BVVL/ FL: Features caused by *SLC52A3* mutations; *WDFY4* and *TNFSF13B* may be novel causative genes

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Abstract

Brown-Vialetto-Van Laere(BVVL) and Fazio-Londe(FL) are disorders with ALS-like features, usually with recessive inheritance. We aimed to identify causative mutations in ten probands. Neurological examinations, genetic analysis, audiometry, MRI, biochemical and immunological testings, and/or muscle histopathology were performed. Mutations in known causative gene *SLC52A3* were found in seven probands. More importantly, only one mutated allele was observed in several patients, and variable expressivity and incomplete penetrance were clearly noted. Environmental insults may contribute to variable presentations. Putative causative mutations in other genes were identified in three probands. Two of the genes, *WDFY4* and *TNFSF13B,* have immune related functions. Inflammatory responses were implicated in the patient with the *WDFY4* mutation. Malfunction of the immune system and mitochondrial anomalies were shown in the patient with the *TNFSF13B* mutation. Prevalence of heterozygous *SLC52A3* BVVL causative mutations and notable variability in expressivity of homozygous and heterozygous genotypes are being reported for the first time. Identification of *WDFY4* and *TNFSF13B* as candidate causative genes supports conjectures on involvement of the immune system in BVVL and ALS.

Key words: Brown-Vialetto-Van Laere (BVVL) syndrome, Fazio-Londe (FL) syndrome, *SLC52A3*, *WDFY4*, *TNFSF13B*

1. Introduction

Brown-Vialetto-Van Laere syndrome (BVVL; MIM 211530) is a rare neurological disorder; approximately 150 cases have been reported(Manole et al., 2017; Sathasivam, 2008). BVVL is characterized by pontobulbar palsy, bilateral sensorineural hearing loss, and involvement of lower motor cranial nerves VII-XII. Upper motor neuron defects may appear with disease progression. Other possible manifestations include limb weakness and atrophy, speech defects, ocular anomalies, sensory symptoms/signs, and respiratory insufficiencies. Age at onset ranges from infancy to the third decade. BVVL is progressive, but progression rate is variable. Survival time ranges from a few years to several decades. Respiratory compromise is the most common cause of demise. The clinical features of BVVL overlap with those of several other motor neuron diseases, most notably juvenile amyotrophic lateral sclerosis (ALS) and Fazio-Londe (FL) syndrome. Absence of hearing loss distinguishes the latter from BVVL(McShane et al., 1992). Absence of hearing loss, less prominent bulbar presentations, later onset, asymmetric early presentations, and usually more rapid progression are ALS features that often allow differential diagnosis with BVVL(Yedavalli et al., 2018).

SLC52A3 that encodes solute carrier 52, riboflavin transporter, member 3, also known as RFVT3, was identified as a BVVL causative gene in 2010(Green et al., 2010). Inheritance was reported to be recessive. There has been a single report of involvement of *SLC52A1*, another member of the riboflavin transporter family(Ho et al., 2011). In 2012, mutations in *SLC52A2* that encodes RFVT2, the only remaining riboflavin transporter in humans, were identified as cause of BVVL in a few patients with childhood onset motor neuron disease(Haack et al., 2012; Johnson et al., 2012). Contrary to *SLC52A1*, *SLC52A2* mutations have been repeatedly found in BVVL patients(Ciccolella et al., 2013; Petrovski et al., 2015). Identification of BVVL causative mutations in riboflavin transporters and repeated documentation of benefits of riboflavin supplementation confirm that riboflavin deficiency

contributes to BVVL etiology(Jaeger and Bosch, 2016). This notwithstanding, BVVL cases exist in whom mutations in the riboflavin transport genes were not identified(Johnson et al., 2012; Manole et al., 2017). Therefore, BVVL may have further genetic heterogeneity.

Here, we present clinical data and results of genetic analysis on ten Iranian BVVL- or FL-diagnosed probands. We discuss the implications of the findings.

2. Methods

This research was performed in accordance with the Declaration of Helsinki and with the approval of the ethics board of the University of Tehran.

2.1 Subjects

BVVL or FL diagnosed patients were referred for genetic analysis by the neurologists who are among the authors. Family members were recruited when possible. BVVL diagnosis was based on presence of motor neuronopathy with prominent cranial nerve involvement accompanied with hearing impairment. FL diagnosis was based on presence of motor neuronopathy with cranial nerve involvement without hearing impairment. Riboflavin was always prescribed at dosage of 10 mg/kg body weight/day. Audiometry assessment, electrodiagnostic (EDX) testing, brain magnetic resonance imaging (MRI), biochemical (including acylcarnitine profile measurements), immunological, fluorescent antinuclear antibody (FANA) testings, and histopathology were performed as described in Supplementary materials Text 1.

2.2 Genetic analysis

Genetic analysis was performed as described in Supplementary materials Text 1. Briefly, the exons and flanking intronic sequences of *SLC52A3* and *SLC52A2* were initially sequenced in

the probands as previously described(Dezfouli et al., 2012). Candidate disease causing variations were screened for segregation with disease status in the respective families and control individuals. Evolutionary conservation of amino acids affected by the mutations was checked. Whole exome sequencing was performed for probands without *SLC52A3* and *SLC52A2* mutations and also for several unaffected family members. Candidate disease causing variations that remained after filtering of the sequence data were screened for segregation with disease status in respective families and in control individuals.

3. Results

3.1 Subjects

One FL and nine BVVL diagnosed patients were referred (Fig. 1, Tables 1,2,S1,S2). The parents of eight probands were consanguineous. Interestingly, involvement of cranial nerve V that is usually not emphasized in BVVL, was observed in nine of the ten probands. Its involvement manifested with mastication problems and masseter muscle weakness, sometimes accompanied with atrophy. Involvement of the masseter muscle (that is responsible for mastication), which is not routinely tested in EMG studies, was also evidenced in EMG results of three (BVVL-102-II6, BVVL-103-II1, and BVVL-113-IV1) of four probands tested for this muscle, and also in four additional BVVL diagnosed relatives of BVVL-113-IV1. Presence of sensorineural hearing loss in the nine BVVL probands and some family members, and its absence in the FL proband were confirmed by audiometric testing. MRI images of individuals with hearing loss did not show structural defects. Brain MRI for the three probands without *SLC52A3* mutations was done to possibly gain insight on disease etiology.

Families with *SLC52A3* mutations

Probands BVVL-102-II6, BVVL-104-II1, BVVL-106-II3, BVVL-108-II1, BVVL-109-II1, BVVl-110-II2, and BVVL-113-IV1 had *SLC52A3* mutations. Their pedigrees are shown in Figure 1A, and their clinical features are presented in Table 1. The data on the probands and their families are briefly elaborated upon here, and further described in Supplementary materials Text 2. An item of note was that genetic findings (see below) and family reports prompted clinical examination of family members of several probands. Results of clinical examinations, EDX evidence of motor neuronopathy, and presence of sensorineural hearing defects suggested that, in addition to proband BVVL-102-II6, her mother (-I2) and two siblings (-II3 and -II5) are also affected with BVVL (Table S1). Neurological examination of family members -I1, -II1, -II2, and -II4 was not possible. However, audiometric testing revealed that the father (-I1) and siblings -II1 and -II4 had sensorineural hearing loss, and that -II2 had normal hearing. Neurological examinations including EDX were normal for siblings -II2 and -II3 of proband BVVL-109-II1, but -II3 had severe sensorineural hearing defects; hearing of -II2 was normal. Family members reported that BVVL-109-II4, who was not examined, also had hearing problems. In family BVVL-110, only -I2 and -II6 consented to clinical examination; both were found to be normal. Hearing difficulties were reported for BVVL-110-II1 who was not examined. BVVL-113 is a large two-branched pedigree. Pedigree members told us that there are six individuals (BVVL-113-II1, -II10, -III1, BVVL-113-III10, -III12, and -III13) distributed in the two branches with hearing problems or other presentations similar to the proband. Five (all except BVVL-113-III1) were recruited and results of clinical examinations, including EDX evidence of motor neuronopathy, supported BVVL diagnosis in four (all except BVVL-113-II1) (Table S2). BVVL-113-III1, based on

presentations described, is also probably affected. The only BVVL associated feature observed in BVVL-113-II1 was hearing loss which was confirmed by auditory testing.

Another notable item of BVVL families with *SLC52A3* mutations, was coincidence of onset of BVVL-associated presentations with upper respiratory tract infections (BVVL-104-II1), tonsillitis (BVVl-110-II2), or severe fever and respiratory tract infection (BVVL-113-IV1) in three of the seven probands. Onset of hearing problems in individual BVVL-113-II1 who has the BVVL-associated genotype of his pedigree also presented immediately after a severe upper respiratory infection.

Families without *SLC52A3* mutations

Probands BVVL-103-II1, BVVL-111-II3, and FL-101-II3 did not have *SLC52A3* mutations. Their pedigrees are shown in Figure 1B, and their clinical features are presented in Table 2. The data on the probands are briefly elaborated upon here, and further described in Supplementary materials Text 2. Brain CT scan or brain MRI images were normal. None of these patients responded positively to riboflavin supplementation. BVVL-111-II3 had hearing problems from when he was 16. He reports that he had no other symptom until three years ago at the age of 34 when he noticed dysarthria, dysphonia and dyspnea after a course of severe and long-lasting respiratory infection. FL-101-II3 experienced an episode of febrile seizure at age of six months before onset of symptoms.

In BVVL-111-II3, mildly elevated serum neutrophil and decreased lymphocyte levels and borderline alpha 1 antitrypsin levels were consistent with presence of an inflammatory response (Table S3)(Stockley, 2015). This was further supported by presence of anti-nuclear antibody evidenced as the few nuclear dot pattern by immunofluorescent microscopy (Fig. 2Aleft)(Damoiseaux et al., 2019). Results of other autoimmune related measurements were negative. Plasma lupus anticoagulant levels were normal.

In FL-101-II3, there were multiple indications consistent with possible immune anomalies (Table S4). The cytotoxic T-cell level was slightly high as compared to normal range, and the helper to cytotoxic T cell ratio was inverted. The B cell level as assessed by CD19 measurement was at the lower end of the normal range. Polyclonal immunoglobulin G and M levels in the serum were elevated. This increase, and elevated serum neutrophil and alpha 1 antitrypsin levels and decreased lymphocyte levels were consistent with presence of an inflammatory response(Stockley, 2015). Measurements of various autoimmune factors, including anti-dsDNA antibodies and lupus anticoagulants, were within normal ranges. However, presence of anti-nuclear antibodies that evidenced with the fine speckled pattern in the nucleus by fluorescent microscopy, is suggestive of an autoimmune and/or inflammatory response (Fig. 2Aright)(Damoiseaux et al., 2019). Mildly elevated LDH and CK levels are consistent with muscle involvement, and elevated lactate and lactate to pyruvate ratio are consistent with mitochondrial dysfunction. Some parameters of the patient's acylcarnitine profile, including methylmalonycarnitine (C4DC), hydroxyisovalerylcarnitine (C5OH), decenolylcarnitine (C10:1), and tetradecadienolycarnitine (C14:1) were not within the normal range. Abnormal acylcarnitine profiles may reflect defects in mitochondrial fatty acid beta-oxidation catabolism(Wanders et al., 2010). Results of muscle histology confirmed presence of neurogenic muscle atrophy and some mitochondrial dysfunction that was evidenced in the laboratory results and clinical examinations. The muscle biopsy from the left vastus lateralis revealed marked muscle atrophy with a fascicular atrophy pattern. The remaining fibers were round and multiple nuclear clumps were associated with hypertrophied fibers with occasional fiber splitting (Fig 2Bleft). Succinate dehydrogenase (SDH) staining showed abnormal peripheral mitochondrial proliferation in some fibers (Fig. 2Bmiddle). The SDH plus cytochrome oxidase (COX) reactions revealed a notable number of fibers with reduced COX activity which is consistent with neurogenic atrophy with some mitochondrial dysfunction

(Fig. 2Bright). Histology of a biopsy from the left sural nerve showed no evidence of vasculitis, neither granuloma nor amyloid deposition (Fig. 2C).

3.2 Genetic analysis

Families with *SLC52A3* mutations

Sequencing of *SLC52A3* in the ten BVVL probands identified mutations in seven (Table 3, Fig. S1A). Mutations in *SLC52A2* were not observed. Homozygous, compound heterozygous, and single heterozygous *SLC52A3* mutation, respectively, were observed in four, one, and two probands. The mutations had an allele frequency of <0.01 in all data bases checked, and were not observed in Iranian control individuals. They were all missense mutations that affected evolutionarily well conserved amino acids in RFVT3 (Table S5). The effects of all but p.Arg212Cys were considered damaging by various prediction tools. p.Arg212Cys was earlier reported as cause of BVVL(Manole et al., 2017). Seven different *SLC52A3* mutations were found, three of which have not previously been reported. P.Asn21Ser was observed in more than one proband. All the patients with *SLC52A3* mutations responded favorably to riboflavin intake.

Segregation analyses in the smaller families BVVL-104, -106, and -108 with *SLC52A3* mutations were straightforward and suggested that their mutations cause BVVL in a recessive fashion (Fig. 1A). Heterozygous carriers were reported to be without BVVL related presentations, and audiometric testing on two carriers (BVVL-104-I2 and BVVL-108-I2) showed normal hearing. Results of segregation analysis in the remaining families with *SLC52A3* mutations were more complicated.

The proband of BVVL-102 carried mutations p.Asn21Ser and p.Ala312Val. The BVVL-diagnosed mother and sibling -II5 had the same genotype as the proband. However, BVVL-diagnosed -II3 carried only the mutated allele p.Asn21Ser. The father (-I1) had the same

genotype as this daughter, and siblings -II1, -II2, and -II4 were each heterozygous carriers of the alternate p.Ala312Val allele. Although family members reported absence of BVVL related clinical features in these four heterozygous individuals, audiometric testing as reported above revealed hearing defects in all except -II2.

Only one heterozygous mutation, p.Tyr329Cys, was found in proband BVVL-109-II1. Her parents and four siblings were available for segregation analysis. Unexpectedly, it was observed that all except the father had the same *SLC52A3* genotype as the proband. The father was homozygous for the wild type allele. The siblings had inherited the mutated allele from their mother, who had recently died due to causes unrelated to BVVL. Family members reported that she did not have hearing problems. As described above, the only BVVL-related symptom among the siblings was hearing loss in BVVL-109-II3 and -II4.

Proband BVVL-110-II2 was homozygous for p.Asn21Ser, one of the two mutations in BVVL-102-II6. Both parents and two siblings were carriers of the mutation, two siblings were homozygous for the mutated allele, and another was homozygous for the wild type allele. Neurological examination of the heterozygous mother and one heterozygous sibling showed that both were normal. Hearing problems were reported for only one of the siblings (BVVL-110-II1) homozygous for the mutated allele.

As with BVVL-109-II1, only one mutated allele was found in BVVL-113-IV1. His mutation was p.Gly13Arg. Each of four BVVL-diagnosed relatives, and also BVVL-113-III1 who presents with BVVL features, had the same heterozygous *SLC52A3* genotype. BVVL-113-II1 who had hearing difficulties but no other BVVL-related symptom, also carried the mutated allele. Brain MRI of BVVL-113-II1 was normal. BVVL-113 members not included in Table S2 did not undergo clinical examination. Screening of the *SLC52A3* mutation in twelve of these (age range: 28-70 yrs.) revealed that seven were homozygous for the wild type allele

and five were heterozygous carriers. Audiometric testing on one of these carriers (-III3) confirmed normal hearing.

Families without *SLC52A3* mutations

Disease inheritance in families BVVL-103, BVVL-111, and FL-101 was consistent with an autosomal recessive pattern. There was only one affected individual in each family. The specifications of the exome sequencing data of a representative sample that reflect high quality sequencing are presented in Table S6. Analysis of exome data of probands and unaffected family members identified eight variations in seven genes in BVVL-103, twelve variations in eleven genes in BVVL-111, and seven variations in four genes in FL-101 that were present in the homozygous or compound heterozygous state only in the DNA of the respective proband (Table S7). Screening each of the variations in additional unaffected family members reduced the number of candidate causative variations to three in BVVL-103, and to one in the other two families.

The candidate variations of BVVL-103 were in *SYCP1*, *VCAN*, and *BAIAP2* (Fig. S1B). These genes encode, respectively, synaptonemal complex protein 1, versican, and BAR/IMD domain containing adaptor protein 2 (alias IRSp53). Although c.3637A>G (p.Thr 1213Ala) in *VCAN* is not reported in the databases, it is predicted to be non-damaging by various prediction tools and is assigned a very low CADD (Combined Annotation Dependent Depletion; https://cadd.gs.washington.edu/) value of 0.74. C.1918G>A (p.Glu640Lys) in *SYCP1* (rs756169485) and c.1516C>T (p.Arg539Trp) in *BAIAP2* (rs149637388) are reported at very low frequencies (maximum: 0.0002 and 0.0023, respectively), and only in the heterozygous state. These two variations are, respectively, assigned CADD scores of 25.5 and 15.78. Evolutionary conservation of affected amino acids of the encoded proteins is shown in Table S5. The amino acid (p.Thr1213) of *VCAN* is conserved among mammals.

Alignment of orthologous proteins encoded by *BAIAP2* suggests the region inclusive of the affected amino acid is present only in primates. The amino acid (p.Glu640) of *SYCP1* is best conserved, being observed in mammals to fish. The single candidate variation of BVVL-111 was c.8851T>A (p.Ser2951Thr) in *WDFY4* (Fig. S1B). The gene encodes WDFY Family Member 4 (WD repeat- and FYVE domain-containing protein 4). This variation in *WDFY4* (c.8851T>A) is not reported in the databases. It is predicted by various prediction tools to deleteriously affect the encoded protein; it has a CADD score of 23.9. The affected amino acid (p.Ser2951) is conserved in mammals and birds (Table S5). And the single remaining candidate variation of FL-101was a six nucleotide deletion c.276_281del(p.A93_G94del) in *TNFSF13B* (Fig. S1B). The variation has not been previously reported. The Proven software that is capable of assessing effects of deletion mutations on protein function predicted that the p.93_94 mutation would be damaging. *TNFSF13B* encodes TNF superfamily, member 13b (tumor necrosis factor (ligand) superfamily, member 13b).

4. Discussion

It is notable that ten Iranian BVVL/FL patients were identified within a period of a few years. Iranian neurologists have become familiar with BVVL/FL presentations and there may be minimum under-diagnosis of adult cases(Dezfouli et al., 2012). Additionally, consanguineous marriages are common in Iran. *SLC52A2* mutations were not identified probably because the probands were referred by adult neurologists. Both adult- and child-onset forms of the diseases, because of rarity and overlap of symptoms with other anomalies, may be under-diagnosed worldwide.

Three of seven *SLC52A3* mutations here identified were novel. But more important are observed inheritance issues. Firstly, only one mutated allele was found in two (-109-II1 and -

113-IV1) probands and in BVVL diagnosed individual BVVL-102-II3. Interestingly, before identification of causative genes, autosomal dominant inheritance for BVVL had been considered(Hawkins et al., 1990). To the best of our knowledge, our group was the first to have reported presence of only one mutated *SLC52A3* mutated allele in BVVL diagnosed individuals(Dezfouli et al., 2012). By now, finding only one mutated allele in BVVL/FL diagnosed patients has been reported several times(Allison et al., 2017; Carreau et al., 2020; Ciccolella et al., 2012; Dezfouli et al., 2012; Manole et al., 2017). It appears justified to conclude that a heterozygous *SLC52A3* mutation may in some cases cause BVVL or FL. The conclusion applies to mutations that affect amino acids distributed in the length of the encoded protein, possibly with some predominance in the first 40 amino acids (see Supplementary materials Text 3).

Another issue brought to light relates to expressivity and penetrance, which can be noted especially when segregation analysis is performed. BVVL-102 had BVVL diagnosed individuals with one or two mutated *SLC52A3* alleles (Table S1). Severity of presentations was comparable in two patients of similar age with one (-II3) or two (-II5) mutated alleles. Age at onset of symptoms in two patients with two mutated alleles (-II5 and -II6) differed by nearly ten years. The father (-I1) and BVVL diagnosed daughter (-II3) both carried one copy of the same mutated allele (p.Asn21Val), but the only BVVL relevant presentation of the father was some sensorineural hearing loss. Hearing status of three siblings who were heterozygous for the alternate mutated allele of the family (-II1, -II2 and -II4; p.Ala312Val) was not the same.

The p.Asn21Val mutation of family -102 was also found in BVVL-110. Among three members of family BVVL-110 who were homozygous for the mutation, one (-II2) was diagnosed with BVVL, one (-II1) only had hearing problems, and the third (-II3) was reported to be asymptomatic. Unlike BVVL-affected BVVL-102-II3, two examined

heterozygous carriers of p.Asn21Val in BVVL-110 (-I2 and -II6) were asymptomatic. These findings evidence variable intrafamilial and interfamilial expressivity, and incomplete penetrance. Apparently, both features may apply to genotypes that are heterozygous or homozygous for mutated alleles.

Among three critically examined heterozygous carriers of p.Tyr329Cys in BVVL-109, -II1 was diagnosed with BVVL, -II3 only presented hearing loss, and -II2 was asymptomatic. Among six critically examined heterozygous carriers of p.Gly13Arg in BVVL-113, five were diagnosed with BVVL, and one only presented hearing loss. Five other heterozygous carriers of the mutation in the extended family were reported by themselves and family members to be asymptomatic. Audiometric testing was done on one of these (-III3) and results were normal. The unlikely possibility that the father (-I1) of BVVL-109 has a large deletion or carries an undetected mutation that was inherited only by offsprings -II1 and -II3 cannot be ruled out. Presence of an undetected mutation in patients of BVVL-113 seems even less likely, as the same mutation was also previously reported in the heterozygous state in patients of another study(Manole et al., 2017).

The totality of observations in BVVL families -102, -110, -109, and -113 argues in favor of variable expressivity and incomplete penetrance for *SLC52A3* mutated genotypes. A practical implication is that family members of BVVL patients with one or two *SLC52A3* mutated alleles should be genotyped and/or regularly examined for presentation of symptoms. Environmental factors can be considered as contributing causes of the described differences. It is noted that disease onset coincided with fever, infection, or tonsillitis in some BVVL diagnosed individuals of families -104, -110 and -113 that had *SLC52A3* mutations, and of families -101 and -111 without *SLC52A3* mutations. Coincidence of BVVL onset with similar insults has also been reported elsewhere. Exposure to such insults may influence presentation and/or severity of disease (see below).

SYCP1, *VCAN*, and *BAIAP2* were retained candidate BVVL-causative genes in BVVL-103-II1. The *SYCP1*-encoded protein is a component of the synaptonemal complex(Zickler and Kleckner, 2015). Available data do not suggest an obvious role for *SYCP1* in neuromuscular pathology. *VCAN* and *BAIAP2* may be better candidate BVVL culprit genes. (Additional references about *VCAN* and *BAIAP2* are given in Supplementary materials, Text 4.) Versican encoded by *VCAN* is a chondroitin sulfate proteoglycan with roles in myoblast proliferation and myotube formation(Stupka et al., 2013). It has been associated with skeletal muscle dystrophy pathology. Versilac, which is a naturally occurring proteolytic product of versican, can be pro-apoptotic or pro-inflammatory in some biological contexts. BAIAP2 (IRSp53) is considered an important regulator of membrane and actin dynamics at actin-rich subcellular structures such as filopodia and lamellipodia, is believed to affect neurite initiation and neuronal branching, and has been proposed to also affect mitochondrial morphology(Chen et al., 2015; Ferrari et al., 2016). The protein is expressed most strongly in the brain. BAIAP2 is concentrated at dendritic spines, in close association with the post synaptic density. Despite potential biological relevance of both *VCAN* and *BAIAP2*, bioinformatics tools predicted the *VCAN* variation in BVVL-103-II1 to be very benign. Therefore, based on the sum of bioinformatics predictions and known functions, the *BAIAP2* mutation by affecting dendritic growth and/or mitochondrial functions may be the cause of BVVL in BVVL-103-II1.

WDFY4 and *TNFSF13B* were, respectively, the putative disease causing gene in BVVL-111-II3 and FL-101-II3. A striking feature of these findings is that the major known functions of both genes are within the immune system. The longest *WDFY4* transcript encodes a protein that has two BEACH domains, six WD40 repeats, and a truncated FYVE zinc finger domain (https://www.uniprot.org/uniprot/Q6ZS81). BEACH domains are implicated in membrane trafficking (https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=cl10511). Disruption of this domain in human lysosomal trafficking regulator leads to Chediak-Higashi syndrome

which presents with severe immunodeficiency and neurologic problems. WD40 repeats have multiple functions including signal transduction, pre-mRNA processing, and cytoskeleton assembly (https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=207648).The mutation (p.Ser2951Thr) found in patient BVVL-111-II3 is positioned within the second WD domain (p.2923-p.2972) of WDFY4. WDFY4 is a membrane protein and the mouse orthologous locates in the endoplasmic reticulum and in endosomes (https://www.uniprot.org/uniprot/Q6ZS81). Although the protein is highly conserved during evolution, very little is known about its functions.

The most frequent attribute for *WDFY4* is association with systemic lupus erythematosus (SLE) susceptibility and other autoimmune diseases including rheumatic arthritis, juvenile rheumatic arthritis, and clinically amyopathic dermatomysitis(Yang et al., 2010). Gene expression profiles show highest expression of WDFY4 in tissues with immune functions (https://www.ncbi.nlm.nih.gov/gene/57705). WDFY4 may also have roles in autophagy. WDFY3 (Gene ID: 23001), the closest paralog of WDFY4, encodes ALFY which interacts with p62 to organize misfolded ubiquitinated protein into bodies that become degraded by autophagy(Clausen et al., 2010). Blue cheese, the WDFY4 ortholog in drosophila, similarly functions in autophagy and also contributes to the development of the nervous system (https://flybase.org/reports/FBgn0043362.html). Direct evidence for involvement of *WDFY4* in autophagy and link between autophagy and SLE susceptibility were recently derived from mouse knock out and cell culture knock down studies.

Indications of an inflammatory response in BVVL-111-II3 are consistent with involvement of the immune system in his disease. These indications may now be mild because the notable BVVL-related clinical features of this patient manifested only three years ago. Furthermore, immune system effects of WDFY4 in BVVL pathology may be subtle and not fully reflected

in the analyses performed. Features pertaining to autophagy were not assessed in the patient. Further research on WDFY4 function may reveal how it contributes to BVVL pathology.

TNFSF13B, also known as BAFF (B cell activating factor from the TNF family), is a member of the TNF gene superfamily of genes which have critical roles in inflammation and immune responses(Dostert et al., 2019). (Additional references on *TNFSF13B* and BAFF are given in Supplementary materials, Text 4.) BAFF is a transmembrane protein, and proteolytic cleavage within a stalk segment located between its transmembrane and extracellular domains produces a soluble form of the protein. The mutation (p.93_94del) in FL-101-II3 is within the stalk segment. BAFF has high expression in immune system related organs (https://www.ncbi.nlm.nih.gov/gene/10673), and is a cytokine ligand for receptors primarily expressed by B cells. It has important roles in the proliferation, differentiation, activation, and survival of B cells, and in T cell co-stimulation and T helper cell associated inflammatory responses(Mackay and Browning, 2002). BAFF is implicated in autoimmunity(Chen et al., 2014; Zhang et al., 2008). It has excessive expression in various autoimmune diseases, and overexpression in transgenic mice causes an autoimmune phenotype. Association studies recently identified a *TNFSF13B* variant associated with multiple sclerosis and SLE.

The aggregate of clinical data presented in the Results section on FL-101-II3 who harbors a *TNFSF13B* mutation are consistent with association of immune system malfunctions to her disease status. Notable features were inverted CD4/CD8 ratio, polyclonal gammapathy, and elevated alpha-1 levels. A declining CD4/CD8 ratio is an indicator of immunosenescence and an inverted ratio (less than 1/1) is considered to be a sign of an impaired immune system(McBride and Striker, 2017). Anomalies of the mitochondria were evidenced by lactate and pyruvate levels and by histopathology. The patient's acylcarnitine profile and presence of ptosis may also be indicators of mitochondrial malfunction(Lee et al., 2018). The mitochondrial anomalies may be secondary to immune dysfunction in the patient. It is of

course of note that the common BVVL/FL causative gene *SLC52A3* also ultimately affects mitochondrial activity.

Although functional studies on BVVL/ FL have largely been limited to riboflavin metabolism, there is reason to consider roles for immune functions in these diseases. In a review on BVVL that was published when only 58 patients had been described and well before identification of a causative gene, seven cases were reported in whom intercurrent infections may have precipitated or worsened BVVL presentation(Sathasivam, 2008). This scenario has since been reported in other publications, and also observed in five of the 10 probands in the present study(Bandettini Di Poggio et al., 2014; Dakhil et al., 2010). Three of the latter had mutations in *SLC52A3* and two had mutations in other genes. Improvement of clinical presentations after immune therapy in some reported cases is also consistent with possible contribution of immune dysfunction to BVVL etiology(Bandettini Di Poggio et al., 2014). Finally, there are several reports of patients originally thought to be affected with a neuroimmune disorder, but ultimately diagnosed with BVVL on the basis of genetic testing(Allison et al., 2017).

Functional studies on ALS, whose etiology may have commonalities with BVVL/FL, have been more numerous. A role for the immune system in the etiology of ALS was considered since decades ago, and this proposal is now being given more attention. In fact, the immune system may have roles in various neurodegenerative disorders thus reflecting the interactions between these two important sensory systems(Lall and Baloh, 2017). The incidence of autoimmune diseases was reported to be higher among ALS patients as compared to controls(Turner et al., 2013). Similar results were reported for patients affected with frontotemporal dementia (FTD) which is related to ALS(Miller et al., 2016). Some epidemiological studies have suggested a nearly fivefold increased risk of ALS associated with prior diagnosis of myasthenia gravis which is an autoimmune disease(de Pasqua et al.,

2017; Turner et al., 2013). Additionally, presence of multiple autoimmune antibodies and infiltration of inflammatory mediators in the CNS are implicated in ALS pathology(Hu et al., 2017; Lai and Ichida, 2019). Most interestingly, it is now considered that the hexanucleotide repeat expansion in *C9ORF72* which is the most common cause of ALS, may also influence immune homeostasis(Lai and Ichida, 2019). Mouse knockouts of the orthologous gene present with various immune related dysfunctions(O'Rourke et al., 2016). Intermediate length repeats that are non-ALS-causing have been observed in individuals affected with progressive multiple sclerosis which is a neurodegenerative autoimmune disease(Tiloca et al., 2018). Involvement of the immune system with ALS pathology may to some extent also apply to BVVL and FL which are related diseases. Identification of *WDFY4* and *TNFSF13B* as potential BVVL causing genes supports this conjecture. Nevertheless, definitive assessment of contribution of these genes to BVVL pathology awaits finding mutations in these genes in other unrelated patients and further functional studies.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1- Pedigrees of BVVL/FL affected probands. A. Pedigrees of seven probands with *SLC52A3* mutations. **B.** Pedigrees of three probands without *SLC52A3* mutations. Probands are identified with arrow. Proband of FL-101 was diagnosed with FL and probands of other families were diagnosed with BVVL. *, individuals who underwent complete neurological examination including EMG and NCS; black filled symbols, diagnosed with BVVL or FL; H, individual whose only BVVL-relevant presentation is sensorineural hearing defect; MM, homozygous mutant genotype; MN, heterozygous genotype; NN, normal genotype; in pedigree of family BVVL-102 that has compound heterozygous *SLC52A3* mutations, genotypes of both mutations are shown; in BVVL-103, the genotypes of proband and parents are for mutations in each of three genes (*SYCP1*, *VCAN*, and *BAIAP2*), and the siblings of the proband were either heterozygous carriers of mutations in the genes or were homozygous for the wild type alleles (see text); the mutations in BVVL-111 and FL-101 are, respectively, in *WDFY4* and *TNFSF13B*.

Figure 2- Fluorescent antinuclear antibody (FANA) testing and histopathology images of muscle and nerve tissue. A. Fluorescent microscope images. **Left:** Presence of antinuclear antibodies in the serum of proband BVVL-111-II3 evidences as few nuclear dots. **Right:** Presence of antinuclear antibodies in the serum of proband FL-101-II3 evidences as the fine speckled pattern. B. Histopathology images of FL-101-II3 muscle biopsy was from left vastus lateralis. **Left:** Hematoxylin and eosin staining reveals marked muscle atrophy with fascicular atrophy pattern. Multiple nuclear clumps are seen associated with few hypertrophied fibers. **Middle:** SDH (succinate dehydrogenase) staining shows abnormal peripheral mitochondrial proliferation. **Right:** Blue fibers (arrows) observed after COX-SDH

643 histochemical staining indicate decrease in COX (cytochrome c oxidase) activity and
644 increased SDH activity, possibly due to mitochondrial hyper proliferation. C**.** Histopathology
645 image of nerve biopsy from left sural nerve. Hematoxylin and eosin staining does not show
646 abnormality.

647

648 **Figure S1- Chromatograms of mutations observed in nine BVVL and one FL proband**.
649 **A.** Chromatograms of mutations observed in *SLC52A*3 in seven BVVL probands. **B.**
650 Chromatograms pertaining to three candidate BVVL causing mutations in BVVL-103
651 proband, putative BVVL causing *WDFY4* mutation in BVVL-111 proband, and putative FL
652 causing *TNFSF13B* mutation in FL-101 proband.

Table S1- Clinical data on four BVVL diagnosed individuals of pedigree BVVL-102 with mutations in *SLC52A3*

Individual ID	BVVL-102-I2	BVVL-102-II3	BVVL-102-II5	BVVL-102-II6*
Genotype	Comp. het.: c.62A>G (p.Asn21Ser)/ c.935C>T (p.Ala312Val)	Het.: c.62A>G (p.Asn21Ser)	Comp. het.: c.62A>G (p.Asn21Ser)/ c.935C>T (p.Ala312Val)	Comp. het.: c.62A>G (p.Asn21Ser)/ c.935C>T (p.Ala312Val)
Sex	female	female	male	female
Age at examination (=present age)	55 yrs	33 yrs	31 yrs	27 yrs
Age at onset	25 yrs	32 yrs	28 yrs	19 yrs
Disease duration	30 yrs	1 yr	3 yrs	8 yrs
Initial presentation	weakness of lower extremities	hearing problem & weakness of lower extremities	hearing problem	dysphagia
Bulbar palsy (CN-9,12)	+ dysarthria , dysphagia & tongue fasciculation	-	-	+ dysphagia, dysarthria, tongue atrophy
CN-5 palsy	-	-	-	mastication problems
Facial weakness (CN-7)	+ weakness	-	-	weakness, atrophy, fasciculation
Hearing problem (CN-8)	+	+	+	+
Ophthalmoplegia (CN3/4/6 palsy)	-	-	-	-
Ptosis	-	-	-	-
Optic nerve atrophy (CN-2 involvement)	-	-	-	-
Limb weakness	+ upper and lower extremities	+ proximal weakness, more prominent in right lower extremity	-	+
Limb muscle atrophy	+ upper and lower extremities	-	-	+, distal atrophy
Spasticity	-	-	-	-
Increased DTR	+	-	+	+
Decreased DTR	-	-	-	-
Sensory symptoms/ signs	-	-	-	-
Tremor	-	-	-	-
Ataxia	-	-	-	-
Vertigo	-	-	-	+
Tinnitus	-	-	-	-
Seizure	-	-	-	-
Mental impairment	-	-	-	-
Psychiatric disorder	-	-	-	+, depression
Autonomic disfunction	-	-	-	-
Upward plantar reflex	-	-	-	-
Respiratory problem	-	-	-	-
Ambulatory state	slower but assstive device or help not needed	no limitation	no limitation	no limitation
Acylcarnitine profile	not done	not done	not done	not done
EMG	motor neuronopathy at extremities and cranial and truncal levels	motor neuronopathy at extremities and cranial and truncal levels	motor neuronopathy at extremities and cranial and truncal levels	motor neuronopathy, more prominent but not restricted to cranial myotomes
NCS	Normal sensory findings	Normal sensory findings	Normal sensory findings	Normal sensory findings

*, Proband of BVVL-102, also described in Table 1. Comp. het, compound heterozygous; Het., heterozygous; CN, cranial nerve; DTR, deep tendon reflexes; EMG, electromyography; NCS; nerve conduction studies

Table S2- Clinical data on five BVVL diagnosed individuals of pedigree BVVL-113 with mutation in *SLC52A3* (c.37G>A; p.Gly13Arg)*

Individual ID	BVVL-113-II10	BVVL-113-III10	BVVL-113-III12	BVVL-113-III13	BVVL-113-IV1**
Sex	male	male	male	female	male
Age at examination (=present age)	53 yrs	30 yrs	25 yrs	23 yrs	19 yrs
Age at onset	20 yrs	15 yrs	9 yrs	13 yrs	9 yrs
Disease duration	33 yrs	15 yrs	16 yrs	10 yrs	10 yrs
Initial presentation	hearing problem and dysequilibrium	hearing problem	hearing problem	dysphonia (partial laryngeal paralysis)	asymmetric facial weakness
Bulbar palsy (CN-9,12)	+ mild dysphagia, dysarthria and tongue atrophy	+ dysphonia	+ dysphagia, dysarthria and tongue atrophy	+ dysphonia, dysarthria and tongue atrophy	+ mild dysphagia, dysarthria, tongue atrophy
CN-5 palsy	-	-	-	-	mastication problems and atrophy
Facial weakness (CN-7)	+ weakness	-	+ weakness	+ weakness	weakness, atrophy
Hearing problem (CN-8)	+	+	+	+	+
Ophthalmoplegia (CN3/4/6 palsy)	-	+ right CN6 paresia and nystagmus at right gaze	-	-	-
Ptosis	-	-	-	-	-
Optic nerve atrophy (CN-2 involvement)	-	-	-	-	-
Limb weakness	-	+ distal lower extremities (foot drop)	-	+ mild symmetric in upper and lower extremities	-
Limb muscle atrophy	-	+ distal lower extremities	-	-	-
Spasticity	-	-	-	-	-
Increased DTR	-	-	-	+	-
Decreased DTR	-	+	-	-	-
Sensory symptoms/ signs	-	-	-	-	-
Tremor	+ hand and head	-	+ hand and head	+ hand	-
Ataxia	+	-	+	+	-
Vertigo	-	-	-	-	-
Tinnitus	-	-	-	-	-
Seizure	-	-	-	-	-
Mental impairment	-	-	-	-	-
Psychiatric disorder	-	-	-	-	-
Autonomic disfunction	-	-	-	-	-
Upward plantar reflex	-	-	-	+	-
Respiratory problem	-	-	-	-	-
Ambulatory state	no limitation	slow, uses braces but does not need help			
Acylcarnitine profile	not done	not done	not done	not done	not done
EMG	motor neuronopathy restricted to cranial myotomes	motor neuronopathy at extremities and cranial and truncal levels	motor neuronopathy at extremities and cranial and truncal levels	motor neuronopathy at extremities and cranial and truncal levels	motor neuronopathy, more prominent but not restricted to cranial myotomes
NCS	Normal sensory findings	Normal sensory findings	Normal sensory findings	Normal sensory findings	Normal sensory findings

*, BVVL-113-II1 aged 40 yrs old, not diagnosed with BVVL, is not included in the Table. Hearing problems, with onset 15 years earlier, are his only BVVL relevant presentation. **Proband of BVVL-113, also described in Table 1.

CN, cranial nerve; DTR, deep tendon reflexes; EMG, electromyography; NCS; nerve conduction studies

Table S3- Clinical laboratory measurements of BVVL-111 proband*

Test item	Measurement in patient	Normal range
RBC	6.7 X 10^6 /µL	4.5 - 6.2 X 10^6 /µL
Hb	17.1 mg/dL	12 -16 mg/dL
Hct	54.70%	38.8 - 46.4 %
Neutrophil differential	73.70%	50 - 70 %
Lymphocytel differential	15.70%	25 - 45 %
Ferritin	13 ng/mL	30 - 300 ng/mL
Blood PO2	42.4 mmHg	75 - 100 mmHg
LDH	400 U/L	180 - 400 U/L
CPK	79 U/L	20 - 180 U/L
CRP	< 2 mg/L	< 10 mg/L
Alpha 1 antitrypsin	0.29 g/dL	0.10 - 0.30 g/dL
Serum IgG	1168 mg/dL	700 - 1600 mg/dL
Serum IgA	340 mg/dL	70 - 400 mg/dL
Serum IgM	98 mg/dL	40 - 230 mg/dL
CD4	46.40%	20 - 65 %
CD8	25.30%	10 - 40 %
CD4:CD8 ratio	1.83	1 - 4
CD19	8.90%	4 - 25 %
CD20	9.00%	4 - 25 %
CH50	100 U	70 - 150 U
Complement C3	105 mg/dL	90 - 180 mg/dL
Complement C4	27 mg/dL	10 - 40 mg/dL
Anti mitochondrial Ab	negative	negative (= <1:10)
Rheumatoid factor	4.0 IU/mL	0 - 30 IU/mL
Anti-dsDNA	2.2 IU/mL	< 12 IU/mL
Anti-nuclear antibody	1:320 titer	< 1:100 negative

* Measurements of items considered most relevant to BVVL or known functions of *WDFY4* are reported in the Table. Blood biochemistry including sugar, cholesterol, and triglyceride levels, thyroid hormones, and vitamin D measurements were within normal range. Plasma lactate and pyruvate, and their ratio were normal. Levels of amino acids associated with metabolic disorders, and acyl carnitine profile that included measurements of 28 compounds were normal. CD4 and CD8 are, respectively, markers for helper and cytotoxic T-cells, and CD19 and CD20 are B cell markers. Percent of cells with T-cell and B-cell markers were assessed by flow cytometry. CH50 is an index for total complemtary level. Anti-nuclear antibody was assessed by fluorescent microscopy.
fluorescent microscopy.

Table S4- Clinical laboratory measurements of FL-101 proband*

Test item	Measurement in patient	Normal range
RBC	5.3 X 10^6 /µL	4.0 - 5.2 X 10^6 /µL
Hb	15.6 g/dL	12 -16 g/dL
Hct	47.30%	36 - 46 %
Neutrophil differential	77.00%	50 - 70 %
Lymphocytel differential	17.00%	25 - 45 %
LDH	440 U/L	180 - 400 U/L
CPK	230 U/L	20 - 160 U/L
Plasma lactate	26 mg/dL	4.5 - 20 mg/dL
Plasma pyruvate	0.9 mg/dL	0.3 - 0.9 mg/dL
Lactate: Pyruvate ratio	28.9	< 20
CRP	2 mg/L	< 10 mg/L
Alpha 1 antitrypsin	0.32 g/dL	0.10 - 0.30 g/dL
Serum IgG	1730 mg/dL	700 - 1600 mg/dL
Serum IgA	145 mg/dL	70 - 400 mg/dL
Serum IgM	240 mg/dL	40 - 230 mg/dL
CD4	37.70%	20 - 65 %
CD8	48.30%	10 - 40 %
CD4:CD8 ratio	0.78	1 - 4
CD19	6.90%	4 - 25 %
CH50	100 U	70 - 150 U
Complement C3	127 mg/dL	90 - 180 mg/dL
Complement C4	22 mg/dL	10 - 40 mg/dL
Anti mitochondrial Ab	negative	negative (= <1:10)
Rheumatoid factor	3.6 IU/mL	0 - 30 IU/mL
Anti dsDNA	2.0 IU/mL	< 12 IU/mL
Anti nuclear antibody	1:160 titer	< 1:100 negative
Methylmalonycarnitine (C4DC)	0.49 µM	< 0.22 µM
Hydroxyisovalerylcarnitine (C5OH)	0.52 µM	< 0.17 µM
Decenolylcarnitine (C10:1)	0.11 µM	< 0.10 µM
Tetradecadienolycarnitine (C14:1)	0.06 µM	< 0.12 µM

* Measurements of items considered most relevant to BVVL/FL or known functions of *TNFSF13B* are reported in the Table. Blood biochemistry including sugar, cholesterol, and triglyceride levels were within normal range. Thyroid hormone measurements were normal. Levels of amino acids associated with metabolic disorders were normal. CD4 and CD8 are, respectively markers for helper and cytotoxic T-cells, and CD19 is a B-cell marker. Percent of cells with T cell and B cell markers were assessed by flow cytometry. CH50 is an index for totalcomplement level. Anti- nuclear antibody was assessed by fluorescent microscopy.

Table S5- Conservation of amino acids affected by missense mutations in SLC52A3, SYCP1, VCAN, BAIAP2, and WDFY4 found in BVVL patients*

Organism	Sequence ID**	SLC52A3						
		p.Gly13Arg	p.Asn21Ser	p.Arg212Cys	p.Ala312Val	p.Tyr329Cys	p.Pro385Ala	p.Leu429Phe
Homo sapiens (human)	NP_001357014.1	LVCVF**G**MGSWV	SWVTI **N**GLWVE	SHLES**R**YLPAH	VAFVN**A**LTNGM	YSCLS **Y**GPVAY	MAVMS**P**CPLLQ	LSRSA**L**LWCGA
Pan troglodytes (chimpanzee)	XP_016792755.2	LVCVF**G**MGSWV	SWVTI **N**GLWVE	SHLES**R**YLPAH	VAFVN**A**LTNGV	YSCLS **Y**GPVAY	MAVMS**P**CPLLQ	LSRSA**L**LWCGA
Pongo abelii (orangutan)	XP_024094639.1	LVCIF **G**MGSWV	SWVTI **N**GLWVE	SHLES**R**YLPAH	VAFVN**A**LTNGV	YSCLS **Y**GPVAY	MAVMS**P**CPLLQ	LSRSA**L**LWCGA
Macaca mulatta (monkey)	NP_001181490.1	LVCVF**G**MGSWV	SWVTI **N**GLWVE	SHLES**R**YLPAH	VAFVN**A**LTNGV	YSCLS **Y**GPVAY	MAVMS**P**CPLLQ	LSRSA**L**LWCGA
Bos taurus (cow)	NP_001014864.1	LVCTF**G**MGSWV	SWVAI **N**GLWVE	IHLES**R**YLPAN	VAFVN**A**LTNGV	YSCLS **Y**GPVAY	MAVMS**P**CPFMQ	HSRSA**L**LWCGA
Mus Musculus (mouse)	NP_081448.2	LVCVF**G**MGSWV	SWVAI **N**GLWVE	WHQES**R**YLAPR	VAFVN**A**LTNGV	YSCLP **Y**GPVAY	MAAMS**P**CPVLQ	RSRSA**L**LWCGA
Rattus norvegicus (rat)	NP_001032275.1	LVCVF**G**MGSWV	SWVAI **N**GLWVE	WHLES**R**YLAPR	VAFVN**A**LTNGV	YSCLP **Y**GPVAY	MAAMS**P**CPILQ	RSRSA**L**LWCGA
Cricetulus griseus (hamster)	XP_027277327.1	LVCIF **G**MGSWV	SWVAI **N**GLWVE	WHLES**R**YLAPR	VAFVN**A**LTNGV	YSCLP **Y**GPVAY	MAAMS**P**CPILQ	RSRSA**L**LWCGA
Oryctolagus cuniculus (rabbit)	XP_008254402.1	LVCTF**G**MGSWV	SWVAI **N**GLWVE	GHLQS**R**YLPAR	VAFVN**A**LTNSV	YSCLS **Y**GTVAY	MAVMS**P**CPLMQ	RSRSA**L**LWCGA
Lonchura striata domestica (finch)	XP_031362383.1	LACAF**G**MGSWV	SWVAI **N**GLWVE	FHMES**R**YLPPN	IAWVS**A**LTNGV	YSCLP **Y**GHTTY	IAVMS **P**CPLLQ	RSHSA**L**VWYGV
Parus major (great tit)	XP_015502662.1	LACAF**G**MGSWV	SWVAI **N**GLWVE	FQLET**R**YLPPN	ITWVS**A**LTNGV	YSCLP **Y**GHTTY	IAVMS **P**CPLLQ	RSRSA**L**VWYGV
Ictalurus punctatus (catfish)	XP_017323199.1	LACAF**G**LGSWV	SWVAV**N**GMWVE	FTLEA**Q**YLPPN	VVCVN**C**ATNGL	FSCMP**Y**GNMVY	MAVMS**P**CPILQ	QSHIA**L**VWCGA
Nothobranchius furzeri (killifish)	XP_015824022.1	LACAF**G**LGSWV	SWVAV**N**GLWVE	WLLQT**E**YLPPN	VLWVN**A**ATNGL	YSCMP**Y**GNLAY	MAAMS**P**CPLLR	RSHSA**L**VWCGA
Danio rerio (zebrafish)	NP_001035447.1	LACAF**G**LGSWV	SWVSI **N**GLWVE	FIVET**Q**YLPPN	VLWVN**S**ATNGL	FSCMP**Y**GNMAY	MAAMS**P**CPLLQ	RSHSA**L**VWCGA

Organism	SYCP1		VCAN		BAIAP2		WDFY4	
	Sequence ID**	Mutation	Sequence ID**	Mutation	Sequence ID**	Mutation	Sequence ID**	Mutation
		p.Glu640Lys		p.Thr1213Ala		p.Arg539Trp		p.Ser2951Thr
Homo sapiens (human)	NP_001269470.1	QLNVY**E**IKVNK	NP_004376.2	P - - EA**T**EKSHF	NP_059345.1	QGPEG**R**EHGDG	NP_065996.1	TTIVT**S**GTSTW
Pan troglodytes (chimpanzee)	XP_016780604.1	QLNVY**E**IKVNK	XP_517667.3	P - - EA**T**EKSHF	XP_016788608.1	S - - - - **-** - - - - -	XP_016773727.2	TTIVT**S**GTSTW
Pongo abelii (orangutan)	XP_002810439.1	QLNVY**E**IKVNK	-	-	XP_024090488.1	PGPEG**G**EHGDG	XP_024109991.1	TMIVT**C**GTSTW
Macaca mulatta (monkey)	XP_028685959.1	QLNVY**E**IKVNK	XP_001112269.2	P - - EA**T**EKSHF	XP_014976021.1	QGPEG**R**EHGDG	XP_015002478.2	TMIVT**S**GTSTW
Bos taurus (cow)	XP_024845781.1	QLNVY**E**IKVNK	NP_851378.1	P - - EV**T**EKSHF	XP_010814760.1	- - - - - **-** - - - - -	NP_001192874.3	TTIIT **A**GTSAW
Mus Musculus (mouse)	NP_035646.2	QLNAY**E**IKVSK	NP_001074718.1	P - - EA**P**GKSHS	NP_001032844.2	S - - - - **-** - - - - -	XP_011243421.1	TMIVT**S**GASAW
Rattus norvegicus (rat)	NP_036942.1	QLNAY**E**IKVNK	NP_001164029.1	P - - EA**T**GKSYS	NP_476544.1	- - - - - **-** - - - - -	XP_008769381.1	TMIIT**S**GASAW
Cricetulus griseus (hamsters)	XP_027249321.1	QLNAY**E**IKVNK	XP_027257574.1	P - - EA**T**EKLHS	XP_027281259.1	- - - - - **-** - - - - -	XP_003495398.1	TMIVT**S**GASAW
Oryctolagus cuniculus (rabbit)	XP_008262840.1	QLNVY**E**IKVSK	XP_017200054.1	P - - VA**T**EKPHL	XP_017195899.1	S - - - - **-** - - - - -	XP_017194119.1	TTIVT**S**GASAW
Lonchura striata domestica (finch)	XP_031363384.1	KANSY**E**GKVNK	XP_031363370.1	- - - - - **-** - - - - -	XP_021389776.1	S - - - - **-** - - - - -	XP_031361106.1	TTIIT **S**GTSSW
Parus major (great tit)	-	-	XP_015470575.1	- - - EP**A**QKILL	XP_015501277.1	- - - - - **-** - - - - -	XP_015489137.1	TTIIT **S**GTSSW
Ictalurus punctatus (catfish)	XP_017335924.1	- - - - - **-** - - LAK	XP_017307860.1	- - - DK**E**VTTIV	AHH41893.1	- - - - - **-** - - - - -	XP_017319576.1	NVIIT**A**GSSTW
Nothobranchius furzeri (killifish)	XP_015814136.1	KSSQL **E**VMINK	-	-	XP_015806086.1	E- - - - **-** - - - - -	XP_015799743.1	TTLIT**A**GASTW
Danio rerio (zebrafish)	NP_001112366.1	- - - - - **-** - - VKE	KTG38361.1	HKLST**N**IRIDV	-	-	XP_701288.6	STIIT**A**GTSTW

*, deletion mutation in *TNFSF13B* not included; **,from https://www.ncbi.nlm.nih.gov › protein

Table S6- Specifications on exome sequencing data of BVVL-111-II3*

Total reads	73,627,784
Total yield (Mbp)	7,436
Average read length (bp)	101.0
Target regions (bp)	60,456,963
Average throughput depth of target regions (X)	123
Initial mappable reads (mapped to human genome)	73,165,346
% Initial mappable reads	99.3
Non-redundant reads	65,068,310
% Non-redundant reads	88.9
On-target reads	47,863,931
% On-target reads	73.5
% Coverage of target regions (more than 1X)	99.6
% Coverage of target regions (more than 10X)	98.2
% Coverage of target regions (more than 20X)	93.4
Mean depth of target regions (X)	68.3
Number of SNPs	93,268
Number of synonymous SNPs	11,731
Number of Missense Variant	11,116
Number of Stop Gained	106
Number of Stop Lost	39
Number of indels	13,313
Number of Frameshift Variant	306
Number of Inframe Insertion	180
Number of Inframe Deletion	179
Number of % Found in dbSNP142	96.3
Het/Hom Ratio	1.3
Ts/Tv Ratio	2.3

*, The quality of data pertaining to all individuals exomed in BVVL-111, BVVL-103 and FL-101 were similar.

Table S7- Sequence variations that segregated with BVVL/FL status among family members whose DNAs were exome sequenced *

BVVL-103 (Proband and unaffected family members -I1, I2, & -II2 were exome sequenced.)					BVVL-111 (Proband and unaffected family members -I1, I2, II1, & -II2 were exome sequenced.)					FL-101 (Proband and unaffected family members -II1, II2, & -II4 - aged 36-43 yrs.- were exome sequenced.)				
Chromosome	Gene	Reference sequence	Variation	Zygosity		Gene	Reference sequence	Variation	Zygosity	Chromosome	Gene	Reference sequence	Variation	Zygosity
1	***SYCP1***	**NM_001282541**	**c.1918G>A (p.E640K)**	**Homo**	1	*NCSTN*	NM_001290186	c.757C>T (p.R253W)	Homo	5	*MCTP1*	NM_001297777	c.169C>T (p.R57X)	Het
1	*ANXA9*	NM_003568	c.938G>A (p.R313K)	Homo	**10**	***WDFY4***	**NM_020945**	**c.8851T>A (p.S2951T)**	**Homo**		*MCTP1*		c.770A>G (p.H257R)	Het
5	***VCAN***	**NM_001164097**	**c.3637A>G (p.T1213A)**	**Homo**	10	*CRTAC1*	NM_001206528	c.1892A>G (p.Y631C)	Homo	5	*APC*	NM_001127511	c.244C>T (p.L82F)	Het
5	*PCDHGB7*	NM_018927	c.1469C>T (p.S490F)	Homo	10	*TCF7L2*	NM_001146283	c.536C>A (p.P179H)	Homo		*APC*		c.7891C>T (p.P2631S)	Het
12	*KNTC1*	NM_014708	c.268G>C (p.V90L)	Het	13	*PARP4*	NM_006437	c.3986C>T (p.P1329L)	Het	9	*ZNF462*	NM_021224	c.4922A>C (p.E1641A)	Het
	KNTC1		c.2911A>G (p.K971E)	Het		*PARP4*		c.2339A>C (p.K780T)	Het		*ZNF462*		c.4905_4906insGAG (p.T1635delinsTE)	Het
17	***BAIAP2***	**NM_017451**	**c.1615C>T (p.R539W)**	**Homo**	19	*FOBS*	NM_001114171	c.434C>T (p.P145L)	Homo	**13**	***TNFSF13B***	**NM_001145645**	**c.276_281del (p.A93_G94del)**	**Homo**
X	*ADGRG4*	NM_153834	c.7637C>G (p.T2546S)	Homo/Hemi	19	*SLC8A2*	NM_015063	c.83T>C (p.L28P)	Homo					
					19	*WDR87*	NM_001291088	c.973G>T (p.E325X)	Homo					
					19	*CPT1C*	NM_152359	c.2077C>A (p.L693M)	Homo					
					19	*IL4I1*	NM_152899	c.1640G>A (p.S547N)	Homo					
					X	*PHEX*	NM_000444	c.1463T>A (p.V488D)	Homo/Hemi					

*, Variations that segregated even after screening in other individuals of the immediate and extended family of the prbands are shown in bold. Het, heterozygous; Hemi, hemizygous

Table 1- Clinical data on BVVL probands with mutations in *SLC52A3*

Family ID	BVVL-102	BVVL-104	BVVL-106	BVVL-108	BVVL-109	BVVL-110	BVVL-113
Proband ID	BVVL-102-II6	BVVL-104-II1	BVVL-106-II3	BVVL-108-II1	BVVL-109-II1	BVVL-110-II2	BVVL-113-IV1
Sex	female	female	female	female	female	female	male
Age at examination	27 yrs	16 yrs	17 yrs	14 yrs	39 yrs	41 yrs	19 yrs
Age at onset	19 yrs	8 yrs	9 yrs	6 yrs	12 yrs	14 yrs	9 yrs
Disease duration	8 yrs	8yrs	8 yrs	8 yrs	27 yrs	27 yrs	10 yrs
Initial presentation	dysphagia	hearing problems	dysphagia & dysarthria	ptosis	hearing problems	hearing problems	asymmetrical facial weakness
Bulbar palsy (CN-9,12)	+	+	+	+	+	+	+
	dysphagia, dysarthria, tongue atrophy	dysphagia, dysarthria, tongue atrophy	dysphagia, dysarthria, severe tongue atrophy	dysphagia, dysarthria, tongue atrophy	dysphagia, dysarthria, tongue atrophy	dysphagia, dysarthria	mild dysphagia, dysarthria, tongue atrophy
CN-5 palsy	mastication problems	mastication problems	mastication problems and atrophy	mastication problems and atrophy	mastication problems	mastication problems	mastication problems and atrophy
Facial weakness (CN-7)	weakness, atrophy, fasciculation	weakness, atrophy	weakness, atrophy	severe weakness, atrophy	weakness, atrophy	weakness, atrophy fasciculation	weakness, atrophy
Hearing problem (CN-8)	+	+	+	+	+	+	+
Ophthalmoplegia (CN3/4/6 palsy)	-	-	-	+	-	+	-
Ptosis	-	-	+	+	-	-	-
Optic nerve atrophy (CN-2 involvement)	-	-	-	-	-	-	-
Limb weakness	+	-	-	-	+, symmetrical, > prominent in distal upper extremities	+, asymmetrical, more prominent at left side	-
Limb muscle atrophy	+, distal atrophy	-	-	-	+	+, distal atrophy	-
Spasticity	-	-	-	-	-	-	-
Increased DTR	+	-	-	-	+	+ in upper extremities	-
Decreased DTR	-	-	-	-	-	+ in lower extremities	-
Sensory symptoms/ signs	-	-	-	-	-	-	-
Tremor	-	+, hand tremor	-	-	-	-	-
Ataxia	-	-	-	-	-	-	-
Vertigo	+	-	-	-	-	-	-
Tinnitus	-	-	-	-	-	-	-
Seizure	-	-	-	-	-	-	-
Mental impairment	-	-	-	-	-	-	-
Psychiatric disorder	+, depression	+, depression	-	+, depression	+, depression	+, depression	-
Autonomic dysfunction	-	-	-	-	-	-	-
Upward plantar reflex	-	-	-	-	+	-	-
Respiratory problem	-	-	-	-	-	-	-
Ambulatory status	no limitation	no limitation	no limitation	no limitation	no limitation	no limitation	no limitation
Acylcarnitine profile	not done	not done	not done	not done	not done	not done	not done
EMG	motor neuronopathy, more prominent but not restricted to cranial myotomes	motor neuronopathy, more prominent but not restricted to cranial myotomes	motor neuronopathy, more prominent but not restricted to cranial myotomes	motor neuronopathy restricted to cranial myotomes	motor neuronopathy, more prominent but not restricted to cranial myotomes	motor neuronopathy, more prominent but not restricted to cranial myotomes	motor neuronopathy, more prominent but not restricted to cranial myotomes
NCS	Normal sensory findings	Normal sensory findings	Normal sensory findings	Normal sensory findings	Normal sensory findings	Normal sensory findings	Normal sensory findings
Response to riboflavin	+	+	+	+	+	+	+

CN, cranial nerve; DTR, deep tendon reflex; EMG, electromyography; NCS; nerve conduction studies

Table 2- Clinical data on BVVL/FL probands with mutations in genes other than *SLC52A3* or *SLC52A2*

Family ID	BVVL-103	BVVL-111	FL-101
Patient ID	BVVL-103-II1	BVVL-111-II3	FL-101-II3
Sex	male	male	female
Age at examination	29 yrs	37 yrs	39 yrs
Age at onset	11 yrs	16 yrs	2 yrs*
Disease duration	18 yrs	21 yrs	37 yrs
Initial presentation	facial weakness	hearing problem	asymmetric distal weakness of lower extremities
Bulbar palsy (CN-9,12)	+ dysphagia, dysarthria, tongue atrophy	+ dysarthria	-
CN-5 palsy	mastication problems and masseter muscle atrophy	-	mastication problems and restriction in opening mouth
Facial weakness (CN-7)	weakness, atrophy	-	asymmetric weakness, atrophy
Hearing problems (CN-8)	+	+	-
Ophthalmoplegia (CN3/4/6 palsy)	-	-	-
Ptosis	+	-	+
Optic nerve atrophy (CN-2 involvement)	-	-	-
Limb weakness	+, asymmetric, more prominent at right side	+, mild distal & symmetric	+, asymmetric, more prominent in right side, significantly more severe in lower extremities
Limb muscle atrophy	+, distal atrophy	-	+, distal atrophy
Spasticity	-	-	-
Increased DTR	-	-	-
Decreased DTR	+	-	+ in lower extremities
Sensory symptoms/ signs	-	-	-
Tremor	+, hand tremor	+, hand tremor	+, mild asymmetric (left > right) hand tremor
Ataxia	-	+	-
Vertigo	-	-	-
Tinnitus	-	-	-
Seizure	-	-	+, one episode in early childhood
Mental impairment	-	-	-
Psychiatric disorder	-	-	-
Autonomic dysfunction	-	-	-
Upward plantar reflex	-	-	-
Respiratory problem	-	+	-
Ambulatory status	slow but device or help not needed	no limitation	slow, uses braces but does not need help
Acylcarnitine profile	not done	normal	normal
Brain MRI/CT Scan	normal	normal	normal
EMG	motor neuronopathy, more prominent but not restricted to cranial myotomes	motor neuronopathy at extremities and cranial and truncal levels	motor neuronopathy at extremities and cranial and truncal levels
NCS	Normal sensory findings	Normal sensory findings	Normal sensory findings

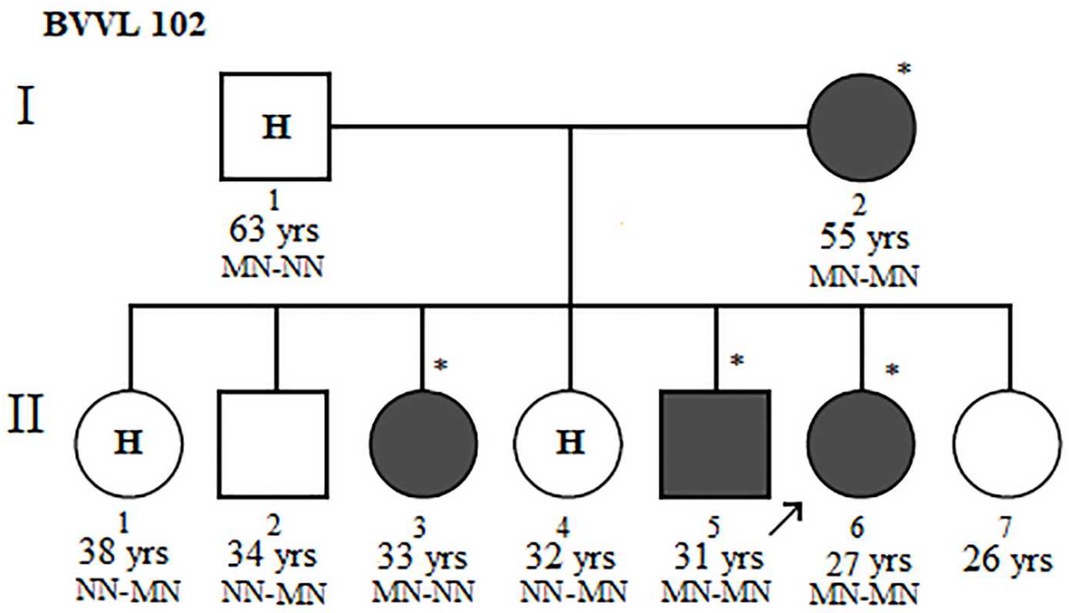
*, no apparent progression between age of 2 yrs. and 16 yrs.; CN, cranial nerve; DTR, deep tendon reflex; EMG, electromyography; NCS; nerve conduction studies

Table 3- Data on *SLC52A3* mutations observed in seven BVVL probands

Mutation no.	Proband ID	Sex	Parental consanguinity	Het/ Comp Het/ Homo	cDNA variation	Effect on protein	PolyPhen prediction	SIFT prediction	PROVEAN prediction	Novel/ non-novel	Report in data bases	Earlier publication	Present/ absent in controls
1	BVVL-102-II6	F	-	Comp Het	c.62A>G	p.Asn21Ser	probably damaging	affected protein function	deleterious	non-novel	dbSNP: rs199588390 HGMD: CM128722	PMID: 27702554 PMID: 22718020	absent
2					c.935C>T	p.Ala312Val	probably damaging	affected protein function	deleterious	non-novel	HGMD: CM128724	PMID: 22718020	absent
3	BVVL-104-II1	F	+	Homo	c.1153C>G	p.Pro385Ala	probably damaging	affected protein function	deleterious	novel			absent
4	BVVL-106-II3	F	+	Homo	c.634C>T	p.Arg212Cys	benign	tolerated	neutral	non-novel	dbSNP: rs778479139	PMID: 29053833	absent
5	BVVL-108-II1	F	+	Homo	c.1285C>T	p.Leu429Phe	probably damaging	affected protein function	deleterious	novel			absent
6	BVVL-109-II1	F	-	Het	c.986A>G	p.Tyr329Cys	probably damaging	affected protein function	deleterious	novel			absent
1	BVVL-110-II2	F	+	Homo	c.62A>G	p.Asn21Ser	probably damaging	affected protein function	deleterious	non-novel	dbSNP: rs199588390 HGMD: CM128722	PMID: 27702554	absent
7	BVVL-113-IV1	M	+	Het	c.37G>A	p.Gly13Arg	probably damaging	affected protein function	deleterious	non-novel	dbSNP: rs146302587	PMID: 29053833	absent

M, male; F, female;Het, heterozygous; Comp.Het, compound heterozygous; Homo, homozygous.

A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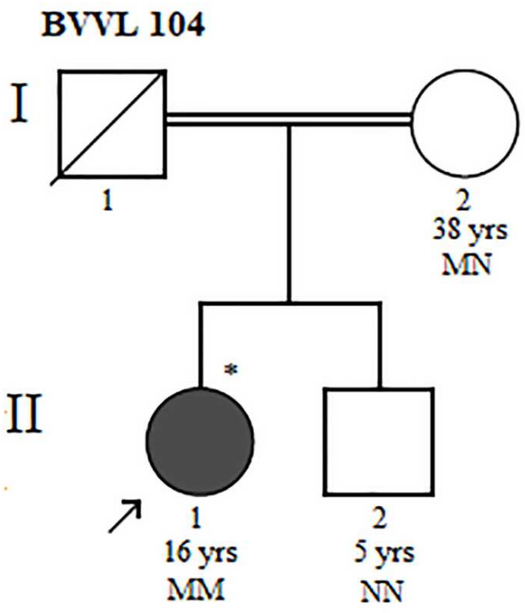
MN-NN
2
55 yrs
MN-MN
II
H
1
38 yrs
NN-MN
2
34 yrs
NN-MN
3
33 yrs
MN-NN
H
4
32 yrs
NN-MN
5
31 yrs
MN-MN
6
27 yrs
MN-MN
7
26 yrs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MN
II
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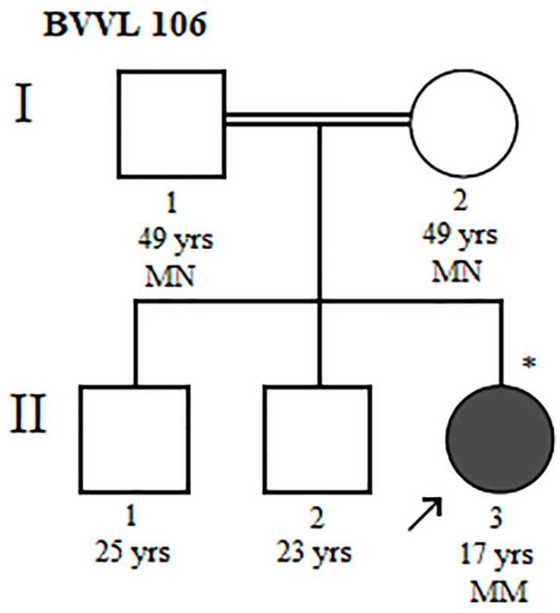
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2
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MN
II
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3
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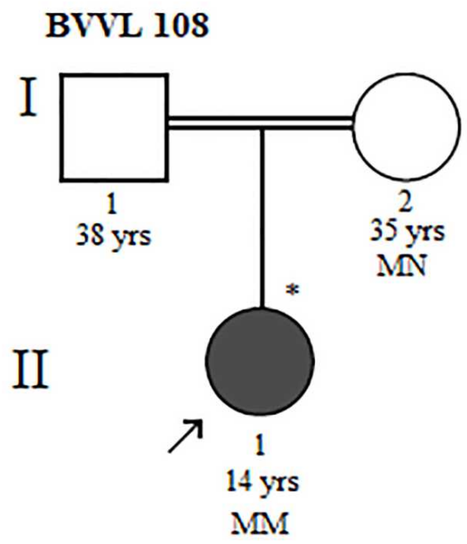
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II
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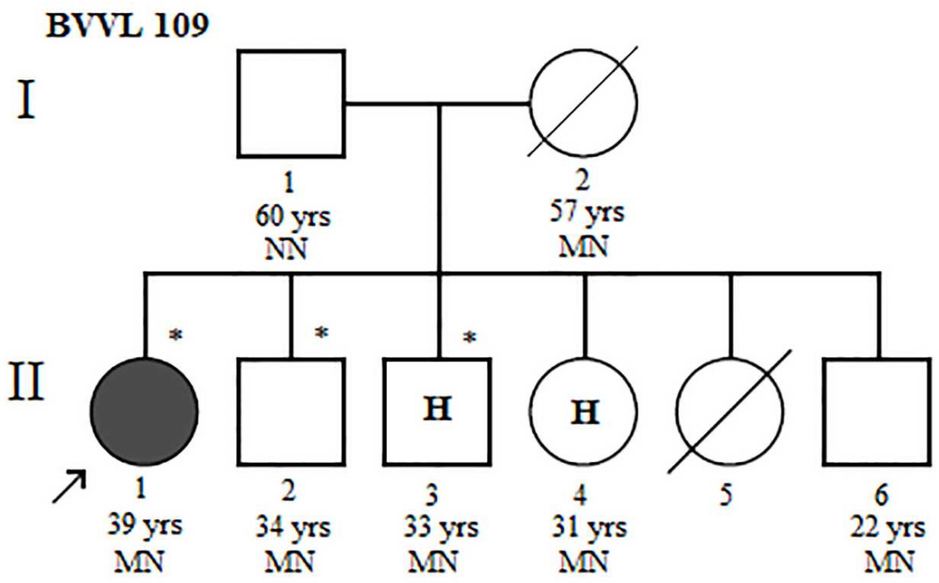
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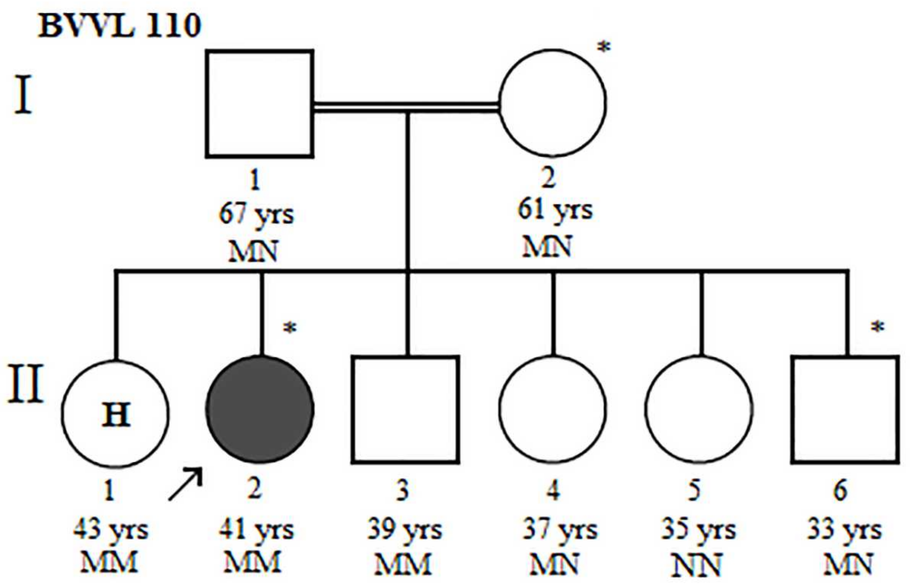
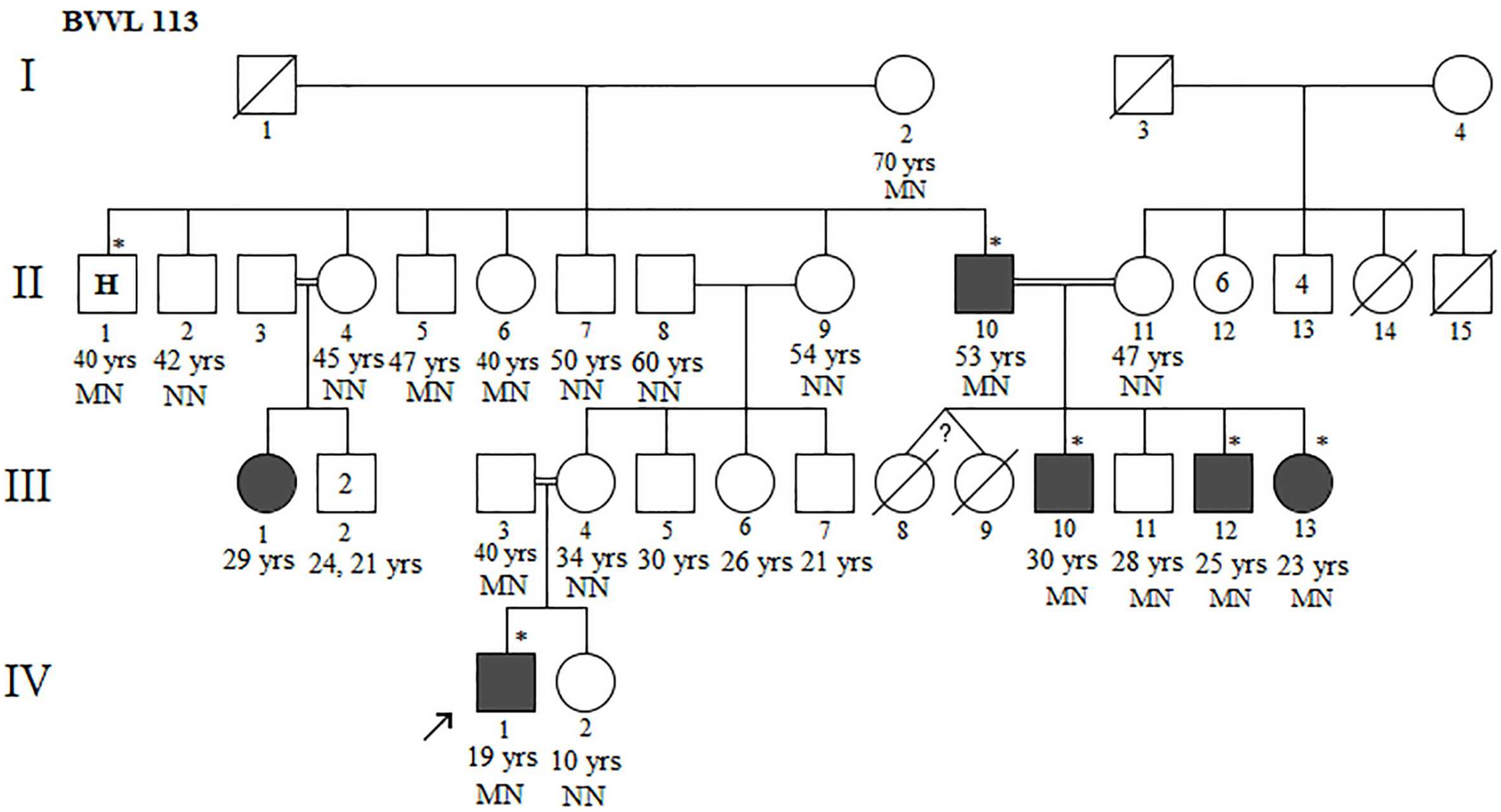
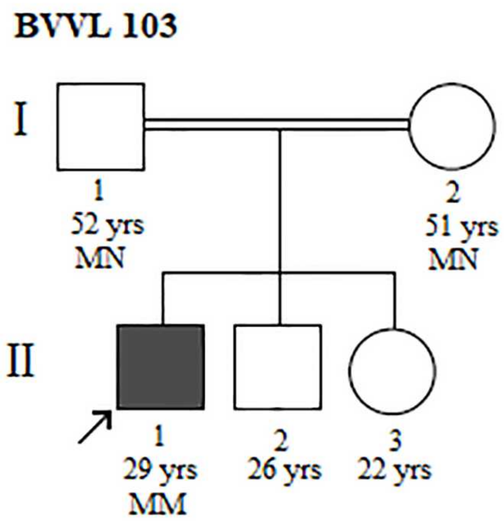
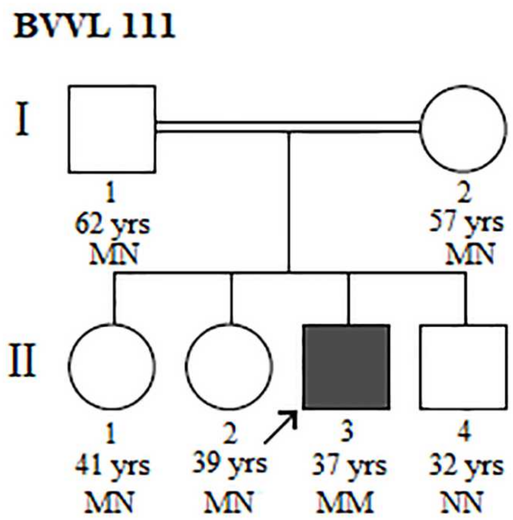
NN
2
57 yrs
MN
II
1
39 yrs
MN
2
34 yrs
MN
3
33 yrs
MN
H
4
31 yrs
MN
5
6
22 yrs
MN

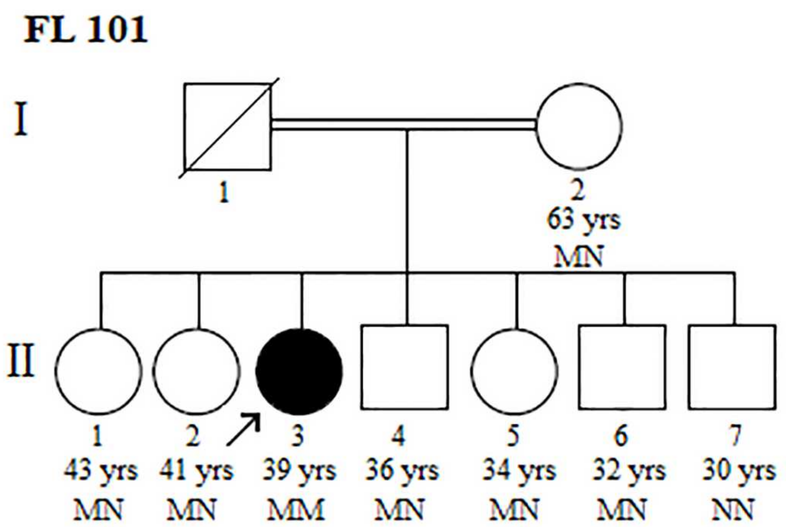

BVVL 110
I
1
67 yrs
MN
2
61 yrs
MN
II
H
1
43 yrs
MM
2
41 yrs
MM
3
39 yrs
MM
4
37 yrs
MN
5
35 yrs
NN
6
33 yrs
MN


BVVL 113
I
1
2
70 yrs
MN
3
4
II
H
1
40 yrs
MN
2
42 yrs
NN
3
4
45 yrs
NN
5
47 yrs
MN
6
40 yrs
MN
7
50 yrs
NN
8
60 yrs
NN
9
54 yrs
NN
10
53 yrs
MN
11
47 yrs
NN
6
12
4
13
14
15
III
1
29 yrs
2
24, 21 yrs
3
40 yrs
MN
4
34 yrs
NN
5
30 yrs
6
26 yrs
7
21 yrs
8
9
10
30 yrs
MN
11
28 yrs
MN
12
25 yrs
MN
13
23 yrs
MN
IV
1
19 yrs
MN
2
10 yrs
NN

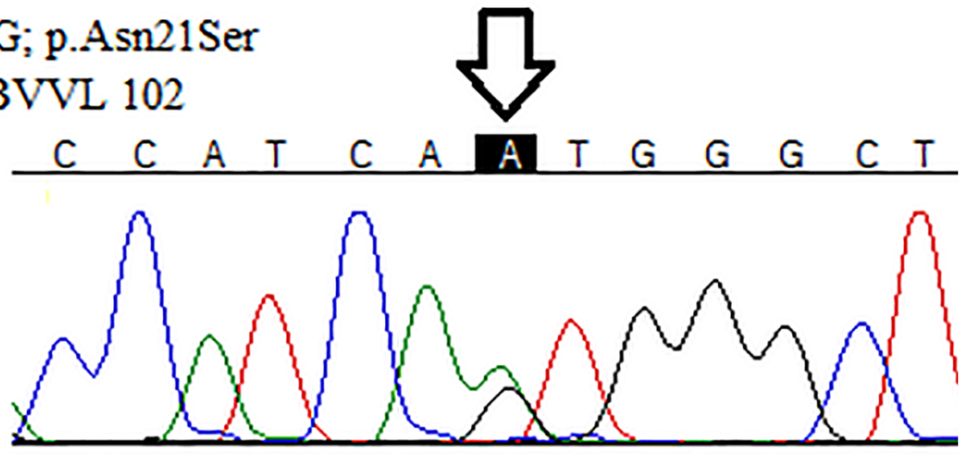
B


BVVL 103
I
1
52 yrs
MN
2
51 yrs
MN
II
1
29 yrs
MM
2
26 yrs
3
22 yrs

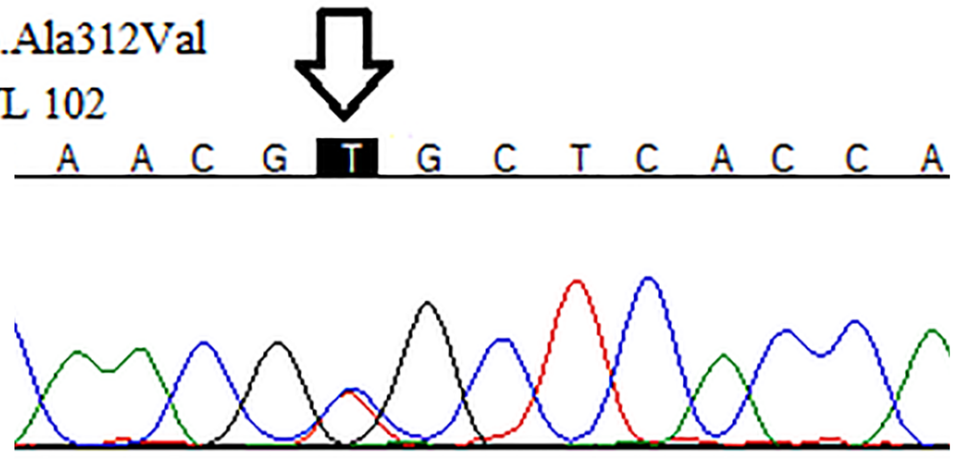

BVVL 111
I
1
62 yrs
MN
2
57 yrs
MN
II
1
41 yrs
MN
2
39 yrs
MN
3
37 yrs
MM
4
32 yrs
NN


FL 101
I
1
2
63 yrs
MN
II
1
43 yrs
MN
2
41 yrs
MN
3
39 yrs
MM
4
36 yrs
MN
5
34 yrs
MN
6
32 yrs
MN
7
30 yrs
NN

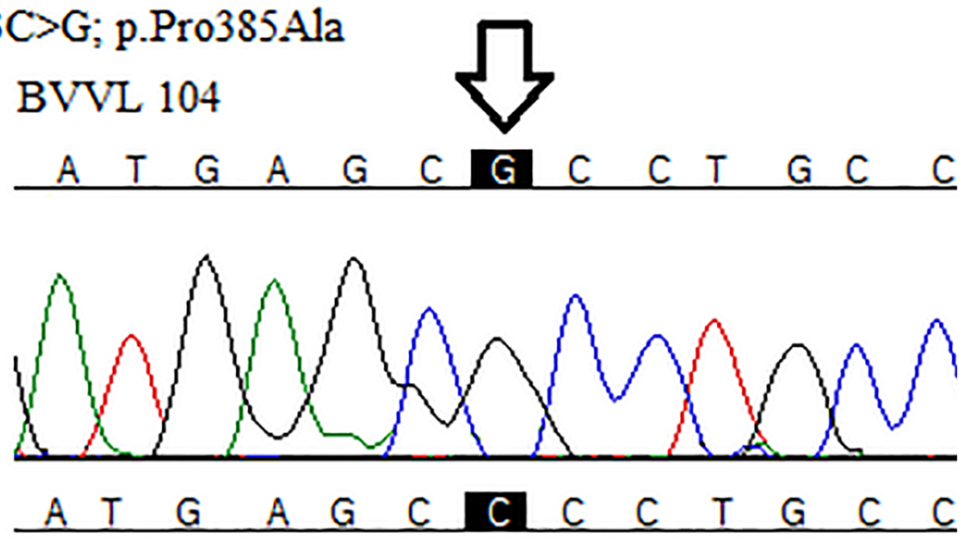
c.62A>G; p.Asn21Ser
BVVL 102



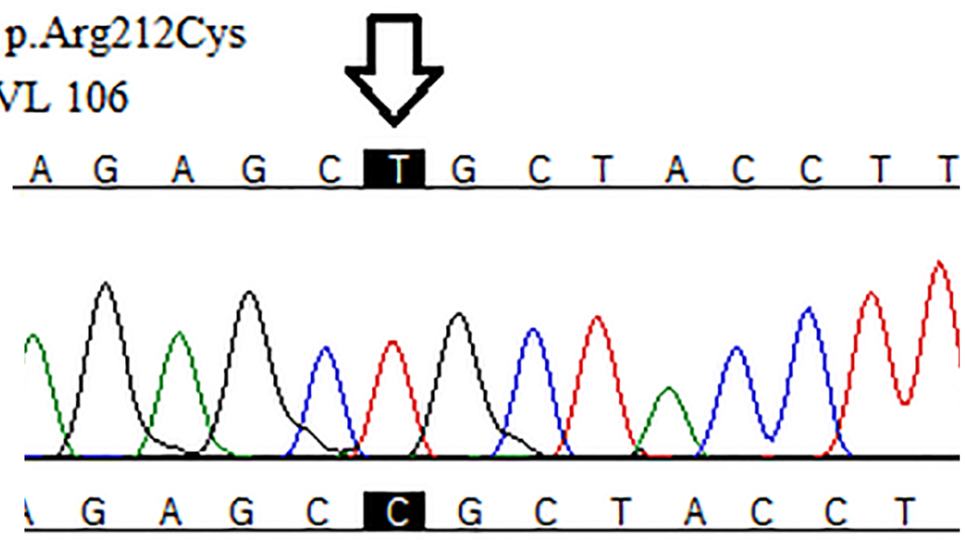
c.935 C>T; p.Ala312Val
BVVL 102



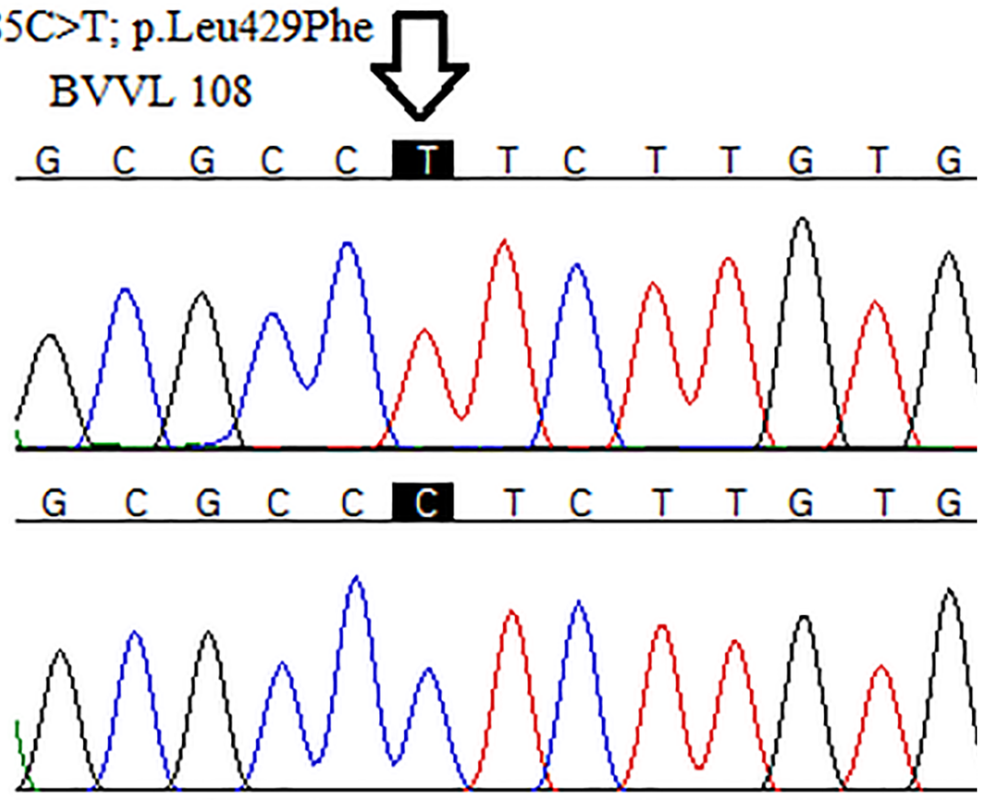
c.1153C>G; p.Pro385Ala
BVVL 104



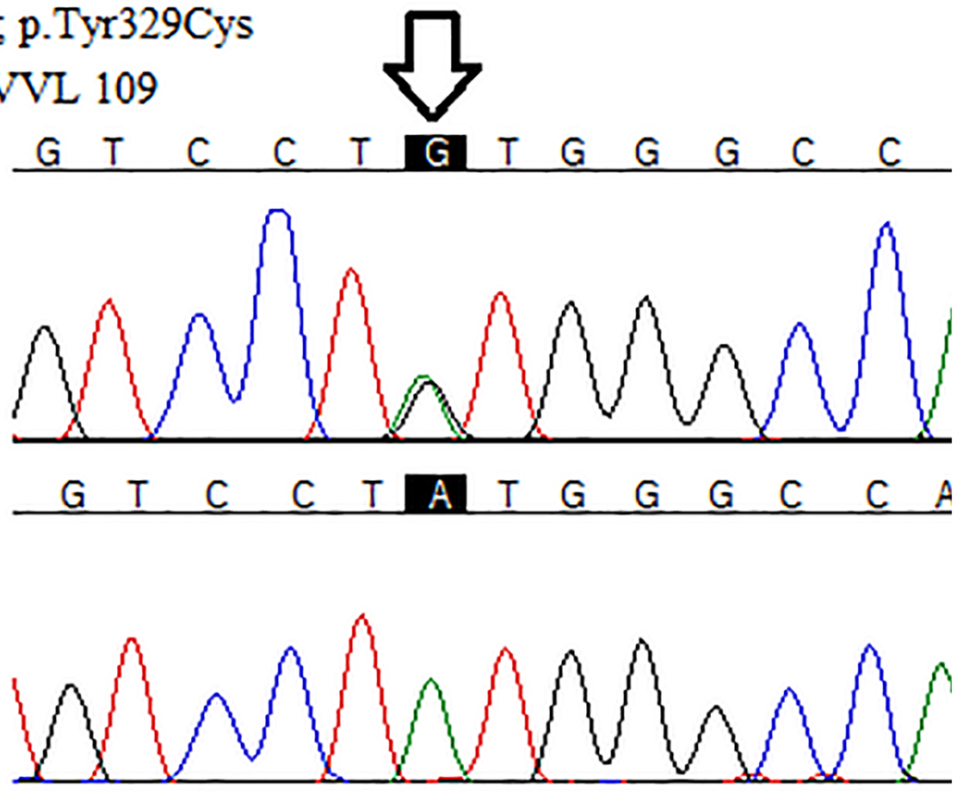
c.634C>T; p.Arg212Cys
BVVL 106



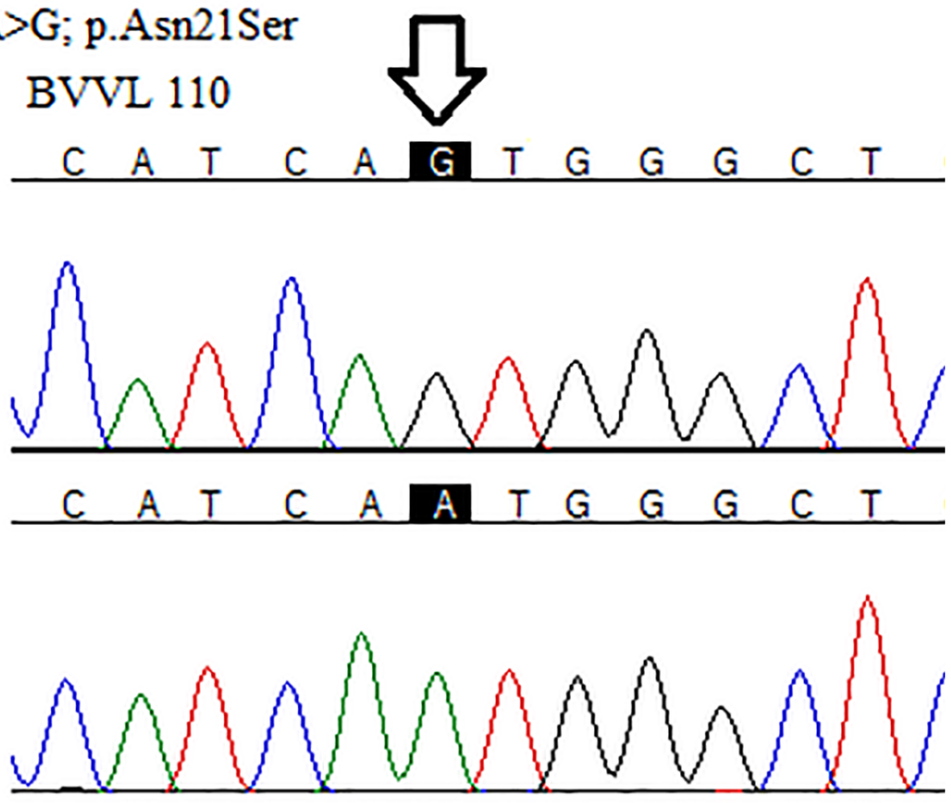
c.1285C>T; p.Leu429Phe
BVVL 108



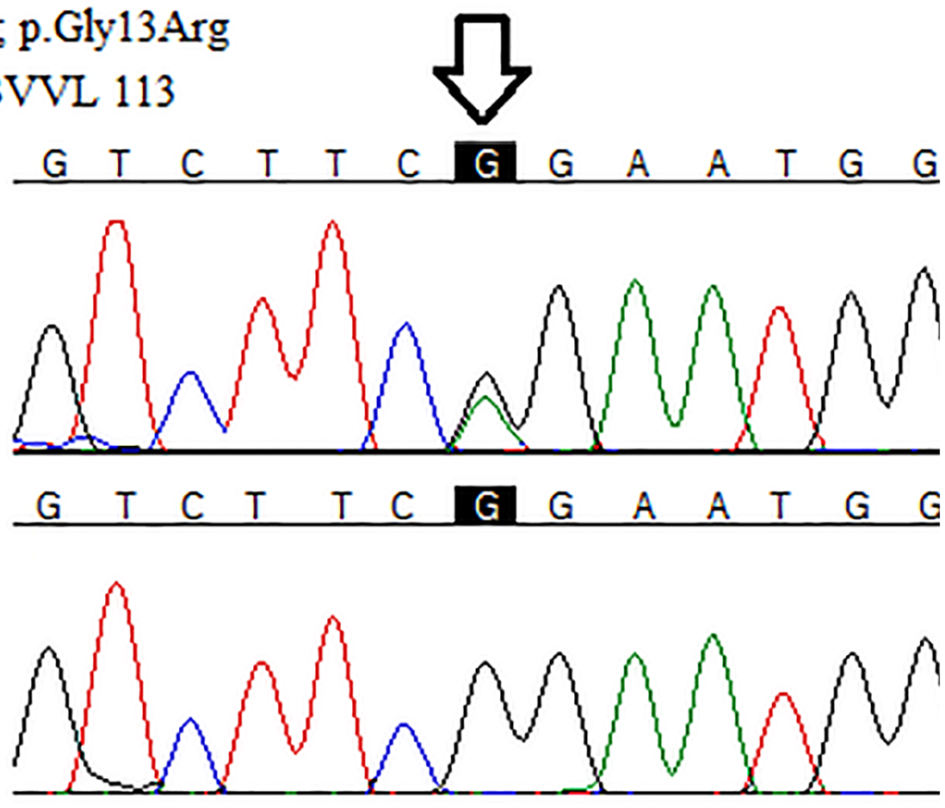
c.986A>G; p.Tyr329Cys
BVVL 109



c.62A>G; p.Asn21Ser
BVVL 110

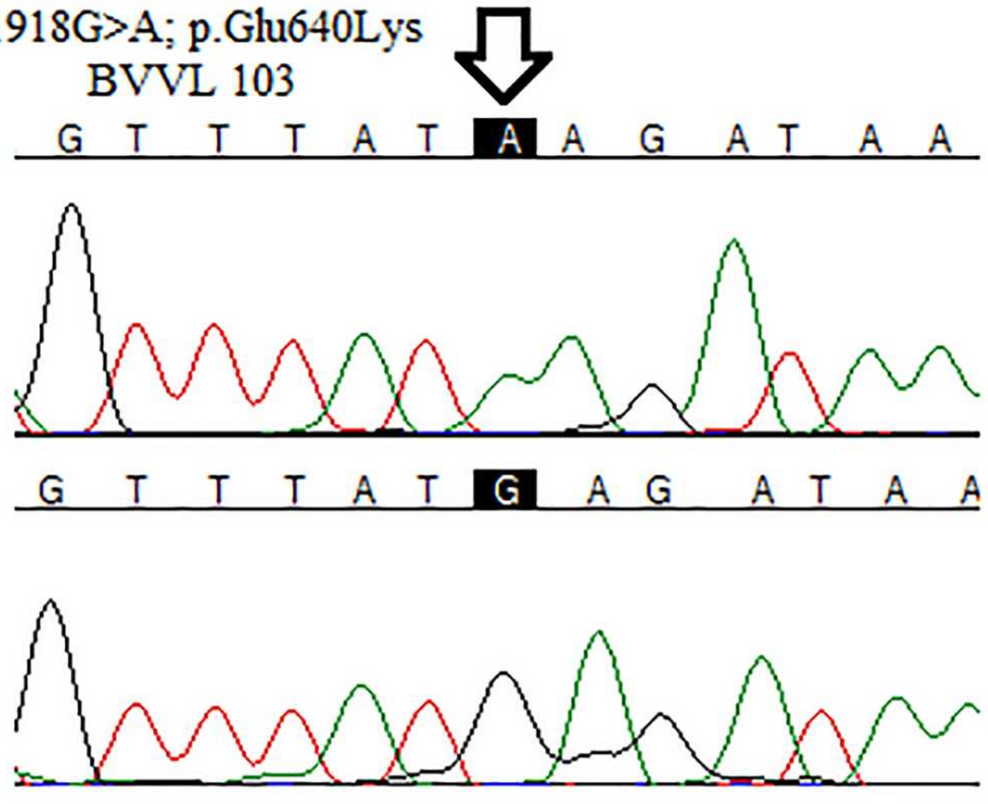


c.37G>A; p.Gly13Arg
BVVL 113



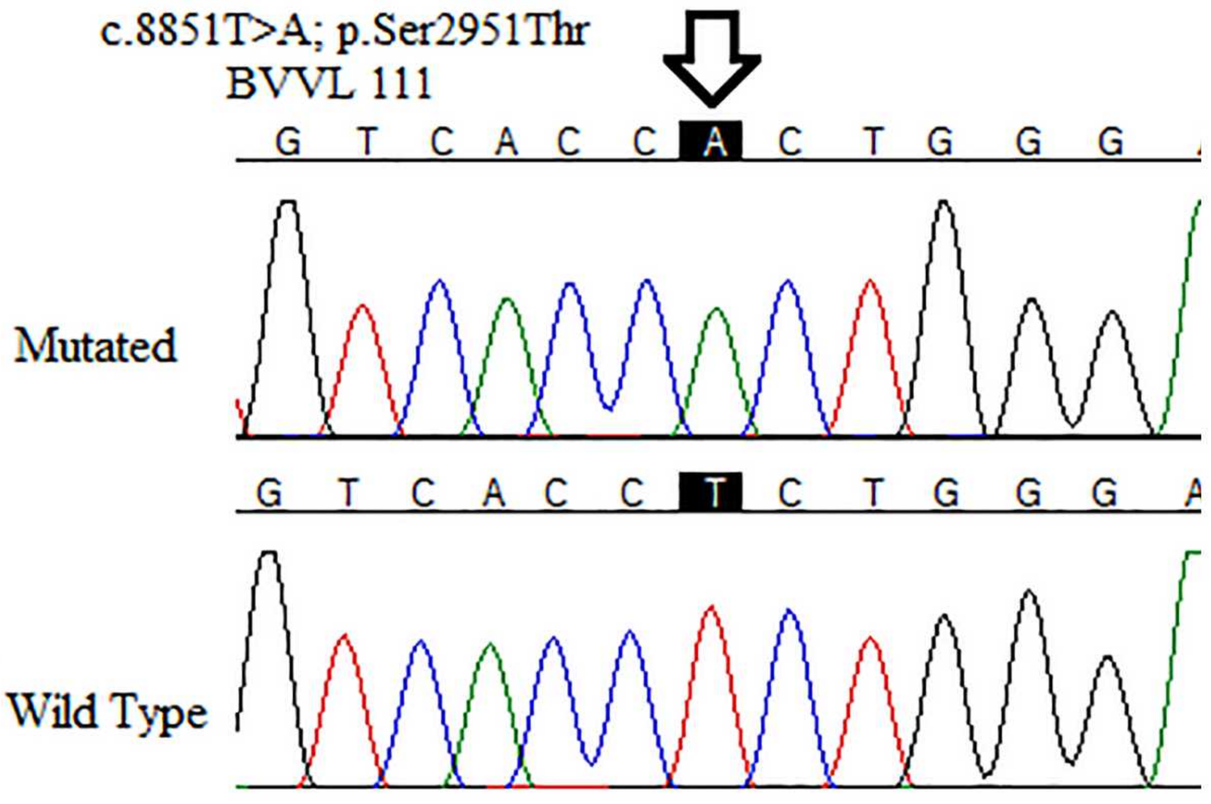
SYCP1

c.1918G>A; p.Glu640Lys
BVVL 103



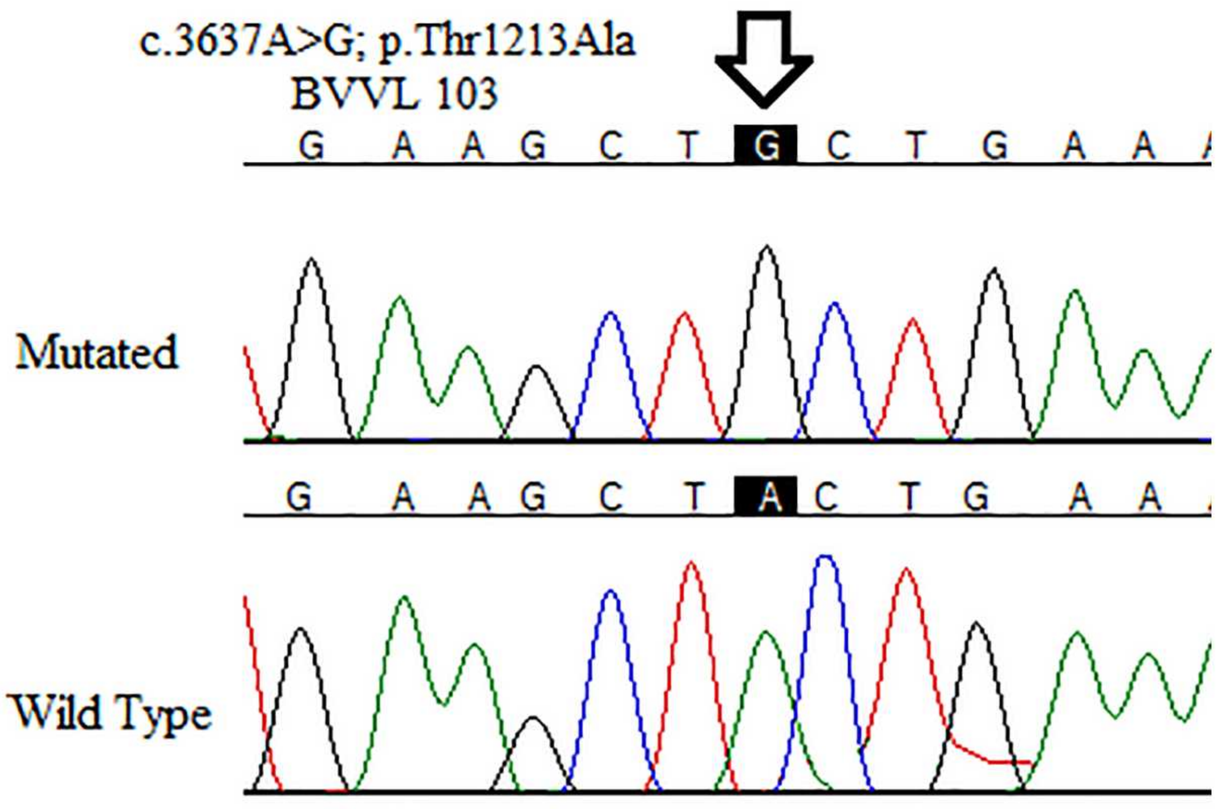
WDFY4

c.8851T>A; p.Ser2951Thr
BVVL 111



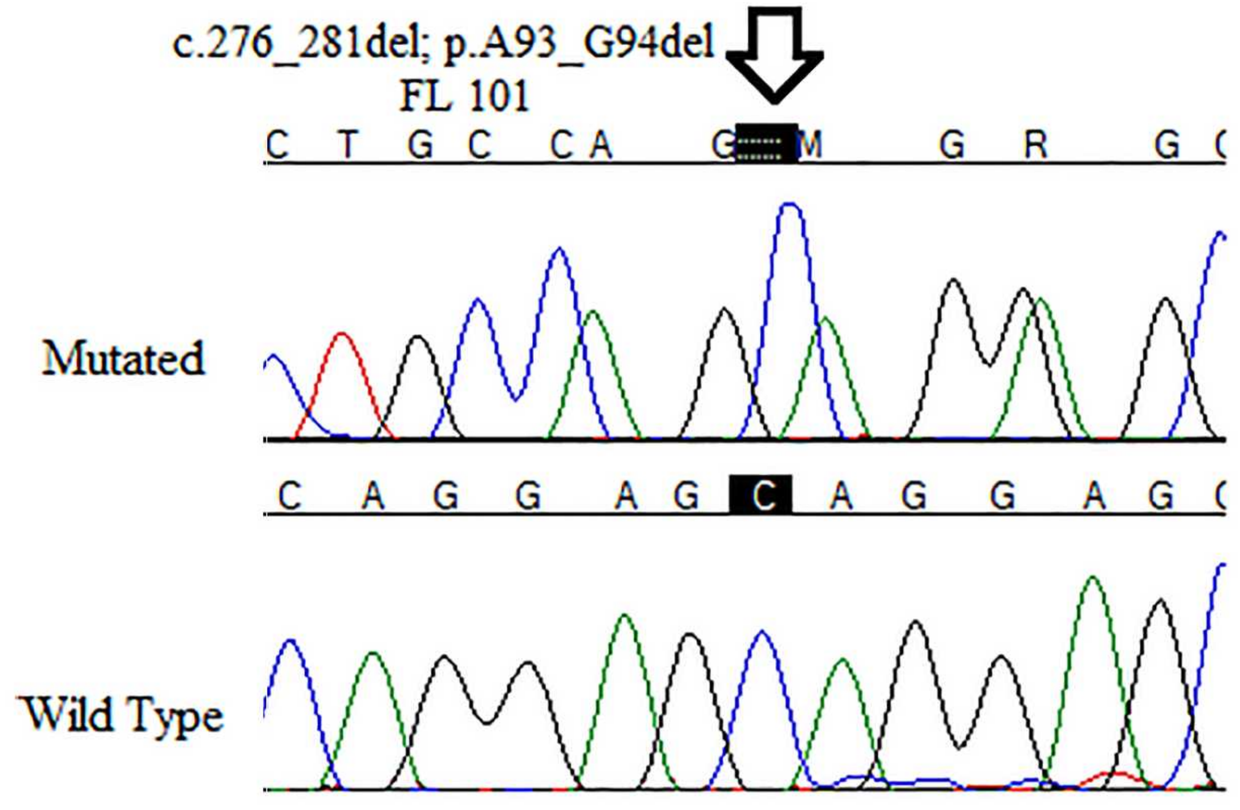
VCAN

c.3637A>G; p.Thr1213Ala
BVVL 103



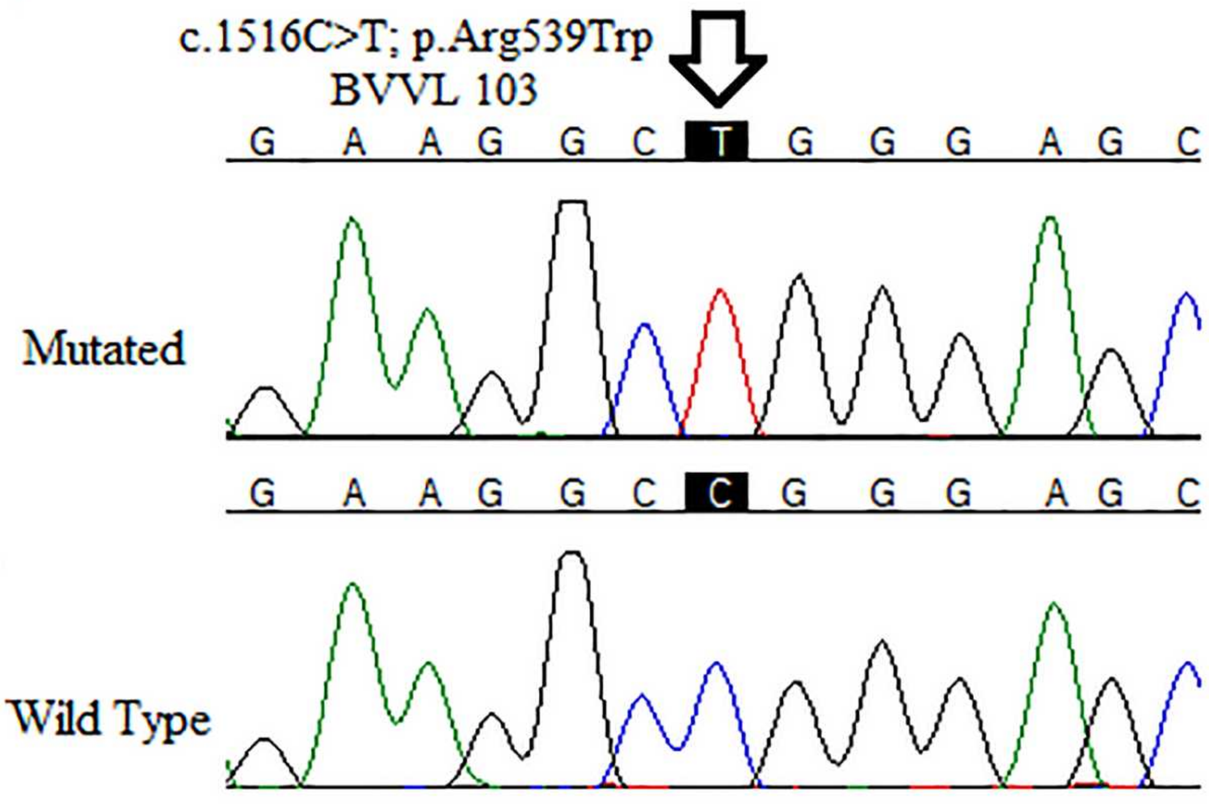
TNFSF13B

c.276_281del; p.A93_G94del
FL 101



BAIAP2

c.1516C>T; p.Arg539Trp
BVVL 103



Supplementary material

TEXT 1: Methods

Subjects

BVVL or FL diagnosed patients were referred for genetic analysis by the neurologists who are among the authors, mostly by SN who is head of the Neuromuscular Division of Shariati Hospital that is associated with the Tehran University of Medical Sciences. Diagnosis in all cases was confirmed by SN and HS. Family members were recruited when possible. BVVL diagnosis was based on presence of motor neuronopathy with prominent cranial nerve involvement accompanied with hearing impairment. FL diagnosis was based on presence of motor neuronopathy with cranial nerve involvement without hearing impairment. Riboflavin was always prescribed at dosage of 10 mg/ kg body weight/ day. Hearing status was based on self or family reports and pure tone audiometry testing on 27 individuals. All probands and some family members underwent electrodiagnostic (EDX) testing including nerve conduction studies (NCS) and electromyography (EMG) in upper and lower extremities, truncal regions, and cranial regions according to standard procedures (Synergy On Nicolet EDX, Natus, CA, USA). Brain magnetic resonance imaging (MRI), biochemical testings, and/or immunological testings were performed on some patients. The biochemical and immunological testings were performed at least two times. Fluorescent antinuclear antibody (FANA) testing on the serum of two patients was done by standard protocols. Plasma acylcarnitine profiles were obtained by tandem mass spectrometry; plasma samples were obtained from blood taken after at least ten days of not having taken riboflavin medication. MRI was done using a 1.5-T system (MAGNETOM Avanto 1.5 Tesla, Siemens, Germany). T1 and T2-weighted spin echo protocols were used. Muscle and nerve histopathology was performed for one proband by standard protocols.

Genetic analysis

Probands and family members were interviewed to obtain information pertaining to BVVL or FL in the families. DNA was isolated from blood cells by standard protocols. Initially, the exons and flanking intronic sequences of *SLC52A3* in the DNA of the probands were amplified and sequenced as previously described (Dezfouli *et al.*, 2012). Reference sequences used for analysis were NC_000020.10, NM_033409.4, and NP_212134.3. Effects of variant sequences on splicing were assessed with NNSPLICE 0.9 (http://www.fruitfly.org/seq_tools/splice.html) and Human Splicing Finder version 3.1 (HSF 3.1) (http://www.umd.be/HSF/HSF.shtml) softwares. Candidate disease causing variations were screened for segregation with disease status in members of respective families by direct sequencing. Previously unreported mutations were also screened in 300 Iranian control individuals by an allele specific PCR protocol and also sought in the Iranome database (http://iranome.com/) that contains exome data on 800 healthy Iranians. Evolutionary conservation of amino acids affected by the mutations was checked. The exons and flanking intronic sequences of *SLC52A2* were screened by the same protocol for patients in whom no or only one *SLC52A3* mutated allele had been found. *SLC52A2* reference sequences used were NC_000008.10, NM_001253815.2 and NP_001240744.1. Whole exome sequencing was performed for probands in whom *SLC52A3* and *SLC52A2* mutations had not been found and for three or four unaffected members of each family. The sequencing was done using the SureSelect V6-Post Kit and an Illumina HiSeq 4000 system (Illumina, CA, USA). Sequence alignment was performed against reference genome GRCh37/hg19, and variant callings were done by using ENSEMBL Variant Effect Predictor (http://www.ensembl.org/Tools/VEP) and wANNOVAR (http://wannovar.wglab.org/). Filtering was performed by removing SNPs with a minor allele frequency (MAF) of > 0.01 in the dbSNP database (http://www.ncbi.nlm.nih.gov/), the Trans-Omics for Precision Medicine program

(https://www.nhlbiwgs.org/), the 1000 Genomes database (www.1000genomes.org), the NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/), the Exome Aggregation Consortium database (http://exac.broadinstitute.org/), the Genome Aggregation database (http://genomad.broadinstitute.org/), the Greater Middle East Variome Project (http://igm.ucsd.edu/gme/), ENSEMBL (https://www.ensembl.org/index.html), the Healthy Exomes database (https://www.alzforum.org/exomes/hex), the Sequencing Initiative Suomi database (http://www.sisuproject.fi/), the VarCards database (http://varcards.biols.ac.cn/), or the Iranome database (http://iranome.com/) , or observed in in-house exome data belonging to approximately 100 unrelated Iranians affected with non-neurological diseases. Among the variations that remained, those that did not affect amino acid change or splicing were removed. Subsequently, a file for each family was prepared that containing retained genes with homozygous or compound heterozygous variations present in the proband and absent in respective unaffected individuals. Though parental consanguinity suggested that causative mutations would most likely be homozygous, compound heterozygous variations were retained for the sake of stringent analysis. Candidate disease causing variations were screened for segregation with disease status by Sanger sequencing in 15 -47 unaffected individuals in the nuclear and extended family of the proband. Segregating mutations were also screened in control individuals as described above.

TEXT 2: Results

Subjects

Families with *SLC52A3* mutations

BVVL-102

The proband (BVVL-102-II6) had normal development until the late teens when the patient began to have difficulty in swallowing food. The patient also reports having experienced vertigo. Difficulty in walking and speech problems ensued within one year. Presently, eight years after onset, she has prominent hearing problems and bulbar palsy presentations including dysphagia, dysarthria, and tongue atrophy. The patient is withdrawn and depressed. Although atrophy in her distal limbs is evident, she is independent in walking and performing daily functions.

Reports from the proband on family members and results of genetic analysis (see section on Genetic analysis/ Families with *SLC52A3* mutations in Results) prompted clinical examination of other family members. The mother (-I2), now in her mid-50s had hearing problems from when she was in her 20s. She presently presents with difficulties in swallowing and more notably in drinking, difficulties in walking, facial weakness, weakness in the limbs and significant atrophy in the legs. The clinical manifestations of the mother are clearly less severe than those of her daughter (-102-II6) who is almost 30 years younger. Sibling -II3 had leg weakness and hearing defects. Sensorineural hearing defects were confirmed in -102-II3 and -102-II5. EDX results evidenced neuronopathy in the proband and in -I2, -II3 and -II5 (Table S1). All four individuals were diagnosed with BVVL. Unfortunately, transport of -I1, -II1, -II2, and -II4 for critical neurological examination was not possible. Audiometric testing on these individuals revealed that the father (-I1) and siblings -II1 and -II4 had sensorineural hearing loss, and that -II2 was normal.

BVVL-104

The mother of the proband reports that her child had hearing problems and frequent episodes of upper respiratory tract infections in early childhood, and that the child regularly snored. The child’s adenoids and tonsils were sequentially removed before the child was five.

Regardless of these interventions, her snoring evolved into a deep and very loud squealing and she experienced voice change and breathing difficulties. Hand tremor, dysphagia, and walking difficulties ensued. Riboflavin treatment was started at age of nearly ten when BVVL was diagnosed. Although the patient responded favorably, she does not use the drug regularly as prescribed. The mother reports that her daughter's father and paternal grandfather both had some hearing problems. Audiometric testing showed that the mother herself has normal hearing.

BVVL-106 and BVVL-108

The clinical profiles of the single affected individual of families 106 and 108 are quite similar. Genetic findings prompted assessment of hearing loss in ostensibly unaffected individuals of these families. Audiometric testing was possible for BVVL-108-I2, and her hearing was found to be normal.

BVVL-109 and BVVL-110

Both probands of BVVL-109 and BVVL-110 had a long disease duration of 27 years. Their clinical features are very similar, although ophthalmoplegia and asymmetry in limb weakness were observed only in BVVL-110-II2. The mother reported that start of hearing loss in BVVL-110-II2 coincided with tonsillitis. Results of genetic analysis suggested that clinical examination of additional members of families 109 and 110 should be performed. In family -109, neurological examinations including EDX were normal for -II2 and -II3, but -II3 had severe sensorineural hearing defects; hearing of -II2 was normal. Family members reported that BVVL-109-II4, who was not examined, also had hearing problems. In family -110, only -I2 and -II6 consented to clinical examination; both were found to be normal. Hearing difficulties were reported for BVVL-110-II1 who was not examined.

BVVL-113

BVVL-113 is a large two-branched pedigree (Fig. 1A). The proband was definitively diagnosed with BVVL (Table 1). The proband's mother reported that onset of his symptoms coincided with an incidence of severe fever accompanied by upper respiratory tract infection. Pedigree members told us that there are six other individuals (BVVL-113-II1, -II10, -III1, BVVL-113-III10, -III12, and -III13) distributed in the two branches with hearing problems or presentations similar to the proband. Five (all except BVVL-113-III1) were recruited and results of clinical examinations, including EDX evidence of motor neuronopathy, supported BVVL diagnosis in four (all except BVVL-113-II1) (Table S2). BVVL-113-III1, based on presentations described, is also probably affected. The only BVVL associated feature observed in BVVL-113-II1 was hearing loss which was confirmed by auditory testing. This individual who is presently in the early 40s old reported that his hearing problems had started fifteen years earlier immediately after a severe upper respiratory infection. The coincidence of BVVL related symptoms and incidence of upper respiratory infection and fever in -113-II1 and the proband of pedigree BVVL-113 is reminiscent of BVVL onset in the proband of family BVVL-104.

Families without *SLC52A3* mutations

BVVL-103

BVVL-103-II1 is the only BVVL affected individual in a large highly inbred pedigree. BVVL-103-II1 was apparently normal until the beginning of second decade of life, at which time he presented with facial weakness. His mother also reports onset of tongue atrophy at that same time. Hearing problems were detected within one year. Weakness in hands and feet, and difficulty in walking ensued. Intrinsic hand muscle atrophy and hand tremor are

now present. His presentations are generally more prominent on the right side. Dysphagia with respect to liquids was more severe than solid food. Brain MRI images were normal. The patient used riboflavin regularly as prescribed for two years from the age of 23, but seeing no improvement, he stopped using it.

BVVL-111

BVVL-111-II3, who is presently in the mid-30s old, is the only affected individual among four siblings. The patient had hearing problems from when the age of 16 and these significantly worsened with passage of time. He reports that he had no other symptom until three years ago, when he noticed dysarthria, dysphonia and dyspnea after a course of severe and long-lasting respiratory infection. Brain CT scan in lieu of MRI was done because presence of cochlear implant precluded MRI. The scan was unremarkable. Results of thorough clinical laboratory measurements are summarized as follows (Table S3). Red blood cell count and hemoglobin and hematocrit measurements were high; these may reflect the patient's respiratory difficulties. Lactate dehydrogenase (LDH) and creatine phospho-kinase (CPK) measurements were within the normal range. C reactive protein and erythrocyte sedimentation measurements, serum immunoglobulin and complement levels, and B-cell and T-cell numbers as assessed by flow cytometry measurements of surface markers were normal. However, mildly elevated serum neutrophil and decreased lymphocyte levels and borderline alpha 1 antitrypsin levels were consistent with presence of an inflammatory response (Stockley RA, 2015). This was further supported by presence of anti-nuclear antibody evidenced as the few nuclear dot pattern by immunofluorescent microscopy (Fig. 2A) (Damoiseaux *et al*., 2019). Results of other autoimmune related measurements were negative. Plasma lupus anticoagulant levels were normal. Amino acid and acylcarnitine

profiles were completely normal. The patient has been using riboflavin for six months and reports no improvement of symptoms.

FL-101

FL-101-II3 is in the late 30s and the only affected individual among seven siblings. The patient's mother reports that her child experienced an episode of febrile seizure at age of six months, that her gait was abnormal from the time she started to walk at the age of two years, and that ptosis was evident from childhood. The proband herself had no complaints until about the beginning of teenage years when it became difficult for her to endure walking long distances. Atrophy became evident in her legs and was progressive. Presently, she has proximal and distal weakness of upper and lower limbs, more prominent in the right side and associated with axial weakness (neck flexor and neck extensor). Distal weakness of lower limbs evidences as foot drop and steppage gait. There is intrinsic muscle atrophy in her right hand. The patient has facial weakness, but does not manifest bulbar palsy. Electromyography study of cranial region showed chronic neurogenic MUP (motor unit potential) pattern in mentalis and trapezius muscles, but normal MUP pattern in tongue and masseter muscles. She does not have hearing problems. She was independent until the age of 31 yrs., but now needs help for some tasks including climbing stairs. The patient was diagnosed with FL at in her early 30s. Brain MRI images were normal. Results of thorough clinical laboratory measurements are summarized as follows (Table S4). There were multiple indications consistent with possible immune anomalies. The cytotoxic T-cell level was slightly high as compared to normal range, and the helper to cytotoxic T cell ratio was inverted. The B cell level as assessed by CD19 measurement was at the lower end of the normal range. Polyclonal immunoglobulin G and M levels in the serum were elevated. This increase, and elevated serum neutrophil and alpha 1 antitrypsin levels and decreased lymphocyte levels were

consistent with presence of an inflammatory response (Stockley RA, 2015). Measurements of various autoimmune factors, including anti-dsDNA antibodies and lupus anticoagulants, were within normal ranges. However, presence of anti-nuclear antibodies that evidenced with the fine speckled pattern in the nucleus by fluorescent microscopy, is suggestive of an autoimmune and/or inflammatory response (Fig. 2B) (Damoiseaux *et al.*, 2019). Mildly elevated LDH and CK levels are consistent with muscle involvement, and elevated lactate and lactate to pyruvate ratio are consistent with mitochondrial dysfunction. Some parameters of the patient's acylcarnitine profile, including methylmalonycarnitine (C4DC), hydroxyisovalerylcarnitine (C5OH), decenolylcarnitine (C10:1), and tetradecadienolycarnitine (C14:1) were not within the normal range. Abnormal acylcarnitine profiles may reflect defects in mitochondrial fatty acid beta-oxidation catabolism (Wanders *et al*., 2010).

Results of muscle histology confirmed presence of neurogenic muscle atrophy and some mitochondrial dysfunction that was evidenced in the laboratory results and clinical examinations. The muscle biopsy from the left vastus lateralis revealed marked muscle atrophy with a fascicular atrophy pattern. The remaining fibers were round and multiple nuclear clumps were associated with hypertrophied fibers with occasional fiber splitting (Fig 3A). Endomysial connective tissue was increased with prominent adipose tissue replacement. There was no evidence of inflammatory cell infiltration. Staining for ATPase showed uniformity of type 2 fibers. Ragged red fibers were not seen in modified Gomori Trichrome staining, but succinate dehydrogenase (SDH) staining showed abnormal peripheral mitochondrial proliferation in some fibers (Fig. 3B). The SDH plus cytochrome oxidase (COX) reactions revealed a notable number of fibers with reduced COX activity which is consistent with neurogenic atrophy with some mitochondrial dysfunction (Fig. 3C).

Histology of a biopsy from the left sural nerve showed no evidence of vasculitis, neither granuloma nor amyloid deposition (Fig. 3D).

The proband has regularly used riboflavin for seven years, but reports no improvement and instead progression of disease presentations.

TEXT 3: Discussion

Single mutated *SLC52A3* alleles in BVVL/FL diagnosed patients previously or now reported include c.37G>A(p.Gly13Arg), c.58A>C(p.Ile20Leu), c.62A>G(p.Asn21Ser), c.106G>A(p.Glu36Lys), c.113G>C(p.Trp38Ser), c.374C>A(p.Thr125Asn), c.403A>G(p.Thr135Ala), c.659C>A(p.Pro220His), c.986A>G(p.Tyr329Cys), c.1124G>A(p.Gly375Asp), c.1296C>A(p.Cys432X), and c.1371C>G(p.Phe457Leu).

References referred to above:

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TEXT 4: Additional references pertaining to main text of article

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References pertaining to ***BAIAP2***:

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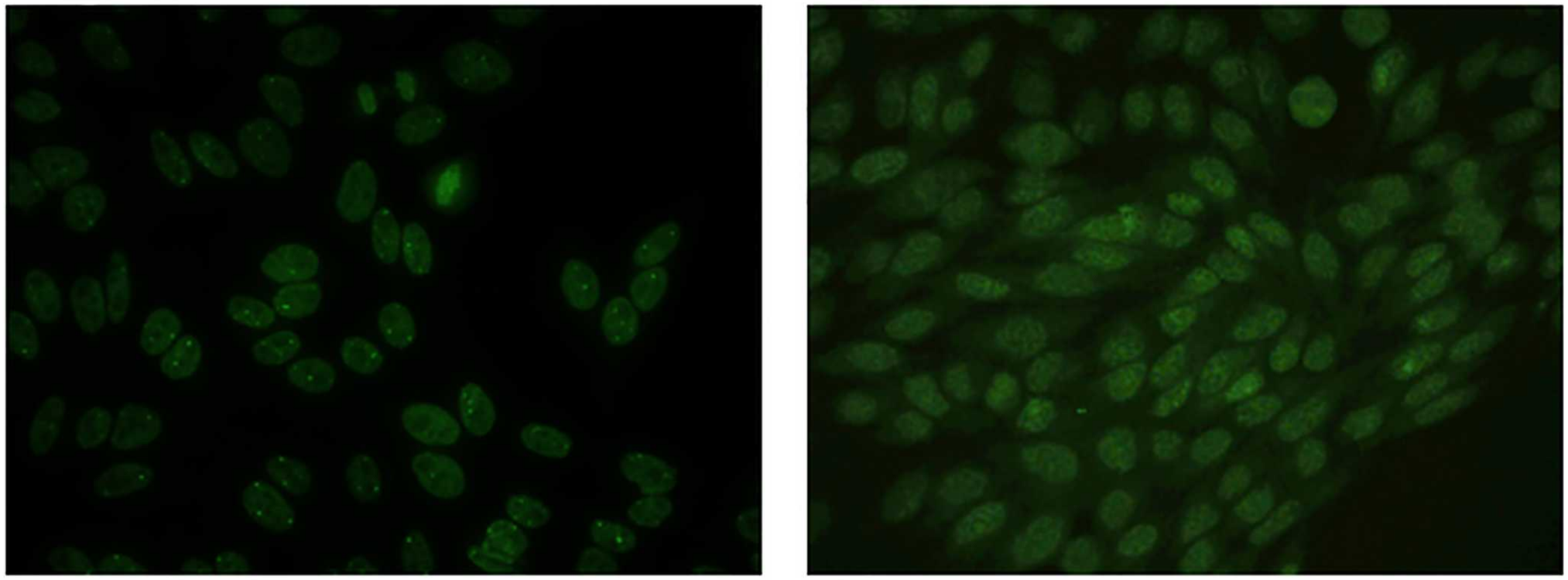

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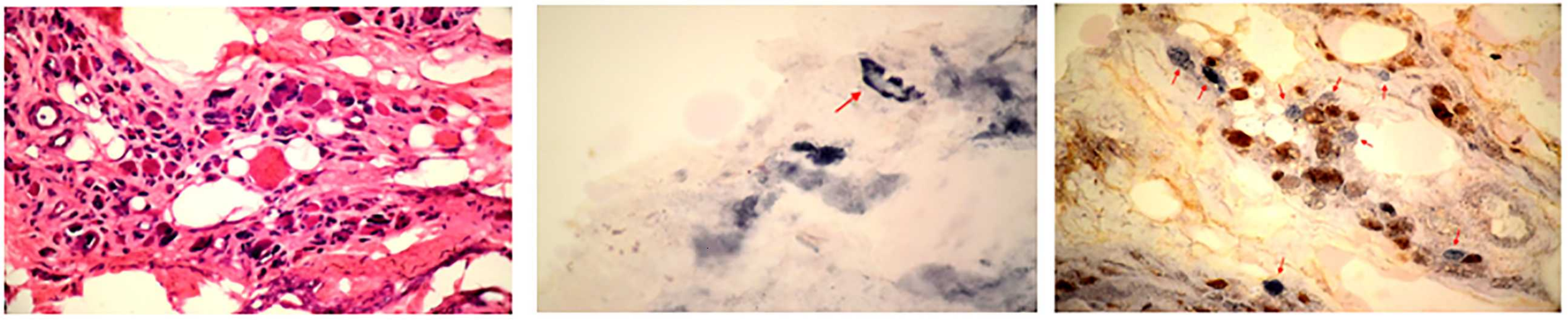

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