunction, which is typical for cerebellar involvement.

NCS were consistant with axonal damage, mainly of sensory fibers, less often of the motor fibers and was present in more than 80% of our patients, which is in accordance with previous descriptions (Pareyson D et al. 1999).

Brain MRI of our patients, similar to the previous descriptions, is characterized by atrophy of the cerebellum, brainstem structures and spinal cord, and in cases with longer history of the disease, cortical atrophy was present (Burk K et al. 1996).

3.1.2 **SCA2**

SCA2 was diagnosed in 18 patients, belonging to 12 pedigrees.

The mean age at onset of the patients in our group was 31.2 (SD 9.8), varying between 7 and 44 years. In 4 of them, independent ambulation was lost 10-17 years after the symptom onset. The frequency of the clinical manifestations at the time of diagnosis is presented in figure 5. Static, locomotor, and dynamic ataxia were present in all patients, along with nystagmus and saccadic eye movements. Limitation of vertical eye movements was present in 9/18 (50%) of the patients. Dysarthria was present in 16/18 patients (88.9%), while dysphagia was found in 10/18 (55.6%). Polyneuropathy with distal hypesthesia with or without hyperpathy was observed in 8/18 (44.4%), while axonal damage on NCS was present only for the sensory nerves in 13/18 (72.2%), and for sensory and motor in 2/18 (11.1%). In 4/18 the EMG displayed fasciculations, fibrillations and neurogenic M-responses, without clinical evidence of progressive involvement of the central and peripheral motor neurons. Myoclonus was found in only 1 patient, with the earliest onset in the cohort - from the age of 7. Cognitive disorders were described in 12/18 (66.7%), and in the patient with an onset at the age of 7 years, the intelligence was borderline with IQ of 75, in 9, MCD with predominant dysexecutive symptoms was found, and in 2, a mild dementia was detected.

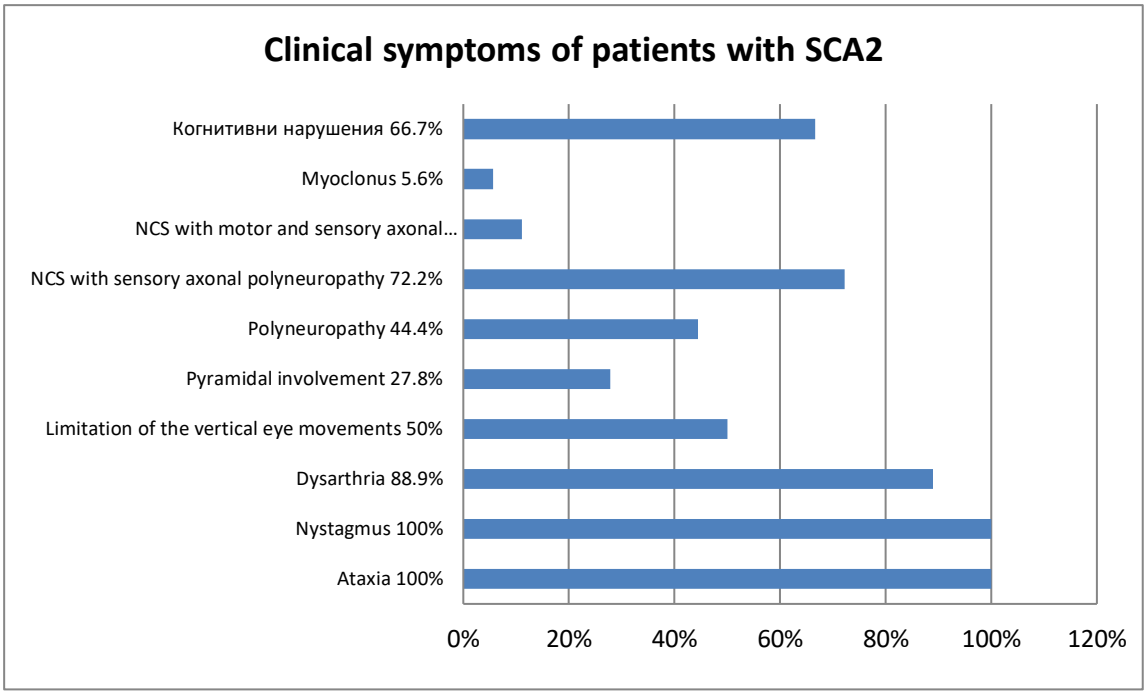


Figure 5. Distribution of the symptoms of the patients with SCA2.

In all of the affected MRI was consistent with cerebellar atrophy, while in 16/18 the brainstem seemed atrophic as well (figure 6).

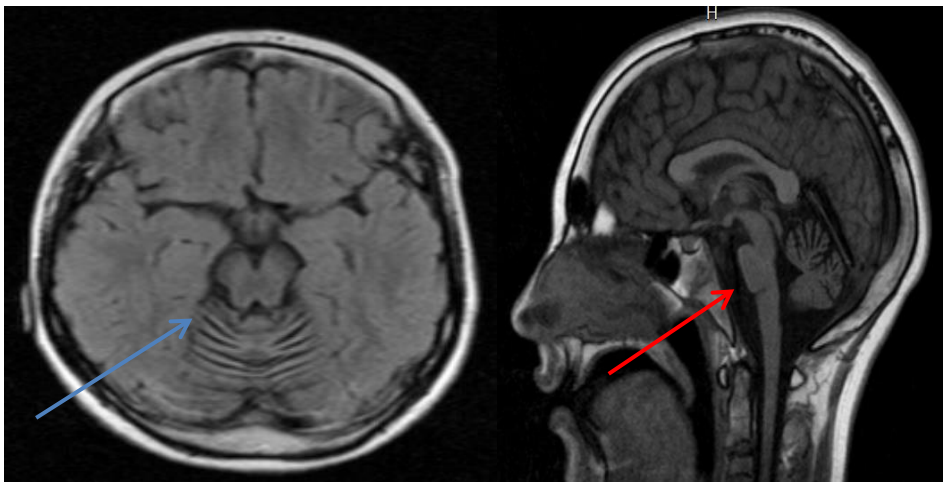


Figure 6. Brain MRI of a 12-year-old patient with a 48 CAG expansion in the *ATXN2* gene and clinical onset at 7 years of age. Atrophy of the cerebellum (arrow) and brainstem (arrow) were prominent. A. AXIAL T2 FLAIR; B SAGGITAL T2 FLAIR.

The CAG expansion in the *AXN2*-gene varies between 38 and 53 with a normal range below 35. In four of the pedigrees, where there is data for the clinical onset in at least two consecutive generations, anticipation with an earlier onset between 2 and 25 years was established for each subsequent generation (Figures 7 and 8).

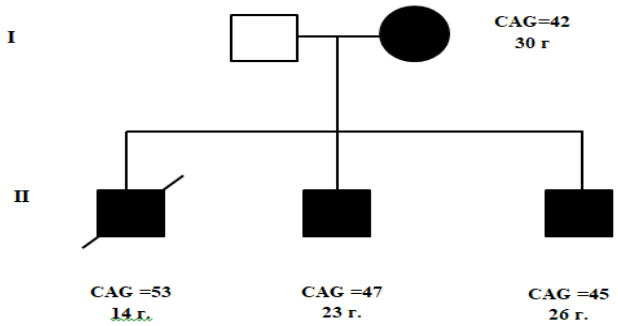


Figure 7. Pedigree of patients with SCA2 demonstrating anticipation in the second generation. The CAG expansion and the age at onset are presented.

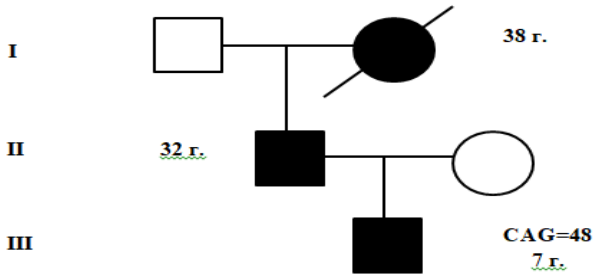


Figure 8. Pedigree of patients with SCA2 demonstrating anticipation in the second generation. The CAG expansion and the age at onset are presented.

Discussion:

SCA 2 is a form caused by a pathological expansion of CAG above 35 in *AXN2* with clinical onset between 2 and 70 years (Auburger GWJ 2012). Among the studied Bulgarian cohort, this is the second most frequent form of AD SCA.

The disease has a complex phenotype, in which the ataxia (Maschke M et al. 2005) is combined with brainstem involvement, encompassing saccadic eye movements, pyramidal involvement, dysphagia, myoclonus, as well as cognitive deficits, which are observed with varying frequency and in the represented group. The larger CAG expansion, is associated with earlier onset, as well as with greater frequency and a greater number of additional manifestations of extracerebellar involvement (Velazquez-Perez L et al. 2004). In our cohort, the CAG number in the *ATXN2* gene was varying between 38 and 53, while the age at onset was 31.2 years (SD 9.8), varying between 7 and 44 years. Only one patient had a childhood onset around 7 years. He had the largest expansion of 53 CAG repeats, being the only patient with extrapyramidal involvement within myoclonus as well.

A typical feature of the NCS is axonal sensory neuropathy, observed in 13/18 (72.2%) of patients in our group. In 4/18 of the needle EMGs, fasciculations, fibrillations and neurogenic M-responses were found, which is probably related to the clinical continuum caused by the expansions in the *ATXN2*-gene, including ALS when amplified up to 34 (Elden AC et al. 2010 ; Sproviero W et al. 2017) and HCA upon amplification above 31 CAG (Antenora A et

al. 2017). In the examined group, there was no clinical data of involvement of the central and peripheral motor neurons, as well as history of relatives with ALS. The combined cerebellar and brainstem atrophy observed in 16/18 of the patients (Maschke M et al. 2005), which correlates with the presence of extracerebellar manifestations represented a typical feature.

3.1.2. **SCA 6**

CCA6 was found in three Bulgarian patients belonging to 2 families. Their clinical characteristics are presented in Table 3.

Table 3. Clinical manifestations of the Bulgarian patients with SCA6.

Patient	№1	№2	№3
Sex	Male	Male	Female
Age at onset (years)	1	32	35
Age of diagnosis (years)	9	45	47
Psychomotor delay	+	-	-
Neurological examination	Ataxia Pyramidal involvement	Ataxia Pyramidal involvement Булбарен синдром с дисфагия и отслабени фарингеални рефлексиBulbar involvement with dysphagia and attenuated pharyngeal reflexes	Ataxia Pyramidal involvement
Brain MRI	Cerebellar atrophy Hyperintense lesions aroung the lateral ventricles	Cerebellar atrophy	Cerebellar atrophy
NCS/EMG	Normal	Normal	Normal
Neuropsychological assessment	Задръжка в експресивната реч IQ 70	Normal	Normal
Molecular	c.4075C≥T;	c.1997C≥T;	c.1997C≥T;

genetic analysis	p.Gly23Trp in exon 25 of *CACNA1A*	p.Thr666Met in exon 16 of *CACNA1A*	p.Thr666Met in exon 16 of *CACNA1A*

Discussion:

We present the first Bulgarian patients with genetically verified SCA 6, caused not by the typical expansions (Jodice C et al. 1997), but by point (missense) mutations in the *CACNA1A* gene. This type of mutation has been described as causing episodic ataxia type 2 and progressive ataxia, clinically indistinguishable from cases with expansies. In our group, the clinical manifestations were consistent with progressive ataxia. In patient #1, the clinical onset was quite early compared to previous descriptions of the disease (Gomez CM et al. 1997), but given the complicated perinatal history, the cognitive deficit and the T2 hyperintense lesions around the posterior horns of the lateral ventricles can be related to comorbidity of cerebral palsy with SCA 6.

The classic onset in patients with SCA 6 is in adulthood - between 19 and 73 years (Gomez CM et al. 1997), which corresponds to the manifestations observed in patients 2 and 3. The dyscoordination syndrome in our group was associated with a quadripyramidal syndrome in all affected, whereas involvement of the pyramidal tracts was found in 40-50% of the cases in the previous descriptions (Gomez CM et al. 1997). Dysphagia is a typical manifestation of this form, as it was present in one patient in our group. Extrapyramidal features can be observed in SCA 6, but were not available in our group (Gomez CM et al. 1997). Cognitive impairment is not typical for SCA 6, unlike SCA 1 and 2, which is also confirmed in patients #2 and #3 (Globas C and 2003).

Brain MRI showed cerebellar atrophy, as in our patients (Butteriss D et al. 2005).

3.1.3. ***SCA 7***

One patient was diagnosed with SCA 7. He is a 21-year-old man with clinical onset at the age of 11-12 years, when blurred vision appeared. Retinal dyspigmentation was suspected. From the age of 16-17 years ataxia of stance was observed, without significant progression in the following years. Sometimes the staggering was accompanied by vertigo. From 18 years he reports a change in speech - it has become slurred.

The family history was positive with a brother diagnosed with cerebral palsy and an aunt, father's sister with an unsteady gait.

From the neurological examination:

- Ataxia of stance and gair was observed. The gait was spastic- ataxic. Dysmetria in the finger to nose and heel to shin tests was present (SARA 7).
- Pyramidal involvement with brisk tendon reflexes in the four limbs, more pronounced in the lower ones.
- Bilateral ambliopia

The NCS revealed axonal damage of the sensory and motor fibers of the lower limbs, while SUDOSCAN proved normal electrochemical conductance.

The neuro-ophthalmological examination proved a greatly reduced visual acuity: VOD=0.1 - 0.15 n.c. VOC=0.1 - 0.15. Oculomotor performance was unbiased in first position, volume-preserved eye saccades, and smooth-tracking eye movements. When examining the fundus, the macules had a normal foveolar reflex, with slight dyspigmentation. Morbus Stargardt (without Fundus flavimaculatus). Cone-rod dystrophy was discussed in terms of differential diagnosis.

Brain MRI in 2019 and 2020 revealed cerebellar atrophy with dilatation of the interfoliar cisterns without progression between the two studies. The patient's cognitive functioning was generally preserved.

The molecular-genetic analysis revealed a pathologically increased number (51) of CAG repeats on one allele of the *ATXN7* gene.

Discussion:

In the presented case, the onset was in adolescence with more pronounced visual impairment and subsequent mild ataxia and dysarthria appeared additionally. Although the number of CAG repeats in ATXN7 (51) is less than 60, which is associated with a classic phenotype with an onset after 30 years of age, in our case the disease had a juvenile onset and initial visual disturbances (Andrew SE et al. 1997; Johansson J et al 1998). Whether cone-rod dystrophy precedes cerebellar involvement is controversial. In the Swedish cohort, in patients with CAG repeats below 59, ataxia preceded visual involvement, in contrast to cases with more than 59 repeats (Johansson J et al 1998), where visual involvement appeared first.

In the course of the disease, ataxia and pyramidal involvement progress, followed by dysphagia and dysarthria, as observed in our case (Martin JJ 2012). Cognitive impairment, auditory hallucinations and behavioral disturbances are also seen in these patients (Benton CS et al. 1998), but were not typical in the patient described.

Brain MRI showed cerebellar atrophy with dilatation of the interfoliar cisterns without pontine atrophy, which is also considered typical (Bang OY et al. 2004), but may occur later in the course of the disease.

3.1.4. ***SCA 14***

Two pedigrees with SCA14 were diagnosed. Family C has three affected from two consequtive generations with SCA 14. Family K has five affected patients from three consecutive generations. The clinical manifestations of those affected are presented in Table 4.

Table 4. Clinical characteristics of patients with SCA14.

Patients	CII.4	CIII.1	CIII.2	KIII.1
Sex	Female	Female	Female	Male
Age at onset (years)	42	23	16	37
Age at diagnosis (years)	65	41	37	40

Neurological examination	Ataxia Pyramidal involvement	Ataxia Pyramidal involvement Extrapyramidal involvement with dystonia in upper limbs	Ataxia Pyramidal involvement Oculomotor impairment	Ataxia Pyramidal involvement
Brain MRI	Cerebellar atrophy Gliosis	Cerebellar atrophy	Cerebellar atrophy	Cerebellar atrophy
NCS	Normal	Normal	Normal	Not performed
Neuroophthalmological examination	Saccadic eye movements	Saccadic eye movements	Infranulear lesion ob n. abducens dex.	Saccadic eye movements and nystagmus
Neuropsychological assessment	Normal	Normal	Normal	Normal
Molecular-genetic analysis	c.67G≥T; p.Gly23Trp in exon 1 of *PRKCG*	c.67G≥T; p.Gly23Trp in exon 1 of *PRKCG*	c.67G≥T; p.Gly23Trp in exon 1 of *PRKCG*	C.380A≥G; p.Gln127Arg in exon 4 of *PRKCG*

The pedigree is presented on figure 9.

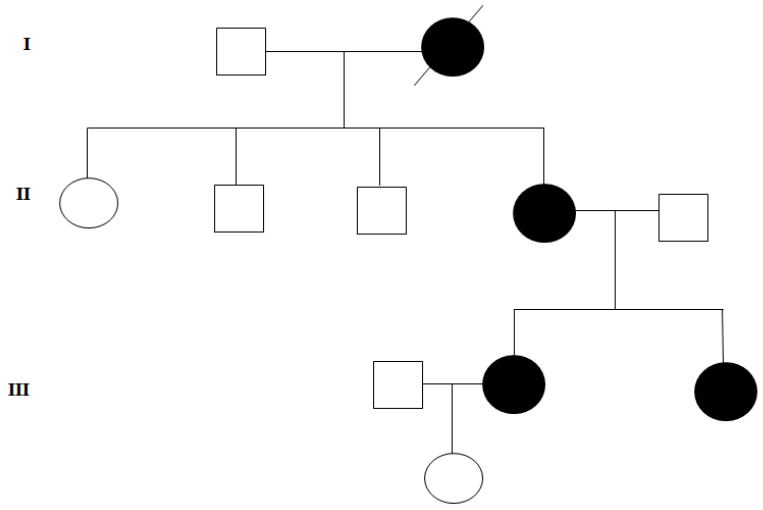


Figure 9. Pedigree of Bulgarian patients with SCA14.

In the patients, a mutation with.67G≥T was found; p.Gly23Trp in exon 1 of the PRKCG-gene.

Discussion:

SCA 14 is an AD disease caused by mutations in the gene encoding protein kinase C gamma (PRKCG), which accounts for between 1%-4% of all AD forms. Although for a long time this form was considered as pure, with the increased the number of diagnosed patients, it was found that cerebellar ataxia is associated with other symptoms, such as myoclonus, spasticity, rigidly increased muscle tone and mild cognitive impairment (Shirafuji T et al.

2019; Chelban V et al. 2018). In the largest genetically verified cohort of 25 patients with SCA14 to date, Chelban V et al. found that more than 1/3 of those affected had a complex phenotype (Chelban V et al. 2018). In the present study, 8% of the total number of diagnosed AD forms were accounted for by SCA14, which is a higher number compared to that found in other studies (Verbeek DS et al. 2005).

In our cohort, the disease started in adolescence and adulthood (between 16 and 42 years of age), with anticipation in family C, despite the fact that SCA14 does not belong to the group of diseases with pathological expansion. Similar observations have been reported in other families with SCA14 (Chelban V et al. 2018). The phenotype in both families was characterized by a combination of ataxia and pyramidal involvement, more pronounced for the lower limbs, which was also described in previous studies, although the percentage of patients with pyramidal symptoms is lower - between 25 and 50% (Verbeek DS et al 2005; Shirafuji T et al 2019; Chelban V et al 2018). Only one patient had evidence of extrapyramidal involvement with dystonic hyperkinesis in the upper limbs, which is consistent with the cases described so far (Shirafuji T et al. 2019; Chelban V et al. 2018). Myoclonus and cognitive impairment were not found in our group.

The two missense mutations found in our patients are novel, undescribed up to now (Chelban V et al. 2018), and similar to the predominant type of mutations in *PRKCG* are missense. The two new mutations (c.67G≥T; p.Gly23Trp in exon 1 and C.380A≥G; p.Gln127Arg in exon 4) are located in the regulatory part of the gene - C1 domain. In vivo experiments demonstrate that mutations in this part of the gene reduce the affinity of protein kinase C gamma for membrane binding and substrate phosphorylation, leading to increased intracellular Ca levels and disturbing the intracellular signaling (Adachi N et al. 2008). In contrast to cases with mutations in the catalytic domain when affecting the regulatory region, the clinical manifestations are of later onset and with a lower rate of extrapyramidal and cognitive involvement, as in our patients (Chelban V et al. 2018).

3.2. Autosomal recessive cerebellar ataxias

The following AR forms are included in the research:

- Friedreich ataxia
- Ataxia teleangiectasia
- Ataxia oculomotor apraxia
- Cerebrotendinous xantomatosis
- Ataxia with vitamin E deficiency
- Desequilibrium syndrome (DES, CAMRQ1)
- ARCA 1
- Spastic ataxia of Charlevoix–Saguenay (ARSACS)
- ARCA3
- SCAR13
- Niemann Pick type B disease
- Niemann Pick type C disease
- Tremor-ataxia and central hypomyelination (HLD7)

- Boucher-Neuhäuser syndrom

3.2.1. Friedreich ataxia

The current study encompassed 31 patients from 20 pedigrees, 15 of them were male and 16 were female. In 30 of the patients, a classic phenotype with an onset before the age of 25 years was observed, and in one patient there was a late form of the disease with an onset at the age of 30.

The mean age at onset in our patients was 14.06 (SD 4.54) years, and in 4/31 (12.1%) the onset of complaints was in the first decade, in 26/31 (83.9%) the onset was in the second decade, and in 1/31 onset was in the fourth decade. The main clinical features are presented in figure 10.

All subjects had ataxia (SARA in the range of 10-24), tendon areflexia for upper and lower limbs, impaired deep sensation in the lower limbs, positive Babinski sign and pes cavus.

Cardiomyopathy (concentric left ventricular hypertrophy) was found in 77.4%, dysarthria- in 80.6%, scoliosis in 48.6%, nystagmus in 32.2%, hearing loss in 22.5%, optic nerve atrophy in 19.3% and diabetes in 12.9%. Brain MRI was consistant with cerebellar atrophy in 6/31 (19.4%), with an evaluation performed after 10 years of clinical onset. In 12/31 patients, an MRI of the cervical spine was performed, and in all of them there was evidence of atrophy of the spinal cord.

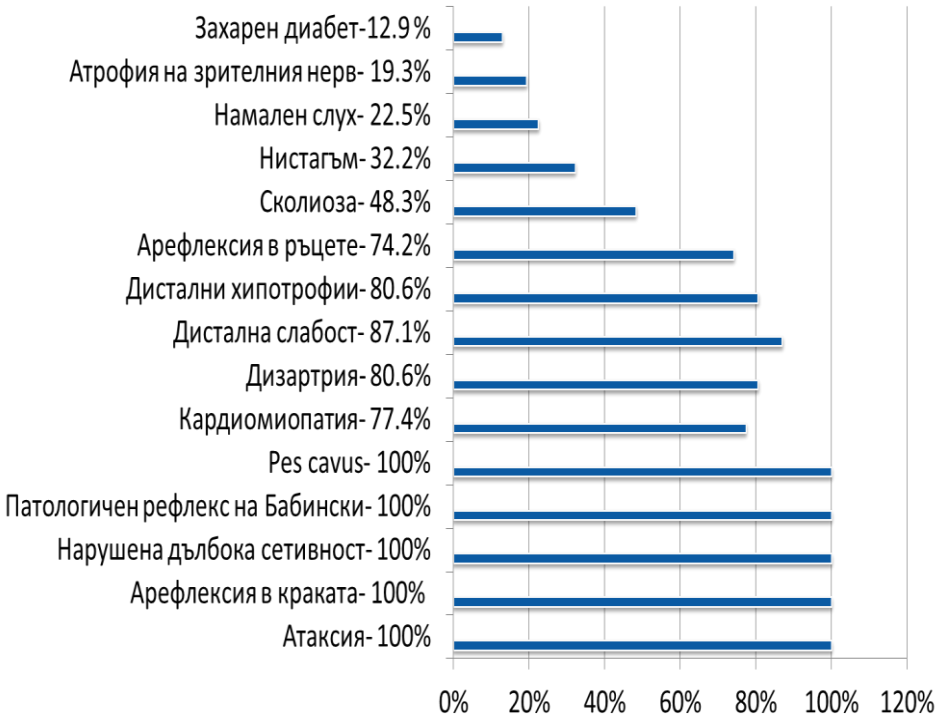
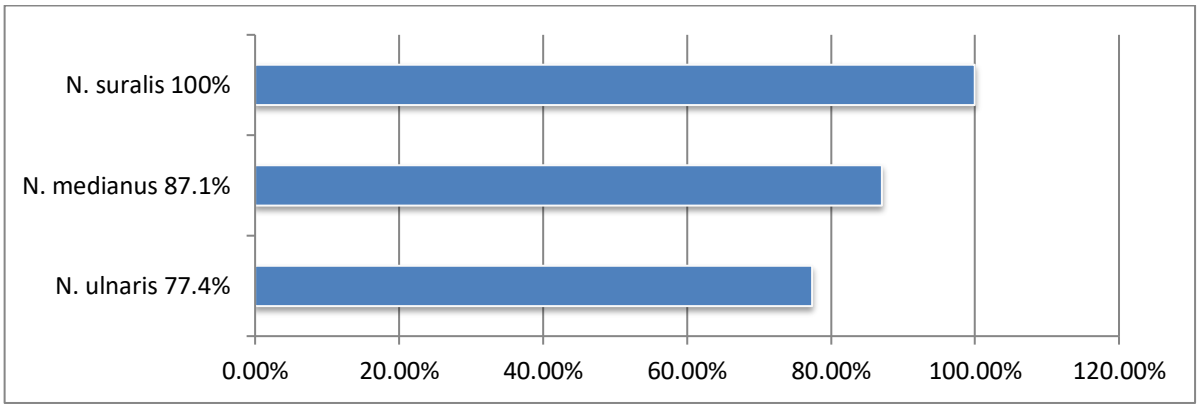


Figure 10. Clinical features of the patients with Friedreich ataxia.

In all the affected, the percentage of involvement of the sensory fibers is very high. Absence of sensory nerve action potential (SNAP) is the main criterion. The findings on this parameter are identical, regardless of the age of the disease. For n. ulnaris SNAP is absent in 77.4% of the patients, and in the remaining 22.6% it has reduced A. SNAP is absent in 87.1% for n. medianus and in 100% for n. suralis (Figure 11).



Фигура 11. Percentages of the absence of SNAP of n. ulnaris, n. medianus, n. suralis.

The second electrophysiologic criterion is the reduced or absent A of the H-reflexIn our cohort, the H-reflex of n. tibialis is missing in 67.7%, and in the remaining 32.3% it has a reduced amplitude. From the NCS normal conduction velocities were present in the majority of patients (figure 12).

- n. ulnaris- normal 87.1%; decreased - 12.9%
- n. tibialis- normal 80.1%; decreased - 19.9%
- n. peroneus- normal 77.4%; decreased - 22.6%

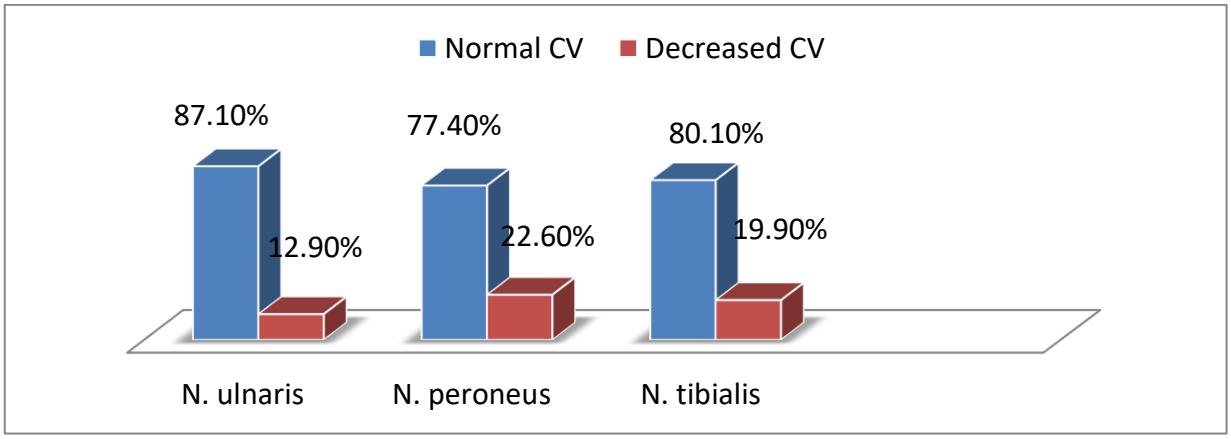


Figure 12. Conduction velocities of the motor fibers of the Bulgarian patients with Friedreich ataxia.

The blink reflex had normal parameters in the studied patients with Froedreich ataxia.

Twenty-nine of those affected were homozygous carriers of a pathological expansion in the GAA in *FXN*-gene, and in two the pathological GAA expansion on one allele was combined with a Q148X point mutation on the other allele.

Discussion:

In accordance with worldwide epidemiological data, patients with Friedreich ataxia were the largest number within the current study (Harding A 1981; Alper G et al. 2003; Vankan P 2013). A classic phenotype was present in 96.7% of our patients, and only 1 patient had a late onset after the age of 25 years, a relatively benign course with loss of independent ambulation at the age of 50 years, and spared cardiac or endocrine functions. According to De Michele G et al. about 17% of patients in the Italian cohort had a late onset (De Michele G et al. 1994). This difference is probably related to the rare evalation of Friedreich ataxia among patients with adult-onset ataxia. Typical neurological manifestations such as ataxia, tendon areflexia for upper and lower limbs, impaired deep sensation in the lower limbs, positive Babinski sign and pes cavus were present in all patients, which is in accordance with previous reports (Harding A 1981; Alper G et al 2003; Vankan P 2013).

In our cohort, cardiac involvement was found in 77.4%, mainly presented by concentric left ventricular hypertrophy. According to some authors, it is possible to find isolated septal hypertrophy (Alper G et al. 2003), but such changes were not found in our group.

Diabetes mellitus was diagnosed in 12.9%, which is consistent with descriptions from previous studies (Alper G et al. 2003; Pandolfo M et al. 1998). In Friedreich ataxia, relatively early in the course of diabetes, it is necessary to start insulin therapy (Harding A 1981; Alper G et al. 2003; Vankan P 2013).

In our cohort, similar to the previously described cerebellar atrophy on brain MRI was present in a small percentage of the patients (19.4%), mainly in the cases when it was performed in the later stages of the disease (Alper G et al. 2003; Pandolfo M and et al. 1998), Cervical spine atrophy was found in all 12 patients where MRI was performed, which correlated with histopathological changes (Alper G et al. 2003; Pandolfo M et al. 1998). Predominant sensory neuropathy was found in NCS. The most consistent electrophysiological finding is the absence of SNAP for n. suralis in all patients, while for n. medianus and n. ulnaris SNAP was greatly reduced or absent. These electrophysiological features correlate with the selective degeneration of neurons in the spinal ganglia (Pandolfo M et al. 1998).

The second electrophysiological criterion is the absence of the H-reflex in most patients, except for three in whom A was greatly reduced, which supports the theory of degeneration of sensory neurons in the spinal ganglia and dorsal roots. The loss of large myelinated fibers, which is also found in young patients, is a consequence of a defect in the maturation and subsequent degeneration of specific neurons and their axons (Bürk K 2017).

The preserved blink reflex in the examined patients with Friedreich's disease confirms (Bhidayasiri R et al. 2015) the theory that the cell nucleus of n. trigeminus remains unaffected. This is further evidence that in Friedreich ataxia the big myelinated fibers are affected, and the fibers transmitting the afferent component of the blink reflex are of medium

size, whose afferent cell bodies are also small or medium in size, and therefore they remain unaffected.

NCS of the motor and sensory fibers of the peripheral nerves and the H-reflex are important for the early diagnosis of Friedreich's disease. Electrophysiological changes are not related to the duration of the disease, but are genetically predetermined. In 6% (2/31-sibling) of the studied patients, the classic pathological GAA expansion in the *FXN*-gene on one allele was combined with a point mutation on the other, without changing the severity or phenotypic characteristics of the disease. Compound heterozygotes for *FXN*-gene mutations are estimated to account for about 4% of patients worldwide and their phenotype is indistinguishable from that of those (Pandolfo M et al. 1999).

3.2.2. Ataxia teleangiectasia

The current research encompassed 28 patients (Bulgarian muslims), 15 of whom were female and 13 male, with genetically verified 2 mutations in the *ATM* gene, which have a much milder course than the classic phenotype of ataxia-telangiectasia, i.e. .is have a clinical picture of a variant of the disease.

Twenty-seven of them belong to 3 large pedigrees from the region of Dospat and Sarnitsa, and one patient No 28 is a sporadic case.

Their main clinical characteristics are presented in Table 5.

Table 5. Clinical features of the Bulgarian patients with ataxia teleangiectasia variant.

Patient N	Mutations in *ATM*-gene	Sex	Date of birth	Age at onset (years)	Dystonic and choreiform hyperkinesia				Tremor			Dystonic dysarthria	Dystonic dysphagia	Ataxia	Pyramidal involvement	Teleangiectasia	Neoplasms	Immune deficiency	AFP	Neuroimaging (CT/MRI/ PET)
					Face	Neck and body	Upper limbs	Lower limbs	Head	Upper limbs	Lower limbs									
1.	p.V2716A/ p.V2716A	F	27.10.1973	17	-	-	-	D	+	+	-	-	-	-	+	-	-	-	НИ	Normal MRI
2.	p.V2716A/ p.V2716A	M	02.10.1976	16	-	-	-	D	+	+	-	+	-	-	-	-	-	-	43.01	Normal MRI
3.	p.V2716A/ p.V2716A	M	17.06.1948	20	-	-	D>L	+	-	-	-	+	-	-	-	-	-	-	ND	ND
4.	p.V2716A/ p.V2716A	F	16.08.1955	7	+	+	D>L	+	-	D	-	-	-	-	+	-	-	-	56.8	MRI with cortical atrophy
5.	p.V2716A/ p.V2716A	F	19.06.1963	2	+	+	D>L	L>D	+	+	-	+	+	-	+	-	-	-	39.3	ND
6.	p.V2716A/ p.V2716A	M	25.10.1970	1	+	+	+	-	+	+	-	+	-	-	-	-	-	-	ND	ND
7.	p.V2716A/ p.V2716A	F	04.06.1971	14 days	+	+	D>L	+	+	+	-	+	+	Ny	-	-	-	-	48.16	Normal MRI and PET
8.	p.V2716A/ p.V2716A	M	19.04.2005	1	-	+	+	-	-	-	-	+	-	-	-	-	-	-	НИ	Normal MRI
9.	p.V2716A/ p.V2716A	M	26.09.1957	18	-	+	D	-	-	-	-	+	-	-	-	+	-	-	8.5	Normal MRI
10.	p.V2716A/	M	03.01.1956	18	-	-	-	-	+	D>L	-	+	-	-	-	-	-	-	ND	Normal

	p.V2716A																		MRI
11.	p.V2716A/ p.V2716A	F	05.06.1986	18	-	+	-	-	+	D>L	-	-	-	-	+	-	-	-	ND	Normal MRI
12.	p.V2716A/ p.V2716A	M	13.08.1975	1.3	-	+	L>D	L>D	-	-	-	+	-	-	-	-	-	-	ND	Normal MRI
13.	p.V2716A/ p.V2716A	F	2012	1	-	+	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND
14.	p.V2716A/ p.V2716A	M	2010.	1	-	+	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND
15.	p.V2716A/ c.4909+1G >A	M	06.06.1952	40	+	+	D>L	-	+	+		+	-	+	-	-	-	-	167	Normal MRI
16.	p.V2716A/ p.V2716A	F	21.10.1985	13	+	+	L>D	-	+	+	+	-	-	-	-	-	-	-	60.8	Normal MRI
17.	p.V2716A/ p.V2716A	F	03.01.1972	1	+	+	+	-	-	-	-	+	-	-	-	-	-	-	ND	ND
18.	p.V2716A/ p.V2716A	M	09.02.1966	1	+	+	-	-	+	+	-	-	-	-	-	+	-	-	ND	ND
19.	p.V2716A/ p.V2716A	F	03.02.1955	14	+	+	-	-	+	D>L	-	+	+	-	-	-	-	-	ND	ND
20.	p.V2716A/ c.4909+1G >A	F	08.01.1965	2	+	+	-	+	+	D>L	+	-	-	-	-	-	-	-	ND	ND
21.	p.V2716A/ c.4909+1G >A	M	24.01.1959	4	-	-	-	+	+	-	-	-	-	-	-	-	-	-	ND	ND
22.	p.V2716A/ p.V2716A	F	18.10.1984	1	-	-	-	-	+	L>D	-	+	-	-	-	-	-	-	112.4	Normal MR
23.	p.V2716A/	F	31.01.2011	1	-	On	-	-	-	-	-	-	-	+/-	-	-	-	-	18.9	Normal

	p.V2716A					the left													MR
24.	p.V2716A/ p.V2716A	F	04/01/1980	5	-	On the left	-	-	-	-	-	+	-	-	-	-	-	-	83.2	Normal MR
25.	p.V2716A/ p.V2716A	M	10.09.1965	17	+	+	+	+	+	+	+	+	+	-	-	-	-	-	32.9	Normal MR
26.	p.V2716A/ p.V2716A	F	05.03.1955	6	+	+	+	+	+	D>L	+	+	+	Intention tremor in the left hand	-	+	-	-	25.4	Normal MR
27.	p.V2716A/ p.V2716A	F	28.05.1967	6	-	-	-	-	+	+	-	+	-	-	-	-	+	-	ND	ND
28.	p.V2716A/ p.Lys119	F	12.05.1987	0.5	+	+	-	-	+	+	-	+	-	+	-	-	-	-	351.8	MRI with cerebellar atrophy

The mean age at onset of the studied patients was 8.3 years ± 9.3 years, with a variation from 14 days to 40 years, despite the relative genetic homogeneity, which suggests the participation genes modifiers, determining the severity of the disease.

The main clinical features include dystonic hyperkinesias, static and postural tremor, mainly affecting the neck and upper limbs, dystonic dysarthria and, in more severe cases, dysphagia. Choreic hyperkinesias were observed in 10/28, and myoclonus - in 5/28. These symptoms slowly progress over time and vary in severity. Mild ataxia of stance and gait was present on 5/28 (SARA 1-4). In 4/28, dilated conjunctival vessels were found (Figure 13), which were not the classic telangiectasias seen in the classic variant of the disease. The cognitive functioning of the patients is within the normal ranges for the respective age.

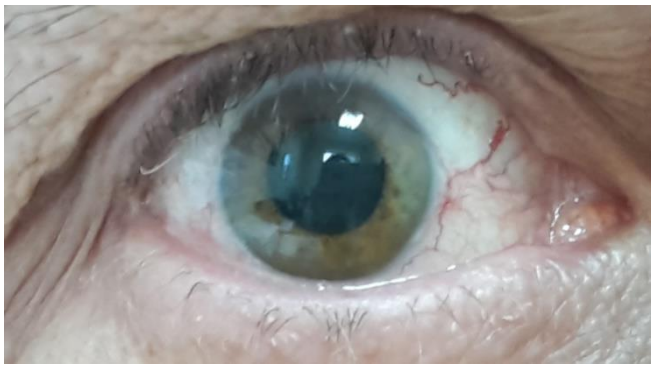


Figure 13. Dilated conjunctival vessels in patient #15.

In 4/17 of the affected, there was evidence of mild axonal neuropathy in the lower limbs with decreased CMAPs and SNAP from nn. reronei and nn. surales.

Brain MRI was normal with no evidence of cerebellar atrophy or degenerative changes in the basal ganglia n 16/17 subjects, with only one patient (number 28) showing cerebellar atrophy (Figure 14). However, she is a compound heterozygous for mutations p.V2716A/ p.Lys119 in the *ATM* gene.

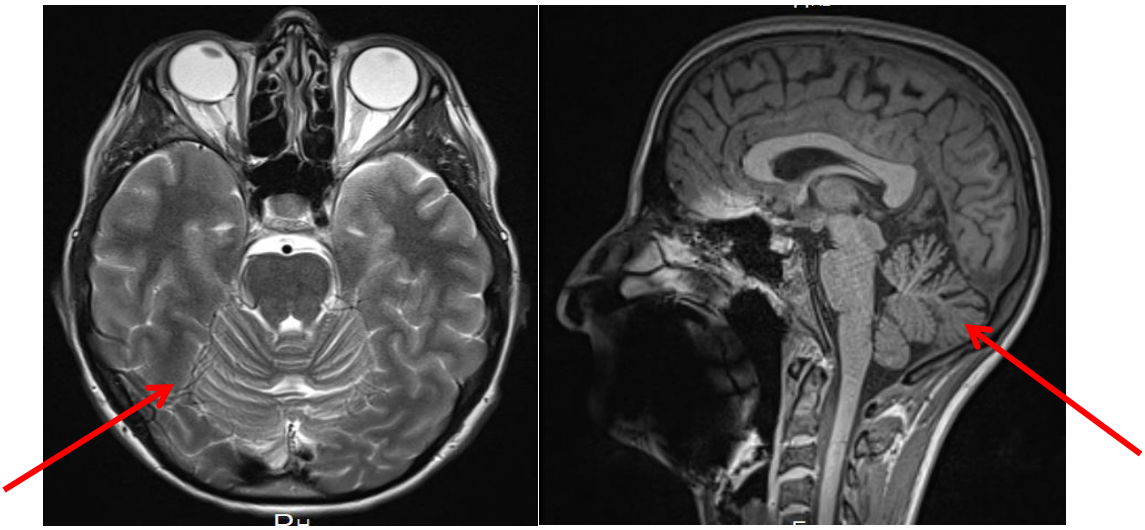


Figure 14. Brain MRI in patient #28 (compound heterozygous p.V2716A/ p.Lys119) with evidence of cerebellar atrophy (arrow). A Axial T2; B Sagittal T1

AFP was elevated in all of the 13 tested patients, which should be used as a screening marker when the disease is suspected. There is no correlation between the severity of the disease and the serum concentration of alpha-fetoprotein.

None of the patients had evidence of very frequent and severe infections in childhood.

In 5 patients with immunological tests, elevated ANA was found, with 3/5 having absolute lymphopenia, without history of frequent infections. There is no history of a higher frequency of neoplasms among the affected and their relatives, which is not typical because even patients with the variant form of AT have a higher frequency of neoplasms. The genetic diagnosis was established by whole exome sequencing (WES), (figures 15, 16, 17). Homozygous mutation c.8147T>C (p.V2716A) in the *ATM* gene was found in 24 of those studied, the remaining 4 were compound heterozygous, in whom the above mutation was combined with p.Lys119 or c.4909+1G>A (figure 18).

In patient #2, the concentration of ATM protein in lymphoblasts compared to controls with other diseases was evaluated by Western blot. Figure 19 shows that the protein concentration is not reduced, but apparently the function is disturbed.

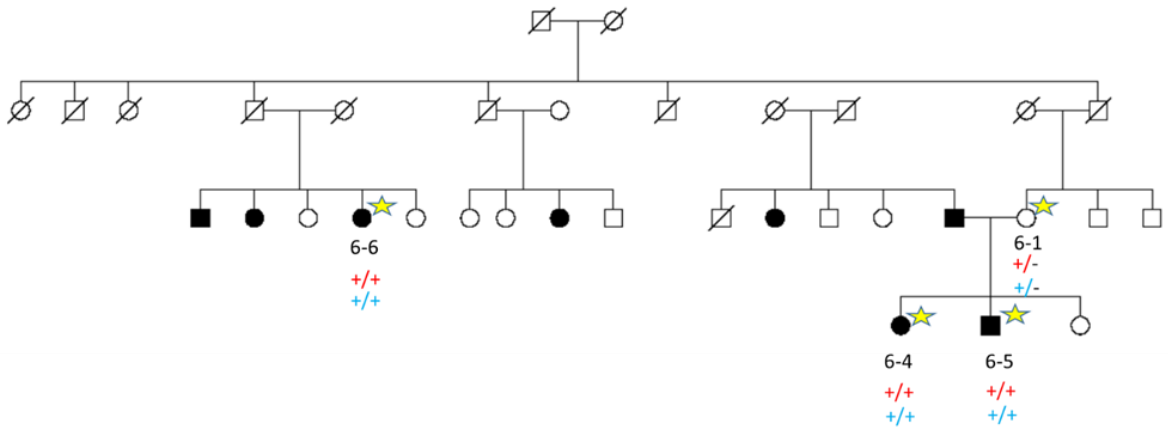


Figure 15. Pedigree 1 with ataxia-teleangiectasia-variant.

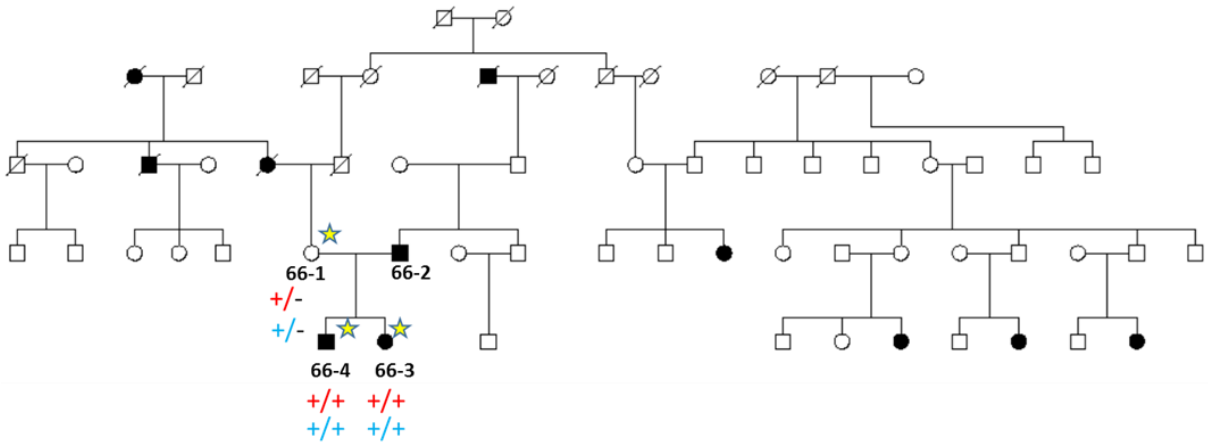


Figure 16. Pedigree 2 with ataxia-teleangiectasia-variant.

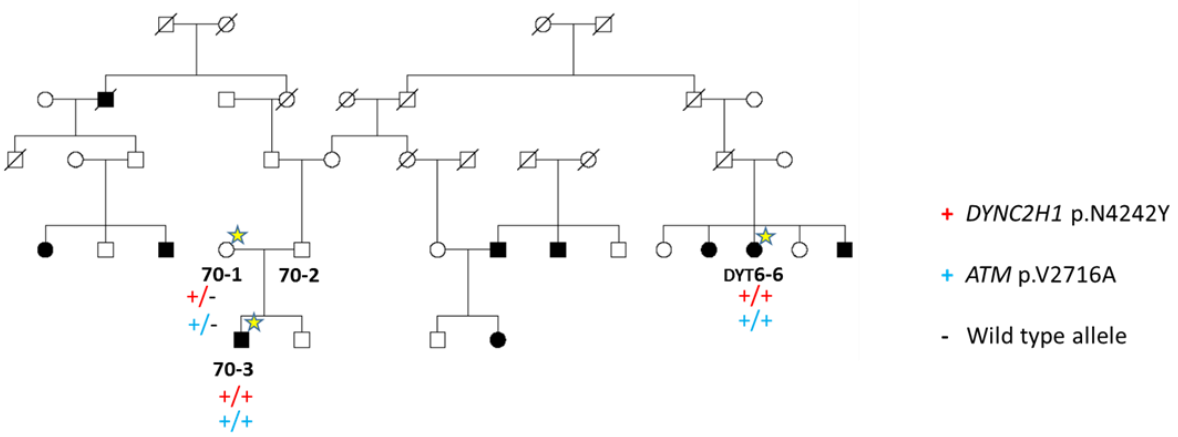


Figure 17. Pedigree 1 with ataxia-teleangiectasia-variant.

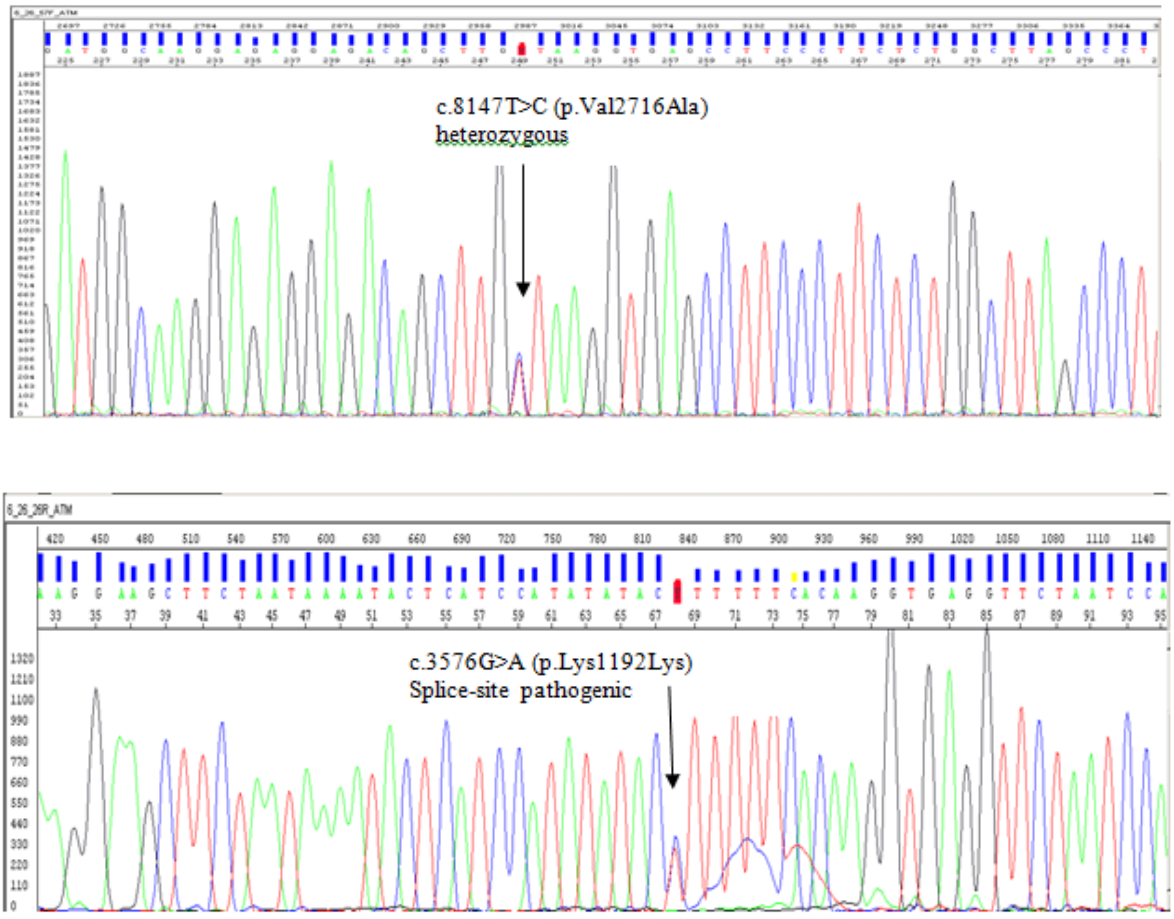


Figure 18. Genotype of patient № 28.

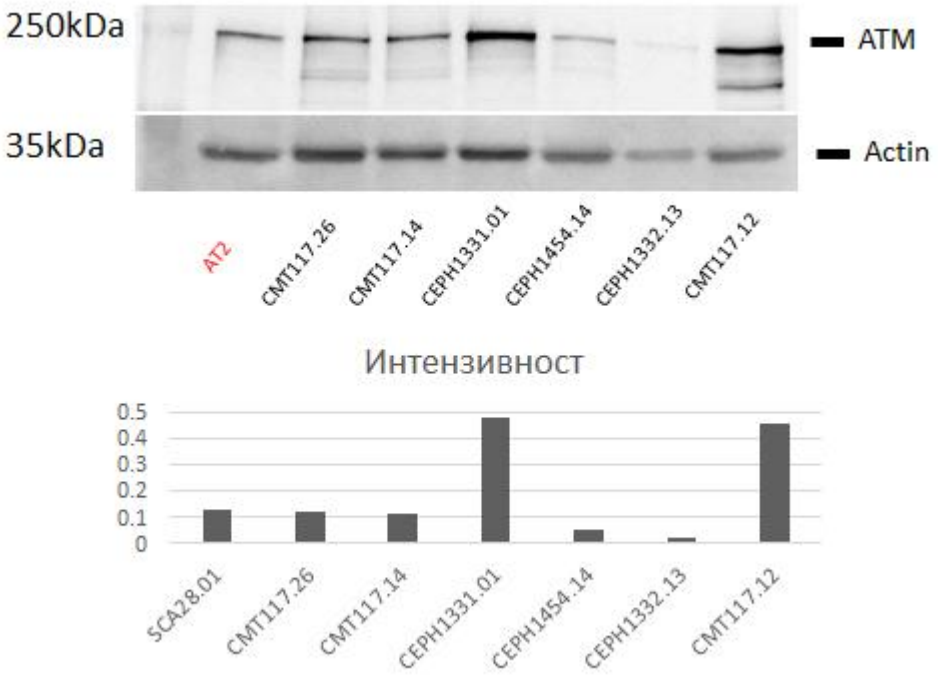


Figure 19. Western blot assessed ATM protein concentration in lymphoblasts compared to controls with other diseases. Patient #2 is marked in red as AT2.

The high prevalence of AT-variant cases in the area of the towns of Dospat and Sarnitsa and the presence of a pseudo-dominant type of inheritance in the three presented pedigrees necessitated a screening study of 100 Guthrie blanks of newborns for carrying the two typical mutations.

From the screening study, 1 carrier was found - 1% carrier of p.V2716A in the *ATM* gene.

Discussion:

In the classic variant, ataxia-telangiectasia is a multisystem disease with childhood onset, due to mutations in the *ATM* gene, leading to progressive cerebellar ataxia, telangiectasias, increased risk of neoplasms, radiosensitivity, immune deficiency, and impaired fertility (Perlman SL et al. 2012; Shiloh Y et al 2013). The advances of the molecular genetic analyses in the last decade made it possible to identify new gene variants in *ATM* in patients with a mild or "atypical" phenotype (Dork T. et al. 2004; Keimling M et al. 2011; Verhagen MM et al. 2012). The clinical spectrum caused by mutations in the *ATM* gene was broadened, including the variant forms with isolated extrapyramidal hypotonic-hyperkinetic syndrome more pronounced in the neck and upper limbs, without ataxia, telangiectasias, but always with increased AFP (Verhagen MM et al. 2009; Saunders-Pullman R et al. 2012; Chamova T et al. 2019).

In Bulgaria, an endemic region of the AT variant was found in the towns of Dospat and Sarnitsa among Bulgarian-muslims, where this AR disease is observed with a pseudodominant type of inheritance in up to 4 consecutive generations. This fact is associated with a high frequency of carrier rate of p.V2716A in the *ATM* gene (1% of newborns) and probably the limited genetic variability in this religious minority. Mild ataxia of snace and

gait was present in a small number of patients 5/28 (SARA 1-4), which differs significantly from classical forms of AT, where early ataxia leading to loss of independent ambulation before the age of 10 years is the main feature of the disease (Crawford TO 1998; Todorova D et al. 2007; Verhagen MM et al. 2009). This phenotypic variability greatly complicates diagnosis, with these patients having prior diagnoses of essential tremor and focal or generalized dystonia (Verhagen MM et al. 2009).

The AT variant has also been described in other inbred populations. Saunders-Pullman R et al. presented 35 Canadian memonite patients with dystonia-tremor syndrome with predominant involvement of the neck and upper limbs and variable age at onset between 1 and 20 years, similar to the Bulgarian cohort (Saunders-Pullman R et al. 2012). In some of the affected, an unstable gait was also present in childhood, which was not observed afterwars. In some cases, dystonia was so severe that it led to captocormia (Paucar M. et al. 2018). Although neurological involvement is milder and predominantly characterized by extrapyramidal hyperkinesias, the incidence of malignancies is higher than in the general population (Saunders-Pullman R et al. 2012). There are no data on increased neoplasms in our patients and their healthy relatives, heterozygous carriers. Може да се спекулира, че ефектът на мутацията p.V2716A е по-доброкачествен поотношение на риска от туморогенеза, но за доказване на тази хипотеза са необходими по-задълбочени функционални изследвания. Schon K. и съавт. описват най-голямата кохорта от пациенти с вариант на A-T, при които началото е предимно през първото десетилетие, по-рядко във второто (Schon K и съавт. 2019). It can be speculated that the effect of the p.V2716A mutation is relatively benign in terms of neoplastic risks, but further functional studies are needed to prove this hypothesis. Schon K. et al. described the largest cohort of patients with the A-T variant, with clinical onset predominantly in the first decade, rarely in the second one (Schon K et al. 2019). The diagnosis was made between 1 and 20 years after the clinical onset. The affected are divided into three main groups based on the leading phenotype: 1. patients with cerebellar ataxia and/or polyneuropathy, without or with minimal extrapyramidal features; 2. patients with ataxia, polyneuropathy and extrapyramidal features; 3. patients with leading extrapyramidal symptoms. Visceral symptoms were very mild, but the risk of neoplasms was significantly increased as well.

The most frequent mutation p.V2716A in the *ATM* gene found in our cohort of patients with an AT-variant was also described in previous studies, but in combination with others c.5932 G_T (p.Glu1978X), (Verhagen MM et al. 2009) and c.7517–7520delGAGA (p.Arg2506ThrfsX3), (Demuth I et al. 2011) within a compound heterozygous state. In all three described patients, the clinical picture is atypical, within the AT-variant. Although the levels of the ATM protein were within normal ranges on the Western blot of patient #2, homozygous for p.V2716A, functional studies demonstrated reduced but still residual ATM kinase activity (Verhagen MM et al. 2009; Demuth I et al. . 2011), as well as moderately reduced phosphorylation activity towards the substrate p-KAP1 (Fiévet A et al. 2019). These observations explain the milder clinical phenotype in our cohort, in which dystonic hyperkinesias and tremor predominate, but immunodeficiency and infertility are absent.

This study provides an opportunity for prevention of new cases in affected families and raises the question of periodic screening for neoplasms after the affected population.

3.2.3. AOA2

Two patients with AOA2 have been diagnosed (table 6).

Table 6. Clinical features of the Bulgarian patients with AOA2.

Patients	№1	№2
Sex	Male	Male
Age at onset (years)	18	20
Age of diagnosis	29	23
Neurological examination	Ataxia Mild polyneuropathy	Ataxia Severe polyneuropathy
SARA	20	18
Neuroophthalmological evaluation	Nystagmus without oculomotor apraxia	Nystagmus without oculomotor apraxia
NCS	Axonal damage	Axonal damage
Neuropsychological assessment	Normal functionning	Mild cognitive impairment
AFP	84.7	94.36
Brain MRI	Cerebellar atrophy	Cerebellar atrophy
Molecular- genetic result	Deletion in exon 7, c.5825T>C of *SETX*	c.6106+1G>T, c.5825 T>C in *SETX*

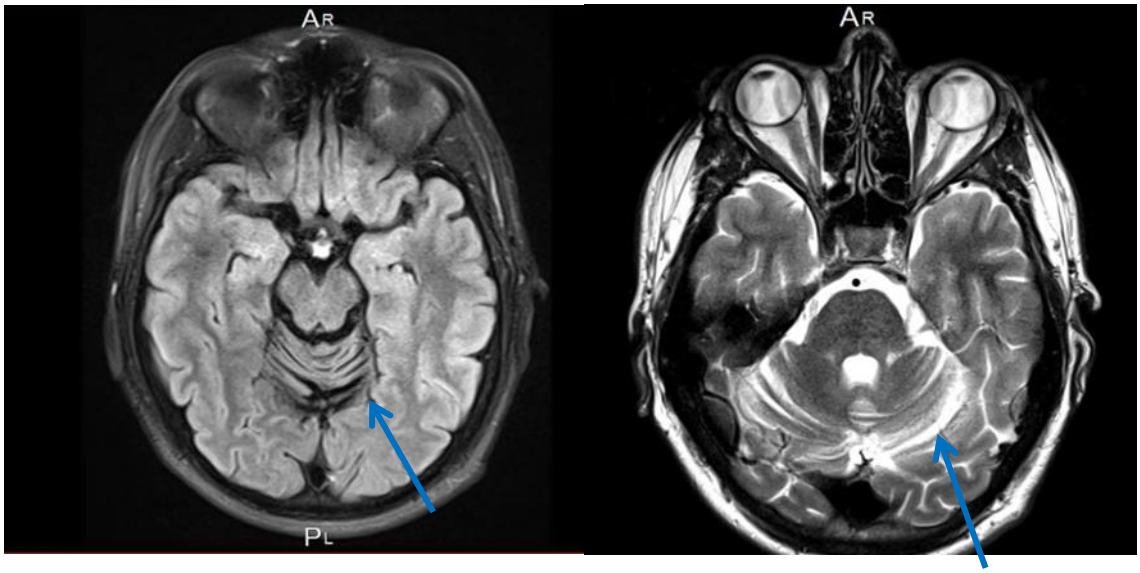




Figure 20. Brain MRI in patient #2 with AOA2. Evidence of atrophy of the cerebellar vermis and hemispheres (arrow). A. Axial T2 FLAIR. B. Axial T2. In Sagittal T2.

Discussion: AOA2 is a disease, characterized by adolescent/young onset, progressive ataxia, axonal polyneuropathy, oculomotor apraxia in about 50% of cases, and elevated AFP (Criscuolo C et al. 2006). The presented patients are compound heterozygous for mutations in the *SETX* gene. One of the mutations c.5825 T>C is present in both patients, although they are unrelated. The onset in the two presented patients is in the second decade, and they have pronounced and progressive ataxia, but they differ in the severity of the polyneuropathy - mildly expressed in the first patient, without distal weakness and hypoesthesia and with peroneal weakness, disturbed superficial and vibration sensation in the second one. AFP levels were elevated in both patients (Anheim M et al. 2010). Given the fact that there are two main forms of AR ataxia with elevated AFP-AOA2 and ataxia telangiectasia, its study in patients with unexplained cerebellar ataxias would facilitate the diagnosis.

Although the name of the disease includes oculomotor apraxia, this symptom is present in half of the affected patients (Tazir M et al 2009). No oculomotor apraxia was found in our two cases.In patients with a combination of ataxia and polyneuropathy, AFP testing is an important element of the diagnostic process.

3.2.4. Cerebrotendinous xanthomatosis

We present two male patients with cerebrotendinous xanthomatosis. They are sporadic cases belonging to different pedigrees. Their clinical features are presented in Table 7.

Table 7. Clinical features of the Bulgarian patients with cerebrotendinous xanthomatosis.

Patient	№1	№2
Sex	Male	Male
Age at onset (years)	8	10
Initial clinical symptoms	Diarrhoea	Weakness in the lower limbs

Age at diagnosis (years)	32	39
Neurological examination	Pyramidal involvement Ataxia Polyneuropathy Cognitive impairment	Pyramidal involvement Ataxia Polyneuropathy Cognitive impairment
SARA	12	15
Xantoma in the Achillis tendons	Yes	Yes
Kyphoscoliosis	Yes	No
Neuroophthalmologist	VOD=0,5; VOS=0,8 Bilateral artephakia	VOD=0,6; VOS=0,7; Bilateral artephakia
NCS	Axonal damage	Axonal damage
EEG	EEG with nonspecific changes	Not performed
Neuropsychologist	IQ 70%; MMSE 26	IQ 65%; MMSE 20
Brain MRI	Cortical atrophy	Cortical and cerebellar atrophy
Abdominal echography	Hepatosplenomegaly	Hepatosplenomegaly
Molecular-genetic analysis	c.776A>G;p.(Lys259Arg) c.1184+1G>A	c.646 G≥C (pAla216Pro) c.1184+1 G≥A

Discussion:

Cerebrotendinous xanthomatosis is an inherited metabolic disorder with lipid accumulation with multisystem involvement (Nie S et al. 2014; Pilo-de-la-Fuente B et al. 2011). Its main clinical encompass chronic diarrhoea, cataracts, tendon xanthomas, osteoporosis, neurological involvement within ataxia, pyramidal symptoms, polyneuropathy, cognitive deficit and psychiatric manifestations with increased levels of cholestanol and bile acids in the serum and urine.

Our two patients have a classic phenotype and pronounced neurological symptoms. In patient #1 the initial manifestations are gastrointestinal with chronic diarrhea, present at the time of diagnosis, and in the second patient the symptoms are neurological - weakness in the lower limbs and ataxia.

From a neurological point of view, none of the patients had epileptic seizures and extrapyramidal involvement, without lesions in the area of the basal ganglia on the MRI (Nie S et al. 2014), which we can speculate is due to the combination of mutations in the CYP27A1 gene in our patients (Stelten BML et al. 2019).

Both patients had evidence of early bilateral cataracts (Dotti MT et al. 2001) requiring surgery and Achilles tendon xanthomas, which are pathognomonic for the disease. Visceral involvement is limited to hepato- and splenomegaly with normal liver enzymes and no evidence of hypersplenism.

In both patients, the diagnosis was made 24 to 29 years after the first manifestations (between 31 and 39 years of age) and at the time of diagnosis they had a developed a neurological involvement. This diagnostic delay is similar to that found in other European

countries (Pilo-de-la-Fuente B et al. 2011), but is important to reduce given treatment options. Our patients are compound heterozygous for mutations in *CYP27A1*, and despite the lack of consanguinity, they have a common splice-site mutation - c.1184+1G>A. Most likely, cerebrotendinous xanthomatosis is a disease that remains hidden in the group of spastic ataxias or the complicated variant of HSP.

3.2.5. Ataxia with vitamin E deficiency (AVED)

We present the first Bulgaria patient with ataxia with vitamin E deficiency. He is a 37-year-old man, born from a normal pregnancy and delivery, with normal neuropsychological development, whose complaints began at the age of 7 with hand tremors. From 20- years of age head tremor was observed. From the age of 34, he began to have seizures during which he stares, turns pale, makes a movement with his right hand as if trying to grab something. He was unaware of all these actions. These seizures occured daily, then the diagnosis of cryptogenic epilepsy was made and therapy with Neurotop Retard 2 x 300 mg was initiated with good effect.

From the neurological status:

- Ataxia of satance and gait, mild dysmetria and intention tremor, dysatria (SARA 15)
- Polyneuropathy with mild tibial and peroneal paresis, absent CP in the lower limbs and weakened in the upper limbs.
- Extrapyramidal syndrome with static and postural tremor in the upper limbs.

The NCS were constant with axonal damage of the sensory fibers of the limbs, the EEG was normal, the brain CT scan was normal. From the neuropsychological examination, the patient has no evidence of cognitive deficit. The neuro-ophthalmological examination did not reveal any evidence of retinitis pigmentosa.

WES established homozygous mutation 552G≥A, p.Thr184 in the *TTPA*-gene. The patient treated by vitamin E 40 mg/kg with stabilization of ataxia.

Discussion: The discovered Bulgarian patient has a known mutation in the *TTPA*-gene, with a classical onset in childhood, but a relatively milder phenotype, with some atypical features, such as epilepsy with complex partial seizures, preserved cognition, normal visual acuity and normal neuroophthalmological status.

Seizures are not reported as a typical feature of AVED, but there is one report. Müller KI et al. described a patient with AVED in whom GTCPs have been present since the age of 11 and are responsive to therapy with Lamotrigine (Müller KI et al. 2011) and vitamin E. They preceded the ataxia. In a study of 100 epileptic children with GTCP and compared with 100 healthy control children, those with epilepsy were found to have lower levels of vitamin E. Deficiency of this vitamin, which is a fat-soluble antioxidant in the CNS, can be discussed as a potential cause of epilepsy (Ogunmekan AO et al. 1979). Although visual disturbances are considered a typical feature of the disease, in a North African cohort of 132 patients only 2.3% had retinitis pigmentosa (El Euch-Fayache G et al. 2014).

Axonal sensory neuropathy from the NCS is present in the patient, which is described in about 34% of patients (El Euch-Fayache G et al. 2014).

In patients with ataxia, pyramidal involvement, polyneuropathy, it is important to examine the serum concentrations of vitamin E, because this is one of the treatable forms of ataxia.

3.2.6. **Desequilibrium syndrome-cerebellar ataxia, mental retardation with or without quadrupedal gait 1 (DES, CAMRQ1)**

We present three patients with DES (two women and one man) from two consecutive generations belonging to a Roma Calderas group, known for its endogamy. The clinical characteristics of the patients are presented in table 8.

Table 8. Clinical features of Bulgarian patients with Desequilibrium syndrome-cerebellar ataxia, mental retardation with or without quadrupedal gait 1 (DES, CAMRQ1)

Patient	Kn II-1	Kn II-2	Kn III-1
Age (years) at examination	Deceased at the age of 40	40	19
Sex	Female	Female	Male
Pregnancy and delivery	Uneventful	Uneventful	Uneventful
Age (years) at walking with support	11-12	10	10
Age (years) at simple sentences	16	16	10
Seizures	No	No	No
Height (cm)		150	154
Weight (kg)		51	42
Head circumference (cm)		50	45
Hypertelorism	No	No	Yes
High arched palate	Yes	Yes	Yes
Neurological examination			
Ataxia (SARA)	Yes[a]	Yes	Yes
Gait (0-8)		7	8
Stance (0-6)		6	6
Sitting (0-4)		1	1
Speech disturbance (0-6)		4	4
Finger chase (R+L mean)/2 (0-4)		Not tested[b]	Not tested[b]
Nose-finger test (R+L mean)/2 (0-4)		1	1
Fast alternating hand movements (R+L		Not tested[b]	Not tested[b]

mean)/2 (0-4)			
Heel-shin slide (R+L mean)/2 (0-4)		Not tested[b]	Not tested[b]
Total SARA score (0-40)[c]		19/28	20/28
Tendon reflexes in the four limbs	Brisk	Brisk	Brisk
Plantar responses	No	No	No
Strabismus	Yes	Yes	No
Horizontal gaze-evoked nystagmus	Yes	Yes	Yes
Intellectual deficit	Moderate	Moderate	Severe
Neuroimaging			
Global small brain		Yes	Yes
Selective cerebral atrophy		No	No
Cortical gyri		Mild simplification	Mild simplification
Cerebellar hypoplasia		Marked generalized	Marked generalized
Pons		Small	Small

a. The deceased patient was examined in 1997 and SARA was not assessed at that time.

b. The severe intellectual deficit did not allow examining these elements of the test in patients #Kn II-2 and #Kn III-1.

c. The maximum score is 28, not 40, due to the items that cannot be assessed.

Pregnancy and delivery in all three patients were normal. The body weight and height of the newborns were normal. There is a severe neuropsychological delay. Patient KnIII-1 was admitted to hospital at the age of 10 months, due to microcephaly and retardation. None of the patients had epileptic seizures. According to their relatives, the condition of those affected is non-progressive.

From the somatic examination, the patients were of short stature and low weight. Microcephaly was also found in patient No. KnIII-1. Dysmorphic features included a high arched palate in all and hypertelorism in #KnIII-1. Patient #KnII-2 has severe comitant, convergent strabismus of the right eye.

The neurological examination revealed severe static and locomotor ataxia, muscle hypotonia, moderate dysarthria and mild dysmetria and intention tremor. Horizontal nystagmus and pyramidal signs were observed. Intellectual deficit is moderate to severe in #KnII-1 and #KnII-2 who were able to form simple sentences and severe in #KnIII-1 who says single words but all of them understand and follow simple commands.

NCS and EMG in patients werre normal. Brain MRI in #KnII-2 (Figure 21) showed reduced brain size, hypoplasia of the cerebellum and pons, more pronounced for the lower part of the vermis and pachygyria. The fourth cerebral ventricle appeared dilated.

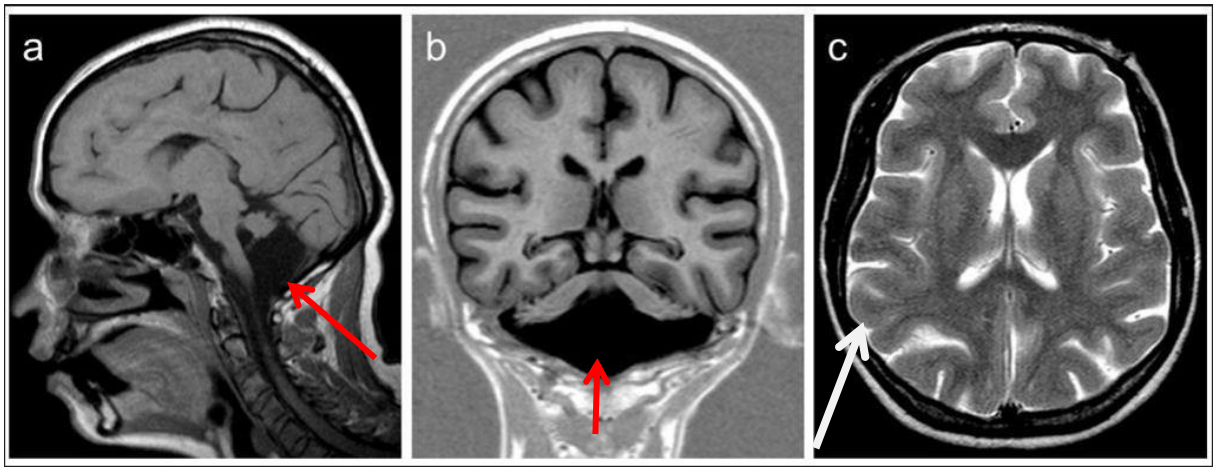


Figure 21. Brain MRI in patient #Kn II-2 with evidence of cerebellar hypoplasia, more pronounced for the lower part of the vermis (arrow) A. Sagittal T1W and B. Coronal T1W C. Axial T2W Pachygyria (arrow).

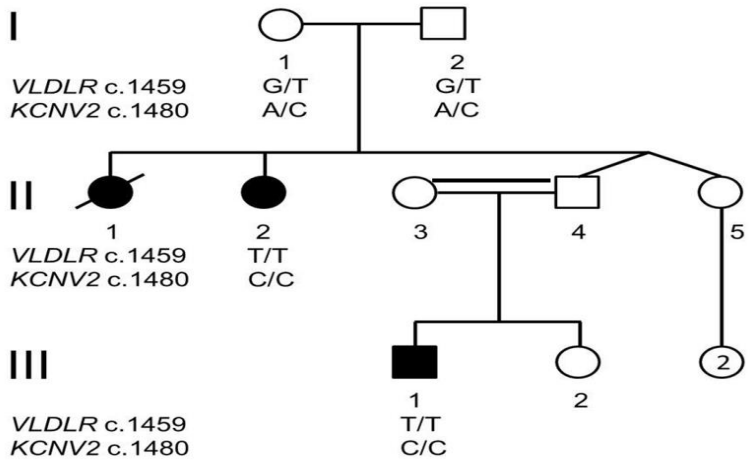


Figure 22. Pedigree of Bulgarian patients with Desequilibrium syndrome-cerebellar ataxia, mental retardation with or without quadrupedal gait 1 (DES, CAMRQ1).

WES detected two gene defects in the homozygous state (Figure 22): G>T (hg19 chr9:2645720; NM_003383.3, exon 10: c.1459G>T (NP_003374.3: p.Asp487Tyr)) in *VLDLR* and A>C. (hg19 chr9:2729569; NM_133497.3, exon 2: c.1480A>C (NP_598004.1: p.Asn494His) in KCNV2, a gene encoding a potassium channel subunit.

Defects in the *VLDLR* gene were associated with cerebellar ataxia, mental retardation and disequilibrium syndrome 1, CAMRQ1 while mutations in *KCNV2* lead to cone retinal dystrophy. The VLDLR p.Asp487Tyr variant was not detected in 566 control subjects, suggesting that it is a private mutation confined to this consanguineous family. By contrast, the KCNV2 variant was very common across subisolates, with 101 carriers (14%) and 8 homozygotes (1.1%) identified among 721 controls, thus it was assumed as a polymorphism.

Discussion:

We present an inbred Roma family with AR disease, clinically characterized by retardation in neuropsychological development, moderate to severe mental retardation, non-progressive severe ataxia, nystagmus and pyramidal signs. On the brain MRI, he main features encompassed reduced brain size, pachygyria and severe hypoplasia of the cerebellum and pons. This phenotype was classified within the broad spectrum of disorders from the lissencephaly and cerebellar hypoplasia (LCH) group. This group is clinically and genetically heterogeneous, with mutations in genes involved in the cycle of Reelin-related neuronal migration, although many cases remain genetically unexplained (Barkovich A. 2012).

The genetic analysis of the Bulgarian family revealed a novel mutation p.Asp487Tyr in the *VLDLR* gene, classifying our patients to the group of patients with DES. The clinical phenotype is similar to that of patients with other *VLDLR* mutations (Ali BR et al. 2012; Boycott KM et al. 2009). The VLDL receptor refers to the family of low density lipoprotein (LDL) receptors. This receptor is known to regulate reelin-related signaling through internalization and conformational changes (Figure 32), (Duit S et al. 2010). The mutation is thought to disrupt the structure of the receptor.

On the other hand, molecules with a p.Asp487Tyr defect can reach the cell surface and disrupt reelin signaling by suppressing ligand dissociation and recycling of the receptor through internalization.

These disorders also account for the clinical similarities between patients with mutations in VLDL and those with mutations in other genes associated with reelin-related signaling.

3.2.7. AR Cerebellar Ataxia 1 (ARCA1)

We present the first ARCA1 patient diagnosed in Bulgaria. He is a man of Roma ethnicity, 45 years old with the onset of complaints at about the age of 31, when his gait gradually became unsteady and he began to stagger.

He reports that he has a first cousin on the paternal side who had similar complaints but died at 45 years.

From his neurological examination:

- Mainly paleocerebellar involvement with ataxia of stance and gait, dysarthric speech, negative Romberg sign, saccadic smooth eye movements. The gait is ataxic, wide based. There is dysmetria in the heel to shin test and intention tremor in the finger to nose test, dysdiadochokinesia.
- Bulbar involvement with dysphagia, dysarthria and attenuated pharyngeal reflexes.
- Lower central paraparesis with spastically increased muscle tone in the lower limbs, brisk tendon reflexes and positive Babinski sign on the right.

EMG revealed damage to the peripheral motor neuron in the limbs and tongue. MRI showed evidence of cerebellar atrophy (Figure 23).

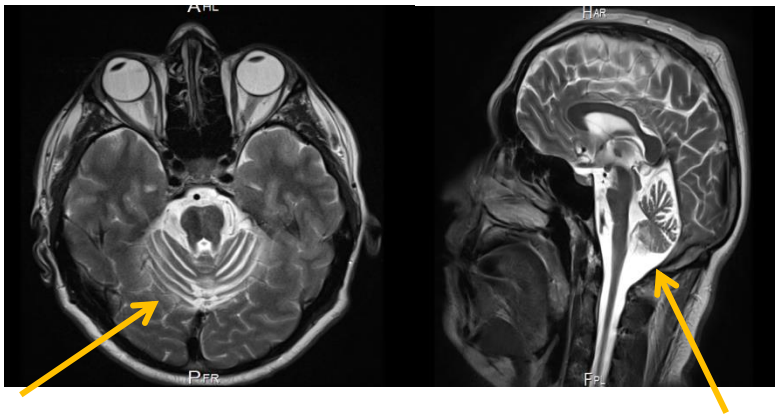


Figure 23. MRI of the Bulgarian patient, homozygous for mutation c.6071+1G≥A in intron 41 of *SYNE1*. Cerebellar atrophy (arrow). A. AXIAL T2; B. SAGITAL T2;

The neuro-ophthalmological examination showed evidence of a mild infranuclear lesion of the n. abducens dex. and saccaded smooth-tracking eye movements in all directions.

Cognitive functioning is within normal ranges.

The patient was found by WES to be homozygous for a s.6071+1G≥A mutation in intron 41 of *SYNE1*.

Discussion: The presented case is the first and, the only one for Bulgaria, due to a novel, so far unreported homozygous mutation in *SYNE1*. The phenotype of our patient is characterized by ataxia plus central and peripheral motor neuron involvement, proven by clinical and electromyographic examination and described by other authors (Mademan et al. 2016; Yoshinaga T

et al. 2016). In our patient, ataxia preceded the clinical involvement of motor neurons, which is present in about 50% of patients (Mademan et al. 2016). There was no cognitive deficit.

A homozygous mutation in a patient belonging to an endogamous group leads to the speculation of the possible higher frequency of this mutation among Roma.

3.2.8. Charlevoix–Saguenay spastic ataxia (ARSACS), (Table 9)

Four patients with ARSACS have been genetically diagnosed in Bulgaria, whose clinical characteristics are presented in table 9.

Table 9. Clinical characteristics of the Bulgarian patients with ARSACS.

Patient №	1	2	3	4
Sex	M	F	M	F
Age at onset (years)	1.8	7	15	Под 1 г.
Age at last examinations (years)	37	44	40	17
Early motor development	Delay in starting to walk	Normal	Normal	Delayed
SARA	19	24	27	21
SPRS	23	6	10	16
Independent ambulation	Possible	Possible with a walker since 35 years	Possible with a cane since 34 years	Possible
NCS	Demyelinating with secondary axonal degeneration	Demyelinating with secondary axonal degeneration	Demyelinating with secondary axonal degeneration	Normal
Additional features	Muscle cramps with neuropathic pain	Epilepsy and mild cognitive impairment	Epilepsy and mild cognitive impairment	No
Brain MRI	Mild cerebellar atrophy, with predominant vermian involvement, hypointense lesions in the pons	Mild cerebellar atrophy, with predominant vermian involvement, hypointense lesions in the pons	Not done	Mild cerebellar atrophy, with predominant vermian involvement
OCT	Absence of physiological foveal depression in both eyes and significant thickening of the global peripapillary retinal nerve fiber layer	Absence of physiological foveal depression in both eyes and significant thickening of the global peripapillary retinal nerve fiber layer	Absence of physiological foveal depression in both eyes and significant thickening of the global peripapillary retinal nerve fiber layer	Normal
Mutations in *ARSACS*	c.13469_13470delAT and c.10062delT	c.13588G>T / NP_055178.3: p.Gly4530Cys in	c.13588G>T / NP_055178.3: p.Gly4530Cys in	p.Asp4192Gly in exon10 and p.Thr904Arg in

		homozygous state	homozygous state	exon 10

Table 10. NCS of patient №1 with ARSACS.

	36 years of age		
Sensory	CV (m/s)	A (μV)	DL (ms)
n. Medianus	0	0	0
n. Ulnaris	0	0	0
n. Peroneus superficialis	0	0	0
n. Suralis	0	0	0
Motor	CV (m/s)	A (mV)	DL (ms)
n. Medianus	36.1	0.69	4.42
n. Ulnaris	49.4	2.5	4.56
n. Peroneus	0	0	0
n. Tibialis	0	0	0

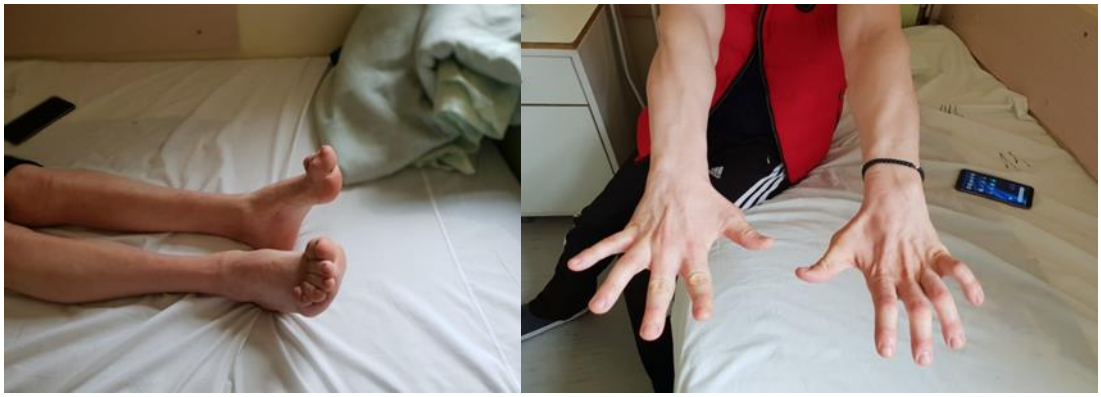
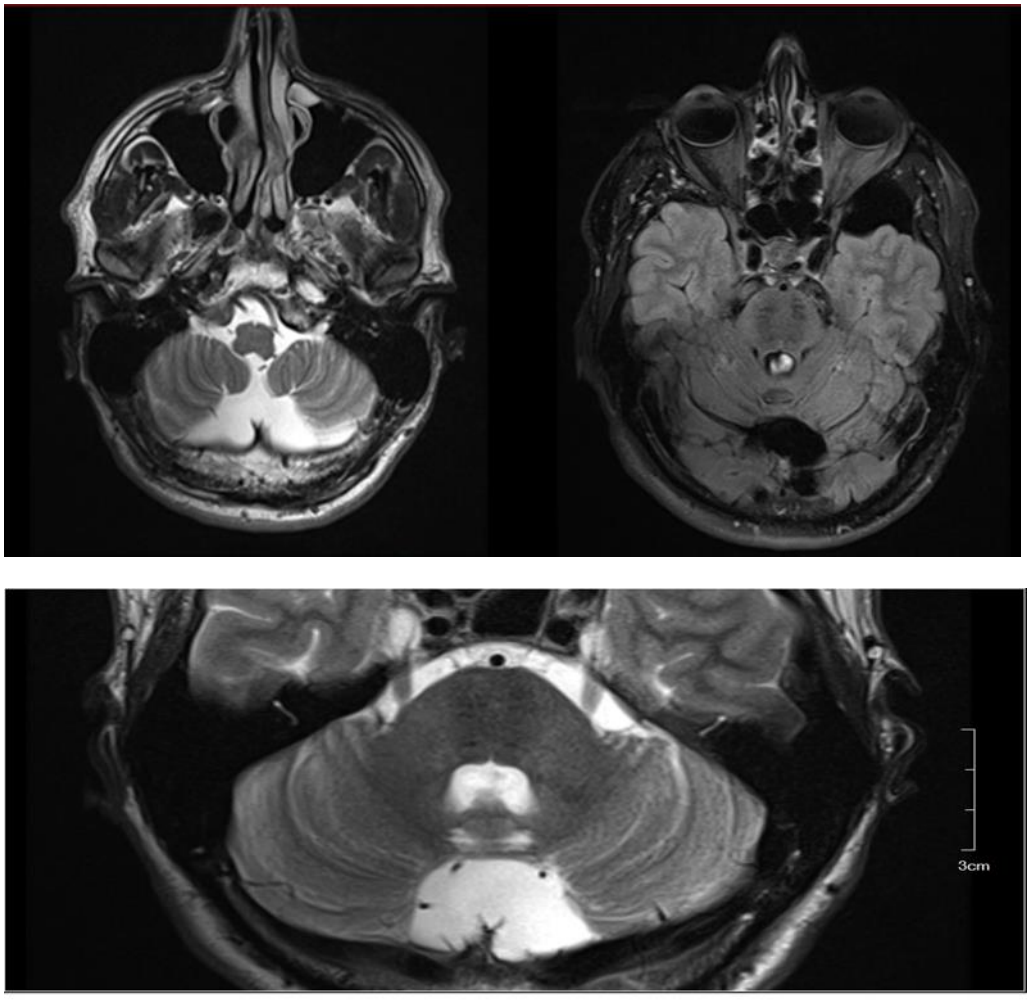




Figure 24. Muscle atrophy in the distal parts of the four limbs with bilateral pes cavus and equinovarus, hammertoe (A) and flexion contractures of the interphalangeal joints of both hands, more pronounced for the left (B and C) in patient #1.



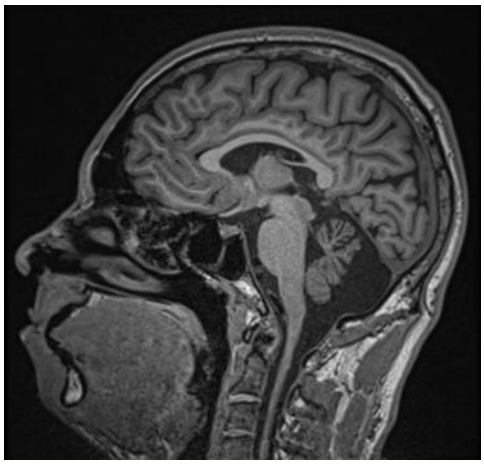


Figure 25. Brain MRI of the Bulgarian patient with ARSACS #1.

A. Axial T2 with evidence of cerebellar predominantly vermis atrophy.

B. Axial FLAIR showing pontine hypointense lesions and cysterna magna permagna

B. Axial T2 with diffuse hyperintensity in the area of the lateral pons.

D. Thinning of corpus callosum

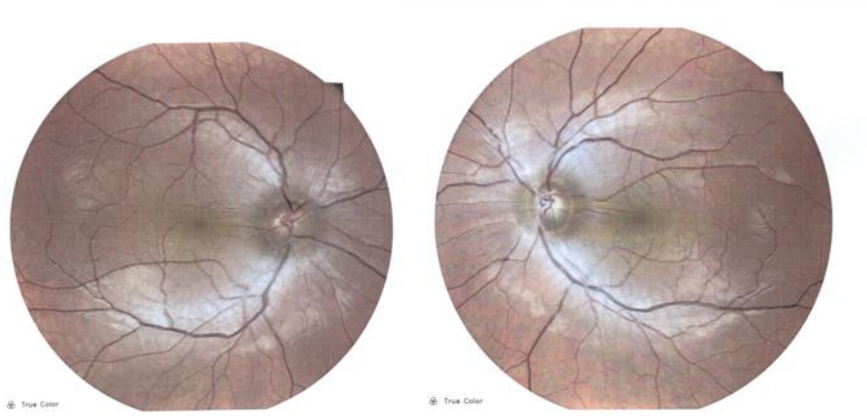


Figure 26. Fundus in patient #1 with presence of white peripapillary striations.

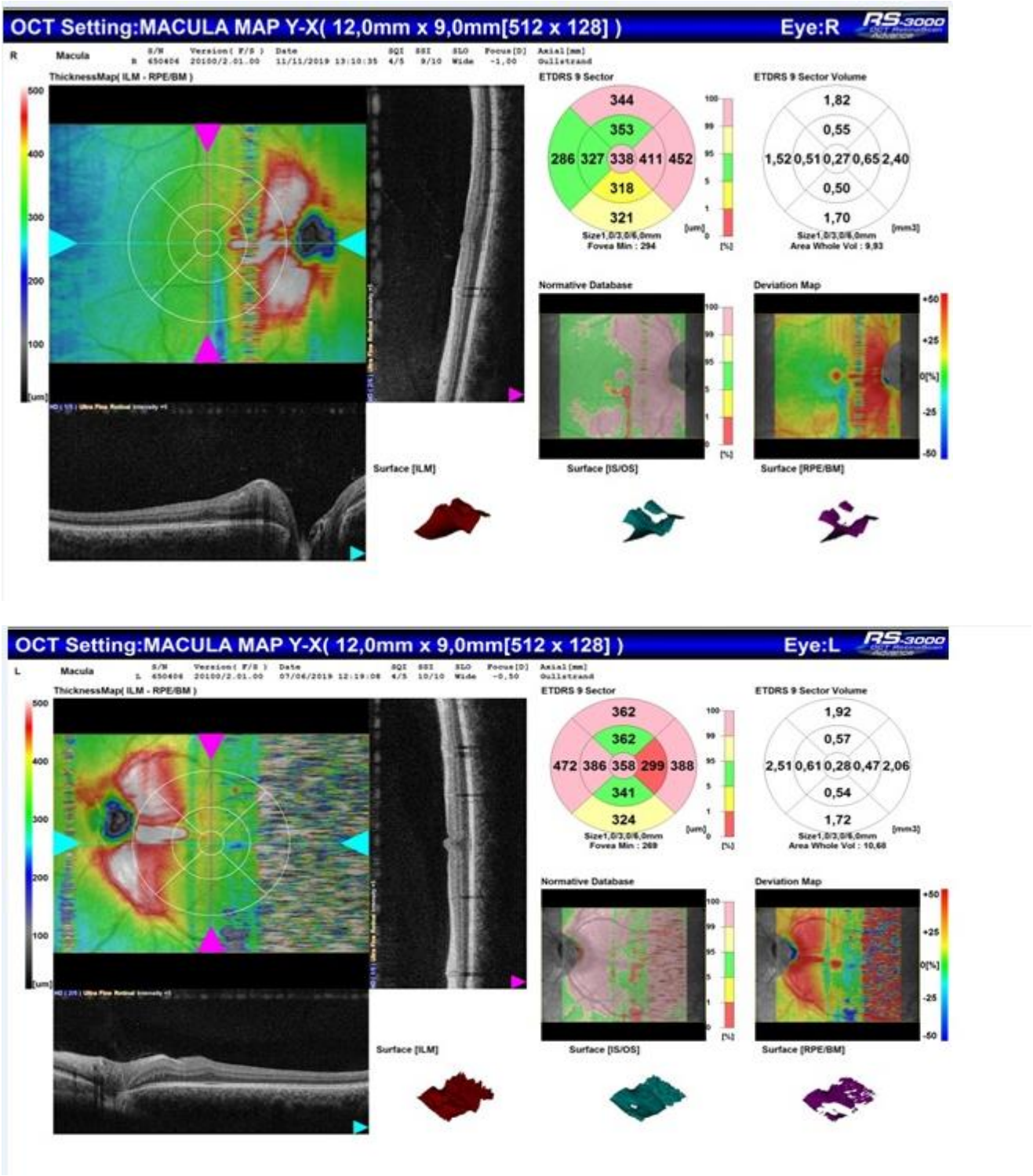


Figure 27. OCT in patient #1 with ARSACS, in whom physiologic foveal depression was absent in both eyes and peripapillary retinal nerve layer (RNFL) thickening was found 196 µm for the right eye and 202 µm for the left eye (norm: 96–99 µm).

Discussion:

Although mutations in the SACS gene were initially and mainly described among Canadian patients from Quebec (Bouchard JP et al. 1978; Synofzik M et al. 2013), in recent years the number of ARSACS patients worldwide has increased (Baets J et al. . 2010; Vermeer S et al. 2008; Kuchay

RAH et al. 2019). In Bulgaria, these are the first four patients with Charlevoix–Saguenay spastic ataxia, carrying 5 new mutations disrupting the reading frame and located in the largest exon 10 of SACS**.**

In Bulgaria, these are the first four patients with Charlevoix–Saguenay spastic ataxia, carrying 5 new mutations disrupting the reading frame and located in the largest exon 10 of SACS. Frameshift variants comprise about 1/3 of all mutations in the gene (ClinVar) and have been shown to lead to the synthesis of a non-functional protein.

The age at onset varied in our cohort from infancy to adolescence, with the typical clinical triad (ataxia, pyramidal involvement, and polyneuropathy) being present in three of the patients. Patient 4 lacked the typical peripheral nerve involvement, which has so far been described in only two patients (Baets J et al. 2010; Synofzik M et al. 2013), with mutations other than the typical Canadian ones.

Patients #2 and #3 had additionally rare epileptic seizures with a good effect from valproic acid therapy. Seizures were reported in 9.3% of Canadian ARSACS patients, a rate higher than the population average (Bouchard JP et al. 2000; Briand MM et al. 2019).

The neuropathic pain and muscle cramps in the lower extremities found in patient #1 are described in a large percentage of ARSACS patients, 23.3% (for pain) and 58.5% (muscle cramps), respectively (Bouchard JP et al. 2000; Briand MM and et al. 2019). These manifestations are the result of the mixed type - demyelinating-axonal polyneuropathy typical of the disease, but in our patient they appeared late, in the fourth decade (Bouchard JP et al. 1978; Synofzik M et al. 2013).

In all patients, the typical MRI characteristics were present - linear hypointense T2 lesions in the pons, diffuse hyperintense lesions at the junction of the pons with the middle cerebellar peduncle and atrophy of the upper part of the cerebellar vermis (Synofzik M et al. 2018; Gazulla J et al 2012). Cognitive functioning within mild mental retardation/mild cognitive impairment is present in 75% of patients, which is a possible manifestation of this syndrome (Bagaria J et al. 2022).

While thinning of the retinal peripapillary neurofibrillary layer (RNFL) is a typical finding in many neurodegenerative diseases (Parkinson MH et al. 2007), Charlevoix–Saguenay is an exception to this rule. RNFL thickening, assessed by OCT, present in 75% of Bulgarian patients, is considered an important biomarker with a sensitivity of 100% and a specificity of 99.4% (Kuchay RAH et al. 2019; Parkinson MH et al. 2007; Desserre J et al. . 2011).

In conclusion, MRI and OCT are important biomarkers for the diagnosis of Charlevoix–Saguenay disease, even in the absence of any of the cardinal symptoms of the triad.

3.2.9. **Autosomal recessive cerebellar ataxia type 3 (ARCA3)**

In Bulgaria, three patients with Roma ethnicity were diagnosed, two men and one woman, belonging to the group of Bowl makers (figure 28).

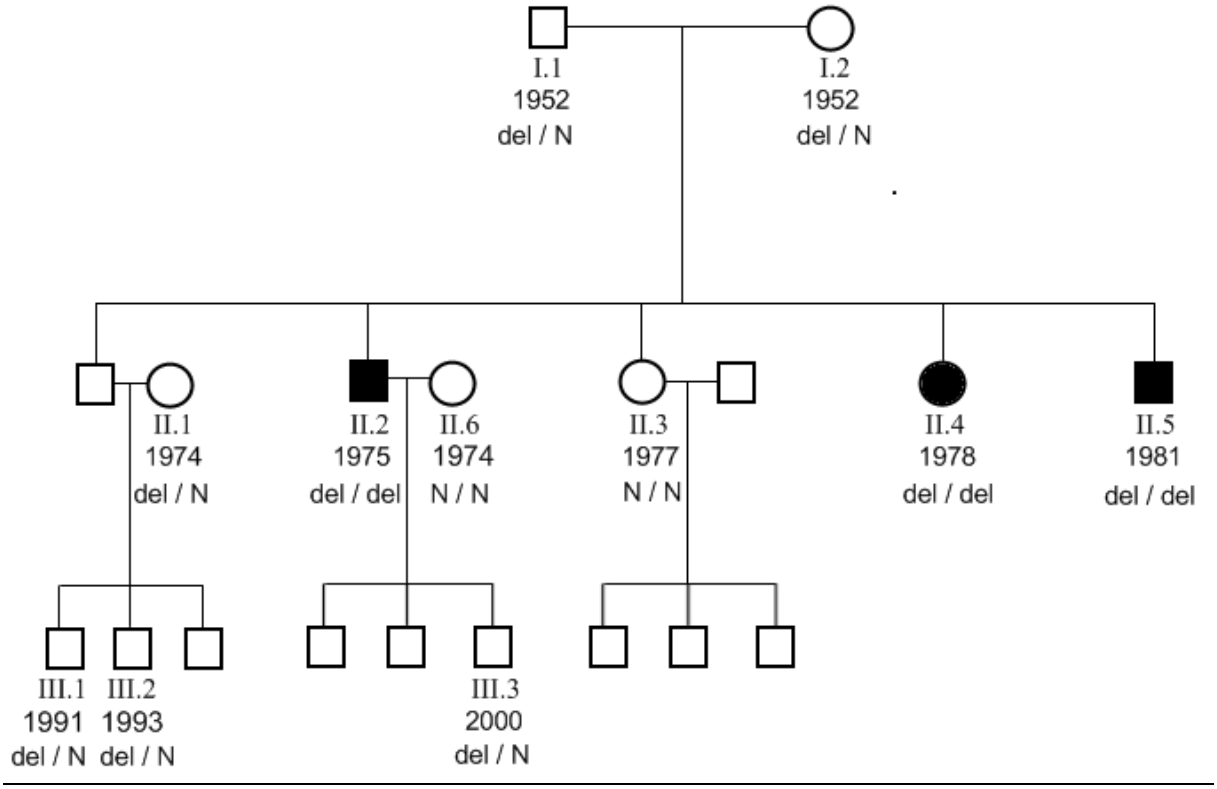


Figure 28. Pedigree of the Bulgarian patients with ARCA3, due to homozygous mutation p.Leu384fs in the *ANO10*-gene. Only patients who participated in the study are marked. Genotypes are designated as N/N (normal homozygotes), N/del (heterozygotes) and del/del (homozygotes for the c.1150_1151 deletion leading to the p.Leu384fs mutation in ANO10).

Table 13 presents the clinical characteristics of the Bulgarian patients with ARCA3.

Table 13. Clinical characteristics of Bulgarian patients with ARCA3.

Patient	**II.2**	**II.4**	**II.5**
Date of birth	07.05.1975	13.10.1978	03.08.1981
Sex	M	F	M
Early development	Normal	Normal	Normal
Age at onset (years)	Dysarthria: 6-7; Ataxia: 16 years	Dysarthria: 6-7; Ataxia: 17	Dysarthria: 7; Ataxia:17
Age at most recent examination	35	32	29
Ocular pursuit	Horizontal and vertical nystagmus	Downbeat nystagmus	Downbeat nystagmus
Cerebellar dysarthria	Moderate	Moderate	Moderate
Dysmetria and dysdiadochokinesia of limbs	Mild	Mild	Mild
Gait ataxia	Severe	Moderate	Moderate
Appendicular ataxia	Mild	Mild	Mild

Ataxia score*	7/16	6/16	6/16
Tendon reflexes: UL	Increased	Increased	Increased
Tendon reflexes: LL (knee)	Increased	Increased	Increased
Tendon reflexes: LL (ankle)	Normal	Increased	Normal
Plantar responses	Normal	Extensor	Normal
NCS	Not done	Normal	Normal
EMG	Not done	Normal (2006) Motor neuron involvement in m.quadriceps femoris (2011)	Normal (2006 and 2011)
Brain MRI findings	Not done	Severe cerebellar atrophy	Not done
Intellectual deficit	After development of ataxia	After development of ataxia	After development of ataxia
Co-morbidity	Gastric ulcer	Syphillis, treated with Benzylpenicillin	No

Pregnancy, birth and early psychomotor development were described as normal in all three affected. They started to walk in the period of 12-14 months, and speech was achieved at around 18 months of age.

Patients #II.2 and #II.4 attended school for 4 years and 2 years, respectively, while #II.5 never attended school. Onset of symptoms in all three siblings was at age 6-7 years, when speech impairment was first noted; the dysarthria progressed slowly in subsequent years. Gait ataxia developed around age 16-17 years and by 28-29 years became so prominent that walking without support was impossible. Upon recent neurological examination, gait ataxia was severe in the oldest patient II.2 (age 35 years) and moderate in the younger siblings. Limb dysmetria and dysdiadochokinesia were mild in all. The speech was moderately dysarthric. Gaze-evoked downbeat nystagmus with hypermetric saccades was observed in the younger patients and horizontal and vertical nystagmus in the oldest one. Tendon reflexes were brisk in all three patients. Pathological extensor responses were present only in II.4. EMG (in patient II.4 detected neurogenic changes in m.quadriceps femoris with spontaneous activity of rare positive sharp waves and fibrillation potentials and some long duration action potentials; tibialis anterior and extensor digitorum brevis showed no changes. The results were normal in patient II.5.

Brain MRI (1.5T MR imager Signa HDxt, GE Healthcare Milwaukee USA) in II.4 showed severe diffuse cerebellar atrophy and diffuse T2/FLAIR hyperintense and T1 hypointense zones in the cerebellar hemispheres, consistent with gliosis. The supratentorial structures were normal.

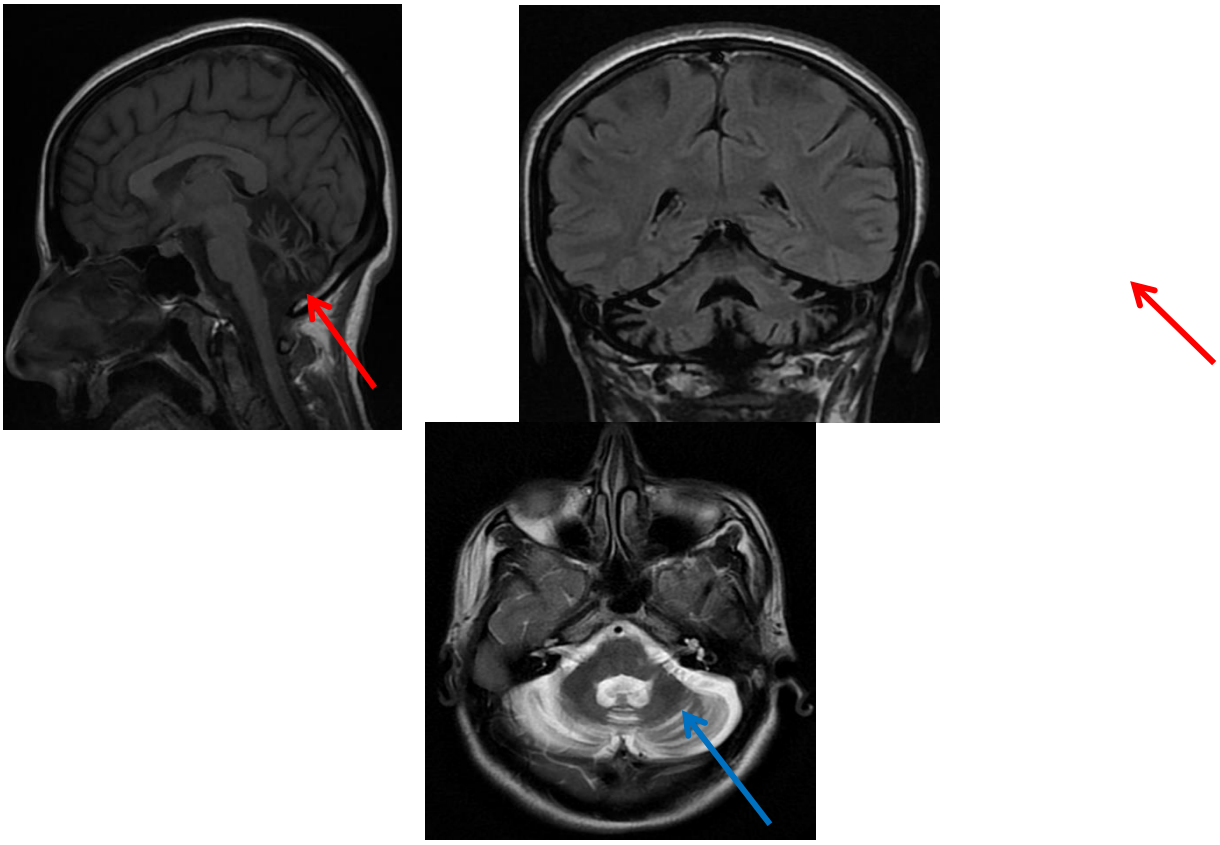


Figure 29. Brain MRI of patient I#I.4 with evidence of severe cerebellar atrophy (arrow) with normal imaging of the supratentorial structures. Diffuse T2 hyperintense and T1 hypointense lesions in the cerebellum consistent with gliosis are also present (arrow). A Sagittal T1, B Axial T2 PROPELLER, C Coronal T2 FLAIR.

From the neuro-ophthalmological examination of patient No. II.2. bilateral macular hypoplasia and tortuosity of the retinal vessels were found.

Given the low educational qualification and language characteristics, the results of the neuropsychological tests were compared with the results of their relatives and age- and sex-matched controls from the same community (table 14). Patients have a moderate intellectual deficit affecting all cognitive domains.

They have been shown to be homozygous for c.1150_1151del deletion in the *ANO10* gene. Of the remaining 7 members of the family that were genetically tested, the parents and four others were heterozygous carriers for the same mutation (Figure 28).

Table 14. Neuropsychological testing of the affected subjects, unaffected siblings and unrelated healthy controls.

Individuals	**Patients**			**Unaffected siblings**		**Healthy controls N=9 Mean values (SD)***
	II.2	**II.4**	**II.5**	**II.1**	**II.3**	
Raven progressive matrices- raw scores	19/60	13/60	12/60	25/60	22/60	24.78 (6.0)
MMSE	21	19	21	27	26	24.56 (1.74)
Digit span						
Forward	2	1	2	6	7	5.56 (1.13)
Backward	2	2	1	7	7	3.89 (1.05)
Verbal Fluency						
Semantic- "animals"	5	4	4	10	12	10.11 (2.26)
Phonemic "M"	2	3	1	4	3	2.56 (1.13)
Rey verbal learning test						
Immediate recall	23/75	24/75	13/75	36/75	36/75	36.78 (6.10)
Delayed recall	2/15	3/15	3/15	7/15	8/15	9.89 (1.83)
Recognition	20/30	20/30	12/30	27/30	29/30	27.56 (1.67)
Tower of London						
Total move score	51	65	61	35	42	46.89 (9.58)
Total correct scores	1	0	0	3	2	2.00 (1.41)

Analyzed by the non-parametric Mann-Whitney U test. Statistical analyses were conducted in SPSS for Windows, version 13.00.

Discussion: Our findings indicate that the ANO10 c.1150_1151 deletion is another founder mutation in the Roma/Gypsy population, leading to AR cerebellar ataxia. While our brief search, following the original publication (Vermeer S et al. 2010; Mišković ND et al. 2016) identified no further patients and families, their existence is suggested by the ancestral origin of the mutation and by the lack of parental consanguinity in our family. Patients described with p.Leu384fs have a relatively homogeneous phenotype that is more severe compared to patients with other mutations (Vermeer S et al. 2010; Chamova T et al. 2012; Chamard L et al. 2016; Maruyama H et al. 2014; Renaud M et al. 2014; Yang SL et al. 2020). Our patients have the earliest onset compared to those described so far. Another characteristic feature is the decline of cognitive functions, which is also present in the Serbian Roma family with the same mutation (Vermeer S et al. 2010). Neuropsychological assessment confirmed a generalized deficit that progressed over time, given evidence of normal early neuropsychological development. This fact is another confirmation of the role of the cerebellum in cognitive processes (Bellebaum C et al. 2007; Laforce R Jr et al. 2010; Middleton FA et al. 1994). ANO10 is expressed in the cerebral cortex and hippocampus, but further studies are needed to clarify its exact role. Pronounced cerebellar atrophy with preserved cortical structure is a typical feature of *ANO10* mutations, while down beat nystagmus and peripheral motor neuron involvement are not mandatory symptoms (Vermeer S et al. 2010; Chamard L et al. 2016; Maruyama H et al. 2014; Renaud M et al 2014; Yang SL et al 2020).

3.2.10. SCAR13

We present 10 patients (4 women and 6 men) belonging to the Roma ethnicity with congenital ataxia SCAR13. They are from 5 unrelated pedigrees. The clinical characteristics of the patients are presented in table 15.

Table 15. Clinical characteristics of patients with SCAR13.

Patient №	Li 1	V III-2	V III-1	B IV-2	P II-2*	B IV-3	P II-1	P II-3	M II-2	M II-1
Age at onset (years)	6	8	10	26	27	32	37	42	47	57
Sex	Female	Female	Male	Female	Male	Female	Male	Male	Male	Nale
Pregnancy and delivery	No data	Normal	Intranatal asphyxia	Normal	Pulmonal atelectasis	Normal	Normal	Normal	Preterm	Preterm
Aga at starting to walk (years)	4	4	5	No data	Never	1	Never	Never	3	No data
Age at starting to talk (years)	4	2	3	No data	Never	No data	Never	Never	Never	Never
Height	120 cm	126 cm	137 cm	147 cm	138 cm	143 cm	151 cm	150 cm	154 cm	152 cm
Weight	22 kg	40 kg	42 kg	42.5 kg	23 kg	42.5 kg	50 kg	60 kg	47.1 kg	45.1 kg
Cerebellar ataxia (SARA)										
Gait (0-8)	4	5	5	5	8	4	7	8	6	7
Standing (0-6)	2	4	4	4	6	2	6	6	5	6
Sitting (0-4)	0	1	1	0	4	0	3	3	1	2
Dysarthria (0-6)	2	2	2	2	6	2	6	6	6	6
Finger chase ((0-4)	1	1	2	1	Not assessed	1	1	1	2	1
Figer to nose (0-4)	1	1	2	1	Not assessed	1	1	2	1	0
Dysdiadochokinesis (0-4)	1	2	2	1	Not assessed	1	2	2	2	1

Heel to shin (0-4)	1	2	2	2	Not assessed	1	2	3	2	2
Total SARA (0-40)	13	18	20	16	24/24	12	28	31	25	25
Oculomotor signs										
Gaze-evoked horizontal nystagmus	No	No	No	Yes	No	Yes	Yes	No	No	No
Hypometric saccades	No	Yes	No	No	Yes	No	Yes	No	No	Yes
Abduction deficit	Yes	No	No	Yes	Yes	No	No	Yes	No	No
Esotropia	No	Yes	Yes	Yes	Yes	No	No	Yes	No	Yes
Ptosis	No	No	No	Yes	No	No	No	No	Yes	Yes
Additional signs										
Intellectual deficit	Mild	Mild	Mild	Moderate	Profound	Moderate	Moderate	Severe	Severe	Severe
Hyperreflexia	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
Spasticity	No	No	No	No	No	No	No	Yes	No	No
Urinarry dysfunction	No	Yes	Yes	No	Yes	No	No	No	No	No
Polyneuropathy	No	No	No	No	Mild	No	No	No data	No data	No data
Epileptic seizures	No	2 years	5 months	No	One abnormal EEG	No	No	No	No	No

In all patients, there was a delay in the heuropsychological development, three patients never started to walk and five never spoke (table 15). The height and body weight were normal in three patients (children), but they are below the normal ranges for the corresponding age in the adult patients.

All affected had a moderate to severe ataxia with a mean SARA value of 11.9, with ataxia being more pronounced in older patients. Mild dysarthria was present in all who can speak. Dysmetria, tremor and dysdiadochokinesia were found in all patients, but they were mild. Pyramidal features were mild with pathologically increased tendon reflexes in the lower extremities.

Neuro-ophthalmologic assessment encompassed gaze-evoked nystagmus, hypometric saccades, abduction deficits, strabismus, and ptosis. All patients had a cognitive deficit ranging from mild to profound (table 15). On neuroimaging evaluation (Table 15, 16 and Figure 30) moderate to severe cerebellar atrophy was observed. A constitutionally smaller lower part of the vermis was found in 3 patients, a small volume of the brain in 5/7. The middle cerebellar peduncle, brainstem, and spinal cord were normal.

Follow-up neuroimaging studies in two patients (table 17 and figure 30) presented progression of atrophic changes of the cerebellum.

Table 16. Characteristics of neuroimaging studies in the Bulgarian patients with SCAR13.

Patient №	**Li 1**	**V 3-2***	**V 3-1***	**B 4-2**	**P 2-2**	**B 4-3**	**P 2-3**
Age at evaluation (years)	6	6	11	24	27	32	37
Evaluation	MRI	MRI	CT	MRI	MRI	MRI	MRI
Modality	T1 FLAIR Sagittal, T1 SPGR Coronal, T2 FSE Coronal, T2 FSE Axial	PD & T2 Axial (JPEG)	Volumetric axial - with orthogonal MPR	T1 Sagittal, T2 propeller Axial, T2 FLAIR Coronal, DWI (b=1000)	T1 MEMP Axial, T2 FSE Coronal, DWI (b=1000), T2* GRE Axial, T1 3D BRAVO with MPR's, T2 FSE Axial, T2 FSEIR Coronal, T2 FLAIR Coronal	Volumetric T1 multiplanar reformats (MPR) - coronal & sagittal	T1 MEMP Axial, T2 FSE Coronal, DWI (b=1000), T2* GRE Axial, T1 3D BRAVO with MPR's, T2 FSE Axial, T2 FSEIR Coronal, T2 FLAIR Coronal
Global brain atrophy	No	No	No	No	No	No	No
Small brain volume (score)	No (0)	No (0)	Mild (1)	Moderate (1)	Mild (1)	Mild(1)	Mild (1)
Ventricular system	Fourth enlarged	Fourth enlarged	Mild to moderate enlargement	Fourth enlarged	No	Fourth enlarged	No
Selective cortical atrophy	No	No	No	No	No	No	No
Selective cerebellar atrophy (score)	Moderate generalized (2)	Moderate generalized (3)	Moderate generalized (2)	Mild for the hemishperes (1)	Marked generalized (3)	Marked generalized (3)	Moderate generalized (2)
Cerebellar hypoplasia (Score)	No (0)	No (0)	Inferior part of vermis (1)	He (0)	Inferior part of vermis (1)	Inferior part of vermis (1)	No (0)

Posterior cranial fossa size (score)	Normal (0)	Normal (0)	Small (1)	Normal (0)	Small (1)	Normal (0)	Normal (0)
Retrocerebellar cyst	No	Yes	Yes	No	Yes	Yes	Yes
Middle cerebellar peduncle size (score)	Normal (0)	Normal (0)	Slightly decreased (1)	Normal (0)	Normal (0)	Normal (0)	Normal (0)
Brainstem size and signal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hippocampal size	Normal	Normal	Normal	Normal	Normal	Normal	Atrophy on the right
White matter	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Basal ganglia and thalami	Normal	Normal	Normal	Нормални	Atrophy in the posterior part of putamen	Normal	Normal
Total imaging score**	2	3	6	2	6	5	3
Total SARA	13/40	18/40	20/40	16/40	18/18	12/40	22/34

* Longitudinal follow-up of the neuroimaging studies in patients V3-2 and V3-1 are presented in Table 17.

**Individual item scores are 0/1 (presence/absence), except for cerebellar atrophy 0/1/2/3, which corresponds to absent/mild/moderate/severe.

Table 17. Longitudinal follow-up of neuroimaging studies of patients #V 3-2 and V 3-I.

Patient №	**V 3-2**						**V 3-I**				
Age (years)	1	1 y 8 mo	3	6	9	9	5	5	8	8	11
Body egion	Глава	Глава	Глава	Глава	Глава	Торакален гръбнак	Глава	Глава	Глава	Лумбален гръбнак	Глава
Modality	CT	MRI	CT	MRI	CT	MRI	CT	MRI	CT	MRI	CT
	Non volumetric axial - 8mm slices	T1 midline sagittal, PD & T2 Axial	Non volumetric axial - 5mm slices	PD & T2 Axial (JPEG)	Non volumetric axial - 3mm slices	T2 frFSE Sagittal, T1 FLAIR Sagittal, T2 STIR sagittal, 2D MERGE Axial	Non volumetric axial - 5mm slices	PD & T2 Axial	Non volumetric axial - 3mm slices	T1 FSE Sagittal, T2 IR - STIR Sagittal, 2D MERGE Axial, T1 FSE Axial, T2 FSE Coronal	Volumetric axial - with orthogonal MPR
Ventricular system	No	No	Fourth dilated	Fourth dilated	Fourth dilated	-	Mild increased	Moderately increased	Moderately increased	-	Moderately increased
Selective cerebellar atrophy	No	No	Moderate generalized	Moderate generalized	Moderate generalized	-	Moderate generalized	Mild to moderate generalized	Moderate generalized	-	Moderate generalized
Cerebellar hypoplasia	No	No	No	No	No	-	Inferior vermis	Inferior vermis	Inferior vermis	-	Inferior vermis
Posterior cranial fossa size	Normal	Normal	Normal	Normal	Normal	-	Small	Small	Small	-	Small
Retrocerebellar cyst	No	No	Да	Yes	Yes	-	Yes	Yes	Yes	-	Yes
Middle cerebellar peduncle	Normal	Normal	Normal	Normal	Normal	-	Mild decrease	Mild decrease	Mild decrease	-	Mild decrease
Comparison			New cerebellar atrophy					Slight increase in ventricular size and cerebellar atrophy			

Spinal cord						Normal				Small lower spinal cord syrinx	

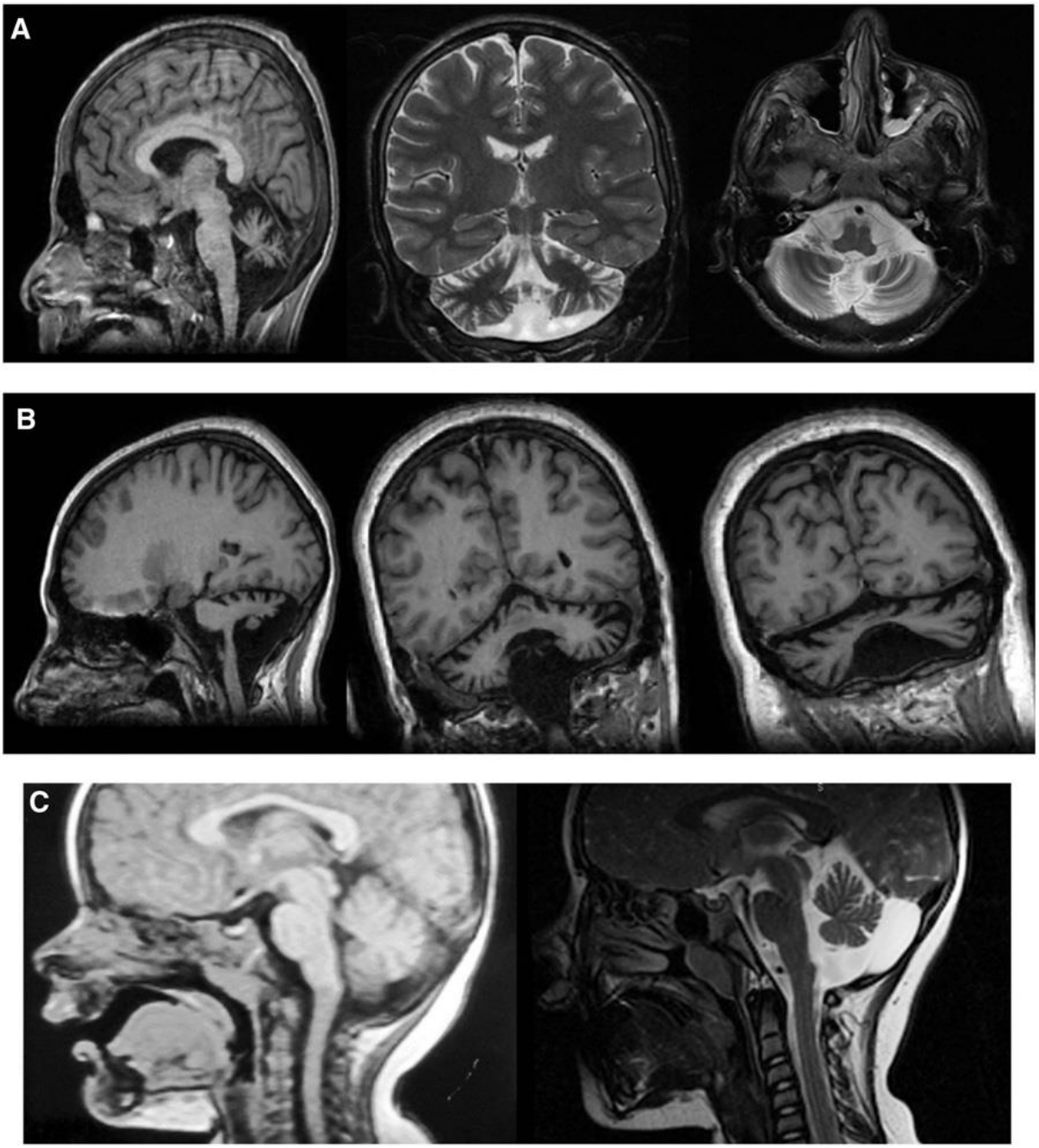


Figure 30. Neuroimaging studies in patients with congenital ataxia.

(A) Cerebellar atrophy and hypoplasia of the lower part of the vermis in patient #PII-2 (T1 3D brain volume [BRAVO], coronal T2, and axial T2).

(B) Moderate to severe cerebellar atrophy and hypoplasia of the lower part of the cerebellar vermis in patient #B IV-3 (volumetric coronal and sagittal T1 MPR).

(B) MRI follow-up in dynamics in patient #VIII-2 at 1 year and 8 months. (no evidence of cerebellar atrophy), and cerebellar atrophy (T1 midline sagittal, T2 sagittal) at 6 years of age.

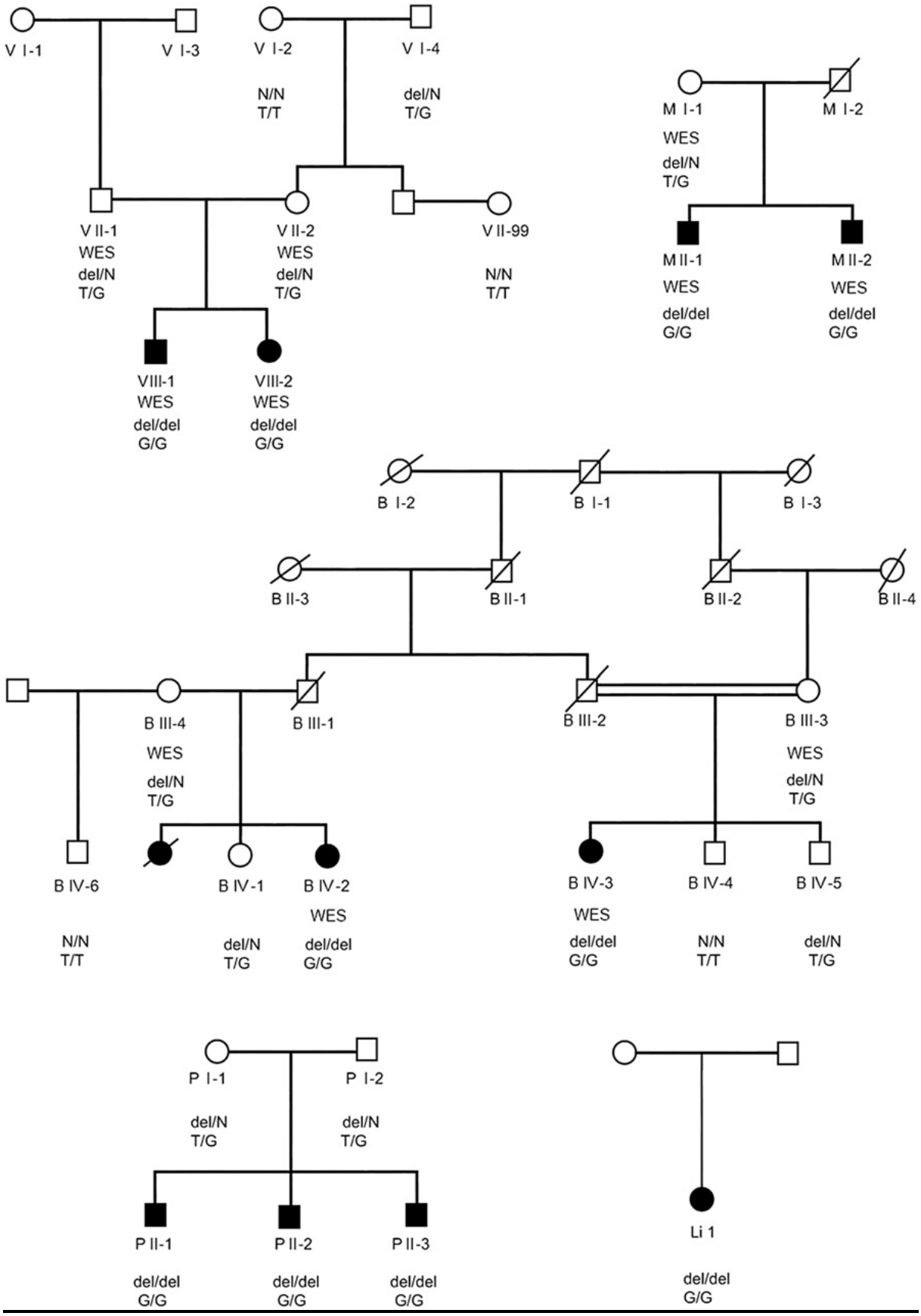


Figure 31. Pedigrees of the Bulgarian patients with SCAR13, caused by mutations in *GRM1*.

Using WES, two homozygous variants were found in the *GRM1* gene encoding metabotropic glutamate receptor 1 (mGluR1): c.2652_2654del and c.2660þ2T>G (figure 31). Prior to this study, mutations in this gene had been associated with ataxia in animal models, but there were no reports of mutations in humans. By Sanger sequencing, all affected were shown to carry two mutations in a homo- or double heterozygous state. In an investigation of carrier frequency among 289 healthy Roma controls from different groups, two heterozygous carriers were identified in the Kopanari group. *GRM1* encodes a metabotropic glutamate receptor mGluR1, which has strong expression in Purkinje cells and plays an important role in cerebellar development and synaptic plasticity.

Discussion: The clinical features of SCAR13 can be summarized as delay in psychomotor development, onset in early childhood with moderate to severe static and locomotor ataxia, dysarthria, mild dysdiadochokinesia and dysmetria, varying severity of intellectual deficit and mild pyramidal signs. Neuroimaging studies showed moderate to marked cerebellar atrophy with or without hypoplasia of the lower part of the vermis with no correlation between the severity of atrophy and ataxia. Until the description of our cohort, the involvement of mGluR1 in the pathogenesis of ataxia has been described in paraneoplastic or autoimmune ataxias (Marignier R et al. 2010).

Subsequently, the genotypic and phenotypic spectrum expanded, as Davarniya B. et al. described three Iranian patients aged 28, 37 and 40 years in whom epileptic seizures and aggressive behavior were added to the already described phenotype (Davarniya B et al. 2015), and Cabet S et al. 2019 described a 6-year-old boy with additional extrapyramidal hyperkinesias (Cabet S et al. 2019).

GRM1 belongs to the group of genes, mutations in which cause congenital ataxias. The description of other cases will also expand the clinical spectrum in the future.

3.2.11. Niemann Pick type B disease (NPB)

Five patients with genetically verified Niemann Pick A/B disease, belonging to the Roma ethnicity, were included in the study. Their clinical manifestations are presented in table 18. The clinical onset was between 5 months and 3 years with visceral manifestations: hepato- and splenomegaly in all, jaundice in 1/5, pneumonia in 3/5 and retardation in psychomotor development in 2/5. The diagnosis was made in childhood in 3/5, and in the rest during adulthood. Neurological involvement was present in 4/5: ataxia of stance and gait were present in 3/5, cognitive deficit was found in 4/5, functioning within borderline intelligence or mild mental retardation.

Neuroophthalmological examination revealed a macular halo, as a "silver gray ring" around the brown-red foveola in all subjects. Psychiatric manifestations were found in 4/5, two were diagnosed with paranoid schizophrenia and were on neuroleptic therapy, while the other two had behavioral disorders with aggressive manifestations and sexual disinhibition. At the last evaluation, all patients had clinical and echographic evidence of hepato-splenomegaly, and an increase in serum transaminases (ASAT, ALAT) was found in only one of the patients. In this patient, there was also a decrease in platelets below the reference limits, as a sign of

hypersplenism. Three of the patients had history of frequent pneumonias, and in them, infiltrative and fibrotic changes were also detected from the lung X-rays.

EEG was abnormal in 1/5 patients, and MRI data of cerebellar atrophy was found in 3/5. In 3/5 of the affected NCS was consistant with axonal damage of the sensory fibers of the four limbs. All of the affected wer homozygous carriers of (1177T>G), p. (W393G) in the *SMPD1* gene.

Table 18. Clinical features of the patients with NPA/B

Patient N	1	2	3	4	5
Age at onset (years)	3	0.7	0.5	2	2
Age at diagnosis (years)	12	0.7	0.5	30	24
Age at last assessment (years)	12	29	26	30	39
Neurological involvement					
Ataxia	+	+	-	-	+
Pyramidal involvement	-	-	-	-	+
Polyneuropathy	-	+	+	-	+
Intellectual deficit	+	+	+	-	+
Psychiatric involvement					
	Behavioral changes	Paranoid schizophrenia	Behavioral changes	-	Paranoid schizophrenia since the age of 35 years
Macular halo	+	+	+	+	+
Vesceral involvement					
Hepato-splenomegaly	+	+	+	+	+
Infiltrative changes in the lungs	-	+	+	-	+
Brain MRI	Normal	Cerebellar atrophy	Asymetric cerebellar atrophy	Normal	Cerebellar atrophy
EEG	Slow waves during hyperventillation	Normal	Normal	Normal	Normal

Discussion:

The intermediate type of NPA/B disease in Bulgaria has been found among the Roma ethnic group, and it is caused by a W391G founder-effect mutation in *SMPD1* (Mihaylova V et al. 2007). From the screening conducted among Roma, the heterozygous carrier of the mutation is 1.1%, and in some of the subgroups it reaches 4%. W391G in *SMPD1* was initially described in Roma families in Serbia (Sperl W et al. 1994; Ferlinz K et al. 1995) and subsequently other countries. The presented patients have the typical phenotype of Niemann–Pick A/B disease with varying severity of involvement of the central and peripheral nervous system in terms of ataxia, polyneuropathy, pyramidal involvement and cognitive deficit combined with hepato- and splenomegaly, which was reported by Mihaylova V et al. 2007 during the first description of patients with this mutation in Bulgaria (Mihaylova V et al. 2007). Given the homogeneity of the genetic background, it has been speculated that this variability may result from the effect of modifier genes.

Ataxia and cerebellar atrophy found in 3/5 of the patients in our cohort have been previously described in humans (Pavlu-Pereira H et al 2005; Mihaylova V et al 2007) as well as in mouse models (Horinouchi K et al 1995), which highlights the great sensitivity of Purkinje cells to acid sphingomyelinase deficiency. Cognitive impairment and psychiatric manifestations are also very typical, necessitating timely diagnosis and therapy.

3.2.12. Niemann Pick type C disease (NPC)

In Bulgaria, 21 patients with Niemann Pick C disease, belonging to 18 pedigrees, have been genetically verified so far. There are 11 affected females and 10 males. In 20 of them, the diagnosis was made after genetic testing, and in one patient, by Filipin staining of skin fibroblasts.

The mean age at onset in the Bulgarian cohort was 12.71+/-7.54 years, ranging from 1.5 to 30 years, while the mean age at diagnosis was 16.98+/-9.91 years. There was a delay in diagnosis of 4.5 +/-4.90 with a variation between 0.5 and 23 years from the initial clinical complaints. During the course of the disease, 4 of those affected died, of food aspiration and asphyxia- 3 and aspiration pneumonia- 1, on average 13 years +/-6.16 years after the onset of clinical manifestations. According to their clinical onset, Bulgarian patients are classified into the following clinical forms:

- Early infantile form - 1 patient
- Late infantile - 3 patients
- Juvenile - 9 patients
- Late-onset form - 8 patients

The genotype and clinical characteristics of the Bulgarian NPC patients are presented in Table 19.

Table 19. Clinical manifestations of Bulgarian patients with Niemann Pick C disease.

Пациент номер	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Инициали	ЙПС	ЗГЯ	СНС	НАР	НАР	МИХ	ДИШ	ДСА	АИА	ЖВВ	ПСС	АСС	ПВЗ	ХЛИ	ИММ	ККР	ДНК	ПНВ	ДАК	КГГ	ХКИ
Възраст на начало	1.5	2.5	4	6	7	8	9	11	9	10	11	11	11	15	15	16	20	19	27	30	24
Възраст на поставяне на диагнозата	2.5	9	5	6	9	8	9.5	21	9.5	14	13	16	34	16	19	19	23	21	34	36	32
Пол	Ж	М	М	Ж	Ж	Ж	Ж	М	М	М	Ж	Ж	М	М	Ж	Ж	Ж	М	Ж	М	М
Мутация в алел 1 на NPC1	c.551G≥A	p.Asn169Ile	p.Asn916del	p.Asn169Ile	p.Asn169Ile	p.(Pro1007Ala)	p.Asn169Ile	p.As1005Val	p.Asn169Ile	p.Asn169Ile	p.Pro1007Ala	p.Pro1007Ala	Cys184Tyr	p.Ala764Val	Asn916del	p.Asn169Ile	p.Pro474Leu	p.Ile409Thr	p.Ser954Leu	p.Ser954Leu	
Мутация в алел 2 на NPC1	c.1070C≥A	p.Ser940Leu	p.Pro1007Ala	p.Ser940Leu	p.Ser940Leu	p.(Gln716*)	p.Ser940Leu	p.Ser954Leu	p.Ser940Leu	p.Asn169Ile	p.Gly1240Arg	p.Gly1240Arg	p.Ser954Leu	p.Ser940Leu	Ser954Leu	p.Asn169Ile	p.Gly992Arg	p.Ser954Leu	p.Ile1061Thr	p.Asn1101Lys	
Клинични прояви																					
Неврологични прояви:																					
Вертикална погледна пареза	да	да	да	да	да	да	да	да	да	да	да	да	да	да	да	да	да	да	да	да	да
Лимитация на хоризонталните верзионни очни движения	не	не	не	не	не	не	не	да	не	не	не	не	не	не	не	не	не	не	не	не	да
Дискоординационен синдром със статична, локомоторна и динамична атаксия	да	не	не	да	не	да	да	да	да	да	да	да	да	да	да	да	да	да	да	не	да
Дистонни хиперкинези	не	не	не	не	не	не	не	да	не	да	да	да	да	да	да	да	да	да	не	да	да
Квадрипирамиден синдром	да	да	да	не	да	не	не	да	да	да	да	да	да	да	да	да	да	да	да	да	да
Катаплексия	не	не	да	не	да	не	не	да	не	не	да	да	не	не	не	не	не	не	не	не	не
Епилептични пристъпи	да	да	да	не	да	не	не	да	да	не	не	не	да	не	не	не	не	не	не	не	не
Антиепилептична терапия	да	да	да	не	да	не	не	да	да	не	не	не	да	не	не	не	не	не	не	не	не
Когнитивен дефицит	Тежка умствена изостаналост	Умерена умствена изостаналост	Лека умствена изостаналост	Лека умствена изостаналост	Тежка умствена изостаналост	Нормален интелект	Лека умствена изостаналост	Лек деменten синдром	Умерена умствена изостаналост	Лека умствена изостаналост	Лека умствена изостаналост	Лека умствена изостаналост	Лек деменten синдром	Лека умствена изостаналост	Леко когнитивно нарушение	Леко когнитивно нарушение	Умерен деменten синдром	Лек деменten синдром	Леко когнитивно нарушение	Леко когнитивно нарушение	Умерен деменten синдром
Дисфагия	да	да	не	да	не	не	не	да	да	не	да	да	да	не	не	не	да	не	не	не	да
Невросензорно намаление на слуха	не	не	не	да	да	не	не	да	да	не	да	да	да	да	не	не	да	да	да	да	не
Периферно нервно засягане	не	не	не	да	да	не	не	не	не	да	не	не	не	не	не	не	не	да	не	да	не
Психични прояви	не	не	не	не	не	не	не	да	да	да	не	не	не	да	да	не	не	не	да	не	да
Висцерални прояви																					
Хепатомегалия	да	да	да	да	да	да	не	не	да	не	да	да	да	не	не	не	не	не	не	не	да
Спленомегалия	да	да	да	да	да	да	не	да	да	да	да	да	не	да	не	да	да	да	не	да	да

Neurological manifestations were the initial symptom in 16 of the patients. Ataxia and tremor of the body and head were the first manifestations in 13/16 of the affected, dystonic involuntary movements were observed in 2/16 and epileptic (atonic, cataplexic seizures) in 2/16.

In four of the patients with NPC, mental and behavioral disturbances were observed at baseline. One of these patients (#21) was diagnosed with schizophrenic disorder and treated with neuroleptics for 10 years. Another patient (#15) was diagnosed with: pseudo-hallucinations, emotional leveling, dysmorphophobic experiences. The remaining two had aggressive manifestations and cognitive deficits with poor school performance (#8 and #9). The patient with early infantile form (No. 1), had an onset was with hepatosplenomegaly and increased values of liver transaminases.

At the time of diagnosis, the following clinical manifestations were found in the Bulgarian cohort (table 18, figure 32):

Neurological manifestations:

Vertical palsy was observed in all patients from the Bulgarian cohort, and horizontal palsy was present in 2/21, in which the diagnosis was made respectively 10 and 23 years after clinical onset. Ataxia of stance and gait, as well as milder dynamic ataxia was found in 17/21 (80%). Dystonic hyperkinesias mainly involving the upper limbs and to a lesser extent the lower limbs and face were present in 12/21 (57%) of the patients, mostly in those with juvenile (5/9) and late (7/8) form of the disease.

Pyramidal involvement with pathologically increased tendon reflexes and pathological reflexes of the Babinski group was observed in 18/21, and in the youngest patient with an early infantile form, spastic quadriparesis was present, while in patient #17, right-sided central hemiparesis was found after Herpes simplex encephalitis in the neonatal period. Cataplexy was present in 5/21 (23%), mostly in those with late infantile and juvenile form. Cataplexy was not observed in the late-onset patients of our cohort. Epileptic seizures (GTCP) were found in 7/21 (33%) patients with onset in childhood and adolescence. However, the EEG was abnormal in 18/21 (85%) and varied from non-specific changes with a delayed basal activity and delta and theta rhythms to a focal slow wave activity with different localization (frontal and temporal) and secondary generalization. Antiepileptic therapy was used in the seven patients with epileptic seizures, and in 4/7 it was polytherapy, but Levetiracetam was included in all patients considering the liver involvement.An intellectual deficit of variable degree was found in 20/21 of the patients at the time of diagnosis. In 13 of them the diagnosis was made before the age of 18 years, with severe mental retardation present in 2/13, moderate in 2/13, mild mental retardation in 8/13 and normal intelligence in 1/13. In the remaining 8 affected moderate dementia in 1/8; mild dementia in 3/8 and mild cognitive impairment in 4/8 with marked involvement of executive functions and attention and to a lesser extent verbal memory was observed.

Dysphagia, with a combination of bulbar and pseudobulbar syndrome was found in 10/21, more typical for those with long history of the disease. Swallowing disorders were also the cause of death in 4 of the patients who died after a suffocation - 3, (#8, #11 and #13) and aspiration pneumonia - 1 (#21). Neurosensory hearing loss was found in 13/21 of the patients, and in patient No. 18 it required a hearing prosthesis. Peripheral damage of the sensory fibers

of the lower limbs with reduced A of SNAP of n. peroneus superficialis and n. suralis were observed in 5/21.

Psychiatric symptoms were found in 7/21 patients. In four of them they were initial manifestations, and in 3 these symptoms developped during the progression of the disease. In patient #21, the visual and auditory hallucinations, which necessitated therapy with neuroleptics without particular effect, were the first manifestations. In patient #14, 1 year after the diagnosis and the initiation of the therapy, visual hallucinations appeared, which were controlled in 6 months with neuroleptic therapy. In patient #15 pseudo-hallucinations, emotional leveling, dysmorphophobic experiences were observed. And the remaining 4 patients had aggressive manifestations and behavioral disorders.

Visceral involvement, in terms of organomegaly was present in 17/21, and in patient #1, in whom hepatosplenomegaly and elevated transaminases were the initial presentation of the disease, history of prolonged neonatal jaundice was also present. At the age of 2, she was already diagnosed with liver cirrhosis. Combined hepato- and splenomegaly was present in 9/21, while in 7 only the spleen was enlarged. Isolated hepatomegaly was observed in only one patient. The visceral involvement is an obligatory manifestation in NPC patients with childhood onset.

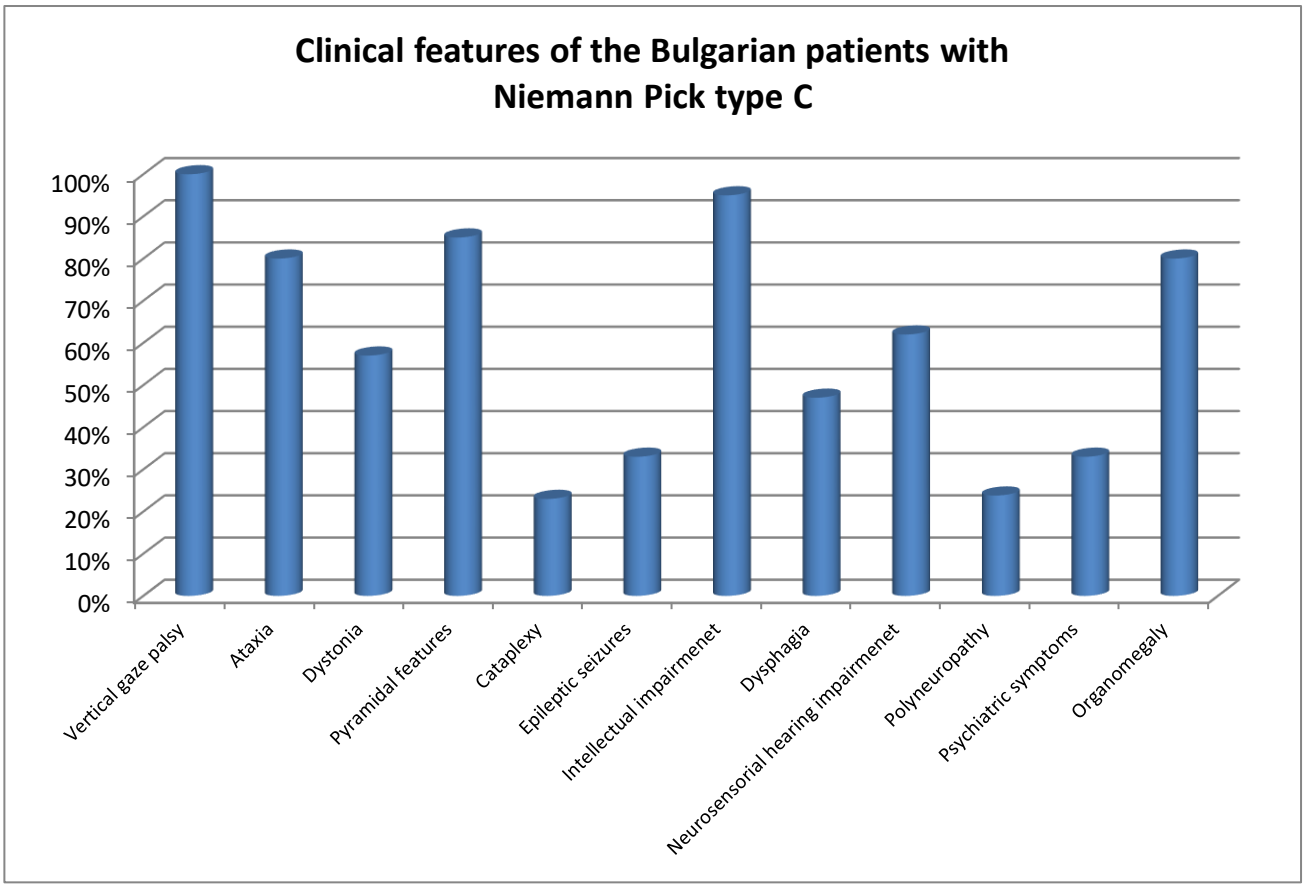


Figure 32. Percentage of the neurological, psychiatric and visceral manifestations in Bulgarian patients with NPC.

Neuroimaging examinations were abnormal in 16/21 patients (Figures 33, 34). Cerebral atrophy, more pronounced for the frontal lobes with dilatation of the lateral

ventricles were present in 6/21. Cerebellar atrophy was found in 5 patients. Hyperintense lesions, ranging from small single to bilaterally confluent, were found in 12 patients. In patient #17, atrophy of the left hemisphere was also present after experiencing meningoencephalitis in the neonatal period.

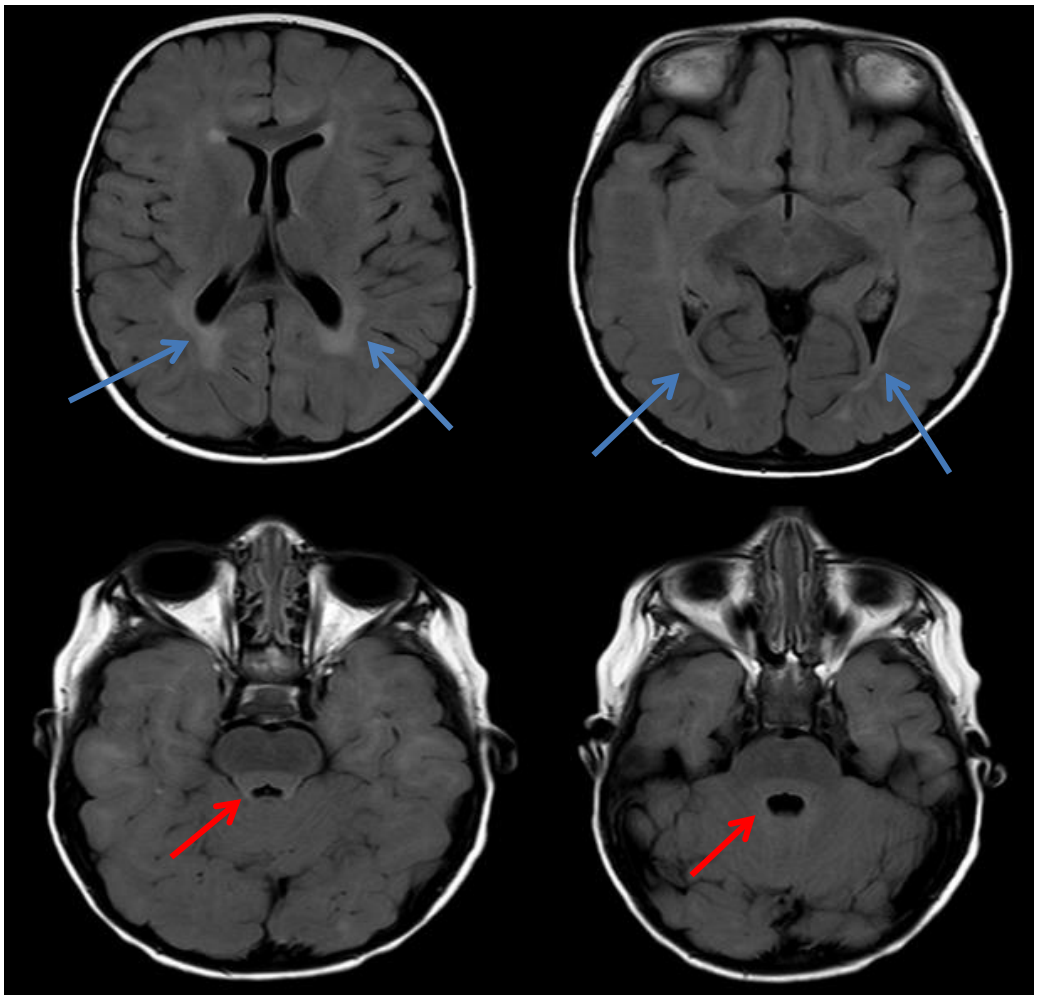


Figure 33. Axial FLAIR of patient #1 showing evidence of periventricular confluent lesions in the white matter of the brain supratentorially (arrows) and in the region of the brainstem (arrows).

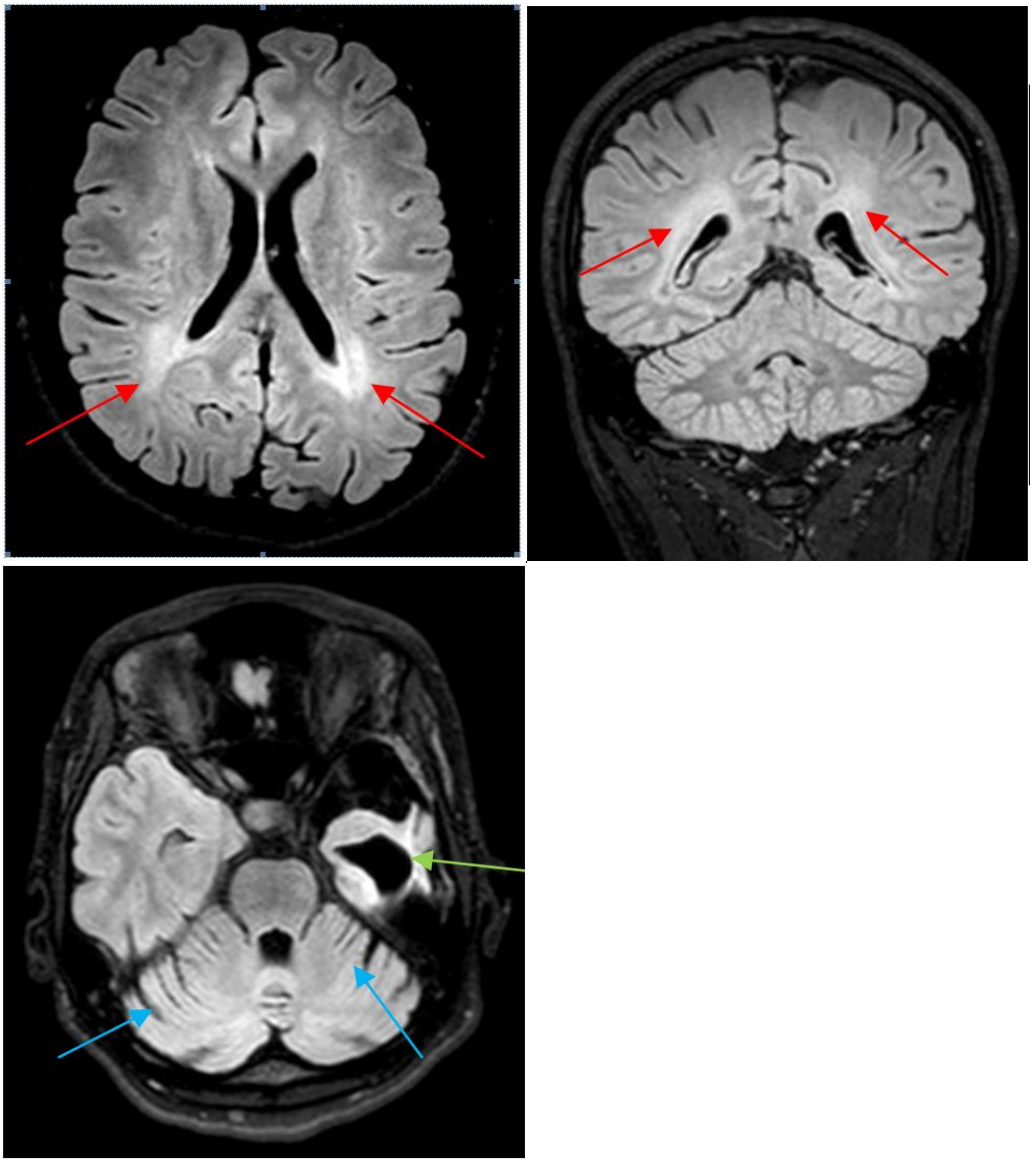


Figure 34. Brain MRI of Bulgarian patients with Niemann Pick type C disease.

A. Axial FLAIR of patient #14 at age 16 showing bilateral confluent lesions in the occipital white matter (arrows).

B. Coronal FLAIR of patient #14 at age 16 showing bilateral confluent lesions in the occipital white matter (arrows).

C. Axial FLAIR of patient #17, aged 23 years with evidence of cerebellar atrophy (arrow) and left hemisphere atrophy following Herpes simplex encephalitis (arrow).

In the Bulgarisn NPC group, there is a marked genetic variability in terms of the mutations located in the *NPC1*-gene, while no mutations in the *NPC2* gene have been detected so far (Figure 35).

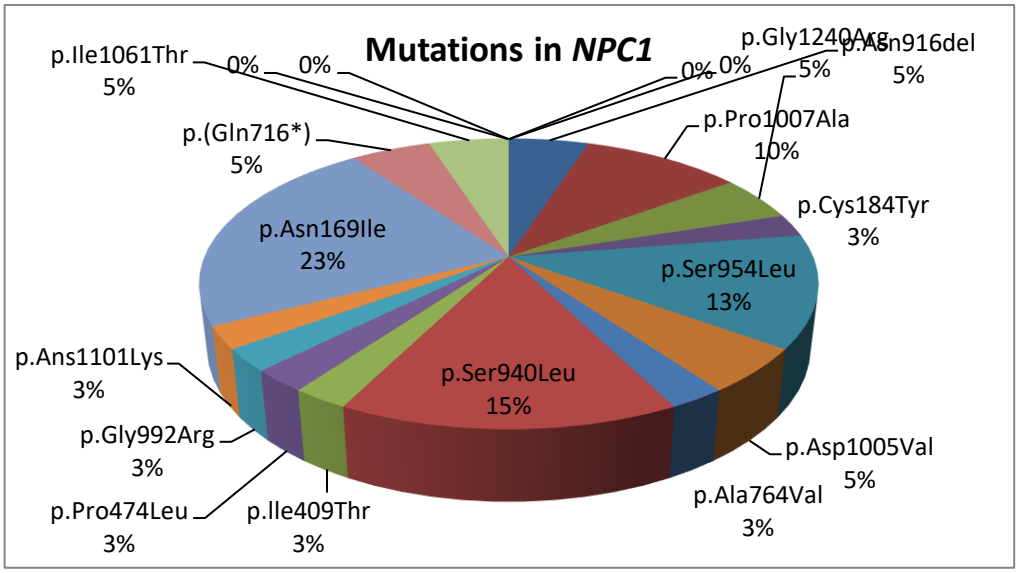


Figure 35. Variability of mutations in the *NPC1* gene in the Bulgarian cohort with Niemann Pick C.

p.Asn169Ile was the most common mutation, found in 23% of mutated alleles. It occured only in patients of the Roma ethnicity, and it is possible that it has an founder effect. In homozygous state, it leads to a juvenile-onset form.

The second most frequent mutation is p.Ser940Leu, consisting of 15% of the mutant alleles, as it was found in Roma patients and in one Bulgarian patient in a double heterozygous state. The remaining mutations account for 2.5-12.5% of the mutant alleles, which once again confirms the broad genetic variability in our group of patients.

Seventeen patients diagnosed with NPC were treated with the pathogenetic substrate-reducing therapy- Miglustat, and 15 patients were subsequently followed up at the Expert Center for Hereditary Neurological and Metabolic Diseases, UMBAL "Alexandrovska". In 14 of these patients, treatment has lasted more than 1 year. The disease progression was assessed by SARA ataxia scale. The safety profile was assessed as well (Table 19). Table 20 demonstrates the clinical characteristics of the patients on Miglustat treatment. The condition was stabilized in 5/14, improved in 4/14 and progressed in 5/14. Progression was present in the patients who were diagnosed later and, respectively, started treatment later (figure 36).

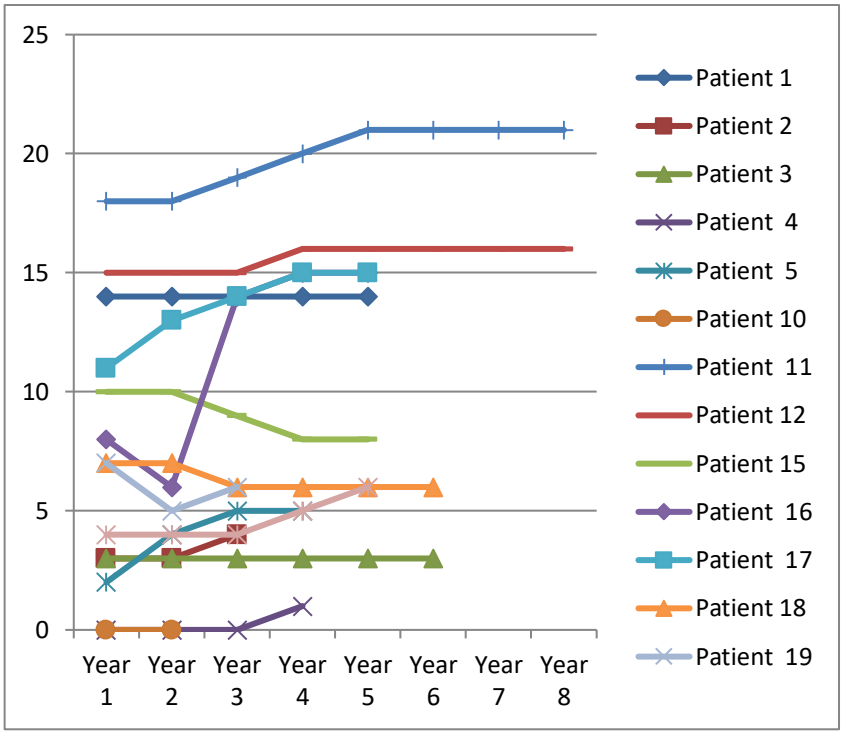


Figure 36. Follow-up of the effect of Miglustat therapy on ataxia assessed by SARA.
Table 20. Results of the treatment of 14 NPC patients with Miglustat in terms of ataxia assessed by SARA.

Patient N	1/ЙПС	2/ЗГЯ	3/СНС	4/НАР	5/НАР	10/ЖВВ	11/ПСС	12/АСС	15/ХЛИ	16/ИММ	17/ДНК	18/ПНВ	19/ДАК	20/КГГ
Age at onset (years)	1.5	2 .5	4	6	7	10	11	11	15	15	20	19	27	30
Age at diagnosis (years)	2.5	9	5	6	9	14	13	16	16	19	23	21	34	36
Sex	F	M	M	F	F	M	F	F	M	F	F	M	F	M
Age at Miglustat initiation (years)	2.7	9	5	6	9	14	15	18	16	19	23	21	34	36
Treatment duration (years)	5	2	6	4	4	2 months	8	8	6	2	6	6	2.5	6
SARA 1	14	3	3	0	2	10	18	15	10	8	11	7	7	4
SARA 2	14	3	3	0	5		21	16	8	6	15	6	5	5

Discussion:

NPC is a fatal neurovisceral lysosomal disease with a wide clinical spectrum, varying from rapidly progressive fatal neonatal forms to slowly progressive late forms with progressive neurological and psychiatric symptoms (Vanier MT 2010).

The present study aims to present the genotypic and phenotypic characteristics of the 21 patients with this lysosomal disease diagnosed so far in Bulgaria.

The prevalence of NPC in Bulgaria is estimated to 0.3 cases per 100,000, which is slightly lower than 0.35-2.2/100,000, which was found in different countries (Burton BK et al. 2021; Labrecque M et al. 2021). Epidemiological studies conducted in diverse countries found the following incidence- 0.47/100,000 for Australia, 0.91/100,000 for the Czech Republic, 2.2/100,000 for Northern Portugal, 0.35/100,000 for the Netherlands, 0.82/100,000 for France and 0.78 /100,000 for Great Britain (Labrecque M et al. 2021). Probably the lower prevalence in our country is the result of the insufficient awareness of the disease, the broad phenotype and the late diagnosis. According to the available data from the international registry for NPC, childhood-onset forms account for about 2/3 of cases worldwide (Vanier M 2010). In the Bulgarian cohort, however, 81% of patients had an onset in adolescence or adulthood. One possible explanations for these differences is the specific mutations observed in our population. On the other hand, the late onset form has predominant psychiatric symptoms and it is possible that the real incidence is higher than reported so far (Wassif CA et al. 2016). The clinical phenotype is extremely heterogeneous, even among patients carrying the same mutations within the same family (Vanier M 2010; Benussi A et al. 2015; Walterfang M et al. 2009). Neurological, psychiatric and visceral manifestations can occur at any age, as was found in the Bulgarian cohort, where the leading initial manifestations were neurological. Despite the variable involvement of the central nervous system, typical features such as vertical gaze paresis, ataxia, dysarthria, cognitive deficits and pyramidal involvement were present in a large proportion of patients with varying degrees of severity at the onset of the disease. Vertical gaze palsy, considered the halmark symptom of the disease (Vanier M 2010), is found in 100% of the patients. It is interesting that in 4 patients it preceded the onset of ataxia, which is not typical.

Similar to other studies and in our cohort, epileptic seizures and cataplexy were more typical in childhood forms of the disease, while dystonia and cognitive decline- in adolescent and late-onset forms (Vanier M 2010; Yanjanin NM et al. 2010; Mengel E et al. 2013). Sensorineural hearing loss was found in 61% of the Bulgarian patients, which is more frequent compared to various reports (Yanjanin NM et al. 2010; Mengel E et al. 2013).

Cholesterol is known to play an important role in the physiology of the auditory system (Purcell EK et al. 2011), so its impaired transport probably affects the processes of sound reception and transmission in the longer course of the disease in patients with juvenile and late onset forms of the disease. This symptom is important because Miglustat is also known to cause hearing loss.

Brain MRI of the Bulgarian cohort revealed cerebellar and cerebellar atrophy in combination with hyperintense lesions in the cerebral white matter - from single small to confluent with involvement of the brainstem structures, which is typical of the disease (Walterfang M et al. 2009; Huang JY et al. . 2011). Brain MRI is an important modality in patients with ataxia, epilepsy, and dementia, but is not specific for NPC.

Visceral involvement was symptomatic in only one patient with early infantile onset, while in 4/21 with onset in adolescence and adulthood, organomegaly was absent at all, which is consistent with the previous reports (Vanier M 2010).

The genotype of Bulgarian patients with NPC is very broad, with 15 different mutations found in 20 patients. p.Asn169Ile was the most common mutation, found in 23% of mutated alleles. It occured only in patients of the Roma ethnicity, so we can speculate it has a founder effect. In homozygous state, it leads to a more benign phenotype. The second most frequent mutation was p.Ser940Leu, accounting for 15% of the mutant alleles, and it was found in patients of various ethnic groups. The remaining mutations account for 2.5-12.5% of the mutant alleles, which confirms the great genetic heterogeneity in our group of patients.

The delay in diagnosis in our cohort was 4.5 +/-4.90, being greater in the late onset forms (1-23 years), possibly due to the wider differential diagnosis and slow clinical course. Early diagnosis is of particular importance because of the available therapeutic options, which are more effective when started timely. Miglustat makes it possible to stabilize and/or slow down the progression of the ataxia (Wraith JE et al. 2010; Vanier MT et al. 2015; Patterson MC et al. 2012), which is also proven during the follow-up of the Bulgarian patients.

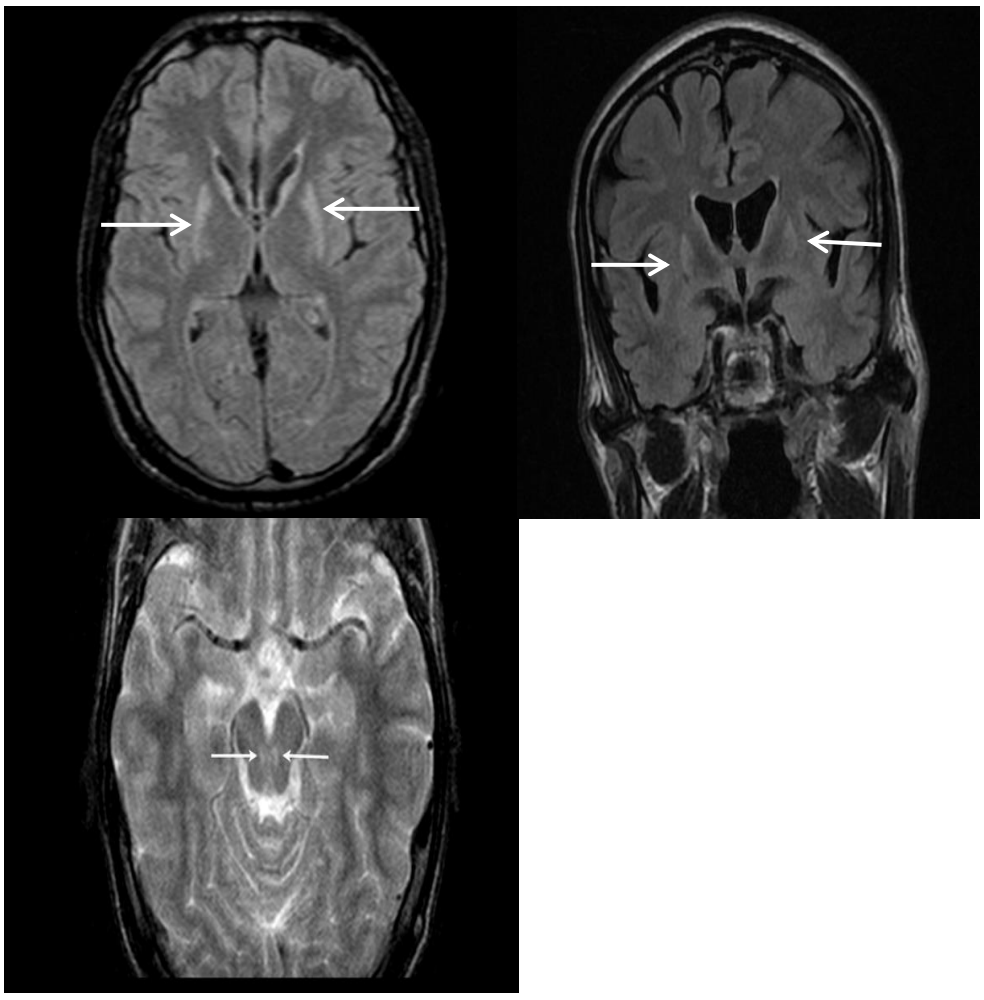
3.2.13. **Tremor-ataxia with central hypomyelination (Hypomelinating leukodystrophy 7- HLD7)**

Three male patients belonging to 2 unrelated Roma pedigrees were diagnosed with this syndrome caused by mutations in the *POLR3A* gene in Bulgaria. The patients were born from uneventful pregnancies and deliveries. They had normal psychomotor development, and all received primary education. Their clinical characteristics are summarized in Table 21.

Table 21. Clinical characteristics of the Bulgarian patients with mutations in the POLR3A gene

Patients	**ShII-1**	**IzII-1**	**IzII-2**
Year of birth	1959	1985	1989
Sex	M	M	M
Age at onset	8	7	8
Age at assessment	52	27	23
Upper Motor Neuron Signs			
Muscle weakness	_	_	_
Spasticity	_	_	_
Brisk tendon reflexes	Upper limbs	_	_
Babinsky sign	+	+	+
Extrapyramidal signs			
Rigidity	LL, R wrist	LL	_
Dystonia	R hand, LL	LL predominantly R	Distal LL
Tremor	_	_	_
Ataxia (SARA scores)*			
Gait (0-8)	6	5	2
Stance (0-6)	5	4	1
Sitting (0-4)	3	1	0
Speech (0- 6)**	5	5	3
Total score (0-40)	24	15	6
Dysphagia	_	+	+

Gaze-evoked nystagmus	+	–	–
Abnormal Smooth Pursuits	+	–	–
IQ	95	85	80
Neuroimaging			
Atrophy of striatal structures	+	+	+
Signal changes in red nucleus	+	+	+
Limb deformities	_	Bilateral pes cavus	Bilateral pes cavus
Hypodontia	_	_	_
Hypogonadotropic Hypogonadism	–	–	–



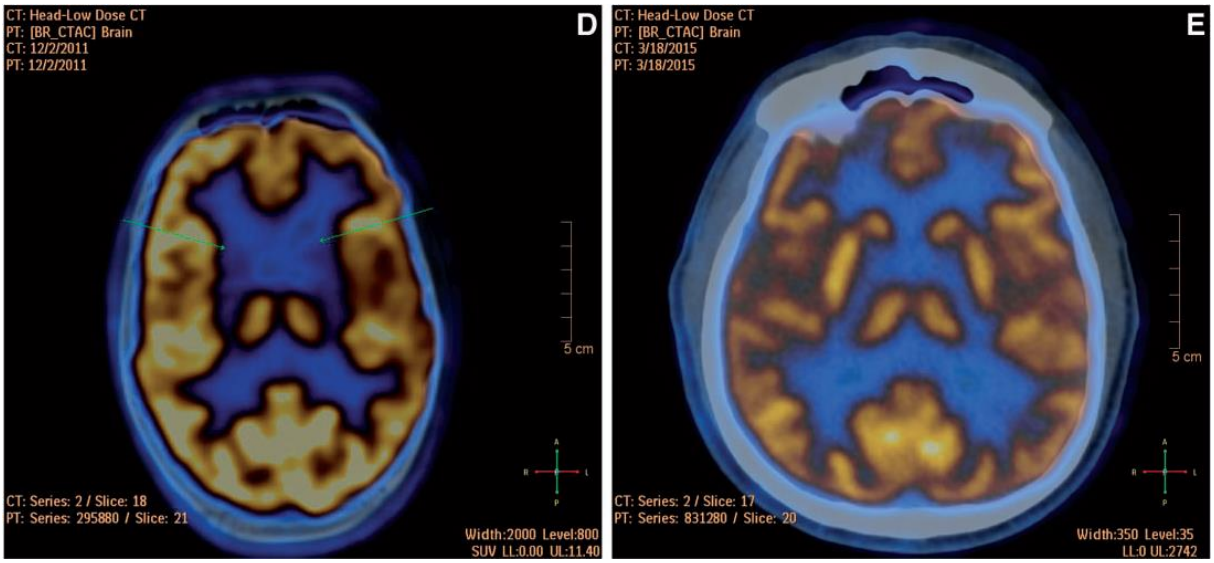


Figure 37. Neuroimaging studies in patient #2.

A. AXIAL T2 FLAIR with bilateral symmetrical atrophy of nucl. caudatus and putamen (arrows)

B. CORONAL T2 FLAIR with bilateral symmetrical atrophy of nucl. caudatus and putamen (arrows)

C. AXIAL TSE T2- in the area of the nucl. ruber and the intraaxial part of n. oculomotorius hyperintensity was detected (white arrows)

D. FDG PET-CT with marked bilateral hypometabolism in the striatum compared to age- and sex-matched healthy control (E)

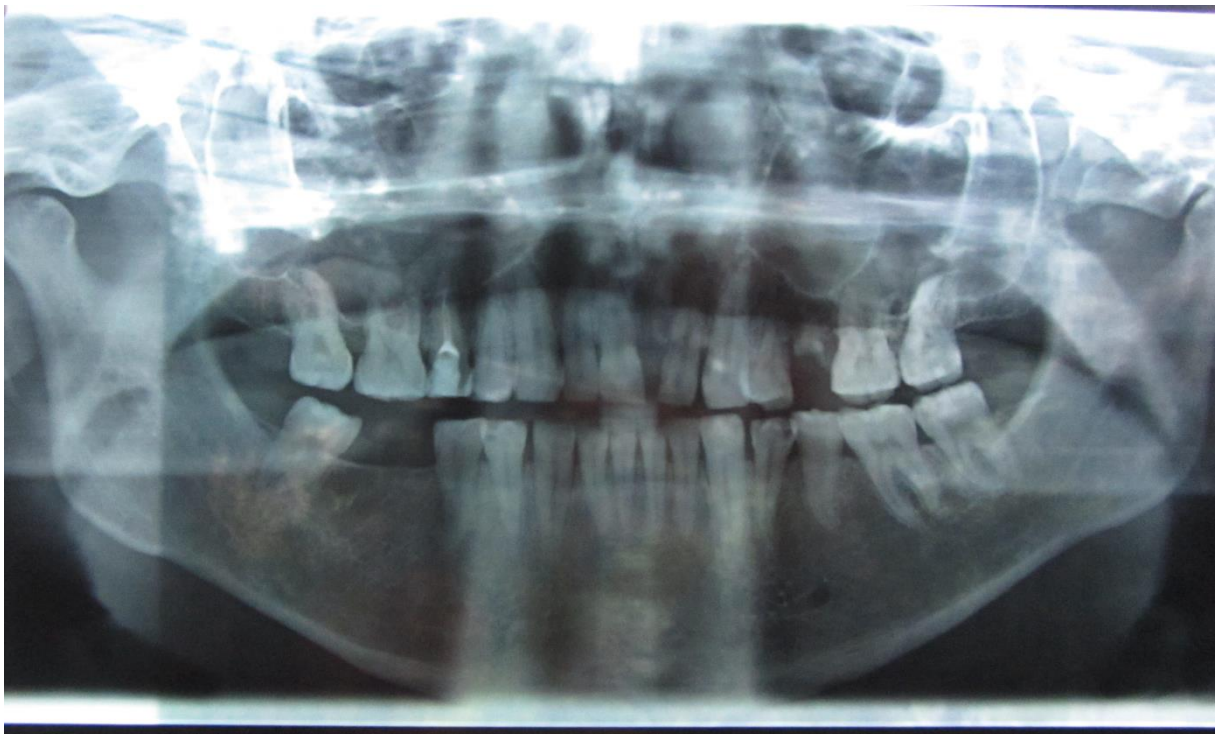


Figure 38. Orthopantomography of patient #3, without evidence of oligodontia.

Table 22. Neuropsychological assessment in the Bulgarian patients with mutations in *POLR3A*. The obtained results were compared with healthy controls from the Roma ethnic group matched by sex, age and education.

Individuals	ShII-1	IzII-1	IzII-2	Controls N=10 Mean values (SD)
IQ (Raven progressive matrices)	95	85	80	
MMSE	28	27	27	27.6 (1.42)
Digit span				
Forward	5	4	4	4.5 (0.85)
Backward	4	3	2	3.2 (1.14)
Verbal fluency				
Semantic "animals"	10	13	12	11.6 (1.71)
Phonemic "M"	5	4	5	5.1 (1.0)
Rey verbal learning test				
Immediate recall	43/75	36/75	34/75	38.0 (3.89)
Delayed recall	8/15	7/15	5/15	5.6 (1.26)
Recognition	30/30	28/30	26/30	26.8 (2.35)
Tower of London				
Total move score	31	34	41	33.0 (2.7)
Total correct scores	6	5	5	4.9 (1.8)

The WES (figure 39) revealed a genetic defect in the homozygous state NC_000010.10: g.79769439G>C; NM_007055.3: c.1771-6C>G located in intron 13 of the *POLR3A* gene. Sanger sequencing proved the presence and segregation of this variant. This mutation is novel, undescribed up to now (Figure 40).

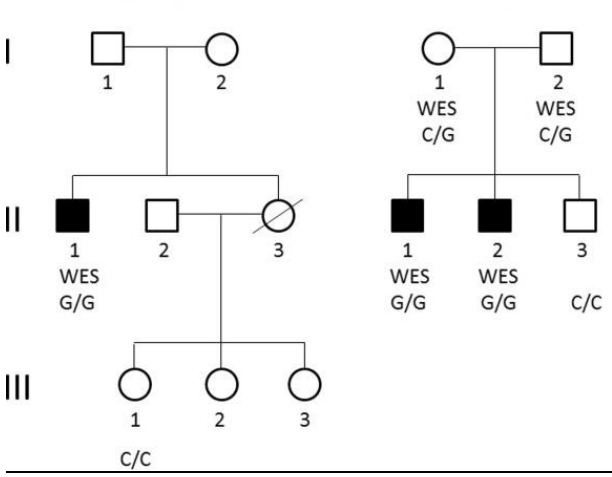


Figure 39. Pedigrees of the Bulgarian patients wit *POLR3A*-mutations (c.1771-6C>G) The individuals, who underwent WES are indicated.

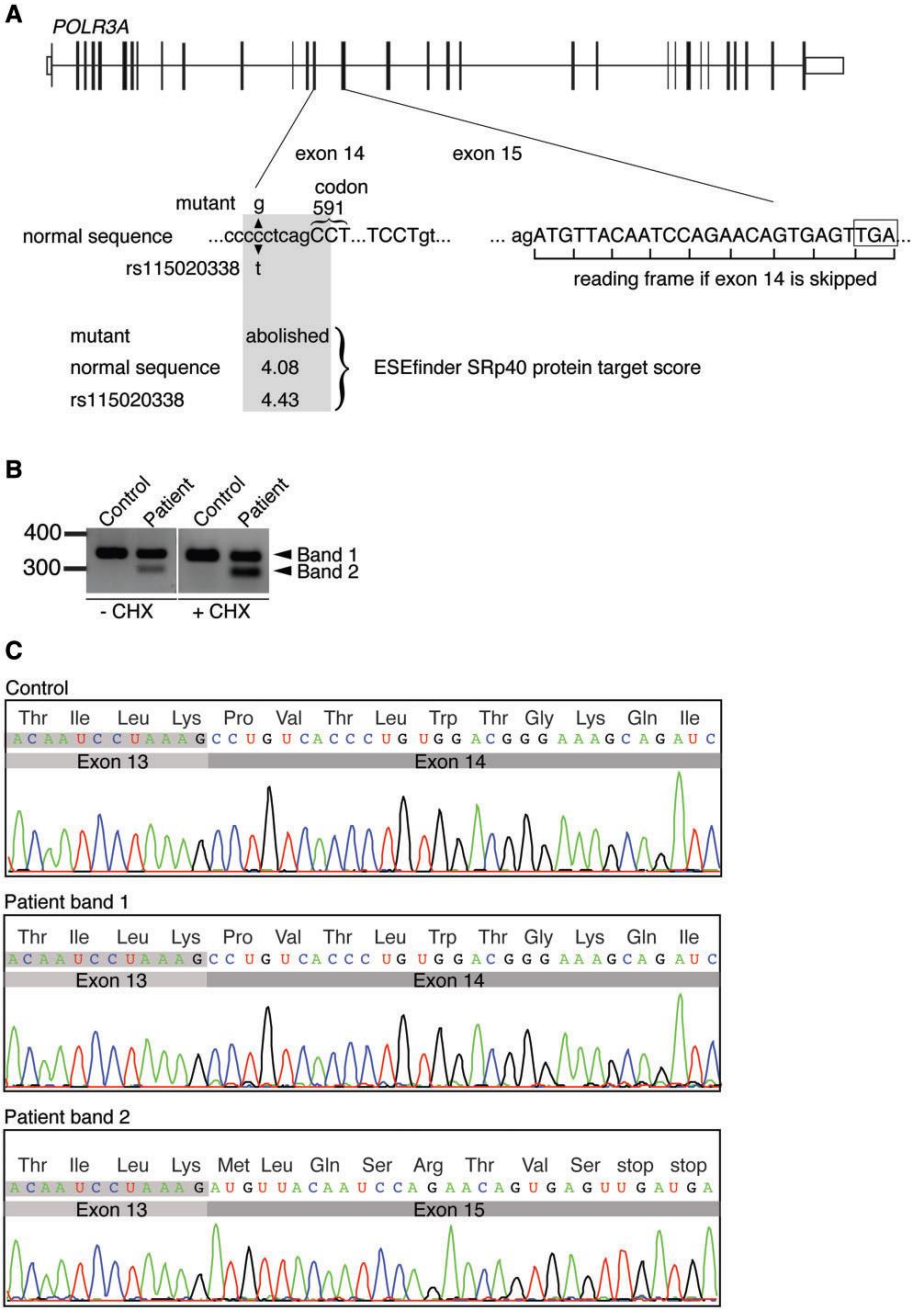


Figure 40. Mutation in *POLR3A* results in a splicing defect.

A. Schematic representation of the predicted functional effect of *POLR3A* c.1771-6C>G mutation

B. RT-PCR with primers in exons 13 and 15 detect exon skipping in fibroblasts

C. Sanger sequencing confirms the occurrence of a stop codon.

The c.1771-6C>G is a mutation located 6 nucleotides from the intron 13/exon 14 boundary of the *POLR3A* gene (Figure 40). This gene defect leads to a 37-63% decrease in the concentration of the POLR3A transcript, which causes a partial deficiency of polymerase III (Pol III), and thus reduced function. mRNA and ncRNA concentrations were decreased. A panel of 703 DNA of healthy Roma controls was evaluated, 202 of whom belonged to the Wallachian group. Ten heterozygous carriers of this gene defect were found among the Wallachian Roma. The carrier rate among Roma was estimated at 1.4%, and among

Wallachian Roma - close to 5%, which is similar to observations for other AR diseases in this population (Kalaydjieva L et al. 2014).

Discussion:

Mutations in the *POLR3A* and *POLR3B* genes, encoding the largest subunits of DNA-dependent RNA polymerase III, lead to a group of AR diseases united under the name hypomyelinating leukodystrophy 7 (Bernard G et al. 2011). Phenotypic heterogeneity in this group of diseases is associated not only with the presence or absence of dental abnormalities and hormonal dysregulation, but also with regard to white matter and cerebellar involvement (Bernard G et al. 2011; Takanashi JI et al. 2014).

In our group, where a novel c.1771-6C>G mutation in *POLR3A* was found in the homozygous state, MRI did not detect white matter lesions, thinning of the corpus callosum, cerebellar or brainstem atrophy. Atypical changes affecting the striatum and nucl. ruber were observed, correlating with the presence of extrapyramidal symptoms.

In our cohort, some clinical features also described in hypomyelinating leukodystrophy 7 were present, but their pathogenesis seamed different. Dysphagia and dysarthria in HLD7 are the result of bulbar weakness (Bernard G et al. 2011), while in the Bulgarian patients dystonia of the laryngeal and pharyngeal muscles is the leading cause. Ataxia in HDL7 is caused by cerebellar atrophy and lesions in the cerebral white matter, while in our patients it is most likely due to the involvement of the dorsal mesencephalon (Bernard G et al. 2011). Therefore, POLR3 dysfunction accounts for a wider clinical spectrum than the one described so far.

Functional studies performed to determine the effect of the mutation on Pol III expression and activity, by isolating RNA from the patients, found a decrease in mRNA and ncRNA. In this way, impaired RNA metabolism leads to impairment of various metabolic pathways and neurodegeneration (Thiffault I et al. 2015). Of course, whether Pol III expression in brain is consistent with that in the peripheral blood needs further investigation.

3.2.14. Boucher-Neuhäuser syndrome

Two patients with Boucher-Neuhäuser syndrome have been diagnosed in Bulgaria.

Patient #1 is a 30-year-old woman born from uneventful normal first pregnancy and delivery, with normal early motor and neuropsychological development, whose complaints date back to adolescence, when, due to amenorrhea, hypogonadotropic hypogonadism was diagnosed and hormonal therapy was initiated. From the age of 19 years, when visual disturbances appeared, more pronounced in the evening hours. Retinitis pigmentosa was then diagnosed. From the age of 21 years, staggering in an undetermined direction appeared, which progressed very slowly over time. She reported no family history.

From the neurological examination, there are:

- Ataxia of stance and gait. There is dynamic ataxia with mild intention tremor and dysmetria and dysdiadochokinesia more pronounced in left limbs. Muscle tone is reduced. The patient had horizontal nystagmus (more pronounced when looking to the right) and saccadic eye movements.

- Pyramidal signs with positive pathological reflexes of Babinski and Chadok bilaterally
- Polyneuropathy with absent Achilles and knee and attenuated styloradial reflexes.

Over the years, multiple NCS have been performed finding mild axonal damage of the sensory and motor fibers in the lower limbs. Brain MRI was performed three times (in 2011; 2017; 2019) and showed evidence of cerebellar atrophy, with reduced volume of the vermis and both cerebellar hemispheres, enlarged intrafoliar sulci and fourth ventricle, cistern magna permagna (Figure 41).

The neuro-ophthalmological examination found:

• VOD=0.7-0.8; VOS=0.7

• Anterior eye segment: nonhomogeneous cortical opacities of the lens.

• Tunnel visual field was observed on computer perimetry

• Fundus: Papillae - vital, with clear outlines. Vessels - diffusely slightly narrowed. Retinas- mid-periphery- scattered pigment clusters, RPE atrophy, clear choroid.

In conclusion: Retino-choroid dystrophy.

The patient has normal cognitive functioning.

By whole exome sequencing (WES), two known heterozygous genetic variants in the *PNPLA6* gene were identified: NM_001166114:c.1883A>G/p.Tyr604Cys (rs1282912583) in exon 14 and NM_001166114:c.3589C>T / p.Arg1173Trp in exon 28 (rs766389806). Both variants involve evolutionarily conserved amino acids and are present at very low frequency in the databases, the latter being found in a patient with spastic paraplegia. Family carrier analysis shows that the two variants were inherited from each parent, and the patient's clinically healthy brother did not carry any of the genetic variants. The patient underwent in vitro fertilization with the subsequent birth of a normal and healthy girl.

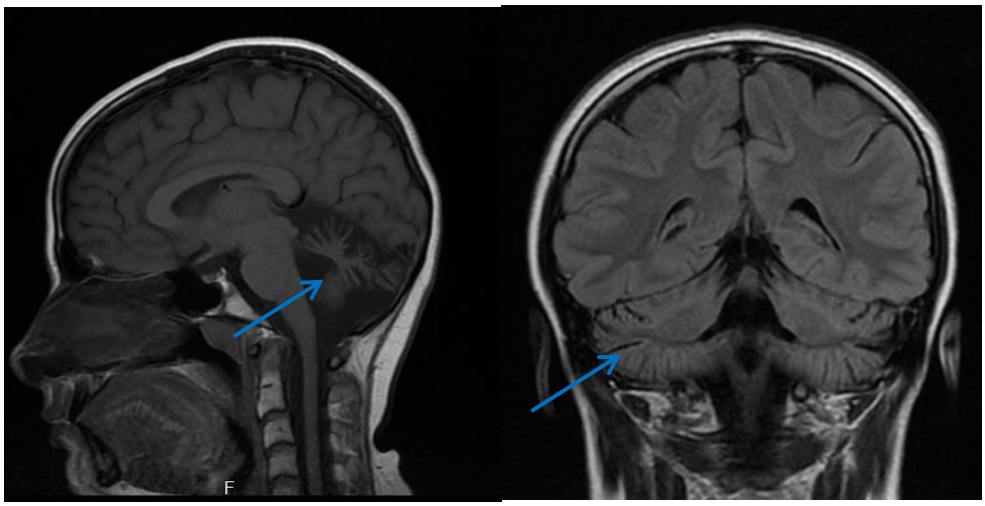


Figure 41. Brain MRI of patient #1 with Boucher-Neuhäuser syndrome. 1A T1, SAG and 1B. T2, COR FLAIR. There is evidence of cerebellar atrophy (blue arrow).

Patient #2 is a 40-year-old woman born from a second uneventful pregnancy and delivery. At the age of 8 years, about a year after experiencing viral meningitis, she started having weakness in her left leg. The patient was diagnosed with amenorrhea and menstruation was hormonally stimulated, then she was diagnosed with hypogonadotropic hypogonadism. Since the age of 23 years, her started having blurred vision, her gait and balance were disturbed - she staggered while walking, her right hand began to tremble. Gradually, the speech also changed, became dysarthric. In the last 2 years, her complaints seem stable. There is a family history for an older sister with similar complaints of impaired balance since age 25 years, who has started to walk with a cane since the age of 31 years and currently has memory impairment and difficulty in swallowing.

From the neurological examination, there are:

• Static, locomotor and dynamic ataxia. The Romberg was negative. There was dysmetria in the finger to nose and heel toshin tests, more pronounced on the right, wit additional dysdiadochokinesia. Horizontal-rotator nystagmus, more pronounced when looking to the left, slurred speech, intermittent postural and intentional tremor, muscle hypotonia in the upper limbs were found.

• Pyramidal involvement with brisk tendon reflexes in the lower limbs, polykinetic knee reflexes, brisk Achilles reflex on the left, clonusoid on the left foot, pathological Babinski sign on the right and slightly spastically increased muscle tone in the lower limbs.

- Polyneuropathy with paresthesias and hypesthesia for superficial sensation in the four limbs.

The NCS revealed mild axonal polyneuropathy with reduced SNAPs of nn. surales and CMAP from nn. peronei. Brain MRI showed cerebellar atrophy and reduced size of the pituitary gland.

The neuroophthalmological examination revealed:

• VOD=0.5; VOC=0.5

• Anterior eye segment: normal.

• Oculomotion was preserved, with bilateral gaze-evoked nystagmus in the horizontal direction.

- The papillae were pale, with clear outlines, with E=0.3 PD. The vessels had a normal course and lumen. Macules – RPE atrophy, clear choroid, pigment dispersion.

  In conclusion: Retinochoroidal dystrophy, with predominantly macular involvement.

  Cognitive functioning was within the normal range.

WES found two heterozygous genetic variants in the *PNPLA6* gene. One of them was novel, previously undescribed variant NM_001166114: c.888_889insACCGTGT / p.Ser272fs in exon 7 associated with a frameshift. The second variant NM_001166114: c.3004G>A / p.Gly1002Ser in exon 24 (rs772547801), was known and resulted in an amino acid substitution in an evolutionarily conserved consensus serine-lipase motif of the protein. These two variants are also present in the patient's older sister, who has the same complaints. The clinically healthy mother of the two sisters carried the variant c.888_889insACCGTGT / p.Ser272fs, but does not carry the other variant c.3004G>A / p.Gly1002Ser. It is assumed that the second variant was inherited by the father, who, according to the mother, had no complaints, but biological material from him was not available for genetic testing.

Discussion:

The two presented cases have the classic phenotypic triad - ataxia, visual disturbances, hypogonadotropic hypogonadism with variable age of onset and different severity at the time of diagnosis. In our patients, there was a slight pyramidal involvement, but due to the chorioretinal involvement, they belong to the group of Boucher-Neuhäuser syndrome (Synofzik M et al. 2014). In the presented cases, clinical and electroneurographic data of mild peripheral nerve involvement of axonal type were observed, which is also considered a hallmark feature of the disease (Deik A et al. 2014; Hufnagel RB et al. 2015; Synofzik M et al. 2014). No paresis were observed. In both patients, primary amenorrhea, diagnosed during adolescence, led to the diagnosis of hypogonadotropic hypogonadism, necessitating hormonal treatment. In patient 1, the clinical symptoms appeared relatively late - the visual disturbances in the second decade and the ataxia in the third one, which is probably related to the functional effect of the mutations in *PNPLA6* (Deik A et al. 2014). Both patients have chorio-retinal dystrophy with different severity.

Molecular genetic testing revealed the cause of the disease in both families- different mutations in the *PNPLA6* gene (Richards S et al. 2015). Boucher-Neuhäuser syndrome should be suspected in patients with a combination of ataxia, visual disturbances, hypogonadotropic hypogonadism.

4. Conclusions

1. SCA1, followed by SCA2 are the most common forms of autosomal dominant cerebellar ataxias in Bulgaria. Both are characterized by a combination of the ataxia, pyramidal involvement and polyneuropathy. Anticipation in every subsequent generation is typical for both forms.
2. Friedreich ataxia turned out to be the most common form of the group of AR cerebellar ataxias. In addition to the classic phenotype with an onset before the age of 25 years, a late form with an onset after the age of 30 was described, without visceral involvement.
3. The most consistent electroneurographic finding in Friedreich ataxia is the absence of SNAP for n. suralis, as well as reduced or absent SNAP of n. medianus and n. ulnaris
4. Ataxia-telangiectasia-variant is a disease caused by mutations in the *ATM* gene, where dystonic hyperkinesias and tremor with variable age at onset are the leading manifestations. In Bulgaria, a high prevalence of mutation p.V2716A was found in *ATM* among Bulgarian muslims in the area of the towns of Dospat and Sarnitsa. The phenotype of Bulgarian patients is not associated with immune deficiency, increased risk of tumors and infertility.
5. AR forms of ataxia are more frequent among the Roma groups, including DES, ARCA3, POLR3A, Niemann Pick type B disease and SCAR13.
6. Mutations in the VLDLR-gene lead to DES. In Bulgaria, an inbred Roma family was identified, clinically characterized by retardation in neuropsychological development, moderate to severe mental retardation, non-progressive severe ataxia, nystagmus and pyramidal signs. On brain MRI the main features are reduced brain size, pachygyria

and severe hypoplasia of the cerebellum and pons. The disease is caused by a novel mutation p.Asp487Tyr in *VLDLR*-gene.

7. ARSACS was diagnosed in four Bulgarian patients with a variable clinical onset from infancy to adolescence, with the typical clinical triad (ataxia, pyramidal involvement and polyneuropathy). All patients had the hallmark characteristics of the brain MRI - linear T2 hypointense lesions in the pons, diffuse hyperintense lesions in the junction of the pons with the middle cerebellar peduncle and atrophy of the upper part of the cerebellar vermis. RNFL thickening assessed by OCT was present in 75% of Bulgarian patients.
8. ARCA3 is a rare form of AR ataxia, described only among Roma in Bulgaria, clinically characterized by clinical onset in adolescence, ataxia with pronounced cerebellar atrophy, downbeat nystagmus and dysarthria. Pyramidal involvement with evidence of pathologically increased tendon reflexes were also present. The disease has a relatively slow progression.
9. The clinical features of SCAR13 can be summarized as neuropsychological retardation, infantile with moderate to severe ataxia of stance and gait, dysarthria, mild dysdiadochokinesia and dysmetria, variable degree of intellectual deficit and mild pyramidal signs. Neuroophthalmologic manifestations were very typical, encompassing gaze-evoked nystagmus, hypometric saccades, abduction deficit, strabismus and ptosis. Neuroimaging studies showed moderate to marked cerebellar atrophy with or without hypoplasia of the lower part of the vermis with no correlation between the severity of atrophy and ataxia. This disease results from mutations in the *GRM1* gene, highly expressed in Purkinjie cells.
10. Niemann Pick C disease has a prevalence of 0.3/100,000 in Bulgaria, which is caused by a wide variety of mutations in the *NPC1* gene and is characterized by a combination of neurological, psychiatric and visceral manifestations. Early diagnosis of the disease is important due to the possibility of pathogenetic treatment.
11. Mutations in *POLR3A* cause a wide range of clinical manifestations, and to the typical features of hypomyelination, hypogonadotropic hypogonadism and hypodontia, extrapyramidal manifestations related to disturbance of the structure and function of the striatum and nucl.ruber are included.

5. Contributions

Scientific and theoretical

1. A novel p.Asp487Tyr mutation in the *VLDLR*-gene has been identified, leading to DES. Main clinical features are neuropsychological retardation, moderate to severe mental retardation, non-progressive severe ataxia, nystagmus and pyramidal signs. Brain MRI revealed reduced brain size, pachygyria and severe hypoplasia of the cerebellum and pons.
2. A new gene - *GRM1* has been discovered. Mutations in this gene cause congenital ataxia, known as SCAR13. Its phenotype was described for the first time.
3. A new mutation c.1771-6C>G in the *POLR3A* gene was identified, which is associated with a novel phenotype within tremor-ataxia with central hypomyelination

(Hypomelinating leukodystrophy 7- HLD7), including ataxia, extrapyramidal syndrome, dysarthria, dysphagia and atrophy of the striatum, nucl. rubber and the dorsal mesencephalon.

Scientific and applied

4. The prevalence of different forms of cerebellar degeneration in the Bulgarian population – has been analyzed.
5. Specificities in prevalence among different ethnic groups have been established, which will optimize the algorithm for the genetic testing of new cases with characteristic phenotypes.
6. A typical mutation p.V2716A in the *ATM*-gene has been found among Bulgarian muslims, causing a specific phenotype (AT-variant) with predominance of extrapyramidal manifestations, and the carrier frequency was estimated. This allows genetic counseling and follow-up of the affected families with a pseudodominant type of inheritance.
7. The effect of the athogeteci therapy in patients with NPS has been analyzed.

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8. Summary

Cerebellar ataxias represent a group of disorders with heterogeneous clinical presentation. Patients may exhibit a pure cerebellar phenotype or various combinations of cerebellar deficits and extracerebellar signs, such as pigmentary retinopathy, extrapyramidal movement disorders, pyramidal signs, cortical symptoms (seizures, cognitive impairment/behavioural symptoms) and peripheral neuropathy.

The aims of the current study is to unravel the different forms of hereditary ataxias in Bulgaria, as well as to define their epidemiology and genotype-phenotype correlations.

The most common autosomal dominant types in Bulgaria are spinocerebellar ataxias (SCA) type 1 and 2, characterized by a complex phenotype, where ataxia is combined with pyramidal, extrapyramidal, peripheral nerve involvement and cognitive impairment. The most frequent autosomal recessive (AR) forms are Friedreich ataxia and Niemann Pick C disease.

AR forms are quite frequent in ethnic or religious minorities with limited genetic diversity.

Ataxia teleangiectasia- variant is a common form in a group of Bulgarian Muslims, residing in the regions of Dospat and Surnitza. It is mainly due to a homozygous mutation c.8147T>C (p.V2716A) in *ATM* gene with a carrier rate of 1% in the above mentioned population. The condition is milder, compared to the classical form and is characterized by tremor and dystonia, predominantly affecting the neck and the upper limbs.

Among the Gypsy/Roma disequllibrium syndrome (DES), ARCA3, *POLR3A*-related disorder were found in the Vlax group, while Niemann Pick B and SCAR13 were typical for the Muslim Roma.

Mutations in *VLDLR*-gene are causing DES. The first Bulgarian patients with DES were reported in this study. They were carrying a novel homozygous mutation, p.Asp487Tyr in *VLDLR*, resulting in a clinical phenotype, encompassing global developmental delay, moderate to severe intellectual deficit, nonprogressive severe truncal ataxia, dysarthric speech, gaze-evoked nystagmus, mild intentional tremor, and pyramidal signs. Neuroimaging showed global small brain, pontocerebellar hypoplasia, and mild to moderate cortical thickening with gyral simplification more pronounced in the frontal and temporal regions.

We report a Gypsy family with autosomal recessive ataxia caused by the same truncating *ANO10* defect, confirming p.Leu384fs as another founder mutation in this population, resulting in clinical onset during childhood/adolescence, slow progression, cerebellar ataxia, pyramidal features and downbeat nystagmus.

Mutations in a novel gene, GRM1, encoding metabotropic glutamate receptor mGluR1, which is highly expressed in cerebellar Purkinje cells and plays an important role in cerebellar development and synaptic plasticity, were described for the first time in human patients. The two mutations affect a gene region critical for alternative splicing and the generation of receptor isoforms (c.2652_2654del and c.2660þ2T>G, respectively). Patients presented with global developmental delay, moderate to severe stance and gait ataxia, dysarthria, mild dysdiadochokinesia, dysmetria and tremors, intellectual deficit, and mild pyramidal signs. Brain imaging revealed progressive generalized cerebellar atrophy, and inferior vermian hypoplasia and/or a constitutionally small brain were observed in some patients. This nover form of congenital ataxia is known as SCAR13.

Niemann Pick type C disease has a prevalence of 0.3/100 000 in Bulgaria and it is caused by a big variety of mutations in *NPC1* gene. The condition is complex, encompassing neurological, psychiatric and visceral involvement. Its early diagnosis is crucial, because there is a pathogenetic treatment available.

A novel and founder mutation c.1771-6C>G in the *POLR3A* gene was described in patients of Roma ethnicity who presented with an unusual clinical and neuroimaging phenotype. These patients showed no MRI evidence of white matter involvement, and did not replicate previous findings of thinned corpus callosum, vermian cerebellar atrophy, prominent cerebellar folia and brainstem atrophy. Instead, they presented with striatal and
red nucleus involvement and clinical manifestations (ataxia, dystonia, pyramidal features and dysphagia), corresponding to the unusual localization of the pathological changes.

In conclusion the number of cerebellar ataxias is still growing as a result of the development of new genetic technologies. The precision of the phenotype is crucial for further diagnosis, treatment and prophylaxis of these various conditions.