Genetic and clinical features of cryopyrin-associated periodic syndromes in Turkish children

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ABSTRACT

Objective. *The aim of this study was to present the genetic and clinical data of the largest cohort of Turkish cryopyrin-associated periodic syndromes (CAPS) patients.*

Methods. *This is a two-centre descriptive study of Turkish children with clinical diagnosis of CAPS. NLRP3 analyses were performed by Sanger sequencing and by massively parallel sequencing. ASC dependent NF-κB activation and transfection-induced THP-1 cell death assays determined the functional consequences of the detected variants. Disease activity and response to anti interleukin 1 (anti-IL-1) treatment was also assessed.*

Results. *Heterozygous germline NLRP3 mutation was detected in 8 of 14 enrolled patients (57.1%). Two novel somatic mutations Y560H and G307D were found which induced both THP-1 cell death and ASC dependent NF-kB activation. With anti-IL-1 treatment the disease activity was improved in all patients except one. Except two patients with macrophage activation syndrome (MAS) attack, there were no serious adverse events requiring hospitalisation.*

Conclusion. *CAPS should be considered in all patients with typical symptoms even if Sanger-based genetic analysis is negative, since a considerable number of patients have mosaicism. Treatment should be patient-tailored and MAS should be considered as a rare complication.*

Introduction

Cryopyrin-associated periodic syndromes (CAPS) are dominantly inherited autoinflammatory diseases as a consequence of *gain-of-function* mutations in the *NLRP3* gene, encoding for cryopyrin. CAPS include three different clinical entities, which represent varying degrees of disease severity along a continuum spectrum: Familial Cold Autoinflammatory Syndrome (FCAS) at the less severe end of the spectrum, Muckle-Wells syndrome (MWS) as an intermediate form of severity, and Chronic Infantile Neurologic, Cutaneous and Articular (CINCA) syndrome, also known as Neonatal-Onset Multisystem Inflammatory Disease (NOMID) as the severest form (1). The usual clinical onset is an early onset of generalised urticaria-like skin rash and recurrent fever associated with a strong acute phase response. As the disease progresses, additional features could be detected such as serositis, joint, eye and neurologic manifestations (2). All CAPS phenotypes respond to anti-interleukin 1 (anti-IL-1) drugs such as anakinra, canakinumab and rilonacept (3-12).

Validated diagnostic criteria are not available for these syndromes. Their heterogeneous presentations and absence of defined germline mutations in substantial number of patients make the diagnosis difficult, preventing initiation of the anti-IL-1 treatments. The definitive CAPS diagnosis currently relies on the identification of *NLRP3* mutation in candidate patients. However, recent reports highlighting the relevance of somatic *NLRP3* mosaicism in CAPS pathogenesis, which is only detected by complex genetic analysis; add additional difficulties to achieve the definitive diagnosis in several patients (13-15).

Herein we present the largest cohort of Turkish patients with CAPS followed at the two main referral centres in Turkey, mainly focusing in the clinical and genetic features of patients and in their responses to treatments.

Patients and methods

Patients

This is a descriptive study recruiting all Turkish children with clinical diagnosis of CAPS followed in Paediatric Rheumatology departments of Hacettepe University Faculty of Medicine and Istanbul University Cerrahpasa Faculty of Medicine, between May 2006 and August 2014. These two centres are referral centres of Paediatric Rheumatology following patients from all over Turkey. The Ethics Committees of these institutions approved this study, which was conducted in accordance with the Declaration of Helsinki. The diagnosis of CAPS was suspected on the basis of early-onset, recurrent episodes of fever and urticaria-like skin rash associated with increased acute phase reactants (APRs) and at least one of the next signs: Presence of sensorineural hearing loss, presence of neurological symptoms (headache, cognitive dysfunction or ventricular dilatation), presence of bone and joint features (bone overgrowth, frontal bossing or arthralgia/arthritis) and presence of eye findings (papilledema, optic atrophy, recurrent conjunctivitis or recurrent scleritis). Patients with any central nervous system (CNS) features (papilledema, optic atrophy, hydrocephalus, seizures or cognitive dysfunction), patellar overgrowth or joint deformities were defined to have severe CAPS.

Clinical characteristics and response to treatment

Initial findings, diagnoses and response to treatments were noted from case notes. Disease activity was assessed with the Autoinflammatory Diseases Activity Index (AIDAI) (16) after May 2011 and with patients/parents' global assessment of disease severity (10 cm visual activity score [VAS]) and physicians' global assessment of disease severity (10 cm VAS). AIDAI score sheet was filled by parents in those patients below 12-year-old and by the patient if older than 12-year-old. This index includes fever (0-1), eye manifestations (0-3), headache (0-3), limb pain (0-3) and skin rash (0-3) and also notes need of pain relief medication. Score below 9 was accepted as inactive disease (17). APRs included complete blood cell count, erythrocyte sedimentation rate (ESR) and serum level of C-reactive protein (CRP). After diagnosis of CAPS, patients were treated with anti interleukin 1 (anti-IL-1) treatments. Initial dose of canakinumab was 2 mg/kg q8w for patients below 40 kg and 150 mg q8w for patients above 40 kg. Anakinra was used at dose of 2 mg/kg/day. Pure tone audiometry, ophthalmological and neurological assessments were performed prior to initiation of anti-IL-1 treatments and on follow-up every 6 months. Side effects were also noted. Hearing loss was graded according to World Health Organisation Criteria (18) and 20dB improvement in one or 10 or greater improvement in 2 adjacent frequencies was defined as 'improvement' (10).

Genetic analysis

Mutation analysis of the *NLRP3* gene was done in all enrolled patients, using genomic DNA extracted from peripheral blood by phenol-chloroform extraction method (19). The *NLRP3* gene was first evaluated to identify germline variants by means of PCR amplification and Sanger sequencing. The PCR and sequencing conditions have been previously reported (20). Somatic *NLRP3* mosaicism was assessed in those patients whom Sanger sequencing did not detect a germline *NLRP3* mutation. For this task, 14 different amplicons were designed to perform targeted deep sequencing of the exons 3, 4 and 6 of the *NLRP3* gene. Library preparation, control quality and quantification were performed according to manufacturer's instructions. Emulsion PCR was performed on a One Touch2 platform, sequencing was performed on a PGM Sequencer using the Ion Torrent PGM 400bp Sequencing kit, and the obtained sequences were analysed using the Torrent Server and the Ion Reporter softwares (Thermo Fisher Scientific Inc, Waltham, MA, USA).

Functional analysis

To determine whether the identified *NLRP3* mutants were disease-causing, two experiments for assessing pathologic functions were performed as previously described (14). Briefly, ASC-dependent NF-κB activation was evaluated using a dual-luciferase reporter assay in HEK293FT cells transfected with *NLRP3* mutants. Transfection-induced cell death in the human monocytic cell line THP-1 was performed by transfecting Green fluorescent protein (GFP)-tagged mutant *NLRP3* into THP-1 cells and then measuring the dead cells with 7-aminoactinomycin D.

Results

A total of 14 unrelated patients (8 male, 6 female) with clinical diagnosis of CAPS were enrolled in this study. Their mean age was 8.29 years (range 3-15 years). Median age at disease onset was 6 months (range 1-84 months) and median age at diagnosis was 3.75 years (range 0.7-9.7 years). All patients were followed by the same medical centres since diagnosis, with a mean of follow-up of 41.5 months (range 9-84 months). All patients had recurrent episodes of fever and urticaria-like skin rash, with five patients suffering from severe CAPS as previously defined (See Table I for a complete summary of the patients' clinical features). None of the patients have cold-induced attacks or FCAS.

Mean delay of diagnosis was 1.9 years (range 0.25-7 years), with systemic-onset juvenile idiopathic arthritis (So-JIA) as the most common initial diagnosis. Initially, before the diagnosis of CAPS was established, twelve patients were treated with prednisolone with or without methotrexate. Once the diagnosis of CAPS was achieved, all patients were treated with anti-IL-1 drugs, with seven patients initially treated with anakinra and the remaining with canakinumab. Anakinra was started at 2 mg/kg/day and had to be increased to 3 mg/kg/day in three patients during the follow-up. Three patients initially treated with anakinra switched to canakinumab because of local reaction at the site of injection (n: 2), and pain at site of injection and noncompliance (n: 1) (Table I). In the group of patients treated with canakinumab (n: 10), the initial dose was 2 mg/kg q8w in all patients. The dose schedule was modified during the follow-up in seven patients according to the AIDAI

Table I. Clinical features of CAPS patients.

Patient	Diagnosis	NLRP3 Mutation	Initial clinical findings+				Treatment			Last visit		
			CNS	Eye	Bone	Hearing	Initial	Last Visit	Dose	CRP	AIDAI	PtGA/ PhGA
1**	Severe CAPS	-	Cognitive delay	-	Arthralgia	Normal	ANA	ANA	2 mg/kg/d	0.6	5	2/2
2	Severe CAPS	I572F	Hydrocephalus+ V/P shunt+ seizure	Papilledema+ Optic atrophy+ scleritis	Frontal bossing+ Patellar overgrowth	Severe hearing loss	CAN	CAN	4 mg/kg/4 wk	15	16	6/7
3	CAPS	Q703K	-	-		Normal	CAN	CAN	2 mg/kg/16 wk	0.8	2	2/0
4**	CAPS	-	-	-	Frontal bossing	Normal	ANA	CAN	2 mg/kg/8 wk	0.4	1	2/3
5	Severe CAPS	D303N	Seizure+ Headache	-	Frontal bossing	Normal	CAN	CAN	3 mg/kg/4 wk	0.5	4	2/4
6*	Severe CAPS	Somatic Y570H	Cognitive delay+ Cerebral atrophy on MRI	Papilledema+ Optic atrophy	Frontal bossing+ arthritis	Mild hearing loss	ANA	ANA	3 mg/kg/d	0.9	3	4/4
7	CAPS	Somatic G307D	-	-	Frontal bossing+ arthritis	Normal	ANA	ANA	3 mg/kg/d	0.7	7	3/4
8	CAPS	E311K	-	-		Normal	ANA	ANA	3 mg/kg/d	0.4	1	4/3
9	CAPS	T436A	-	Severe iridoscleritis	Arthralgia	Mild hearing loss	CAN	CAN	4 mg/kg/8 wk	0.14	1	2/2
10	CAPS	A439V	-	-		Mild hearing loss	ANA	CAN	3 mg/kg/4 wk	0.4	1	3/3
11	CAPS	I313V	-	-		Normal	CAN	CAN	150 mg/8 wk	0.1	0	3/2
12**	CAPS	-	-	Uveitis		Mild hearing loss	CAN	CAN	150 mg/8 wk	0.4	0	3/3
13	Severe CAPS	G569R	Hydrocephalus+ V/A shunt+ cerebral atrophy	Papilledema+ Optic atrophy	Frontal bossing+ patellar overgrowth	Mild hearing loss	ANA	CAN	4 mg/kg/8 wk	0.4	2	7/8
14	CAPS	T436A	-	Uveitis		Normal	CAN	CAN	4 mg/kg/8 wk	0.4	0	5/4

CAPS: Criyopyrin associated periodic syndrome; ANA: Anakinra; CAN: Canakinumab; CRP: C reactive protein; AIDAI: Autoinflammatory Diseases Activity Index; PtGA: Patient/Parents' assessment of disease severity; PhGA: Physicians'global assessment of disease severity; SN: Sensorineural; V/P: ventriculoperitoneal; V/A: ventriculoatrial; Upper. limit of normal CRP is 0,8 mg/dl; PtVAS(0-10) and PhVAS(0-10) higher scores represent more severe disease activity,

+All patients have recurrent fever and urticarial rash. This table demonstrates additional clinical manifestations.

*This patient has also genetically confirmed Duchenne Muscular Dystrophy (DMD). **Patients with neither germline nor somatic NLRP3 mutations.

scores and CRP levels. The dose was increased in three patients (pt 9, 13 and 14), dose increment along with interval decrement was needed in three patients (pt 2, 5 and 10) and the dose interval was increased to 16 weeks in one patient (pt 3). Presently ten patients are treated with canakinumab, with a mean duration of 23.5 months (range 12-39 months) and four patients are on anakinra treatment, with a mean duration of 29.75 months (range 6-57 months).

Growth retardation (<3rd percentile) was observed in eight patients before anti-IL-1 treatment was started. During this treatment, four of these patients had significant percentile increments and catch-up growth, with only three patients being below the 3rd percentile at the last visit (patients 2, 6, 7). On follow up, AIDAI scores decreased below 9 with anti-IL-1 treatment in all patients except patient 2 (AIDAI score: 16). This patient had a severe phenotype, with severe mental retardation and hearing impairment. Despite the high dose of canakinumab (4 mg/kg q4w) administered, he still has high levels of acute phase reactants. Patient and physician's VAS score was also improved in all patients except patient 2 with the anti-IL-1 treatment (Table I). Interestingly, hearing improved in four of seven patients (pt 9, 10, 12 and 13).

With regard to adverse events, two patients (5 and 10, one from each centre) were diagnosed as macrophage activation syndrome (MAS) on the basis of persistent fever, hyperferritinemia (443 ng/ml, 3349 ng/ml), low platelet count (23000 and 154000 /mm^3), low fibrinogen levels (325 and 154 mg/dl) and haemophagocytosis in bone marrow while being treated with canakinumab and anakinra, respectively. Patient 5 was born from a first degree consanguineous couple but family history was unremarkable for familial haemophagocytic lymphohistiocytosis. Functional studies for NK cell and T cell degranulation as well as intracellular expression of perforin and other granule constituents in the NK cells were fully normal. After MAS was subsided, canakinumab and anakinra were re-started with no additional side effects. In the remaining patients, during anti-IL-1 treatment no serious adverse events or severe infections requiring hospitalisation were detected.

Heterozygous germline *NLRP3* mutation was detected in 8 of enrolled patients (57.1%) (See Table II for a detailed summary of genetic data). Those

Table II. Summary of genetic data of enrolled patients.

Patient	Nucleotide Exchange[1]	Protein Exchange	Type of mutation (% mutated allele)	Population Genetics		Bioinformatics analyses		Reference
				1000 Genome Project	NHLBI ESP	SIFT	Polyphen-2	
1	-	-	-	-	-	-	-	-
2	c.1714A>T	p.I572F	Germline	Absent	Absent	Tolerated	Possibly damaging	INFEVERS database
3	c.2107C>A	p.Q703K	Polymorphism	3-7%	3.5%	Tolerated	Uncertain significance	32
4	-	-	-	-	-	-	-	-
5	c.907G>A	p.D303N	Germline	Absent	Absent	Damaging	Probably damaging	33
6	c.1708T>C	p.Y570H	Somatic (11.9%)[2]	Absent	Absent	Tolerated	Benign	Present study
7	c.920G>A	p.G307D	Somatic (4.5%)[2]	Absent	Absent	Tolerated	Possibly damaging	Present study
8	c.931G>A	p.E311K	Germline	Absent	Absent	Tolerated	Possibly damaging	34
9	c.1306A>G	p.T436A	Germline	Absent	Absent	Damaging	Probably damaging	35
10	c.1316C>T	p.A439V	Germline	Absent	Absent	Damaging	Possibly damaging	20
11	c.937A>G	p.I313V	Germline	0.03%	0.02%	Tolerated	Benign	INFEVERS database
12	-	-	-	-	-	-	-	-
13	c.1705G>C	p.G569R	Germline	Absent	Absent	Tolerated	Possibly damaging	33
14	c.1306A>G	p.T436A	Germline	Absent	Absent	Damaging	Probably damaging	35

[1]Reference Sequence NM_001243133.1. [2]Frequency of mutated NLRP3 allele in DNA extracted from peripheral blood.

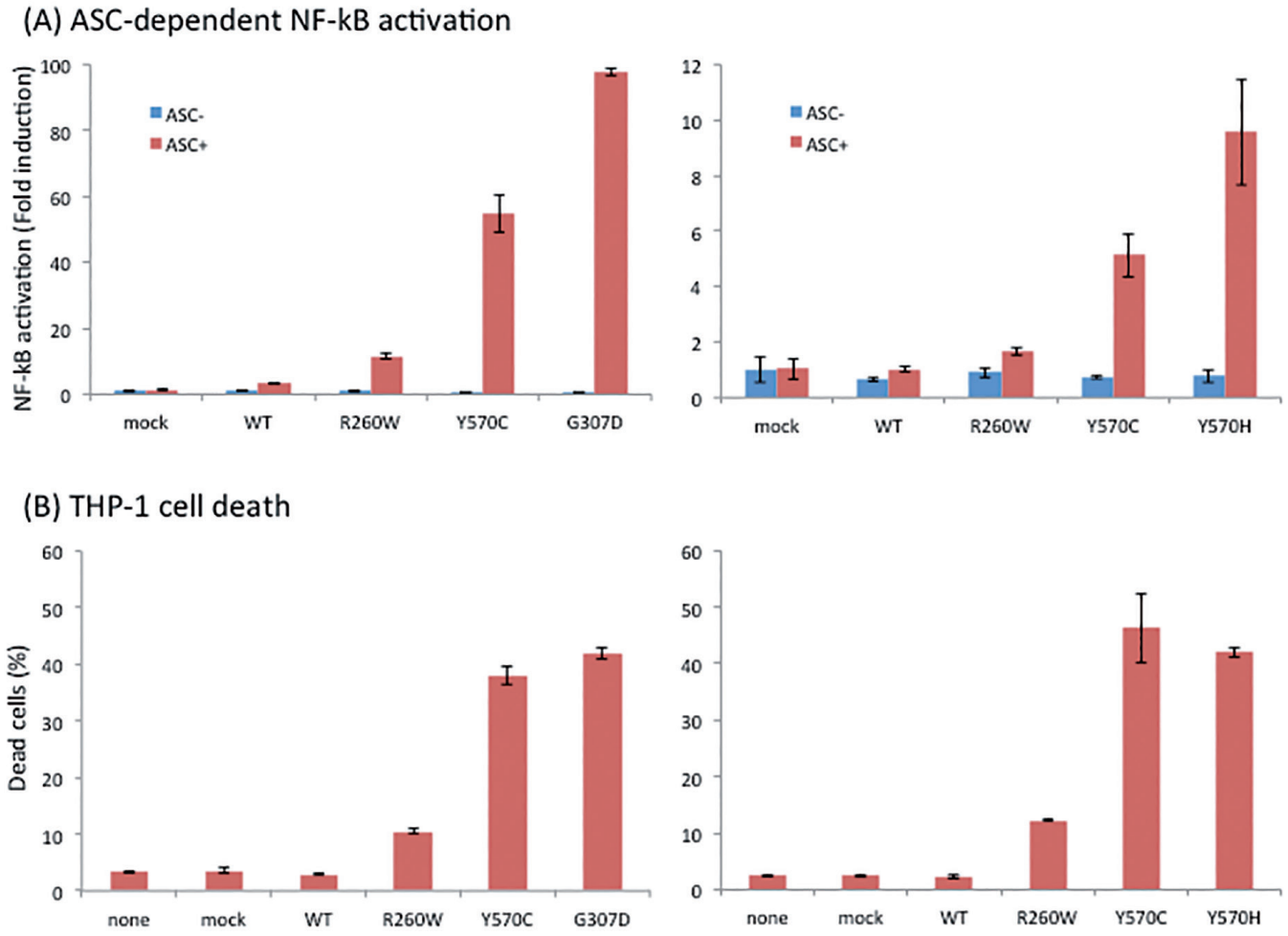

Fig. 1. *In vitro* functional analysis of the novel detected NLRP3 mutations. (A) ASC-dependent NF-kB activation induced by the NLRP3 mutations. HEK293FT cells were co-transfected with WT or mutant NLRP3 in the presence or absence of ASC. The induction of NF-kB is shown as the fold change compared with cells that were transfected with a control vector without ASC. (B) THP-1 cell death induced by the NLRP3 mutations. Green fluorescent protein (GFP)–tagged mutant NLRP3 was transfected into THP-1 cells, and the percentage of dead cells (7-aminoactinomycin D positive) among GFP-positive cells is shown. Values are the mean ± SD of triplicate experiments, and data are representative of two independent experiments. None: nothing transfected; Mock: vector without NLRP3; WT: wild type NLRP3.

patients who were negative for germline *NLRP3* mutation (patient 1, 4, 6, 7, and 12) or with variant of uncertain significance p.Q703K (patient 3) were assessed for somatic *NLRP3* mosaicism. These analyses identified two patients (patient 6 and 7) carrying two novel mutations (p.G307D and p.Y570H) with variable degree of somatic mosaicism (4.5 and 11.9%, respectively) (See Table II). These new variants have not been previously registered in different genomic databases, suggesting a potential pathogenic behavior.

These new mutations induced both THP-1 cell death and ASC dependent NF-κB activation as compared with previously reported *NLRP3* mutations and had shown to have pathogenic effects (See Fig. 1). A blood sample taken in 2010 from patient 6 was also studied and no significant difference in mutation frequency (11.5%) was observed. Neither germline nor somatic *NLRP3* mutations were detected in four patients (pt 1, 3, 4 and 12) and patients 1, 4 and 12 were diagnosed as mutation-negative CAPS according to their clinical and genetic findings.

Discussion

The present study describes the largest Turkish cohort of patients with CAPS, representing also the largest one from the Eastern Mediterranean. Fourteen unrelated patients were enrolled, and the definitive diagnosis could be confirmed in ten patients by means of the detection of pathogenic germline or somatic *NLRP3* mutations. Recently a classificatşon criteria was published by Federici *et al*., and according to these criteria, all of our patients including mutation negative patients fulfill diagnosis of CAPS (21). Since the same *NLRP3* mutation may result in different phenotypes, we have used the general terminology "CAPS" for all enrolled patients and refrained the use of the subgroups of FCAS, MWS and CINCA-NOMID. In turn, we have labelled the patients with severe manifestations such as CNS features or patellar overgrowth or joint deformities as "severe CAPS" with the aim of clearly distinguish them from other less severe patients.

CAPS syndromes are caused by monoallelic, *gain-of-function NLRP3* mutations, which could be dominantly inherited from one progenitor or could appear in the patient as a *de novo* mutation. Up to date 176 disease causing mutations have been registered into the INFEVERS database (22), being most of them found in the exon 3 of the gene. In the present study, ten different missense *gain-of-function NLRP3* mutations located in exon 3 were detected among the Turkish patients. Eight of them were previously reported as true disease-causing mutations in patients with different phenotypes of CAPS, and the remaining two were novel (p.G307D and p.Y570H). Interestingly, these novel mutations were located on amino acid residues where recurrent *NLRP3* mutations have already been described (p.G307V, p.Y570C), and in both cases the mutation was detected as a somatic mutation. In one patient (pt 3) the heterozygous, germline p.Q703K variant was detected. This gene variant has been identified in patients with mild phenotypes in the Eurofever database (23). However, this variant is currently considered as a variant of uncertain clinical significance of the *NLRP3* gene (24).

The rare phenomenon of low-level somatic *NLRP3* mosaicism was described in nearly 35% of patients with CINCA-NOMID patients who were considered as mutation-negative in conventional screening and has been also recently described in patients with MWS (13, 15). Our data confirms previous data regarding the relevant role of somatic *NLRP3* mosaicism in CAPS and highlight the importance of clinical judgment and also the need to search for somatic mutations in those patients in whom conventional studies gave negative results.

Initially a substantial proportion of CAPS patients were diagnosed and treated as SoJIA (25). This was also the case in some of our patients where the partial clinical benefit associated with the improvement of laboratory markers with steroids and other immunosuppressive drugs complicated the differential diagnosis. Paediatricians must consider the monogenic autoinflammatory syndromes, and specially CAPS syndromes, in the differential diagnosis of young patients with fever and high acute phase reactants. In CAPS the urticaria-like skin rash is typical at the disease onset and very helpful to distinguish this illness from other hereditary periodic fever syndromes. Additional features to include in differential diagnosis are the presence of familial history of the disease, hearing impairment, headache, papilledema, frontal bossing, clubbing and joint findings. CAPS and SoJIA share some inflammatory markers such as increased serum levels of IL-6 and S100 proteins (26). Consequently, further research is needed to identify specific biomarkers or certain functional analysis that may help the differential diagnostic process.

The retrospective data collected in our study confirms the long-term efficacy and safety of different anti-IL-1 drugs in patients with CAPS. Initially we used the currently licensed dose (2 mg/kg/day for anakinra and 2 mg/kg q8w for canakinumab). During the follow-up, the dose and the frequency of drug administration needed to be arranged according to the patient's symptoms and laboratory parameters. Patients with severe CAPS needed higher doses of anti-IL-1 drugs than those with non-severe phenotypes, thus confirming previous published data (8, 10, 27). Our data supports the data of the Eurofever registry regarding those patients who have disease onset after 3 years of age, which have milder course of disease with fever, urticaria and mild articular involvement as main features (23). We have also observed that those patients with somatic mutation also had a milder course and needed less dose of anti IL1 treatment. However, the upper dose limit of both anti-IL-1 drugs is currently unknown and government policies have restrictions on the use of anti IL1 therapies.

With regard to the safety, two patients developed MAS while receiving anti-IL-1 drugs. The MAS episodes were well controlled with conventional treatments and did not recur after re-introduction of anti-IL-1 drugs. MAS is a rare complication of the clinical course of inherited autoinflammatory diseases, with only three cases with CAPS were reported (10, 28, 29). Consequently, its detection of in two cases of our co-

hort was absolutely unexpected and we cannot obtain conclusive evidence regarding their association with the administered anti-IL-1 therapies. The IL-1β overproduction is considered the cause of the clinical features detected in patients with CAPS and represents the rationale to treat them with the anti-cytokine drugs. However, additional research is warranted to identify novel therapeutical approaches for CAPS targeting the inflammasome aggregation or upstream IL1β production (30, 31).

In conclusion, we herein retrospectively describe the largest known cohort of patients with CAPS in the Eastern Mediterranean. The CAPS diagnosis should be considered in all patients with typical symptoms even if Sanger-based genetic analysis is negative. In these cases, the search for low-level somatic *NLRP3* mosaicism should be seriously considered. The data collected from the different administered treatments strongly suggest a patient-tailored therapy along with the care for specific complications such as hearing loss, papilledema or the fortunately less frequent MAS.

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