EJR European Journal of Rheumatology

DOI: 10.5152/eurjrheum.2022.21049

Original Article

Next-Generation Sequencing Analysis of MVK, NLRP3, TNFRSF1A, and PSTPIP1 Genes in Patients without MEFV Gene Variation and Genotype–Phenotype Correlation

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Abstract

Objective: In this study, we aimed to evaluate other interleukin-1β-mediated monogenic autoinflammatory diseases (AIDs) (tumor necrosis factor receptor-1-associated periodic syndrome, hyperimmunoglobulin D syndrome, cryopyrin-associated periodic syndrome (CAPS), pyogenic arthritis, pyoderma gangrenosum, and acne syndrome) by the next-generation sequencing method (NGS) in cases with clinical Familial Mediterranean Fever symptoms, and no variant detected in the MEFV gene.

Methods: The cases included in this study and their parents were interviewed and filled in a survey form. The targeted genetic panel for interleukin-1β-mediated AIDs covering four genes (*MVK*, *NLRP3*, *TNFRSF1A*, and *PSTPIP1*) was studied for cases with a negative result from the MEFV gene analysis. The genetic analysis was conducted using the targeted NGS method.

Results: Variants were found in 16 out of the 40 patients in the study sample. These variants were priorly reported in variant databases, and three of them were identified as definitely pathogenic (V377I of the MVK gene, C52Y of the TNFRSF1A gene, and I313V of the NLRP3 gene), four as a variant of uncertain significance (VUS) (R92Q of the TNFRSF1A, A372V of the PSTPIP1, and V198M and Q703K of the NLRP3), and one as benign polymorphism (S52N of the MVK gene). The median age of onset among variant-positive cases was 10.5 (3.5-18) years. The most common clinical findings in the variant-positive group were arthralgia, fever, and abdominal pain. While three out of 40 patients met the classification criteria before genetic analysis, only one patient was diagnosed with CAPS as a result of genetic analysis, and other patients were considered as nonspecific phenotype.

Conclusion: The use of NGS gene panels seems beneficial in diseases with heterogeneous clinical manifestations such as systemic AIDs. Although the number of variants detected is high, clinical diagnosis rates remain low. The genotype–phenotype relationship in these diseases is still unclear.

Keywords: Autoinflammatory disease, genotype–phenotype, next-generation sequencing, periodic fever

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Cite this article as: Vuran G, Berdeli A. Next-generation sequencing analysis of MVK, NLRP3, TNFRSF1A, and PSTPIP1 genes in patients without MEFV gene variation and genotype–phenotype correlation. *Eur J Rheumatol.* 2022;9(2):62-67.

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Submitted: February 21, 2021
Accepted: November 22, 2021

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Introduction

Systemic autoinflammatory diseases (sAIDs) are a group of rare genetic diseases,[1] resulting from deficiencies in the natural regulation of the immune system and characterized by recurring systemic inflammatory episodes. Recurrent fever, abdominal pain, joint pain, rash, and lymphadenopathy are the most common findings in these diseases. The most common is Familial Mediterranean Fever (FMF) caused by the *MEFV* gene variant.[2] In the hyperimmunoglobulin D syndrome (HIDS) caused by a variant in the mevalonate kinase (*MVK*) gene, unlike these common findings, such as vomiting, diarrhea, and mouth ulcers, are observed,[3] Migratuar erythematous plaques, focal myalgia, periorbital edema, and conjunctivitis are also observed in tumor necrosis factor receptor-1 associated periodic fever syndrome (TRAPS) caused by TNF receptor 1A (*TNFRSF1A*) gene variant.[4–6] Cryopyrin-related periodic fever syndromes (CAPSs) caused by *NLRP3* gene variant; Familial cold autoinflammatory disease has attacks triggered after exposure to cold, while progressive sensory neural hearing loss in Muckle-Wells syndrome (MWS) and growth retardation, abnormal facile appearance, and chronic meningeal involvement are seen in neonatal-onset multisystem autoinflammatory disease (AID) or chronic infantile neurological cutaneous articular syndrome.[7,8] Clinicians made classification criteria for these diseases based on differences in clinical findings.[9–11] In the diagnosis of these diseases, it is recommended to evaluate the patients according to these classification

criteria in order to identify suspicious cases and then to confirm the diagnosis with genetic tests. Genetic analysis gains importance in cases with an incomplete phenotype that do not fully meet the clinical diagnostic criteria or in cases with symptoms overlapping with more than one AID. Until 2012, these analyses were performed by the Sanger sequencing method as whole gene analysis, which was a waste of time. In 2012, the next-generation sequencing (NGS) method was started to be used in the diagnosis of diseases with genetic heterogeneity. This method has a higher diagnosis rate than the Sanger sequencing method[12]; it also provides faster results of genetic analyzes in centers where many patients apply.

With the development of genetic analysis methods and the increase in the number of patients with variants, the number of studies examining the genotype–phenotype relationship has increased, but the genotype–phenotype correlation in these diseases is still unclear.[13]

Since it is common in Turkey and is well known by physicians, cases with recurrent inflammatory attacks are primarily evaluated in terms of FMF. Therefore, in this study, we aimed to evaluate the possibility of TRAPS, HIDS, CAPS, and PAPA from other interleukins 1β (IL-1β)-mediated monogenic AIDs in patients with clinically suspected FMF but no MEFV gene variant detected in genetic analysis, and to examine the genotype–phenotype relationship in cases with variants.

Methods

Patients and study design

This study is a cross-sectional study. Patients referred to our University Faculty of Molecular Medicine Laboratory between January 2013 and April 2017 for *MEFV* gene analysis were determined, considering FMF in the foreground due to the symptoms of recurrent febrile attacks and abdominal pain, joint pain, lymphadenopathy, rash, etc. Patients with negative MEFV gene analysis were included in this study. This study excluded those cases that indicated primary immunodeficiency as a result of relevant examinations. The patients and their parents were informed of the study, and a written informed consent was collected from each of them. The cases were asked to fill out a survey form that featured questions on the diseases (age of onset of episodes, symptoms accompanying episodes, duration of episodes, and family history). Clinical diagnosis of CAPS, HIDS, and TRAPS was made on the basis of Eurofever clinical diagnostic criteria/classification criteria-Federici Score.[9] When the patients were evaluated according to the classification criteria, two patients met the criteria for HIDS, and one patient met the CAPS criteria, while the other patients did not fully meet the criteria.

Main Points

- Targeted NGS panels have impact on diagnosing IL-1B-related monogenic autoinflammatory diseases.
- Although the number of variants detected by the next-generation sequencing (NGS) method is high, the number of cases with a definitive diagnosis after evaluation with the clinical classification criteria of autoinflammatory diseases is low.
- The genotype-phenotype correlation in autoinflammatory diseases is still unclear.

The targeted NGS genetic panel for AIDs covering four genes (*MVK*, *NLRP3*, *TNFRSF1A*, and *PSTPIP1*) was studied on genomic DNA for cases with a negative result from the *MEFV* gene analysis. The NGS method was employed for genetic analysis. For a final definitive diagnosis, the clinical findings and genetic analysis results of the patients were re-evaluated according to the Eurofever classification criteria.[11]

Methods

DNA isolation and control

The genomic DNA was isolated from peripheric venous blood using a Purelink Genomic Blood DNA Purification DNA isolation mini kit (İnvitrogen Co, Carlsbad, UK). The DNA extraction procedures were followed according to the directions given in the prospectus of the kit. DNA purity was measured by a NanoDrop Spectrophotometer at a wavelength of 260/280. The DNA molecules, once checked, were stored at +4°C to start DNA sequence analysis.

Next-generation sequencing

The relevant library was built through the amplification of 10 ng DNA (measured with the combined use of Qubit dsDNA HS Assay kit and Qubit fluorometer) through the panel for AIDs (Ampliseq™, Life Technologies) and Ion Ampliseq HiFi Master Mix (Ion Ampliseq™ Library kit 2.0). An amplicon library was established for the sequencing of the variant in the exons and exon-intron boundaries (±20 bases) of four genes (*NLRP3* (NM_001243133.1), *TNFRS1A* (NM_001065.3), *MVK* (NM_000431.3), and *PSTPIP1* (NM_003978.3)).

Each library was multiplied through ion sphere particles (ISPs) emulsion PCR with the Ion PGM™ Hi-Q™ OT2 kit and Ion One Touch 2. Finally, the ISP template was enriched with the use of the Ion PGM™ Enrichment Beads kit and the Ion One Touch ES device and was then loaded on an Ion PGM™ chip and sequenced through the use of an Ion PGM™ Hi-Q™ Sequencing Kit and the Ion PGM device. The reference genome was identified as hg19 (Genome Reference Consortium GRCh37). The data were evaluated in the Integrative Genome Viewer (http://software.broadinstitute.org/software/igv/) software, and the relevant analyses were performed in Variant Caller and Ion Reporter software.

All variants were searched in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and Infevers (https://infevers.umai-montpellier.fr/web/). The results of the genetic analysis were assessed in line with Sorting Intolerant from Tolerant (SIFT, http://sift.jcvi.org/) and MutationTaster (http://www.mutationtaster.org/) bioinformatics databases. This study planned to evaluate the genotype–phenotype relationship by comparing the results of such genetic analyses with those in the clinical picture of the cases and the relevant literature.

The local ethics committee approved the study for Clinical Research of the Faculty of Medicine.

Statistical analysis

Data were analyzed using SPSS (Statistical Package for the Social Sciences) version 17 (SPSS Inc, Chicago, IL, USA). Categorical variables were shown as frequencies and percentages. Numeric values were presented as the median and interquartile range (IQR).

Results

Eight different variants were identified in 16 patients by giving a variant detection rate of 40%. All of these variants were missense. Most of the variants were detected in the *NLRP3* gene. Three variants (3/40 patients; 7.5%), which were detected in MVK gene (V377I), TNFRSF1A gene (C52Y), and NLRP3 gene (I313V), were reported as pathogenic or likely pathogenic in Clinvar and Infevers databases. Additionally, four variants (8/40 patients; 20%) were noted as a VUS in these databases. These variants were detected in the TNFRSF1A gene (R92Q), PSTPIP1 gene (A372V), and NLRP3 gene (V198M and Q703K). Only the S52N variant in MVK gene (5/40 patients; 12.5%) was reported as a benign polymorphism. The most common clinical findings in the variant-positive group were arthralgia (12/16 patients; 75%), fever (11/16 patients; 68.7%), and abdominal pain (9/16 patients; 56%). None of the cases included in this study

Table 1. Demographic Data of the Patients Included in the Study and Clinical Findings during the Attacks

	Variant Positive Patients (n = 16)	Patients with NLRP3 Gene Variants (n = 7)	Patients with MVK Gene Variants (n = 6)	Patients with TNFRSF1A Gene Variants (n = 2)	Patients with TNFRSF1A Gene Variants (n = 1)
Age (years) [median, (IQR)]	10.5 (3.5-18)	12 (6-18)	7.5 (3.5-16)	10.2 (5-15.5)	17
Age at onset (years) [median, (IQR)]	7 (1.5-14)	7 (1.5-12)	7.2 (1.5-14)	7.7 (1.5-14)	2
Male/female	10/6	5/2	4/2	1/1	0/1
Family history [n (%)]	6 (37)	3 (75)	2 (33)	1 (50)	0
Fever [n (%)]	10 (62.5)	5 (71)	2 (33)	2 (100)	1 (100)
Disease pattern [n (%)]					
Recurrent	12 (75)	6 (86)	3 (50)	2 (100)	1 (100)
Chronic	4 (25)	1 (14)	3 (50)	0 (0)	0
Duration of attacks (day) [median, (IQR)]	3 (1-7)	3 (2-7)	3 (3-4)	2	1
Mucocutaneous involvement [n (%)]	8 (50)	3 (43)	3 (50)	1 (50)	1 (100)
Oral aphthae	0	0	0	0	0
Urticarial rash	3 (19)	2 (28)	1 (17)	0	0
Exudative pharyngitis	2 (12)	0	1 (17)	1 (50)	0
Maculopapuler rash	3 (19)	1 (15)	1 (17)	0	1 (100)
Musculoskeletal involvement [n (%)]	14 (87.5)	6 (86)	6 (100)	1 (50)	1 (100)
Arthralgia	14 (87.5)	6 (86)	6 (100)	1 (50)	1 (100)
Myalgia	3 (19)	1 (14)	2 (33)	0	0
Monoarthritis	0	0	0	0	0
Oligoarthritis	4 (25)	3 (43)	1 (17)	0	0
Polyarthritis	0	0	0	0	0
Eye involvement [n (%)]	3 (19)	2 (28)	1 (17)	0	0
Periorbital edema	0	0	0	0	0
Conjunctivitis	3 (19)	2 (28)	1 (17)	0	0
Gastrointestinal involvement [n (%)]	9 (56)	3 (43)	3 (50)	2 (100)	1 (100)
Abdominal pain	9 (56)	3 (43)	3 (50)	2 (100)	1 (100)
Diarrhea	6 (37)	3 (43)	2 (33)	1 (50)	0
Vomiting	5 (31)	3 (43)	1 (17)	1 (50)	0
Reticuloendothelial involvement [n (%)]	3 (19)	0	2 (33)	0	1 (100)
Lymphadenopathy	3 (19)	0	2 (33)	0	1 (100)
Splenomegaly	0	0	0	0	0
Cardiovascular involvement [n (%)]	2 (12)	2 (28)	0	0	0
Chest pain	2 (12)	2 (28)	0	0	0
Neurological involvement [n (%)]	2 (12)	1 (14)	1 (17)	0	0
Headache	2 (12)	1 (14)	1 (17)	0	0

IQR, interquartile range.

Table 2. Demographic and Clinical Characteristics of Patients with NLRP3 Gene Variants

Patient	Clinical Manifestations	NLRP3 Gene Variants	Age at Disease Onset (Years)	Recurrent/ Chronic	Duration of Attacks (Day)
1	Fever, arthralgia	Q703K	1.5	Recurrent	3
2	Fever, arthralgia myalgia	Q703K	11	Recurrent	7
3	Fever, arthralgia oligoarthritis, abdominal pain, vomiting, and diarrhea	Q703K	4	Recurrent	3
4	Arthralgia oligoarthritis, conjunctivitis, and rash	Q703K	9	Recurrent	2
5	Fever, arthralgia, oligoarthritis, and urticarial rash	Q703K	7	Recurrent	–
6	Fever, arthralgia, abdominal pain, conjunctivitis, and chest pain	V198M	12	Recurrent	3
7	Fever, abdominal pain vomiting, diarrhea, and chest pain	I313V	5	Recurrent	2

exhibited hearing loss, severe neurological involvement, and amyloidosis. The demographic characteristic of the patients and their clinical findings are shown in Table 1.

NLRP3 gene variants

Three different variants were identified in seven patients (7/16 patients; 43.7%). The most common variant of this gene was Q703K, which was noted in five patients. The median age of these patients was 9 (6-18) years, and their median age of onset was 7 (1.5-11) years. The most common features of these patients were arthralgia (5/5 patients), fever (4/5 patients), and oligoarthritis (2/5 patients). The patients' clinical characteristics are summarized in Table 2.

MVK gene variants

Two different variants were identified in six patients (6/16 patients; 37.5%). The most common variant of this gene was S52N, which was noted in five patients. The median age of these patients was 8 (3.5-16) years, and their median age of onset was 7.5 (1.5-14) years. The most common features of these

Table 3. Demographic and Clinical Characteristics of Patients with MVK Gene Variants

Patient	Clinical Manifestations	MVK Gene Variants	Age at Disease Onset (Years)	Recurrent/ Chronic	Duration of Attacks (Day)
1	Arthralgia, oligoarthritis	S52N	6	Chronic	–
2	Arthralgia, myalgia abdominal pain, vomiting, diarrhea, and lymphadenopathy	S52N	7.5	Chronic	–
3	Fever, abdominal pain arthralgia, and lymphadenopathy	S52N	1.5	Recurrent	3
4	Arthralgia, oligoarthritis urticarial rash	S52N	14	Chronic	–
5	Arthralgia, abdominal pain, diarrhea, conjunctivitis, headache	S52N	13	Recurrent	4
6	Fever arthralgia, myalgia exudative pharyngitis	V377I	3	Recurrent	3

patients were arthralgia (5/5 patients), abdominal pain (3/5 patients), diarrhea (2/5 patients), and oligoarthritis (2/5 patients). Demographic and clinical manifestations of HIDS cases are given in Table 3.

TNFRSF1A gene variants

A variant in the TNFRSF1A gene was detected in two patients. The R92Q variant was detected in a 5-year-old girl who had 2 days of attacks accompanied by fever, arthralgia, abdominal pain, and exudative pharyngitis since the age of 1-and-a-half-year-old. C52Y variant was also detected in a 15-year-old male patient with 2 days of fever, abdominal pain, vomiting, and diarrhea for the last 1 year.

PSTPIP1 gene variants

A 17-year-old female exhibiting fever episodes of 1-2 days, arthralgia, episodes of abdominal pain since age 2, and acneiform rash for the past 1-2 years was identified to carry an A372V variant in the *PSTPIP1* gene.

When evaluated with clinical diagnostic criteria, S52N variant was detected in one of the two cases meeting the HIDS criteria, and no variant was found in the other case. Q703K variant was also detected in the case meeting the CAPS criteria.

Discussion

The clinical spectrum of sAIDs ranges from mild inflammatory attacks to severe chronic diseases. It is thought that the use of methods that enable the study of multiple genes such as NGS gene panel in diseases with clinical and genetic heterogeneity of this type will be more effective in diagnosing the diseases. In our study, 40 patients with suspected sAIDs and negative MEFV gene analysis were evaluated with an NGS gene panel. Pathogenic variant or VUS was detected in 11 (27%) of these patients. In the study of De Pieri et al.,[14] in which 42 patients with undifferentiated sAIDs were evaluated with the NGS gene panel, no pathogenic mutation was detected in any patient, and VUS was detected in 24 patients. In the study by Karacan et al.,[15] in which 140 patients with suspected sAIDs were evaluated with the NGS gene panel, pathogenic variants were detected in 41 (20.9%) cases, and when the cases were re-evaluated according to the classification criteria, 10% of them could be definitively diagnosed. In the Eurofever study, in which 159 patients with undefined sAIDs were evaluated, pathogenic variant or VUS was detected in 15 (9.4%) patients.[16]

The most frequently detected variant in our study was the Q703K variant in the NLR3 gene. The Q703K variant identified in the *NLRP3* gene is regarded as a VUS under the Infevers database. It was initially considered a functional polymorphism due to the high allele frequency in the healthy population.[17] However, relevant studies established that a Q703K variant increased the production of IL-1B and IL-18 through the activation of *NLRP3* inflammasome owing to the functionalization in the *NLRP3* gene and exhibited the characteristics of a low-penetrance variant.[18,19] Naselli et al.[20] indicated that the great majority of cases carrying a Q703K variant suffered from fever, urticarial rash, arthralgia, and conjunctivitis but recovered with the use of steroid treatment only during episodes without needing an anti-IL-1 treatment. The Eurofever study, on the other hand, concluded that all of the cases identified to carry a Q703K variant indicated an age of onset later than 6 months, while none of them exhibited severe neurological involvement, a severe musculoskeletal involvement, or hearing loss.[21] Only one of the five cases was diagnosed as CAPS; the other cases were evaluated as a nonspecific AID.

The V198M variant is classified as a VUS under the Infevers database. Aganna et al.[22] indicated that cases carrying V198M variants exhibited the clinical picture for MWS, but a medical screening is undertaken with the families of the cases also pointed out to asymptomatic individuals carrying this variant, adding that healthy cases in the control groups were also observed to have V198M variants. In the Eurofever study, the V198M variant was considered to be a low-penetrance variant referring to the lack of severe neurological involvement or severe musculoskeletal involvement among the patients identified to carry V198M variants.[21] Our case was established as the phenotype for nonspecific AID phenotype with episodes accompanied by fever, arthralgia, abdominal pain, conjunctivitis, and chest pain. The I313V variant is regarded as pathogenic in the Infevers database and is reported to cause the Magic Syndrome characterized by inflamed cartilage accompanying ulcers in the mouth and the genital area. The case study published by Omenetti et al.[23] reported a moderate CAPS phenotype in two cases identified to carry I313V variants and higher Il-1B levels in these two cases when compared to the healthy group. In our study, the case identified to carry a I313V variant was of the phenotype for nonspecific AID phenotype.

The V377I variant identified in the *MVK* gene is considered to be pathogenic under the Infevers database. The Eurofever study indicated that cases with an identified V3771 variant exhibited a disease pattern dominated by episodes without severe musculoskeletal or gastrointestinal involvement.[24] According to the New Eurofever classification criteria, our case was evaluated in the nonspecific AID phenotype. The S52N variant, considered a benign polymorphism in the Infevers database, was reported to be observed among the patient population and the group of healthy controls at the same incidence.[25] Stabile et al.[26] specified that symptomatic cases with an identified S52N variant in the *MVK* gene exhibited a later age of onset and invariably suffered from arthralgia, abdominal pain, and lymphadenopathy. The authors also pointed out the uncertainty as to whether this polymorphism represents a decreasing effect on enzyme activity or a trigger for inflammatory pathways. Although the S52N variant is stated as benign in the literature, it appears to be associated with clinical findings in our ethnic population and responds well to short-term treatment.[27,28] The present study observed a late age of onset and an invariable symptom of arthralgia among the cases that carried S52N variants. These cases were considered to represent the clinical picture of a nonspecific AID.

The R92Q variant identified in the *TNFRSF1A* gene is regarded as a VUS under the Infevers database. This was primarily considered as a polymorphism due to the common incidence in both healthy controls and asymptomatic cases. As a finding, the lower TNF receptor level observed among cases with R92Q variants also supports the argument that this variant bears the characteristics of very low penetrance rather than a polymorphism.[29,30] The Eurofever study indicated that cases with R92Q variants exhibited an earlier age of onset, a lower rate in terms of family history, and skin and ocular findings; no amyloidosis; but a higher rate of oropharyngeal symptoms.[31] By way of long-term follow-up, the cases carrying this variant were also reported to enjoy spontaneous remission more commonly.[32] The C52Y variant affects cysteine residues and is regarded as pathogenic under the Infevers database. Cases that carry variants that affect cysteine residues are reported to exhibit longer attacks, a higher risk of amyloidosis, and more common skin lesions and ocular involvement.[31] As for our case, the age of onset was 14, and attacks of 2 days were accompanied by fever, abdominal pain, and vomiting. The age of onset and the duration of the attack observed in this case were not compatible with the findings reported in the literature for cases carrying this mutation. It was evaluated in the nonspecific AID phenotype in two of our cases with variants in the TNFRSF1A gene.

In the present study, one patient was established to carry an A372V variant in their *PSTPIP1* gene. The A372V variant is considered to be a variant of uncertain pathogenic significance in the ClinVar database. There was no case report or clinical information specified in studies related to patients with A372V variant. Our case was accepted as a nonspecific AID phenotype.

Some of the cases included in the study had symptoms compatible with the diagnosis of PFAPA syndrome. The diagnosis of PFAPA is based on clinical criteria, but these criteria have not been confirmed in a patient cohort. In addition, Gattorno et al.[33] emphasized that a significant number of patients with monogenic periodic fever also met the diagnostic criteria for PFAPA syndrome, and that the current classification criteria had poor specificity. Therefore, it is recommended that patients be screened clinically or genetically for other known periodic syndromes before diagnosing PFAPA. In the light of this information, we did not want to diagnose PFAPA without ruling out AIDs with obvious genetic causes.

A patient with suspected sAID may not always have specific clinical findings of monogenic diseases. Evaluation of these patients with NGS gene panels is thought to be helpful in diagnosis. However, although many variants (pathogenic or VUS) were detected with NGS gene panels, the number of patients diagnosed was low (4-10%) in studies using the criteria specified in the final guideline for definitive diagnosis.[15,34,35] In our study, VUS or pathogenic variant was found in 11 (27.5%) patients with whom we had a genetic screening, one (2.5%) patient with Q703K variant was diagnosed as CAPS, and other patients were evaluated as nonspecific AID phenotype. We think that the reason why the diagnosis rate was found to be lower in our study compared to other studies is that the patients were from the group with no MEFV gene variants and who did not fully meet the clinical diagnostic criteria for CAPS, HIDS, and TRAPS.

The main limitations of this study are that it is a single-center study, the number of our patients is small, the study group is heterogeneous, and the detected variants are predominantly VUS or benign polymorphism. Due to the small number of patients included, a clear genotype–phenotype correlation could not be established. Another limitation is that detected variants were not verified by Sanger sequencing.

Conclusion

The use of NGS gene panels in the diagnosis of clinically and genetically heterogeneous sAIDs is becoming increasingly common. Although the number of variants detected by the NGS gene panel is high, the rate of patients with a definitive diagnosis is low. Therefore, multicenter studies in which more genes are evaluated in more patients are needed.

Ethics Committee Approval: Ethical committee approval was received from the Clinical Research Ethics Committee of the Ege University of School of Medicine (17-1.1/12).

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - G.V., A.B.; Design - G.V., A.B.; Data Collection and/or Processing - G.V.; Analysis and/or Interpretation - G.V., A.B.; Critical Review - G.V., A.B.

Declaration of Interests: The authors have no conflicts of interest to declare.

Funding: The authors declared that this study has received no financial support.

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