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Extended Phenotype of VPS45 Defect with Additional Features of Combined Immunodeficiency and Neuromotor Developmental Delay Along with Severe Congenital Neutropenia

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ABSTRACT

Objective: Genetic aberrations of VPS45 (Vacuolar protein sorting 45 homolog) protein, a member of the Sec1/Munc18 (SM) family, results in congenital neutropenia, bone marrow fibrosis, and extramedullary renal hematopoiesis. VPS45-associated severe congenital neutropenia is an extremely rare disorder, with only 20 cases reported so far. Patients present in the first few months of life with severe recurrent bacterial infections caused by deep neutropenia, typically unresponsive to G-CSF therapy. Here we report a cohort of 4 additional cases bearing *VPS45* mutations and review the current literature data.

Materials and Methods: Next generation sequencing was performed for all patients. To determine the impact of VPS45 deficiency on in vivo lymphocytic differentiation, we performed further flow cytometric analyses.

Results: All four patients presented with severe neutropenia during the neonatal period; three out of four experienced neonatal sepsis, two had omphalitis, and one had temporal mastoiditis. Three out of four patients died before hematopoietic stem cell transplantation, typically between three months and one year of age. In addition to neutropenia, our cohort exhibited lymphopenia, prompting an investigation into the impact of VPS45 deficiency on lymphocytic differentiation. Flow cytometric analyses revealed significantly reduced absolute counts of CD4 and CD8 positive lymphocytes, with a more pronounced reduction in the CD8 population. Activation responses to phytohemagglutinin and anti-CD3 antibody were markedly decreased. Although neurological symptoms are not common in VPS45 mutation cases, all patients in our study demonstrated neuromotor delay, atypical facial features, nystagmus, cortical blindness, and generalized hypotonia. The previously reported common feature of nephromegaly was absent in our patients.

Conclusion: Our findings suggest that VPS45 deficiency should be considered in patients with progressive bone marrow failure as well as combined immunodeficiency with neuromotor retardation. Early diagnosis is crucial since early hematopoietic stem cell transplantation with myeloablative conditioning is the only therapeutic option.

Keywords: Congenital neutropenia, bone marrow failure, neuromotor retardation, combined immunodeficiency

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INTRODUCTION

Severe congenital neutropenias (SCN) comprise a variety of genetic disorders leading to defect in maturation, homeostasis and function of the neutrophils. After Kostmann first described the SCN, numerous genetic defects have been described elucidating the pathogenesis (1,2). Although SCNs were classically categorized under inherited bone marrow failure syndromes, emerging novel molecular mechanisms reveal that ubiquitous cellular processes are impaired alongside the particular impact on hematopoietic cells. Consequently, clinical phenotypes have also been extended from merely neutropenia to a syndromic presentation. In 2013, two independent groups defined mutations in the vacuolar protein sorting 45 (*VPS45*) gene in a total of 14 patients with SCN, having recurrent bacterial infections, infant onset myelofibrosis, and nephromegaly (3-5). To date, a total of 20 patients with *VPS45* mutations have been reported (3-10). Here we report 4 additional patients from 4 Turkish families bearing *VPS45* p.E238K and p. L406P mutations, presenting as a combined immunodeficiency with neutropenia and additional multisystemic symptoms extending the initial clinical description of VPS45 deficiency.

MATERIALS and METHODS

Patients

Four patients, who were under follow-up at Pediatric Allergy and Immunology Departments of Ankara University School of Medicine, University of Health Sciences, Dr Behçet Uz Research and Training Hospital and University of Health Sciences, Dr Sami Ulus Research and Training Hospital, were assessed retrospectively in this study. Informed consent was taken from all parents according to the Declaration of Helsinki. Complete blood counts, immunoglobulin levels, lymphocyte subsets, lymphocyte activation responses and genetic aberrations were analyzed as well as the clinical data and histopathological findings of biopsy specimens.

Genetic Analyses

Exome sequencing and filtering

Genomic DNA (gDNA) was isolated from peripheral blood samples using the DNeasy Blood and Tissue Kit (Qiagen). Whole-exome sequencing (WES) of P3 and P4 involved library preparation and exome enrichment utilizing the Truseq Exome kit (Illumina) and Nextera flex v1.2, respectively, followed by paired-end sequencing on the Illumina HiSeq3000 system. For the other two patients, P1 and P2, we employed custom-designed targeted enrichment based on Haloplex technology (Agilent, Santa Clara, CA) followed by massively parallel sequencing of 625 genes implicated in immune functions including known primary immunodeficiency (PID) genes and additional PID genes recently published or presented at conferences at the time of the gene panel design. Sequenced DNA reads were mapped to the human reference genome (GRCh37/hg19 assembly by means of the Burrows-Wheeler Aligner (BWA) (11). Following variant calling with the Genome Analysis Toolkit (GATK) HaplotypeCaller, the Variant Effect Predictor (VEP) was used for annotating single-nucleotide variants (SNVs) and small insertions/deletions (12). From the obtained variant calls, non-synonymous (nonsense, missense, small insertions and deletions) as well as splice-region variants (+/-8 bp from the intron/exon boundaries) were then filtered to exclude those with a minor allele frequency >0.01 in gnomAD (v2.1.1) (13).

An in-house database including sequencing data from >1200 individuals was used to further exclude recurrent variants with an allele frequency >0.01. The remaining variants were prioritized based on literature research and their Combined Annotation Dependent Depletion (CADD) pathogenicity prediction score in version GRCh37-v1.6 (14).

RESULTS

Clinical and Immunological Characterization of Patients

Patient 1 (P1, female) was born to consanguineous parents. Family history revealed early death of a sibling having neutropenia (Figure 1A). The patient presented to the clinic on the 15th day of life, and hearing loss was detected in the right ear and temporal bone MRI revealed a contrasting lesion. Temporal bone biopsy showed a chronic inflammation leading to temporal bone destruction. She exhibited syndromic face features with prominent forehead, almond shaped palpebral fissures, bulbous nasal tip, and high palate. Axial hypotonia with normal reflexes and horizontal pendular nystagmus with poor pupillary light reflex were present on neurological exam. Mild anemia and lymphopenia together with profound neutropenia were detected in the blood count while immunoglobulin levels were within age references (Table I). Peripheral lymphocyte subgroup analysis revealed CD8 lymphopenia, and

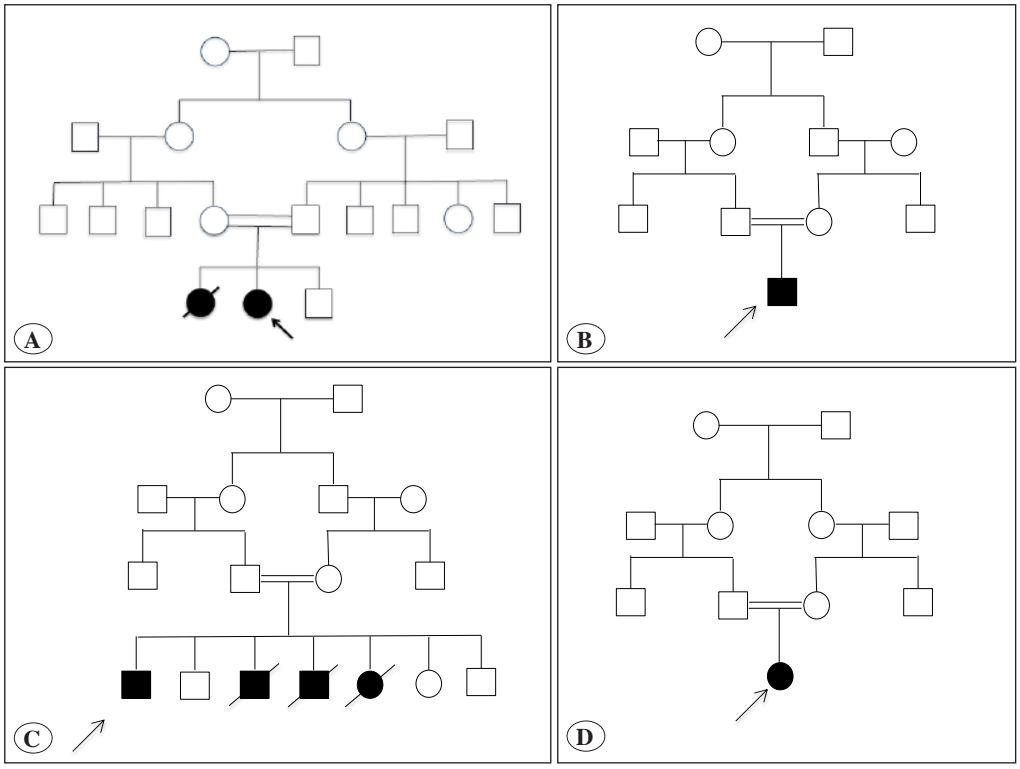

Figure 1. Pedigrees of the patients **A)** P1, **B)** P2, **C)** P3 and **D)** P4

Table I: Immunological parameters of the patients

	P1		P2		P3	P4	Age references
	2 months	6 months	3 months	7 months	1.5 months		
Hb (g/dL)	9.3	9.6	10.2	7.7	10.4	11.7	10.5-14
Absolute lymphocyte count (/mm^3)	1190	1190	830	1340	1200	1040	>3000
Absolute neutrophil count (/mm^3)	10	670*	360	220*	1000	750	>1500
Thrombocyte (/mm^3)	432000	49000	480000	81000	126000	129000	150000-450000
IgA (mg/dL)	95	-	161	323	152	191	7-123
IgG (mg/dL)	508	-	761	1460	937	634	304-1231
IgM (mg/dL)	114	-	192	150	237	214	32-203
CD3+CD16-56- (%)	52	36	11.7	10.1	55	13	51-79
CD3-CD16+56+ (%)	7	6	10.7	8.2	9	5	5-23
CD3+CD4+ (%)	48	34	14.4	7.8	52	9	31-54
CD3+CD8+ (%)	5	4	1.6	1.4	5	2.3	10-31
CD19+ (%)	34	58	74.9	78.9	40	44	14-44
CD20+ (%)	34	57	-	-	36	36	13-40
CD4+CD45RO+ (%)	8	6	-	-	9	7	6-21
CD4+CD45RA+ (%)	39	21	-	-	92	10	25-45
CD4+CD45RA+CD31+ (%)	49	38	-	-	62	51	
Activation by phytohemaglutinin							
CD3+CD25+ (%)	37				25	10	52-94
CD3+CD69+ (%)	37				22	11	48-85

*Under G-CSF.

recent thymic emigrants (CD3+CD4+CD45RA+CD31+) cell counts were normal. Activation responses to phytohemaglutinin and anti-CD3 were low (Table I). Maternal engraftment of T cells was absent in chimerism analysis. Profound neutropenia (<500/L) was persistent, and both the nitroblue tetrazolium (NBT) test and dihydrorhodamine (DHR) oxidative burst test were negative. During the follow-up, hemoglobin levels decreased without reticulocyte response, and thrombocyte counts lowered gradually. Hepatosplenomegaly was detected on abdominal ultrasound. Bone marrow aspirations were dry tap, and bone marrow biopsy revealed hypercellular granulocytic cells with no maturation arrest, while megakaryocytes and erythroid progenitors were normal (Figure 2A,B) She had recurrent abscesses in deep tissues (perianal, orbital and intraabdominal), whereby granulocyte colony stimulating factor (G-CSF) was initiated, but neutropenia was resistant to G-CSF. Metabolic screening, performed for neuromotor retardation and horizontal nystagmus, was normal. Orbitocranial magnetic resonance imaging (MRI) was normal other than chronic mastoiditis in the right temporal bone. Fundoscopic evaluation of the eyes revealed optic atrophy and the visual evoked potential latencies were prolonged suggesting cortical blindness. The patient succumbed to a sepsis episode during the donor survey for an unrelated allogeneic hematopoietic stem cell transplantation (HSCT).

Patient 2 (P2, male) born at 34th week of gestation to consanguineous parents, presented to the clinic with severe neutropenia and lymphopenia. The medical history revealed that he was previously hospitalized three times due to neonatal sepsis, severe pneumonia ,and diarrhea episodes. Facial features similar to P1, polydactyly of the feet, and right inguinal hernia were detected with the physical exam. Mild anemia, lymphopenia and profound neutropenia were present in the blood count. Peripheral lymphocyte subgroup analysis revealed prominent T cell lymphopenia suggesting a T-B+NK+ severe combined immunodeficiency (SCID) phenotype (Table I). Cytomegalovirus (CMV) polymerase chain reaction (PCR) was positive, and ganciclovir and regular intravenous immunoglobulin (IVIG) replacement was initiated. Hepatosplenomegaly was detected om abdominal ultrasound. Bone marrow aspiration and biopsy showed normocellular bone marrow with grade 2-3 reticulin fibers having no collagen fibrosis. No maturation arrest was present in the granulocytic cells but an increase in the myeloid to erythroid ratio was detected (Figure 2C,D). The neutropenia was refractory to G-CSF, and the thrombocytopenia and anemia became worse in the meantime. Due to the presence of axial hypotonia, syndromic face features and horizontal nystagmus; further neurologic evaluation was performed. Metabolic screening, cranial MRI, and fundoscopic evaluation were normal. Sensorineural hearing loss was detected. HSCT was planned, but the family rejected the transplantation and the patient succumbed to a pneumonia episode.

Patient 3 (P3, male) born to consanguineous parents was admitted to the neonatal intensive care unit with sepsis and omphalitis. Atypical facial features with prominent forehead and cathedral palate were noted. The family history was remarkable for early death of three siblings, two of which had neutropenia and myelodysplasia associated with combined immunodeficiency (Figure 1C). Anemia, lymphopenia, and neutropenia were prominent in the complete blood count. CD8 lymphopenia and low activation responses to phytohemaglutinin were documented (Table I). Bone marrow aspiration revealed trilineage normal maturation, despite being hypocellular. The patient was discharged from the hospital with trimethoprim-sulfamethoxazole antibiotics and IVIG replacement. HSCT was planned, but the patient died in a local hospital following a sepsis episode while waiting for the donor survey.

Patient 4 (P4, female) born to consanguineous parents (Figure 1D) was admitted to the neonatal intensive care unit with omphalitis and intraabdominal abscess. Atypical face features including bulbous nasal tip and retromicrognathia were noted on physical exam. Anemia, lymphopenia, and neutropenia were prominent in the complete blood count. Profound T cell lymphopenia and low activation responses to phytohemaglutinin were documented (Table I). Bone marrow aspiration revealed normocellular bone marrow with trilineage normal maturation. With a clinical diagnosis of T-B+NK+ SCID, the patient underwent allogeneic hematopoietic stem cell transplantation from her Human Leukocyte Antigen (HLA)-matched mother (10/10) without a conditioning regimen. The lymphocyte engraftment took place but the patient still needed regular G-CSF treatment as well as transfusion of thrombocyte suspensions. Meanwhile whole exome sequencing results revealed the *VPS45* pE238K mutation and the patient was re-transplanted from her mother following fludarabine (30mg/m^2/d) and treosulfan (12mg/m^2/d) conditioning. Myeloid and thrombocyte engraftments oc-

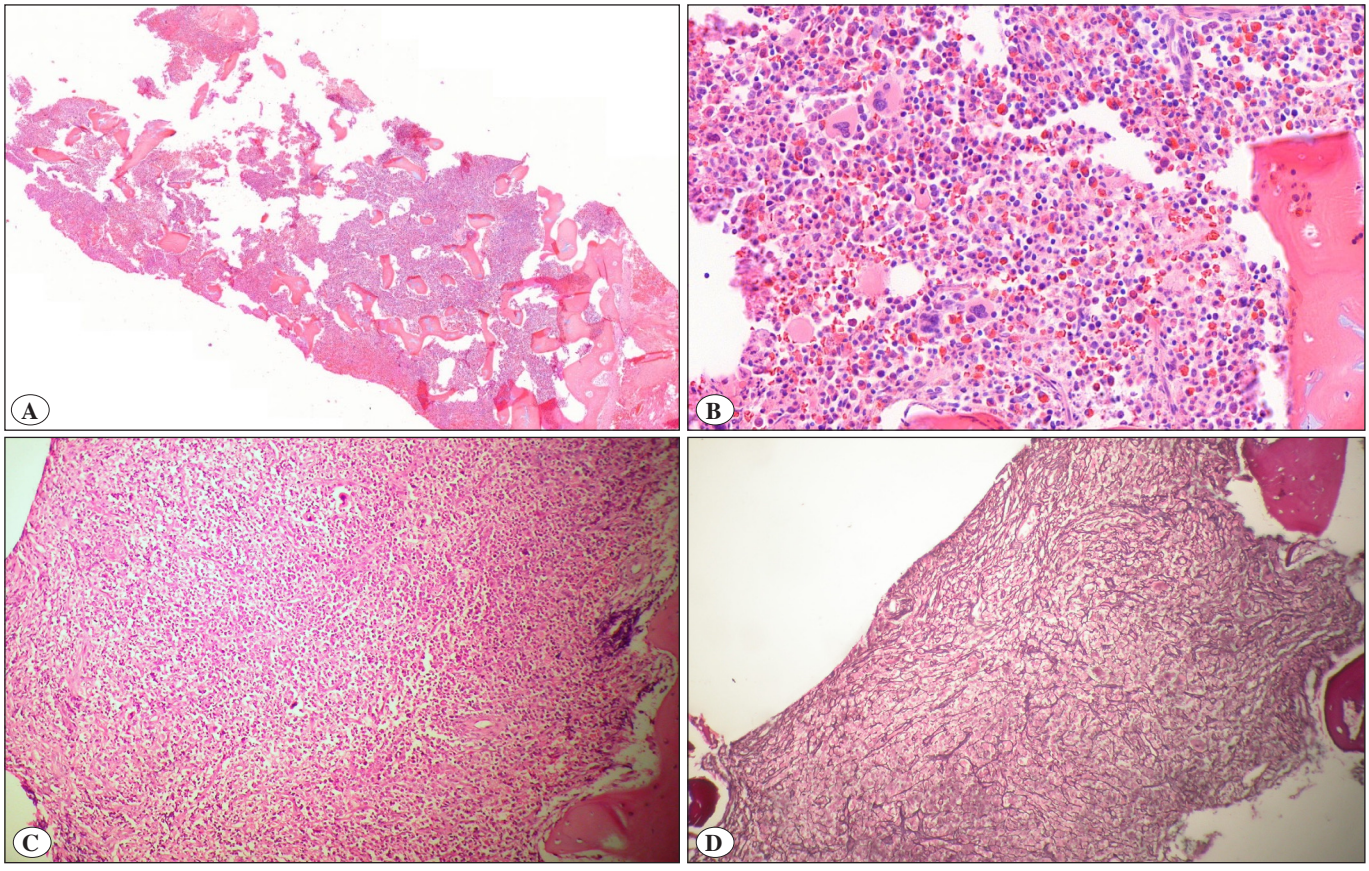

Figure 2. Bone marrow biopsies of patients. **A,B)** Hypercellular bone marrow of patient 1 having granulocytic predominance without maturation arrest. **C,D)** Hypercellular bone marrow of patient 2 with increased reticulin fibers.

curred on posttransplant days 16 and 13 respectively. During the hospitalization, strabismus was noted and cortical visual impairment was detected. The patient is currently under follow-up during post-transplant 4th year with full myeloid and T lymphocyte chimerism, normal lymphocyte and neutrophil count, and is free from infections.

We performed whole exome sequencing to identify the underlying molecular disease etiology, and identified two different homozygous missense mutations in *VPS45* (ENST00000369130.3:c.712G>A, p.E238K in P1 and P4, and ENST00000369130.3:c.1217T>C, leading to p.L406P in P2 and P3) deemed disease-causing based on functional predictions. The p.E238K mutation (CADD score: 32) was previously described as disease-causing in three patients and has an allele frequency of 0.000008228 in GnomAD. The one in p.L406P (CADD score: 26,2) was identified as a novel mutation and is not found in the GnomAD database.

Clinical Phenotype

Consistent with previous reports on VPS45 deficiency, the most common clinical features of the patients were profound neutropenia in the first few months of life that fails to respond to G-CSF therapy, failure to thrive, and infections consistent with neutropenia. All our patients presented with severe neutropenia during the newborn period, to the point that 3/4 patients had experienced a neonatal sepsis episode. Two patients had omphalitis and 1 patient had temporal mastoiditis. Neutropenia was typically unresponsive to G-CSF administration. The *VPS45* defect carries an exceptionally poor prognosis, and similar to the previous reports, 3 out of 4 patients died before hematopoietic stem cell transplantation. Death occurred early, with all patients dying between three months and one year of age.

Immunophenotyping

Although previous reports focused mainly on neutropenia, in our cohort lymphopenia was detected in all four patients with the complete blood count. Thus we performed flow cytometric analyses to determine the impact of VPS45 deficiency on *in vivo* lymphocytic differentiation. Absolute counts of CD4 and CD8 positive lymphocyte populations were much lower than the age references, with a more prominent impact on CD8 fraction. Activation responses of the lymphocytes to phytohemaglutinin and anti-CD3 antibody were reduced remarkably.

Extrahematological Manifestations

Whilst most patients bearing *VPS45* mutations do not manifest neurological symptoms, all of the here-described patients demonstrated neuromotor delay. Atypical facial features including prominent forehead and generalized hypotonia, nystagmus, cortical blindness, and unusual facial features only noted in patients bearing p.E238K mutations (rounded facies, a prominent forehead, long almond-shaped palpebral fissures, and 11 pairs of ribs) Nephromegaly, which was a common feature in previously reported cases, was absent in our patients.

DISCUSSION

VPS45-associated SCN was previously described in patients with neutropenia, myelofibrosis, progressive bone marrow failure, osteosclerosis and nephromegaly (3,4). VPS45 is a highly conserved protein involved in intracellular vesicle transport and is a member of the SEC/MUNC protein family, which binds soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) (15). The lack of VPS45 results in impaired protein trafficking required for neutrophil functions and triggers early apoptosis of neutrophils (4,15,16). Previously reported patients with homozygous p.T224N mutations in VPS45 presented with profound neutropenia resistant to G-CSF, thrombocytopenia, and transfusion-dependent anemia progressing to infant-onset bone marrow failure with myelofibrosis (3,4,6). Organomegalies, including nephromegaly secondary to extramedullary hematopoiesis and osteosclerosis, were also prominent disease characteristics in this patient cohort (3,4). The findings and the follow-up of these patients implied a survey of solitary hematological involvement.

Poor response to G-CSF is an important disease feature of patients having VPS45 deficiency. Recent data have demonstrated that, complete loss of VPS45 results in the perturbed intracellular organization of endolysosomal vesicles and cargo mistrafficking through the early endosomal compartment. VPS45 deficiency causes aberrant trafficking of the G-CSF receptor, which might be associated with the severe neutropenic phenotype of VPS45-deficient patients and their poor response to G-CSF therapy (16).

To date, a total of 20 patients with *VPS45* mutations have been reported (Table II). The majority of these cases (13 out of 20) had the p.T224N mutation (remaining variants pE238K 6/20, pP468L 1/20), which led to a phenotype of infant-onset myelofibrosis with isolated myeloid lineage involvement. We now have reported additional 4 patients and a novel variant. (pE238K 2/4, pP406L 2/4) Despite severe neutropenia being the prominent finding, lymphopenia was also documented in all our patients, suggesting a severe combined immunodeficiency (SCID)-like phenotype rather than pure SCN as the initial clinical presentation, which means *VPS45* defects should be kept in mind during the diagnostic process of such patients. The description of additional 4 patients with other mutations including p.E238K and p.P406L, exhibiting lymphopenia and/or impaired lymphocyte function, reveals that the lymphoid lineage is also affected in VPS45 deficiency (7). Compared to the CD4 fraction, CD8 cytotoxic T cell numbers were lower. One plausible explanation for the more severe phenotypic impact on CD8 T cells might be the extent of vesicular compartment in CD8 cytotoxic T cells. However currently there is no literature data to support this hypothesis, and further research is warranted.

As a SNARE binding protein, VPS45 is ubiquitously expressed in various tissues, including the brain, although initially described as an etiologic factor of infant-onset myelofibrosis, current data reveal that neurologic impairment is a consistent clinical feature of certain *VPS45* mutations such as p.E238K (17). Previously, a total of three patients from two different families with SCN and progressive myelofibrosis accompanied by neuromotor retardation were reported to have p.E238K mutations in the *VPS45* gene (3,5). These patients exhibited developmental delay and cortical blindness. In 2015, Meerschaut et al. described a case of a patient with p.E238K having atypical facial features, horizontal nystagmus, and neuromotor retardation associated with progressive bone marrow failure, suggesting a specific genotype-phenotype correlation for p.E238K mutations highlighting the difference to the canonical p.T224N mutations (5). Similarly, the presen-

Table II: Comparative analysis of clinical and immunological features of currently reported VPS45 mutations

	p.T224N (n=13)	p.E238K (n=8)	p.P406L (n=2)	p.P468L (n=1)
Impact	Missense	Missense	Missense	Missense
Age onset	Neonatal-3 mo	Neonatal	Neonatal	Neonatal
Infections				
Bacterial infections	++	++	++	++
Viral infections	-	-	+	-
Dysmorphia				
Prominent forehead	-	++	+	-
Cathedral palate	-	++	+	
Skeletal abnormalities	-	++	+	-
CNS involvement	-	++	+	+
Immunological features				
Anemia	++	++	++	++
Lymphopenia	-	++	++	-
Neutropenia	++	++	++	++
Thrombocytopenia	++	++	++	++
Immunoglobulins	Normal/high	Normal/low	Normal	Normal
CD3 lymphocytes	Normal	Normal/low	Normal/low	Normal
CD4 lymphocytes	Normal	Normal/low	Normal	Normal
CD8 lymphocytes	Normal	Low	Low	Normal
Recent thymic emigrants	Normal	Normal	Normal	Low
CD19+ B cells	Normal	Normal	Normal	Normal

CNS: Central nervous system

tation of P1 and P4 with neutropenia, lymphopenia, and progressive bone marrow failure is in line with the aforementioned case. The striking resemblance of clinical and laboratory findings among these patients underscores the importance of genotype-phenotype correlation for different mutations, resulting in distinct phenotypic expressions of VPS45 deficiency.

A structural homology model for the protein, using known structures of the Sec 1/Munc18 homologs Munc18a (PDB ID 3c98), was previously created (7). The mapping of the P468L together with previously reported T224N and E238K residues onto the model indicates that although they are not near each other in the primary sequence, all lie within close proximity in three-dimensional space. They are located in a functionally important hinge region of *VPS45*, that possibly controls endosomal membrane fusion (7). Hence, all three of these mutations produce a common structural and functional defect in SNARE regulation, although it is possible that all three could lead to decreased stability and *in vivo* levels of mutant VPS45 proteins. The newly identified p.406L is also localized in close proximity to the aforementioned variants, underscoring the fact that the phenotypic differences observed in the patients are independent of the position of the variants on structure. To clarify the etiology of the genotype-phenotype correlation, larger cohorts and further studies are needed.

Patients with *VPS45* gene mutations can be supported for some time with antimicrobial therapy and regular transfusions of blood and platelets. However, HSCT is reported to be the only definitive therapeutic option for these patients. Mortality is very high due to G-CSF-resistant neutropenia and life-threatening thrombocytopenia with a bleeding tendency. Including the cases reported here, 11 of the 24 so far reported patients have undergone HSCT, with 8 surviving the procedure and 3 dying post-transplant (3,5,6). All non-surviving patients demonstrated engraftment failure (8).

The syndromic characteristics of *VPS45* mutations are partially compatible with SCN syndromes such as Cohen (*VPS13B*), Kostmann (*HAX1*), and reticular dysgenesis

(*AK2*) in terms of neurodevelopmental deficits. However, early-onset myelofibrosis is a unique characteristic of VPS45 deficiency, and the pathogenesis underlying this process still needs to be identified. Although the diagnosis can be challenging, it should be considered in patients with G-CSF unresponsive neutropenias and progressive bone marrow failure as well as combined immunodeficiency with neuromotor retardation. Early diagnosis is crucial since early HSCT with myeloablative conditioning is the only therapeutic option for these patients.

Conflict of Interest

All the authors concur with the current version of the manuscript and declare no conflict of interest.

Authorship Contributions

Concept: **Sevgi Kostel Bal, Figen Dogu, Aydan Ikinciogullari,** Design: **Sevgi Kostel Bal, Figen Dogu, Aydan Ikinciogullari,** Data collection or processing: **Sevgi Kostel Bal, Candan Islamoglu, Sule Haskologlu, Sait Karaman, Raul Jimenez-Heredia, Anna Segarra Roca, Ferah Genel, Nesrin Gülez, Caner Aytekin, Ferah Genel, Nesrin Gulez,** Analysis or Interpretation: **Sevgi Kostel Bal, Ferah Genel, Nesrin Gulez, Caner Aytekin, Kaan Boztug,** Literature search: **Sevgi Kostel Bal,Raul Jimenez-Heredia,** Writing: **Sevgi Kostel Bal, Raul Jimenez-Heredia, Anna Segarra Roca,Ferah Genel, Nesrin Gulez, Caner Aytekin, Kaan Boztug, Figen Dogu, Aydan Ikinciogullari,** Approval: **Sevgi Kostel Bal, Candan Islamoglu, Sule Haskologlu, Sait Karaman, Raul Jimenez-Heredia, Anna Segarra Roca, Ferah Genel, Nesrin Gulez, Caner Aytekin, Kaan Boztug, Figen Dogu, Aydan Ikinciogullari.**

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