

# Bulgarian mutation spectrum in Cadasil: Our experience

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## ABSTRACT

### Introduction

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL, OMIM 125310), is a hereditary autosomal dominant disease, caused by mutations in the NOTCH3 gene. The aim of this study was to describe our experience with the molecular diagnostics of CADASIL in a group of Bulgarian patients in the period of 2015-2020.

### Materials and Methods

Screening for germline mutations was performed by Sanger sequencing of the NOTCH3 gene in eleven subjects provided written and signed informed consent forms. Mutation negative patients were further subjected to multiplex ligation-dependent probe amplification (MLPA) to detect deletions/duplications in the NOTCH3 gene.

### Results

We present eleven Bulgarian patients (3 males and 9 females, median age 49.7 years) exhibiting clinical symptoms of possible CADASIL, referred to our laboratory for genetic testing of the NOTCH3 gene in the period of 2015-2020. Sequencing revealed missense mutations in five patients (one male and four females) following the typical pattern of cysteine-altering mutations in CADASIL. Four of the mutations have been previously described in the literature. One patient presented with a novel mutation, classified as “likely pathogenic” by the criteria of the ACMG. No large deletions or duplications were detected by the use of MLPA analysis.

### Conclusions

This study shows the need for future research to clarify the genotype-phenotype correlation between pathological variants in the NOTCH3 gene and the clinical manifestation of the disease, which would greatly improve current diagnostic and treatment guidelines for patients with CADASIL.

**KEYWORDS:** Bulgarian population, CADASIL, NOTCH3, Sanger sequencing

## SAŽETAK:

SPEKTAR BUGARSKIH MUTACIJA U CADASILU: NAŠE ISKUSTVO

### Uvod

Cerebralna autosomno dominantna arteriopatija s subkortikalnim infarktom i leukoencefalopatijom (CADASIL, OMIM 125310), nasljedna je autosomno dominantna bolest, uzrokovana mutacijama gena NOTCH3. Cilj ovog istraživanja bio je opisati naša iskustva s molekularnom dijagnostikom CADASIL-a u skupini bugarskih pacijenata u razdoblju 2015.-2020.

### Materijali i metode

Provjera mutacija provedena je Sangerovim sekvenciranjem gena NOTCH3 u jedanaest ispitanika pod uvjetom da imaju potpisane obrasce za informirani pristanak. Mutacijski negativni pacijenti su dalje

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podvrgnuti metoda višestrukog umnažanja vezanih sonda (MLPA) kako bi se otkrile delecije / duplikacije u genu NOTCH3.

### Rezultati

Predstavljamo jedanaest bugarskih pacijenata (3 muškarca i 9 žena, srednja dob 49,7 godina) koji pokazuju kliničke simptome mogućeg CADASIL-a, koji su upućeni u naš laboratorij za genetsko ispitivanje gena NOTCH3 u razdoblju 2015.-2020. Sekvencioniranjem su otkrivene pogrešne mutacije kod pet pacijenata (jedan muški i četiri ženske) slijedeći tipični obrazac mutacija koje mijenjaju cistein u CADASIL-u. Četiri od mutacija prethodno su opisane u literaturi. Jednom je pacijentu predstavljena nova mutacija, klasificirana kao "vjerojatno patogena" prema kriterijima ACMG. Korištenjem MLPA analize nisu otkrivena velika brisanja ili dupliciranja.

### Zaključci

Ova studija pokazuje potrebu za budućim istraživanjima kako bi se razjasnila korelacija genotip-fenotip između patoloških varijanti u genu NOTCH3 i kliničke manifestacije bolesti, što bi uvelike poboljšalo trenutne dijagnostičke i smjernice liječenja za pacijente s CADASIL-om.

**KLJUČNE RIJEČI:** bugarsko stanovništvo, CADASIL, NOTCH3, sekvencioniranje Sangera

## INTRODUCTION

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL, OMIM 125310), is a hereditary autosomal dominant disease, caused by mutations in the *NOTCH3* gene. Patients with this condition usually present with a history of migraines with aura, subcortical transient ischemic attacks (TIA), mood disorders, cognitive decline progressing to dementia, white matter lesions and encephalopathy<sup>1</sup> cognitive decline progressing to dementia, a history of migraine with aura, mood disturbance, apathy, and diffuse white matter lesions and subcortical infarcts on neuroimaging. The diagnosis of CADASIL is established in a proband either by identification of a heterozygous pathogenic variant in *NOTCH3* by molecular genetic testing or, if molecular genetic testing is not definitive, by detection of characteristic findings by electron microscopy and immunohistochemistry of a skin biopsy. Treatment of manifestations: There is no treatment of proven efficacy for CADASIL. Standard supportive treatment for stroke; the effect of thrombolytic therapy for the treatment of stroke remains unknown. Migraine should be treated symptomatically. Standard treatment for psychiatric disturbance. Supportive care (practical help, emotional support, and counseling.

The reported prevalence of CADASIL causing *NOTCH3* mutations is estimated to be between 1 and 9 per 100,000 people<sup>2,3</sup>. However, studies have shown that this number could be deflated based on the severity of the symptoms, with mutations causing milder phenotypes being as frequent as 1 in 300<sup>4</sup> thereby explaining an important part of CADASIL disease variability." "container-title": "Genetics in Medicine", "DOI": "10.1038/s41436-018-0088-3", "ISSN": "1530-0366", "issue": "3", "language": "en", "note": "number: 3\npublisher: Nature Publishing Group", "page": "676-

682", "source": "www.nature.com", "title": "The effect of NOTCH3 pathogenic variant position on CADASIL disease severity: NOTCH3 EGFr 1–6 pathogenic variant are associated with a more severe phenotype and lower survival compared with EGFr 7–34 pathogenic variant", "title-short": "The effect of NOTCH3 pathogenic variant position on CADASIL disease severity", "volume": "21", "author": [{"family": "Rutten", "given": "Julie W."}, {"family": "Van Eijsden", "given": "Bastian J."}, {"family": "Duering", "given": "Marco"}, {"family": "Jouvent", "given": "Eric"}, {"family": "Opher", "given": "Christian"}, {"family": "Pantoni", "given": "Leonardo"}, {"family": "Federico", "given": "Antonio"}, {"family": "Dichgans", "given": "Martin"}, {"family": "Markus", "given": "Hugh S."}, {"family": "Chabriat", "given": "Hugues"}, {"family": "Lesnik Oberstein", "given": "Saskia A. J."}], "issued": {"date-parts": [{"2019, 3}]}], "schema": "https://github.com/citation-style-language/schema/raw/master/csl-citation.json". The condition itself has been known and reported as early as 1977 as a form of vascular encephalopathy<sup>5</sup>. The first studies revealing the dominant inheritance of the disease came out in 1991<sup>6-8</sup> including transient ischemic attacks, and minor or major strokes starting between the fourth and sixth decades, with neuroimaging evidence of small, deep infarcts and a widespread white matter disorder. Other symptoms included migraine (three, followed by the first study describing mutations in *NOTCH3* as causative of CADASIL in 1996<sup>9</sup>). In the following years, genetic testing has allowed for the identification of a plethora of *NOTCH3* mutations with a yet to be fully explained effect on disease progression and severity. Because of this varied prevalence and non-uniform age of onset, current diagnostic criteria for CADASIL are based on a multidisciplinary approach. Specifically, a combination of the following symptoms is sought in the process of the differential diagnosis: unexplained cerebral ischemic events,

cognitive decline at a young age, brain MRI abnormalities and a family history of stroke or dementia with a dominant pattern of inheritance<sup>10</sup>. Neuroimaging methods (CT, MRI) can characterise the diffuse ischemic lesions of the white matter of the brain substance, subcortical lacunar infarctions and leukoencephalopathy. Lesions in the anterior temporal pole and in the external capsule have the highest diagnostic sensitivity and specificity (Fig 1). To confirm the diagnosis, either molecular testing<sup>9</sup> for the detection of a characteristic cysteine-altering mutation and/or electron microscope examination of a skin biopsy to confirm the presence of granular osmiophilic material (GOM) deposition around vascular smooth muscle cells are required<sup>11</sup>. Testing for GOM has a sensitivity of 45-96% and a specificity of 100% for the diagnosis of CADASIL<sup>12,13</sup>. Currently, the guidelines for the diagnosis and treatment of CADASIL do not cover the specific genotype-phenotype interaction in disease progression and management, however, there has been observable difference in the effect of *NOTCH3* mutations and the exhibited clinical symptoms<sup>4,14</sup> thereby explaining an important part of CADASIL disease variability.”, container-title:”Genetics in Medicine”, DOI:”10.1038/s41436-018-0088-3”, ISSN:”1530-0366”, issue:”3”, language:”en”, note:”number: 3\npublisher: Nature Publishing Group”, page:”676-682”, source:”www.nature.com”, title:”The effect of *NOTCH3* pathogenic variant position on CADASIL disease severity: *NOTCH3* EGFr 1–6 pathogenic variant are associated with a more severe phenotype and lower survival compared with EGFr 7–34 pathogenic variant”, title-short:”The effect of *NOTCH3* pathogenic variant position on CADASIL disease severity”, volume:”21”, author:[{”family:”Rutten”, given:”Julie W.”}, {”family:”Van Eijdsden”, given:”Bastian J.”}, {”family:”Duering”, given:”Marco”}, {”family:”Jouvent”, given:”Eric”}, {”family:”Opherk”, given:”Christian”}, {”family:”Pantoni”, given:”Leonardo”}, {”family:”Federico”, given:”Antonio”}, {”family:”Dichgans”, given:”Martin”}, {”family:”Markus”, given:”Hugh S.”}, {”family:”Chabriat”, given:”Hugues”}, {”family:”Le snik Oberstein”, given:”Saskia A. J.”}], issued:[{”date-parts:”[”2019”, 3]”}], {”id:”240”, uris:[{”http://zotero.org/users/5045968/items/CL5S9HYL”}], uri:[{”http://zotero.org/users/5045968/items/CL5S9HYL”}], itemData:[{”id:”240”, type:”article-journal”, abstract:”Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). To date, more than 200 CADASIL mutations have been reported<sup>10</sup>, with the majority localized in exons 2-24 of the gene. These exons encode for 34 EGF-like repeats (EGFr), which are essential for the *NOTCH3*<sup>ECD</sup> (extracellular domain). Each EGFr domain has 6 cysteine residues, forming 3 disulphide bridges; mutations which add or subtract a cysteine in one of the domains disrupt the 3-dimensional structure of the *NOTCH3*<sup>ECD</sup>, leading to protein aggregation and the typical CADASIL phenotype.

## AIM

In this study we aim to describe our experience with the molecular diagnostics of CADASIL in a group of Bulgarian patients in the period of 2015–2020.

## MATERIALS AND METHODS

We present eleven Bulgarian patients (3 male and 8 female) exhibiting clinical symptoms of possible CADASIL and referred to our laboratory for genetic testing. The median age of the patients at time of referral was 49.7 years. Written and signed informed consent was obtained from the patients. Genomic DNA was extracted from the peripheral white blood cells by standard protocols and direct sequencing of the *NOTCH3* gene was performed to screen for germline mutations. Primers were designed to specifically amplify all coding exons and exon–intron boundaries. The mRNA reference sequence was based on information available from RefSeq Homo sapiens *NOTCH3*, NM\_000435.3. Mutation negative patients were further subjected to multiplex ligation-dependent probe amplification (MLPA) to detect deletions/duplications in the *NOTCH3* gene. MLPA was performed using the commercially available SALSA MLPA P071-B1 probe mix (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer’s instructions<sup>15</sup> MSH2 and MLH1 genes, detection of trisomies such as Down’s syndrome, characterisation of chromosomal aberrations in cell lines and tumour samples and SNP/mutation detection. Relative quantification of mRNAs by MLPA will be described elsewhere. In MLPA, not sample nucleic acids but probes added to the samples are amplified and quantified. Amplification of probes by PCR depends on the presence of probe target sequences in the sample. Each probe consists of two oligonucleotides, one synthetic and one M13 derived, that hybridise to adjacent sites of the target sequence. Such hybridised probe oligonucleotides are ligated, permitting subsequent amplification. All ligated probes have identical end sequences, permitting simultaneous PCR amplification using only one primer pair. Each probe gives rise to an amplification product of unique size between 130 and 480 bp. Probe target sequences are small (50–70 nt. Data were analysed with Coffalyser. Net data analysis software<sup>16</sup>).

## RESULTS

Among the 11 referred patients, only 5 of them (one man and four women) showed typical neuroimaging results (Fig. 1). Direct sequencing methodology revealed 5 missense mutations in the *NOTCH3* gene (Table 1). In three of the patients, mutations were localized in exon 4, known for being a hot-spot for missense mutations. A single case (case number 4) presented with a missense mutation in exon 19. In case number 5, the *NOTCH3* analysis led to the identification of a heterozygous mutation in the exon 5 *NOTCH3* gene, NM\_000435:c.752G>C (p.Cys251Ser, Figure 2). This is a novel mutation, unreported in gnomAD v.2.1.1<sup>17</sup> whereas non-essential genes will tolerate

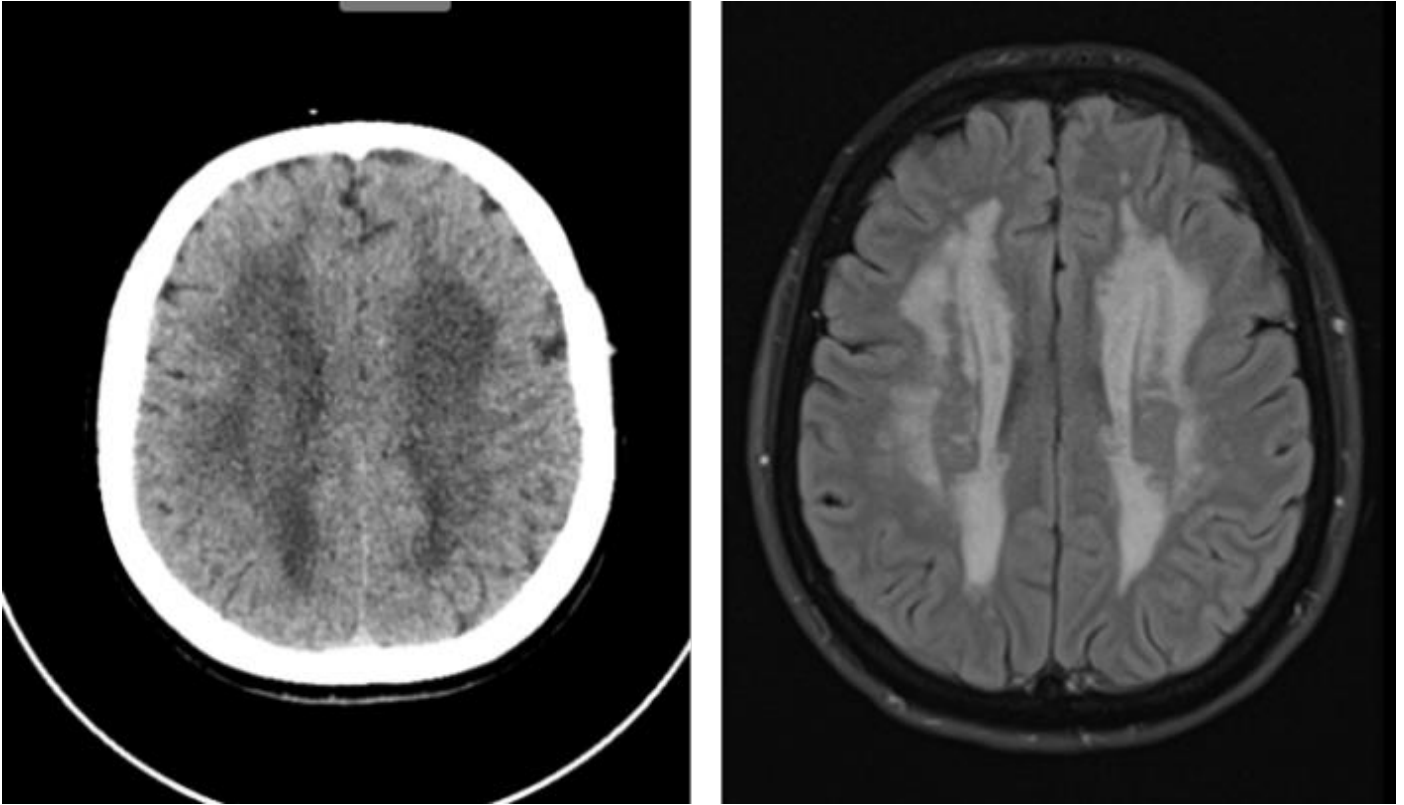


Figure 1. A typical CT/MRI imaging in a patient with CADASIL. High signal sections are visible bilaterally temporopolarly and in semioval centers (21).

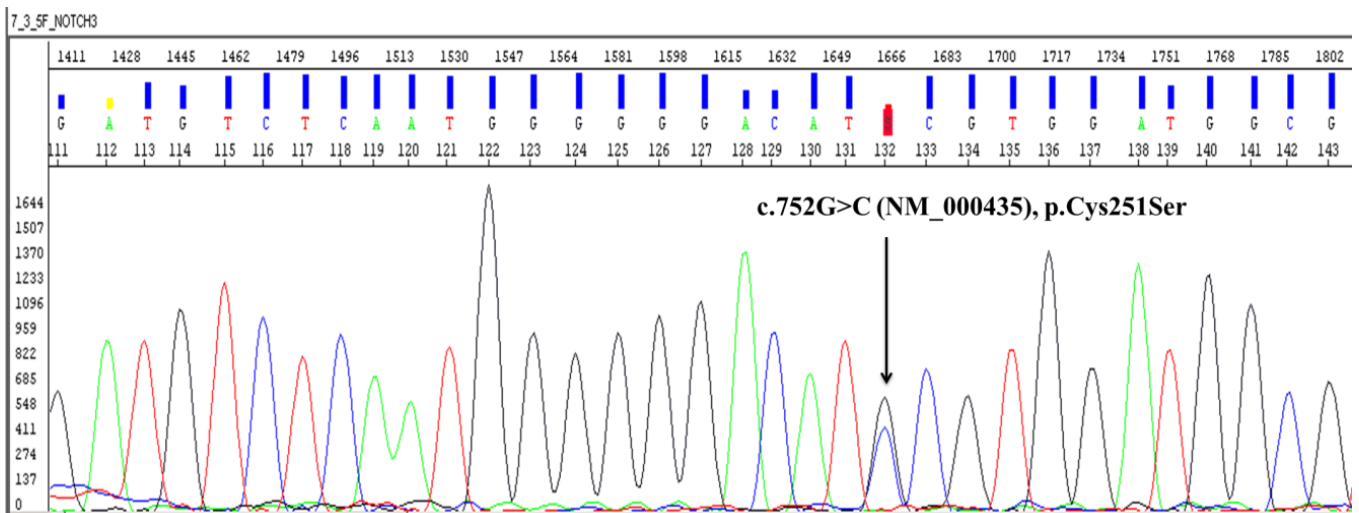
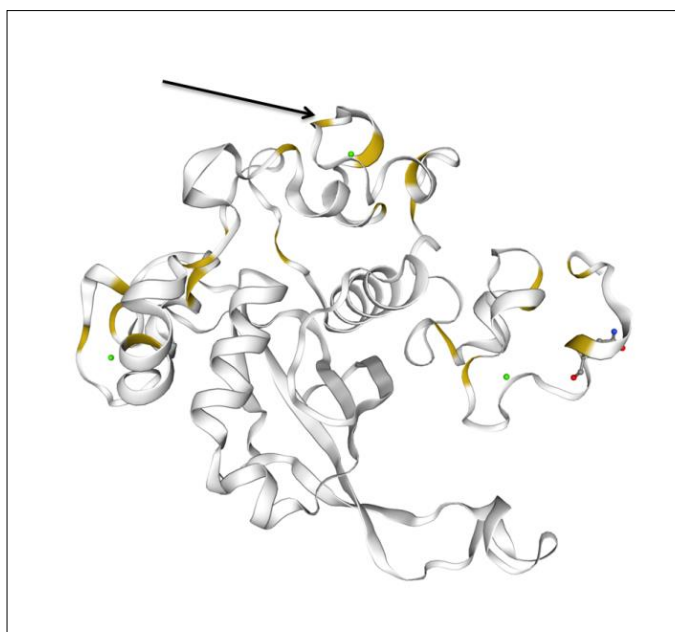


Figure 2. A novel missense mutation, c.752G>C (p.Cys251Ser), discovered in the examined Bulgarian population.



*Figure 3. The novel missense mutation, c.752G>C (p.Cys251Ser) represented in the protein structure of the NOTCH3 receptor.*

*Table 1. NOTCH3 mutations discovered in the Bulgarian population (age refers to age at which the genetic testing was performed).*

CASE	SEX	AGE	ORIGIN	MUTATION	TYPE OF MUTATION	EXON	EGFR DOMAIN
1	M	52	Bulgarian	c.581G>A, p. Cys194Tyr	Missense	4	4
2	F	27	Bulgarian	c.581G>A, p. Cys194Tyr	Missense	4	4
3	F	68	Bulgarian	c.581G>A, p. Cys194Tyr	Missense	4	4
4	F	39	Bulgarian	c.3062A>G, p.Tyr1021Cys	Missense	19	26
5	F	35	Bulgarian	c.752G>C, p.Cys251Ser	Missense	5	6

their accumulation. However, predicted loss-of-function variants are enriched for annotation errors, and tend to be found at extremely low frequencies, so their analysis requires careful variant annotation and very large sample sizes<sup>1</sup>. Here we describe the aggregation of 125,748 exomes and 15,708 genomes from human sequencing studies into the Genome Aggregation Database (gnomAD samples and controls as well as ENSEMBL<sup>18</sup> and the Human Genome Mutation Database<sup>19</sup>). The mutation is leading to the gain of a cysteine residue in one of the 34 epidermal growth factor-like repeat (EGFr) domains of the protein encoded by *NOTCH3*. This results in an uneven number of cysteine residues in the given EGFr domain, most likely modifying the tertiary structure of the protein (Fig. 3).

According to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology 2015 guidelines, the pathogenicity potential of the p.Cys251Ser variant is “likely pathogenic” based on the following criteria: (1) it is located in a hot-spot region for CADASIL-causing mutations (100% of mutations found in this region have been reported as pathogenic, PM1), (2) the variant is not found in the control patients of the Exome Aggregation Consortium (PM2), (3) several alternative variants on this and neighbouring positions have

been classified as pathogenic or likely pathogenic (PM5), (4) 13 in silico bioinformatics tools (BayesDel\_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, LIST-S2, M-CAP, MVP, MutationAssessor, MutationTaster, PrimateAI, REVEL and SIFT) predicted that the variant causes a deleterious effect on the gene (PP3) because it occurs in a highly conserved area across multiple species with no benign predictions and (5) the patient’s phenotype is highly specific for the disease (PP4).

In six out of the 11 patients in which the entire coding sequence of the *NOTCH3* gene was evaluated, no small deletions/ insertions nor missense variants were found. No large deletions or duplications were detected by the use of MLPA analysis.

### DISCUSSION

CADASIL often starts with attacks of migraine with aura during the third decade, followed by ischemic strokes 10-15 years later and subsequently dementia two decades after the initial onset, followed by death approximately during the sixth decade<sup>8</sup> progressive or stepwise subcortical dementia with pseudobulbar palsy (31%). Most of the ischemic events, transient or permanent, are classic lacunar infarcts that arise in the absence of hypertension or any other recognised vascular risk factors<sup>20</sup>. Different

Table 2. General spectrum of genotype-phenotype associations (10).

Specific mutation	Phenotype associations
EGFR domains 1-6	<ul style="list-style-type: none"> <li>• 12 years earlier onset of stroke</li> <li>• lower survival rate</li> <li>• greater loss of white matter</li> </ul>
EGFR domains 7-34	<ul style="list-style-type: none"> <li>• Milder phenotype</li> <li>• Possible non-penetrance</li> </ul>
EGFR domains 10-11	<ul style="list-style-type: none"> <li>• Conflicting interpretations</li> <li>• Both more severe and milder phenotypic manifestations</li> </ul>

genotype-phenotype associations have been reported in the literature, most of them are summarized in table 2<sup>21</sup>.

Patients examined in this study exhibited symptoms concordant with a typical CADASIL phenotype as well as typical genetic manifestations. This can be confirmed by the localization of the mutations, as exons 2-6 are a frequent hotspot for missense mutations, with most of the reported *NOTCH3* genetic alterations falling into this region<sup>21</sup> OMIM#125310. This can be further confirmed by the mutation spectrum in the reported cohort, which albeit small in number, is still significant for such a rarely diagnosed monogenic disorder.

It should also be noted that mutations which arise in domains 2-6 are causative of a more severe CADASIL phenotype, typically with a 12-year earlier onset of stroke, lower survival, and increased white matter hyperintensity volume while patients with pathogenic cysteine-altering mutations in domains 7-34 have a milder phenotype with a mean survival time of 76.9 years<sup>4</sup> thereby explaining an important part of CADASIL disease variability.”, container-title: “Genetics in Medicine”, DOI: “10.1038/s41436-018-0088-3”, ISSN: “1530-0366”, issue: “3”, language: “en”, note: “number: 3\npublisher: Nature Publishing Group”, page: “676-682”, source: “www.nature.com”, title: “The effect of NOTCH3 pathogenic variant position on CADASIL disease severity: NOTCH3 EGFr 1–6 pathogenic variant are associated with a more severe phenotype and lower survival compared with EGFr 7–34 pathogenic variant”, title-short: “The effect of NOTCH3 pathogenic variant position on CADASIL disease severity”, volume: “21”, author: “[{“family”: “Rutten”, given: “Julie W.”}, {“family”: “Van Eijdsden”, given: “Bastian J.”}, {“family”: “Duering”, given: “Marco”}, {“family”: “Jouvent”, given: “Eric”}, {“family”: “Opherk”, given: “Christian”}, {“family”: “Pantoni”, given: “Leonardo”}, {“family”: “Federico”, given: “Antonio”}, {“family”: “Dichgans”, given: “Martin”}, {“family”: “Markus”, given: “Hugh S.”}, {“family”: “Chabriat”, given: “Hugues”}, {“family”: “Lesnik Oberstein”, given: “Saskia A. J.”}], issued: {“date-part s”: [“2019”, “3”]}], schema: “https://github.com/citation-style-language/schema/raw/master/csl-citation.json”}. Interestingly, the mutation frequency can vary dramatically when cases are split in those two categories - with mutations affecting exons 7-34 being a 100 times more frequent compared to the classic estimated prevalence of CADASIL pathogenic mutations<sup>22</sup> 706 exomes of the exome aggregation consortium (ExAC). Adding to the complexity, mutations affecting EGFRs 10 and 11 (the ligand-binding domain) have been reported with both a milder phenotype, exhibiting a lower volume of lacunar infarcts<sup>23</sup> and a profound manifestation with an unusually early stroke median age of 31<sup>24</sup> including a second Colombian kindred with an R1031C mutation.”, container-title: “Neurology”, DOI: “10.1212/wnl.59.2.277”, ISSN: “0028-3878”, issue: “2”, journalAbbreviation: “Neurology”, language: “eng”, note: “PM

ID: 12136071”, page: “277-279”, source: “PubMed”, title: “C455R notch3 mutation in a Colombian CADASIL kindred with early onset of stroke”, volume: “59”, author: “[{“family”: “Arbol eda-Velasquez”, given: “J. F.”}, {“family”: “Lopera”, given: “F.”}, {“family”: “Lopez”, given: “E.”}, {“family”: “Frosch”, given: “M. P.”}, {“family”: “Sepulveda-Falla”, given: “D.”}, {“family”: “Gutierrez”, given: “J. E.”}, {“family”: “Vargas”, given: “S.”}, {“family”: “Medina”, given: “M.”}, {“family”: “Martinez De Arrieta”, given: “C.”}, {“family”: “Lebo”, given: “R. V.”}, {“family”: “Slaugenhaup t”, given: “S. A.”}, {“family”: “Betensky”, given: “R. A.”}, {“family”: “Villegas”, given: “A.”}, {“family”: “Arcos-Burgos”, given: “M.”}, {“family”: “Rivera”, given: “D.”}, {“family”: “Restrepo”, given: “J. C.”}, {“family”: “Kosik”, given: “K. S.”}], issued: {“date-pa rts”: [“2002”, “7”, “23”]}], schema: “https://github.com/citation-style-language/schema/raw/master/csl-citation.json”}.

Furthermore, the reported prevalence of CADASIL causing *NOTCH3* mutations is estimated to be between 1 and 9 per 100,000 people<sup>2,3</sup>. However, studies have shown that this number could be deflated based on the severity of the symptoms, with mutations causing milder phenotypes being as frequent as 1 in 300<sup>4</sup> thereby explaining an important part of CADASIL disease variability.”, container-title: “Genetics in Medicine”, DOI: “10.1038/s41436-018-0088-3”, ISSN: “1530-0366”, issue: “3”, language: “en”, note: “number: 3\npublisher: Nature Publishing Group”, page: “676-682”, source: “www.nature.com”, title: “The effect of NOTCH3 pathogenic variant position on CADASIL disease severity: NOTCH3 EGFr 1–6 pathogenic variant are associated with a more severe phenotype and lower survival compared with EGFr 7–34 pathogenic variant”, title-short: “The effect of NOTCH3 pathogenic variant position on CADASIL disease severity”, volume: “21”, author: “[{“family”: “Rutten”, given: “Julie W.”}, {“family”: “Van Eijdsden”, given: “Bastian J.”}, {“family”: “Duering”, given: “Marco”}, {“family”: “Jouvent”, given: “Eric”}, {“family”: “Opherk”, given: “Christian”}, {“family”: “Pantoni”, given: “Leonardo”}, {“family”: “Federico”, given: “Antonio”}, {“family”: “Dichgans”, given: “Martin”}, {“family”: “Markus”, given: “Hugh S.”}, {“family”: “Chabriat”, given: “Hugues”}, {“family”: “Lesnik Oberstein”, given: “Saskia A. J.”}], issued: {“date-parts”: [“2019”, “3”]}], schema: “https://github.com/citation-style-language/schema/raw/master/csl-citation.json”}, with mutations affecting exons 7-34 being a 100 times more frequent compared to the classic estimated prevalence of CADASIL pathogenic mutations<sup>22</sup> 706 exomes of the exome aggregation consortium (ExAC). Because of this considerable variation of disease prevalence is in contrast to the number of reported mutations related to CADASIL, it can be deduced that the disease is probably underdiagnosed. Current diagnostic guidelines would benefit if patients with recurrent small subcortical infarcts, TIAs, migraines, mood disturbances and abnormalities in the subcortical white matter and basal ganglia (confirmed by MRI) are referred for genetic

testing of the entire coding sequence of the *NOTCH3* gene. Of note, all but one of the patients in the examined cohort was referred for genetic testing well beyond the typical age of onset of the disease. In the author's opinion, current screening methods would greatly benefit from an earlier consideration for molecular testing in the presence of symptoms.

### CONCLUSION

This study shows the need for future research to clarify the genotype-phenotype correlation in Bulgarian population between pathological variants in the *NOTCH3* gene and the clinical

manifestation of the disease, which would greatly improve current diagnostic and treatment guidelines for patients with CADASIL.

### AUTHOR CONTRIBUTIONS:

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

### DECLARATION OF CONFLICTING INTERESTS:

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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