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Longitudinal Analysis of Somatic Mosaicism and Clonal Hematopoiesis in Cryopyrin-Associated Periodic Syndrome

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Conflict of Interests

NK received canakinumab free of charge and consulting fees from Novartis in relation to an investigator-initiated clinical trial conducted for a condition unrelated to this study. MY is the CEO and sole shareholder of Live Cell Diagnosis Ltd. SO received consulting fees from Chordia

Therapeutics Inc., Eisai Co., Ltd., and Montage Bio, Inc.; and holds stocks in Asahi Genomics Inc.

RN and TY received lecture fees from Novartis Inc. KI received consulting fees from SOBI and lecture fees from Novartis Inc.

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Data availability

Datasets generated and analyzed during the present study are partly provided in the Supplementary Material. The remaining datasets are available from the corresponding author upon reasonable request.

Abstract

Objective: Cryopyrin-associated periodic syndrome (CAPS) is an autoinflammatory disease caused by gain-of-function mutations in *NLRP3*. Although somatic *NLRP3* mosaicism is increasingly recognized, it remains unclear how variant allele frequency (VAF) changes over time and whether disease onset age reflects differences in somatic mutation dynamics. This study aimed to analyze the longitudinal VAF trends and their correlation with clonal hematopoiesis (CH) in patients with CAPS mosaicism.

Methods: We analyzed *NLRP3* VAF in blood and various tissues, including dried umbilical cord (DUC) using digital PCR and deep amplicon sequencing. Whole-exome and single-cell genome sequencing were performed to evaluate CH-related mutations.

Results: We included 15 patients with VAFs of 4.3% to 34.9%. In the 12 early-onset cases, VAFs were generally consistent across various tissues and did not increase over time, including the DUC. Conversely, in the three late-onset cases, VAFs were particularly high in myeloid cells. Notably, in one late-onset case, no mutations were detected in DUC. After disease onset, VAFs exhibited 2.9%-to-10.6% and 6.2%-to-16.4% increase in the whole blood and neutrophils over 6.4 and 4.4 years, respectively, showing a clonal expansion of *NLRP3*-mutant cells. Single-cell genome sequencing revealed frequent co-occurrence of *NLRP3* and *TET2* mutations in the same cells, with a gradual increase in both VAFs over 3 years, in one late-onset case.

Conclusion: In early-onset cases, the VAFs were comparable across various tissues and did not increase over time. Mutations in late-onset cases were enriched in myeloid cells, and VAFs were increased, suggesting a link to CH.

INTRODUCTION

Autoinflammatory diseases are disorders characterized by systemic inflammation resulting from dysregulation of the innate immune system.¹ Cryopyrin-associated periodic syndrome (CAPS) comprises autosomal dominant inherited systemic autoinflammatory syndromes, including familial cold autoinflammatory syndrome, Muckle–Wells syndrome, and chronic infantile neurological cutaneous articular/neonatal-onset multisystem inflammatory disease (CINCA/NOMID). The main symptoms of CAPS are fever, urticarial rash, hearing loss, aseptic meningitis, arthritis, and epiphyseal overgrowth. Most patients with CAPS carry heterozygous gain-of-function mutations in *NLRP3*, resulting in activation of the NLRP3 inflammasome and subsequent inappropriate release of inflammatory cytokines, including IL-1 β . The standard treatment for CAPS involves IL-1 blockade, with most patients showing favorable responses when adequately dosed.²⁻⁴

Although most patients with CAPS carry germline heterozygous *NLRP3* mutations, there are so-called “mutation-negative” cases where no apparent disease-causing *NLRP3* variants are detected using conventional Sanger sequencing. In 2005, we reported the first case of CAPS caused by *NLRP3* somatic mosaicism in a “mutation-negative” case.⁵ Since then, additional cases of CAPS caused by somatic mosaicism have been identified.⁶⁻¹²

In general, early-onset CAPS caused by somatic mosaicism shows even distributions of mutations across multiple cell types, including blood cells and buccal mucosa tissues,⁶ whereas late-onset CAPS, with initial symptoms appearing at or after age 30–40, has been attributed to myeloid-restricted or myeloid-enriched *NLRP3* mosaicism.⁹⁻¹² However, we currently have limited data on the longitudinal changes in *NLRP3* VAF over time, in both early-onset and late-onset CAPS mosaicism cases. The detailed mechanisms underlying somatic mosaicism in

patients with CAPS remain unclear. In a patient with NLRC4-related late-onset autoinflammatory disease, clonal hematopoiesis (CH), characterized by skewed myelomonocytic differentiation, may have contributed to the pathogenesis and VAF changes.¹³ However, no related studies have been reported in patients with CAPS.

Thus, we aimed to elucidate the time course of *NLRP3* VAF. Given the limited number of reports on this topic, we decided to establish the dynamics of *NLRP3* VAF, including the timing of mutation occurrence and changes over the course of the disease. We also investigated the contribution of CH to CAPS somatic mosaicism.

Specifically, we conducted a comprehensive analysis of CAPS somatic mosaicism using longitudinal samples and a single-cell analysis. Our findings reveal possible differences in *NLRP3*-mutant clone evolution depending on disease onset. The insights gained from this study are crucial for understanding CAPS, as well as have broader implications for other somatic mosaic diseases.

MATERIALS AND METHODS

Study Design and Patients

We enrolled all patients with CAPS mosaicism in Japan based on the results of the nationwide survey on CAPS.¹⁴ Additionally, we included a patient with somatic NLRC4-related autoinflammatory disease, as this condition can exhibit phenotypes similar to those of CAPS.¹⁵ Clinical information was obtained through a questionnaire administered during the nationwide survey. Three healthy volunteers, who participated on a voluntary basis from among hospital staff, and one patient with CAPS carrying an *NLRP3* R260W germline mutation were recruited for reference data.

Ethics Statement

Clinical information and samples were obtained after obtaining informed consent for participation in this study. Consent to participate in the research and publish the findings was obtained either from the patients or, in the case of underage patients, from their respective parents or guardians. This study was approved by the Kyoto University Ethical Committee on Human Research (protocol numbers R2259, G1118, G432, R2831, R2524, G457, G1233, and G1091) and was conducted in accordance with the Declaration of Helsinki.

Sample Collection

The following samples were collected from each patient and healthy control and evaluated over time.

Whole blood, neutrophils, peripheral blood mononuclear cells (PBMCs), T cells, B cells, monocytes, and NK cells

Whole blood was collected, and PBMCs were isolated using Lymphoprep (Axis-Shield, Oslo, Norway). Neutrophils were also isolated during this process, as previously described.¹⁶ PBMCs were stained with 7ADD (BD Biosciences, San Jose, CA, USA) to monitor viability, CD3 APC (BD Biosciences) for T cells, CD19 BV510 (BD Biosciences) for B cells, CD14 FITC (BD Biosciences) for monocytes, and CD56 PE (BD Biosciences) for NK cells. Cell sorting of PBMCs was performed using FACS Melody™ (BD Biosciences). DNA was extracted from each blood sample using a Nucleo Spin Blood kit (Macherey-Nagel, Düren, Germany).

Nail, hair, DUC, buccal mucosa, and dried blood spot

For nail, hair, and DUC samples, 20–30 mg of each sample was processed, and DNA was isolated using the ISOHAIR kit (Nippon Gene, Tokyo, Japan). Buccal mucosa samples were collected, and DNA was isolated using the Isohelix Swab and BuccalFix Plus DNA Isolation Kit (SciTrove, Tokyo, Japan). Dried blood spot samples obtained at birth were provided by the municipality, and DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Digital PCR

Digital PCR (dPCR) was performed using a QuantStudio 3D Digital PCR platform (Thermo Fisher Scientific, Waltham, MA, USA). Mutation analysis of genomic DNA was conducted using an allele-specific TaqMan® probe targeting known *NLRP3* mutations. WT *NLRP3* alleles were detected using a VIC fluorescent probe, whereas mutant *NLRP3* alleles were detected using a FAM fluorescent probe. All 3D dPCR reagents were obtained from Thermo Fisher Scientific, and the custom-ordered primers are listed in **Supplementary Table 1**. The TaqMan SNP Genotyping reagent was added to each DNA sample along with the Digital PCR V2 Master Mix. The samples were then loaded onto a Digital PCR 20 K chip, which was sealed using a QuantStudio 3D Digital PCR Chip Loader. dPCR was performed using a Thermo Fisher ProFlex PCR system. Chip reads were obtained using a QuantStudio 3D Digital PCR Chip Reader (Thermo Fisher Scientific). The samples were individually analyzed online using the QuantStudio 3D Analysis Suite.

Amplicon-Based Deep Sequencing

Library preparation and sequencing methods

The concentration of the DNA solution was measured using a Synergy LX (BioTek, Winooski, VT, USA) and QuantiFluor dsDNA System (Promega, Madison, WI, USA). Libraries were prepared using a two-step tailed PCR method (primers are listed in **Supplementary Table 2**). The concentrations of the prepared libraries were measured using Synergy H1 (BioTek) and the QuantiFluor dsDNA System. The quality of the prepared libraries was assessed using a Fragment Analyzer and the dsDNA 915 Reagent Kit (Advanced Analytical Technologies, Ankeny, IO, USA). Sequencing was performed using the MiSeq system and MiSeq Reagent Kit v3 (Illumina) with 2 × 300 bp reads.

Bioinformatic analysis

Quality filtering of reads was conducted using the Fastq_barcode_splitter of the Fastx toolkit (ver. 0.0.14). Sequences with reads starting from the primers were extracted. Sequences with a quality value of less than 20 and those with a length of 40 bases or less, along with their paired sequences, were removed using Sickle (ver. 1.33). High-quality sequences were joined using the paired-end join script FLASH (ver. 1.2.11) under the following conditions: a merged sequence length of 409 bases, a read sequence length of 280 bases, and a minimum overlap length of 10 bases. The combined sequences were mapped using BWA (ver. 0.7.18), and the mutation sites were manually inspected using Integrated Genome Viewer.

Whole-Exome Sequencing

For whole-exome sequencing, three late-onset cases and three early-onset cases were selected. Whole blood and neutrophils from all patients were used, while patient 13 also provided T cells, buccal mucosal tissues, and additional whole blood from different time points.

Genomic DNA was extracted from peripheral blood using NucleoSpin Blood (Takara, Kyoto, Japan). Sequencing libraries were prepared using the Lotus DNA Library Prep Kit, xGen Hybridization and Wash Kit, and xGen Exome Research Panel v2 (Integrated DNA Technologies, Coralville, IA, USA). Sequencing was performed using the MGI DNBSEQ platform in the 150-bp paired-end mode. The target sequencing depth was 200×, with an actual average coverage of 198× (range: 164×–231×). Variant calling was performed using the Genomon2 pipeline (<https://github.com/Genomon-Project/genomon-docs>). Sequencing reads were aligned to the hg19 human reference genome using BWA (v0.7.10), and PCR duplicates were removed using Picard-tools (v1.39). To detect somatic mutations, low-quality reads and artifacts were filtered out based on mapping quality (<20), base quality (<15), or excessive variant calls (≥5 SNVs or indels). Variants were retained if supported by ≥8 total reads and ≥4 variant reads, with VAF ≥2% in the sample and ≤10% in control, and without extreme strand bias. Statistically significant variants ($P \leq 10^{-5}$) were identified using the EBCall algorithm, based on an empirical VAF distribution from non-paired blood samples processed concurrently.

Subsequently, CH-related genes were extracted according to previously reported methods.¹⁷ Mapping errors were removed by visual inspection using the Integrated Genome Viewer browser. Copy number variations were analyzed using the in-house software CNACS.

Single-Cell Genotyping of NLRP3 and Clonal Hematopoiesis-Related Genes

PBMCs from selected patients were isolated as previously described and sorted into 96-well plates using a SONY SH800S cell sorter (SONY, Kanagawa, Japan). In total, 32 single cells were analyzed. Whole-genome amplification of the single cell was performed using the PicoPLEX® Single Cell WGA Kit v3 (Takara). One microliter of the amplified genome was used for deep

amplicon sequencing (primers are listed in **Supplementary Table 4**). Subsequent analyses were conducted as previously described. For frameshift deletions, we applied the following modified pipeline. Duplicate marking was intentionally excluded because all fragments originated from targeted PCR amplicons. Variant calling was performed using two GATK (v4.2.0.0) callers that were configured for high-depth amplicon sequencing. For Mutect2, positional down-sampling was disabled and soft-clipped bases were excluded; the minimum allele fraction threshold was set to 0.001 to retain mutations present at approximately 0.1% VAF. Subsequently, variant filtering was performed using FilterMutectCalls. HaplotypeCaller was run with the same down-sampling and soft-clip settings; however, the sample ploidy was increased to 200 to accommodate very low-frequency alleles. Variant filtering was then performed using VariantFiltration. Variants identified by both callers were retained. For variants detected by only one caller, the filtering reasons were carefully reviewed, and those deemed reliable based on manual inspection with IGV were reinstated.

Allelic depths for SNV were subsequently quantified using GATK CollectAllelicCounts. For each locus, the VAF was calculated as $\text{ALT count} / (\text{ALT count} + \text{REF count})$ and used in all downstream analyses. For variants involving deletions, local realignment was performed using ABRA2 (v2.20) to standardize indel representation and minimize alignment artifacts. VAF were subsequently recalculated by parsing the pileup output from samtools mpileup (v1.20). At each deletion locus, the VAF was computed as the number of reads supporting the deletion divided by the total read depth.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (version 9.4) for Windows (GraphPad Software, San Diego, CA, USA). To confirm that the variant allele frequency (VAF) in the patients with mosaic did not vary due to the selection pressure associated with aging, we applied a linear mixed-effects model. VAF was set as the dependent variable and age at sampling (in years, including months as decimals) as the explanatory variable. In cases wherein multiple samples were available for the same patient; the patient was included as a random effect. The model can be expressed as follows:

$$VAF_{ij} = \beta_0 + \beta_1 \times Age_{ij} + (u_{0i} + u_{1i} \times Age_{ij}) + \epsilon_{ij}$$

where VAF_{ij} represents the VAF for sample j in patient i ,

β_0 and β_1 are fixed effects, u_{0i} and u_{1i} are random effects associated with patient i , and ϵ_{ij} is the residual error term. A significance level of 5% was used. Age: Age at sampling

RESULTS

Clinical and genetic characteristics

In total, 15 Japanese patients with CAPS with somatic *NLRP3* mosaicism, including 12 early-onset and three late-onset cases, were included in this study. Clinically, eight patients showed the CINCA/NOMID phenotype, whereas the remaining seven showed the Muckle–Wells syndrome phenotype. The variant allele frequency (VAF) at diagnosis ranged from 4.3% to 34.9%. Additionally, we included one patient who initially presented with a CINCA/NOMID-like phenotype but was later diagnosed with NLRC4-related autoinflammatory disease by somatic mosaicism. The clinical and genetic profiles and specific mutation sites are summarized in **Table**

1 and **Figure 1**, respectively. Details of the clinical information have been reported previously,^{5,6,7,14,18,19,20} and the **supplementary materials** offer further details regarding patient 13 (G564D), patient 14 (I572F), and patient 15 (Y563C).

Temporal Changes in *NLRP3* VAF

Among the patients with early-onset CAPS, 11 patients underwent time-course analysis, including five patients assessed for dried umbilical cords (DUCs) (**Figure 2A**). In certain Asian countries, including Japan, preserving DUCs in a small box is a traditional custom. It has been reported that DUCs can be used for genetic analysis.²¹ We used these samples for evaluating VAF at the time of birth. Mutations were observed in all patients with evaluable DUC samples, and the VAFs remained stable over time. VAFs observed in the buccal mucosal tissues, which originate from the ectoderm, exhibited similar patterns.

We further examined various blood cell lineages and found that the VAFs were generally comparable across different cell types and time points. Although certain cases (e.g., patients 2 and 9) exhibited a decreasing trend in VAF, a linear mixed-effects model did not detect statistically significant changes among all patients ($p = 0.22$; **Figure 2A, Supplementary Table 3**). Patient 16, with an *NLRP4*-related autoinflammatory disease, exhibited the same trend (**Supplementary Figure 2, Supplementary Table 3**).

Three patients with late-onset CAPS showed higher VAFs in myeloid cells, specifically neutrophils and monocytes (**Figure 2B, Supplementary Table 3**), than in other cell types, consistent with previous findings. In patient 13 (G564D) with available DUC, no mutations were detected at birth using dPCR and deep sequencing, supporting the idea that mutation acquisition events occur late in life in these cases. An increased VAF was observed in patient 13

(G564D), for which longitudinal analysis was available: from 2.9% to 10.6% in whole blood cells over 6.4 years and from 6.2% to 16.4% in neutrophils over 4.4 years (**Figure 2B**). Patient 14 also showed a modest increase in *NLRP3* VAF from the age of 71 to 74. Notably, *NLRP3* VAF in whole blood slightly decreased between ages 68 and 71. At age 68, the patient had marked leukocytosis (17,300/ μ L) with 91.3% neutrophils. In contrast, at age 71, the WBC count had decreased to 7,900/ μ L, with neutrophils comprising 68.8%, and at age 74, to 7,500/ μ L with 74.8% neutrophils, suggesting that shifts in white blood cell composition—particularly neutrophil predominance—may influence VAF measurements (**Figure 2B**). In contrast, patient 15 (Y563C) showed a decrease in monocyte VAF from 11.2% to approximately 5.0% over 2.2 years. (**Supplementary Table 3**) This decline occurred concurrently with a period of clinical deterioration as a result of septic shock. Although speculative, it is notable that monocytes harboring *NLRP3* mutations have been reported to undergo rapid pyroptosis in response to LPS stimulation²², which may have influenced the observed VAF.

CH in Patients with Late-Onset CAPS Mosaicism

Recent studies have proposed the involvement of clonal hematopoiesis-related genes (CH-related genes) in the increased VAFs and adult-onset autoinflammatory disease associated with *NLRP3*.¹³ To explore this further in our patients, we conducted whole-exome sequencing in both patients with late-onset and early-onset CAPS. We identified three candidate mutations in CH-related genes, *DNMT3A* and *TET2*, exclusively in patients with late-onset CAPS: patient 13 (G564D) and patient 14 (I572F) (**Table 2**). This mutation corresponds to a previously reported CH-related mutation, and in silico functional analysis suggested a high impact on the mutation burden (**Table 2**). Additional deep amplicon sequencing confirmed the presence of these

mutations (**Figure 3A**). No candidate mutations were detected in patient 15 (Y563C) and in patients with early-onset CAPS. To assess clonal dynamics, we performed a time-course analysis of patient 14 (I572F), who harbored somatic mutations in *NLRP3*, *DNMT3A*, and *TET2*. VAFs were evaluated across multiple time points (ages 68, 71, and 74) and cell types. Between ages 71 and 74, modest increases in VAFs were observed for both *NLRP3* and *TET2* mutations across multiple myeloid compartments. In whole blood, *NLRP3* VAF increased from 26.2% to 37.0%, and *TET2* from 21.2% to 28.1%. In neutrophils, the VAFs increased from 34.3% to 36.7% (*NLRP3*) and from 14.7% to 23.8% (*TET2*), whereas in monocytes they increased from 34.1% to 41.1% (*NLRP3*) and from 20.1% to 30.6% (*TET2*). In contrast, the *DNMT3A* mutation, primarily detected in B cells, showed a relatively high VAF increase from 10.1% to 26.4% over the same period. These findings suggest the potential for a coordinated expansion of *NLRP3* and *TET2* mutant clones within the myeloid lineage, indicating a synergistic clonal advantage.

Single-Cell Analysis of CH-Related Gene Mutations and *NLRP3* Somatic Mosaicism

Next, we aimed to elucidate the association between CH and *NLRP3* somatic mosaicism. Notably, the *DNMT3A* mutant clone identified in patient 13 (G564D) was considerably smaller than the *NLRP3*-mutant clone (**Figure 3A**), although both VAFs in neutrophils increased over time, from 7.6% to 12.6% for *NLRP3* and from 0.6% to 1.4% for *DNMT3A* between ages 44 and 47. Conversely, the *DNMT3A* and *TET2* mutant clones in patient 14 (I572F) were similar in size to the *NLRP3*-mutant clone (**Figure 3A**). To further investigate this, we analyzed the genotypes of single cells in patient 14 (I572F) (**Figure 3B**). Our findings revealed that monocytes and B cells carrying *DNMT3A* mutations did not carry *NLRP3* mutations, whereas all *TET2* mutation-carrying cells harbored *NLRP3* mutations.

Notably, although both monocytes and B cells exhibited a high co-occurrence of *NLRP3* and *TET2* mutations within the same single cell, the co-occurrence rate in B cells was relatively lower than that in monocytes. This suggests that the *NLRP3* mutation occurred first, followed by the secondary acquisition of the *TET2* mutation, which may have led to a growth advantage, especially in monocytes. *TET2* is known to be involved in methylation processes. However, our analysis of whole blood and neutrophils from patient 14 (I572F) did not reveal any significant methylation changes (**Supplementary Data File, Supplementary Figure 3.**).

All these results indicate that in the early-onset type, the mutations occur relatively early after fertilization, and VAFs are stable for years, whereas in the late-onset type, the mutations predominantly occur later in life, are restricted to myelomonocytic blood cells, and can expand over time. The frequent occurrence of *TET2* mutations in *NLRP3*-mutant cells in one patient implies a potential association between CH and disease pathogenesis.

DISCUSSION

To our knowledge, this is the largest study to explore the longitudinal patterns of VAF in patients with CAPS with somatic mosaicism. In the early-onset group, mutations were uniformly detected across various tissues, including the DUC, and remained stable over time. This suggests that *NLRP3* mutations likely occurred early in embryogenesis in these cases. Conversely, in late-onset cases, mutations were predominantly confined to the myeloid lineage, and no mutations were observed in the DUC. These findings indicate that in late-onset cases, mutations arise later during myeloid lineage differentiation, possibly before disease onset.

Notably, we included one patient with *NLRP4*-related early-onset disease who exhibited stable *NLRP4* VAFs, similar to those of patients with early-onset CAPS mosaicism. This finding contrasts

that of a previous report, which indicated that VAFs in patients with late-onset *NLR4* mosaicism increased over time.¹³ These results suggest that patients with early-onset and late-onset autoinflammatory disease may exhibit different patterns of clonal evolution, a distinction that may be applicable to patients with CAPS, as well as to other cases of somatic mosaicism; however, further analysis is required to confirm this conclusion.

In one late-onset case, we observed a significant increase in VAF over time. Although this phenomenon is intriguing, previous reports are scarce. Notably, Rowczenio et al. reported an increase in VAF from 5% to 45% in whole blood over a 5-year period for patients with late-onset CAPS with the Y563C mutation, which correlated with disease worsening.¹⁰ This poses a curious scenario since *NLRP3* mutations are typically associated with cell death and unlikely to confer cellular growth or clonal advantage. In a related autoinflammatory condition, *NLR4*-related autoinflammatory disease, De Langhe et al. reported cases of mutant clonal expansion.¹³ They proposed that CH-related mutations, observed in hematologic diseases such as myelodysplastic syndrome, lead to a substantial increase in specific clones of the myelomonocytic lineage, suggesting a potential driver mechanism. In contrast, Gutierrez and Rodrigues et al. recently conducted high-throughput screening for CH-related mutations in patients with Vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic syndrome known as VEXAS syndrome, a typical autoinflammatory disease caused by somatic mutations.²³ They concluded that CH-related gene mutations occurred secondarily and did not have significant implications. In our study, we identified three patients with late-onset CAPS with varying profiles of CH-related mutations. We detected a *DNMT3A* mutation, a typical CH-related mutation, in patient 13 (G564D), who exhibited an increase in *NLRP3* VAF. However, the clone size was considerably smaller than that of the *NLRP3*-mutant clone, suggesting that the *DNMT3A* mutation likely occurred after the

NLRP3 mutation, possibly due to aging, an inflammatory microenvironment,²⁴ or specific treatments such as methotrexate.²⁵

Notably, although patient 13 (G564D) experienced an increase in VAF over time, the clinical symptoms were relatively well-controlled with canakinumab. This observation contrasts the findings of Rowczenio et al., who reported that increased VAF correlated with disease worsening.¹⁰ Conversely, in patient 14 (I572F), frequent co-occurrence of the *NLRP3* and *TET2* mutations in the same single cell, along with gradual clonal expansion over time was observed. As previously reported,¹³ this finding may support a type of driver-passenger model, in which variant *TET2* drives expansion and variant *NLRP3* is a pathogenic “passenger” in the expanding clone. Stimulation of Tet2-knockdown hematopoietic stem cells with IL-1 β enhances self-renewal and promotes myeloid lineage differentiation in mouse models²⁶. Additionally, Caiado et al. demonstrated in a murine model that aging-associated inflammation, particularly IL-1 signaling, promotes the expansion of Tet2+/- clones, suggesting a synergistic interaction between IL-1 and TET2 in clonal hematopoiesis²⁷. The *TET2* mutation identified in patient 14 (I572F) was a stop-gain mutation, suggesting a loss of function of *TET2*, while the *NLRP3* mutation clearly leads to overproduction of IL-1 β . Based on these findings and the previous report^{26,27}, we hypothesize that cells harboring the *TET2* mutation in this patient may have acquired a growth advantage due to the synergistic effect of IL-1 β overproduction. This may have led to clonal expansion with a myeloid bias. Indeed, we observed a modest increase in both *NLRP3* and *TET2* VAFs over a 3-year period in this patient.

Additionally, the co-occurrence of *NLRP3* and *TET2* mutations at the single-cell level is primarily observed in monocytes. In contrast, B cells exhibited a relatively low co-occurrence of the *TET2* mutation, despite the presence of the *NLRP3* mutation. The finding that the VAF of

NLRP3 is relatively high despite the relatively low VAF of *TET2* in B cells warrants further investigation. Future studies, including cell line validation, are required to confirm this hypothesis and provide further insights into the observed mutation patterns. In another case, no CH-related gene mutations were identified. In patients with CAPS mosaicism, CH involvement can vary, and further analysis is needed to determine its clinical relevance. Notably, restoration of *TET2* function with agents such as vitamin C can block aberrant self-renewal and prevent leukemia progression.²⁸ It is intriguing to consider applying these findings to late-onset cases with CAPS mosaicism carrying *TET2* loss-of-function mutations.

It is unlikely that *NLRP3* mutations in patients with late-onset CAPS have a growth advantage distinct from that of early-onset mutations. In our cohort, no mutations were shared between the early- and late-onset cases. However, considering our cohort and previous reports, some mutations are present in both types (**Figure 1**). For instance, the E567K mutation was previously reported in late-onset cases¹⁰ and in the four early-onset cases in the present study. In these patients, the VAF has remained stable for many years. Therefore, the E567K mutation itself is not considered to possess a growth advantage. Although a systematic analysis of variant-specific behavior has not yet been reported, the clinical and genetic variability observed in certain variants may suggest intrinsic differences. For example, E567K is the most frequently reported somatic mutation in CAPS, with nine cases including ours, and is generally associated with early-onset disease and low-to-moderate VAF (5.0%–13.0%). In contrast, germline E567K mutations are rare, with only two reports to date, suggesting that this variant may be incompatible with life or result in severe disease when present constitutionally. Consistent with this, a recent systematic functional study classified E567K as a strongly activating mutation²⁹. Furthermore, in our previous report, a patient with a germline E567K mutation failed to achieve disease

control despite treatment with high-dose canakinumab (12 mg/kg every 2 weeks), highlighting its clinical severity¹⁴. The limited number of cases precludes firm conclusions; however, larger cohort studies are warranted to explore potential variant-specific effects on mosaicism dynamics and phenotypes.

This study had certain limitations. Although the DUC serves as a valuable sample for detecting the genotype at the time of live birth, DUC samples do not precisely represent blood cells. However, the umbilical cord is an organ of fetal origin, originating from the same mesoderm as blood cells, and DUC has been commonly used for genetic testing.³⁰⁻³² To ensure sufficiently high quality of DUC samples for genotyping, we conducted quality evaluations using both negative and positive control samples to ensure the quality of DUC as a sample for genotyping (Supplementary Table 3). In the future, alternative samples, such as dried blood spots collected at birth, may provide more accurate insights for genotyping.

Another limitation of this study is the relatively small sample size, although it represents the largest cohort study of CAPS patients with *NLRP3* mosaicism to date. Given the limited number of cases, we were unable to perform robust statistical analyses of the relationship between VAF fluctuations and clinical or treatment-related variables. Inter-patient heterogeneity in inflammatory status, variant type, and treatment regimen further complicates interpretation. Caution should therefore be exercised when drawing conclusions about longitudinal VAF changes, particularly in the context of disease activity or therapeutic response. Nevertheless, we note an interesting observation. Among three patients harboring the same *NLRP3* variant E567K (patient 3, 4, 5), VAFs remained relatively stable over time, despite differing inflammatory profiles: patients 3 and 4 showed rising CRP levels, whereas patient 5 maintained low CRP. Although limited to a small subset, this finding may suggest that, at least for the E567K

variant, VAF dynamics are not strongly influenced by inflammatory activity (data not shown). Future studies involving larger, prospectively followed cohorts are required to validate our observations and clarify whether certain *NLRP3* variants are associated with clonal expansion under persistent inflammatory conditions or suboptimal treatment response. Finally, technical challenges in single-cell sequencing, such as allele dropouts and unbalanced amplification of alleles, which compromise the accuracy of genotyping, remain significant concerns. Although we verified that our target mutation sites were adequately amplified to detect the intended mutations, we cannot completely rule out the possibility that mutant cells were misclassified as wild-type by allele dropout. Eliminating this possibility entirely is difficult, and further advancements in sequencing methodologies are required to address this issue.

This comprehensive analysis of patients with CAPS mosaicism highlights the evolutionary differences in mutant clones based on disease onset type. These findings contribute to our understanding of CAPS mosaicism and provide insights into other genetic diseases caused by somatic mutations.

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FIGURE LEGENDS

Figure 1. Mutation sites

Schematic representation of the amino acid sequences of *NLRP3*- and *NLRC4*-encoded proteins, showing mutation sites. Mutation symbols in red indicate patients from our cohort, while those in black represent cases previously summarized in reference [7]. # denotes the number of patients listed in Table 1.

PYD, pyrin domain; NACHT, nucleotide-binding and oligomerization domain; LRR, leucine-rich repeat domain; CARD, caspase recruitment domain

Figure 2. VAF changes in patients with cryopyrin-associated periodic syndrome mosaicism

(A) Results for patients with early-onset CAPS showing mean data obtained from two measurements using dPCR without any specific annotations (see **Supplementary Table 3** for detailed information). The year below the graph indicates the age of the patients when each sample was collected.

VAF, variant allele frequency; DUC, dried umbilical cord; Neu, neutrophils; PBMC, peripheral blood mononuclear cell; T, T cells; B, B cells; Mono, monocytes; NK, NK cells; dPCR, digital PCR; y, year; WB, whole blood

(B) Results for patients with late-onset CAPS showing mean data obtained from two measurements using dPCR without any specific annotations (see **Supplementary Table 3** for detailed information). The years above the upper graphs indicate the age of the patient when each sample was collected. The lower graph shows the change in VAF over time for patient 13 and 14.

VAF, variant allele frequency; DUC, dried umbilical cord; WB, Whole blood; Neu, neutrophils; PBMC, peripheral blood mononuclear cells; T, T cells; B, B cells; Mono, monocytes; NK, NK cells; dPCR, digital PCR; y, year

Figure 3 Analysis of clonal hematopoiesis-related genes

(A) Validation with deep amplicon sequencing. The years above the upper graphs indicate the age of the patient when each sample was collected.

VAF, variant allele frequency; WB, Whole blood; Neu, neutrophils; T, T cells; B, B cells; monocytes; NK, NK cells; y, year

(B) The two left panels show the results of genotyping 32 single monocytes and B cells from patient 14. White boxes indicate wild-type cells, whereas gray boxes indicate mutant cells. The right panels summarize the genotyping results.

Mono, monocytes; B, B cells; WT, wild-type; Mut, mutant type

Tables

Table 1. Summary of patient characteristics

Patient No.	Sex	Gene	Nucleotide exchange*	Amino acid exchange	VAF at initial diagnosis (%)	Phenotype	Type of onset	Age at onset (y)	Age at diagnosis (y)	Age at therapy (y)	Age at the last follow-up (y)	Reference No.
1	Male	<i>NLRP3</i>	c.1000 A>G	I334V	34.9	MWS	Early onset	2.0	13.0	14.0	22.6	7, 14
2	Female	<i>NLRP3</i>	c.1064 A>C	K355T	20.2	MWS	Early onset	0.0	6.5	9.6	18.8	7, 14
3	Female	<i>NLRP3</i>	c.1699 G>A	E567K	5.6	MWS	Early onset	4.0	15.5	15.9	25.0	7, 14
4	Male	<i>NLRP3</i>	c.1699 G>A	E567K	5.5	MWS	Early onset	0.6	13.3	15.4	23.5	7, 14
5	Female	<i>NLRP3</i>	c.1699G>A	E567K	5.0	MWS	Early onset	0.0	6.9	7.3	9.8	14
6	Female	<i>NLRP3</i>	c.1721T>G	I572S	6.2	MWS	Early onset	0.2	5.1	5.1	6.7	14
7	Female	<i>NLRP3</i>	c.790C>T	L264F	4.3	CINCA/NOMID	Early onset	0.0	19.7	32.7	33.5	6, 14
8	Male	<i>NLRP3</i>	c.907G>C	D303H	11.9	CINCA/NOMID	Early onset	0.0	0.1	2.7	13.2	6, 14

		3				D	-						
							onset						
9	Male	<i>NLRP</i>	c.919G>A	G307S	10.7	CINCA/NOMI	Early	0.1	4.0	15.0	25.8	6, 14	
		3				D	-						
							onset						
10	Femal	<i>NLRP</i>	c.1699G>	E567K	6.5	CINCA/NOMI	Early	0.0	26.0	32.5	44.7	6, 14	
	e	3	A			D	-						
							onset						
11	Male	<i>NLRP</i>	c.1709A>	Y570C	12.2	CINCA/NOMI	Early	0.0	12.0	22.1	32.5	5, 6, 14	
		3	G			D	-						
							onset						
12	Male	<i>NLRP</i>	c.2269G>	G755R	32.0	CINCA/NOMI	Early	0.0	0.3	0.3	0.9	14	
		3	A			D	-						
							onset						
13	Femal	<i>NLRP</i>	c.1691G>	G564D	4.6	MWS	Late-	39.0	44.4	44.6	47.5	14	
	e	3	A				onset						
14	Male	<i>NLRP</i>	c.1714A>	I572F	29.1	MWS	Late-	55.0	67.0	67.0	72.0	14, 18	
		3	T				onset						
15	Male	<i>NLRP</i>	c.1694A>	Y563C	12.0	CINCA/NOMI	Late-	42.0	64.5	64.7	died at	14, 19	
		3	G			D	onset				67.5		
16	Male	<i>NLRC</i>	c.529A>G	T177A	30.3	CINCA/NOMI	Early	0.0	0.0	19.0	31.0	20	
		4				D	-						
							onset						

CINCA/NOMID, chronic infantile neurological cutaneous articular syndrome/neonatal-onset multisystem inflammatory disease; MWS, Muckle–Wells syndrome; VAF, variant allele frequency; y, year

*NCBI Reference Sequence NM_0012431

Table 2. Detected clonal hematopoiesis-related genes

Patient No.	Type of onset	Sample	Gene	Nucleotide exchange*	Amino acid exchange	VAF (%)	Effect on protein ACMG Guidelines	Population Frequencies gnomAD (Max)
13	Late-onset	Neutrophil					Pathogenic very	< 0.01%
			<i>DNMT3A</i>	c.2330delC	P777fs	0.6	strong	
14	Late-onset	Whole					Pathogenic very	N/A
		blood	<i>DNMT3A</i>	c.1601_1605del	Q534fs	10.6	strong	
14	Late-onset	Neutrophil	<i>DNMT3A</i>	c.1601_1605del	Q534fs	12.0	Pathogenic very	N/A

14	Late-onset	Whole	<i>TET2</i>	c.2502T>A	C834X	21.2	strong	N/A
		blood					Pathogenic very strong	
14	Late-onset	Neutrophil	<i>TET2</i>	c.2502T>A	C834X	14.7	strong	N/A
							Pathogenic very strong	

ACMG, American College of Medical Genetics; VAF, variant allele frequency

*NCBI Reference Sequence

N/A: This variant is not present in the gnomAD database.

DNMT3A NM_022552

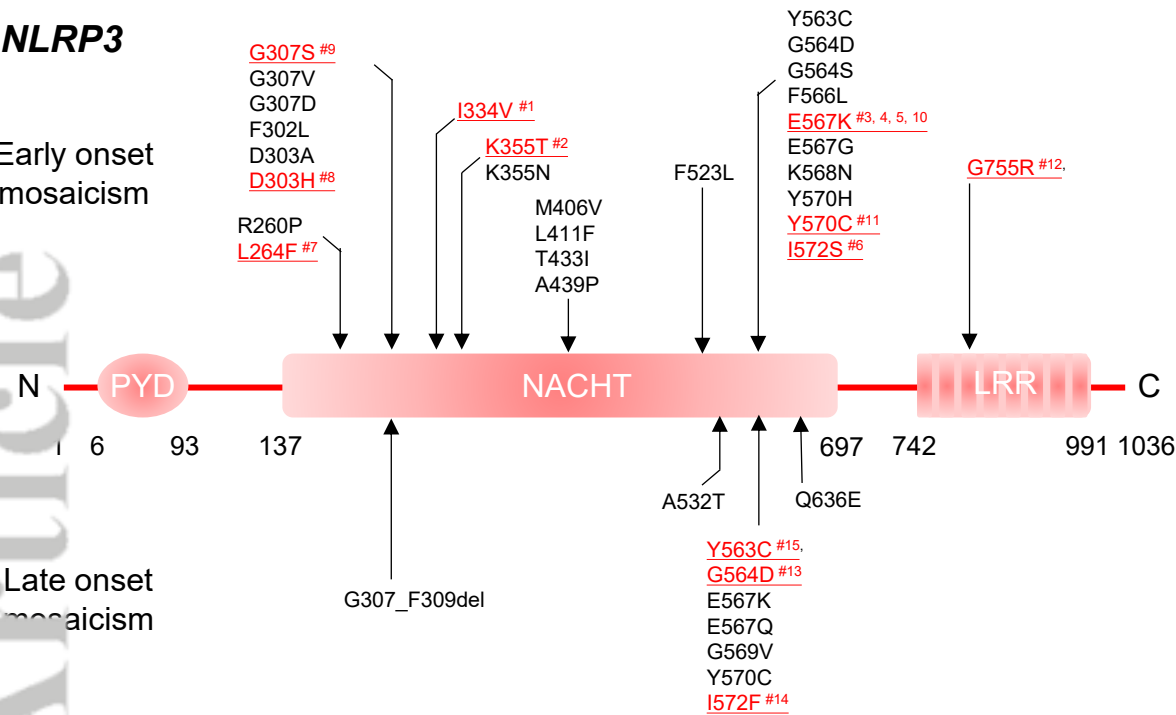
TET2 NM_00112720

Figure 1.

NLRP3

Early onset
mosaicism

Late onset
mosaicism



NLRP4

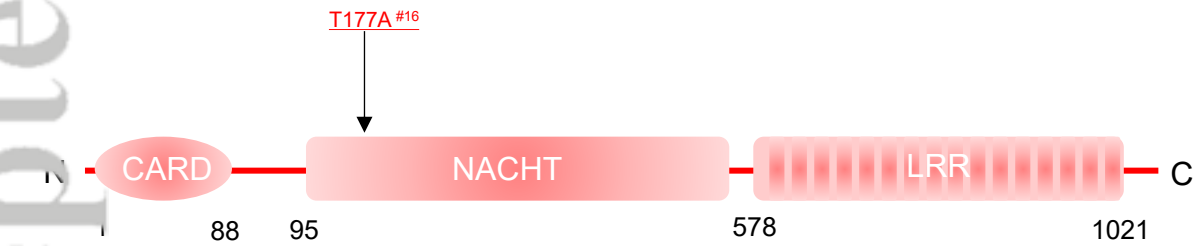


Figure 2. (A)

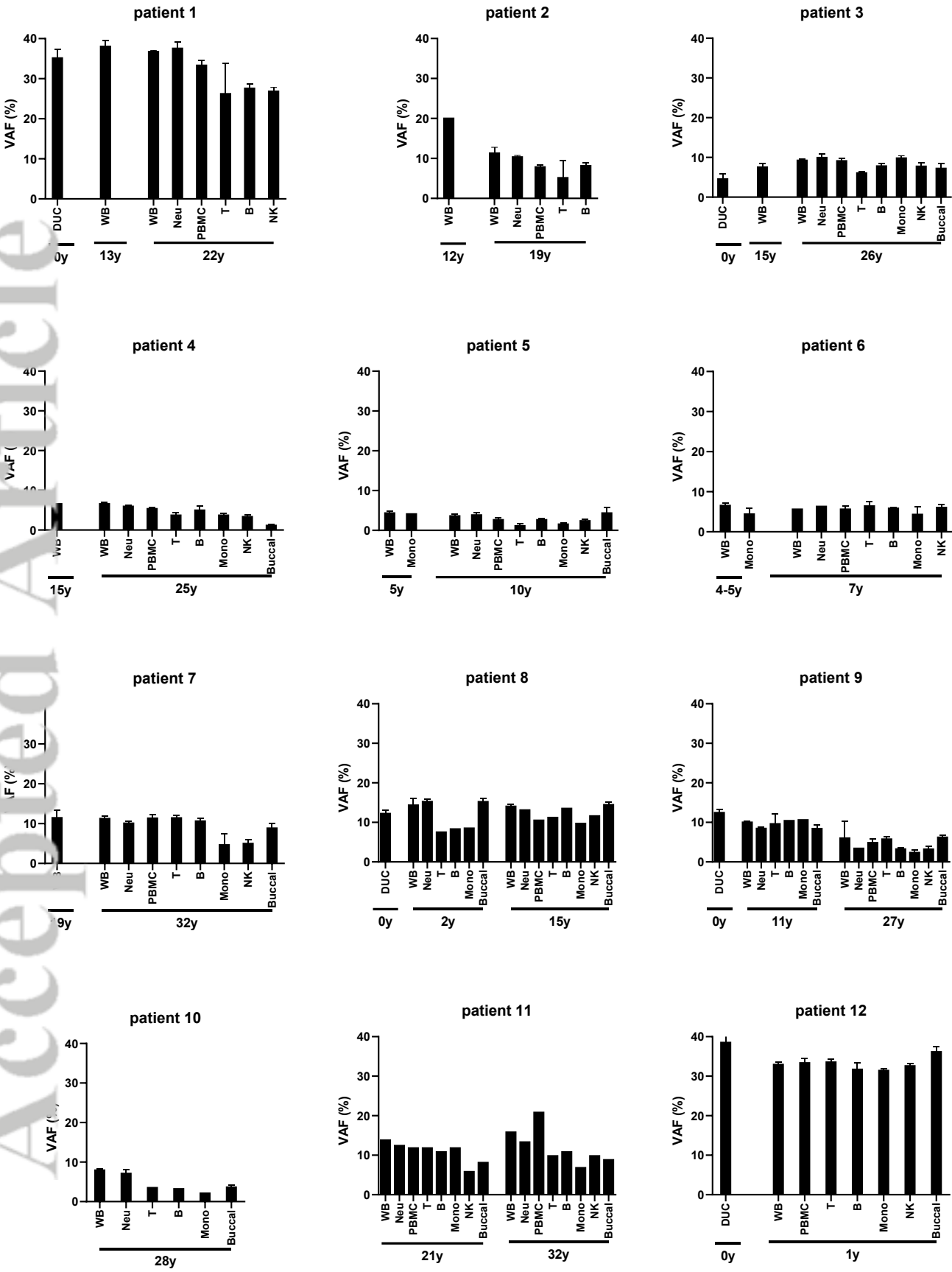


Figure 2. (B)

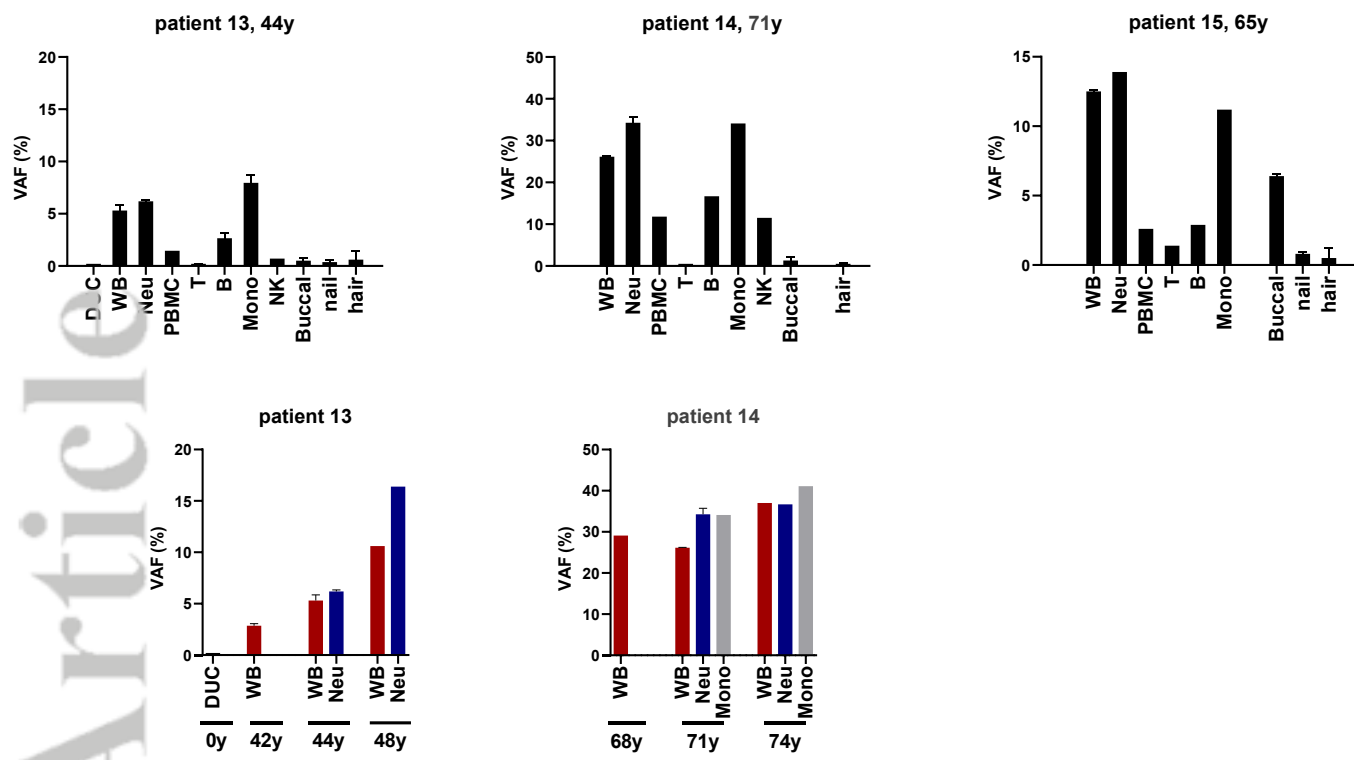
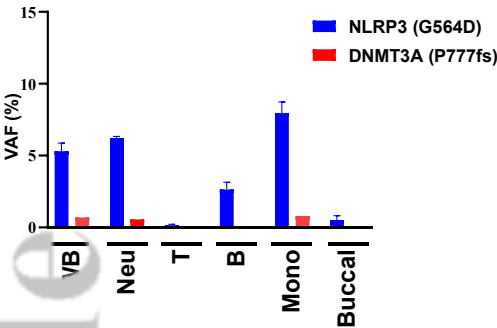


Figure 3. (A)

patient 13, 44y



patient 14

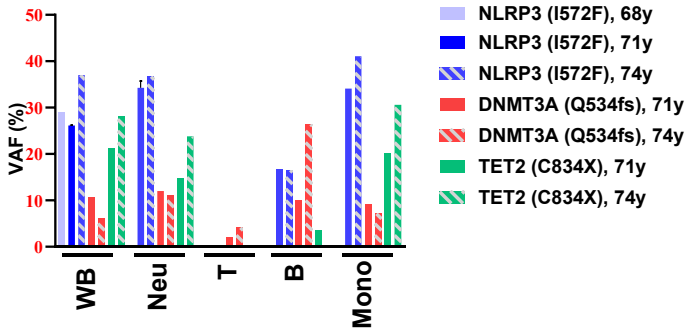
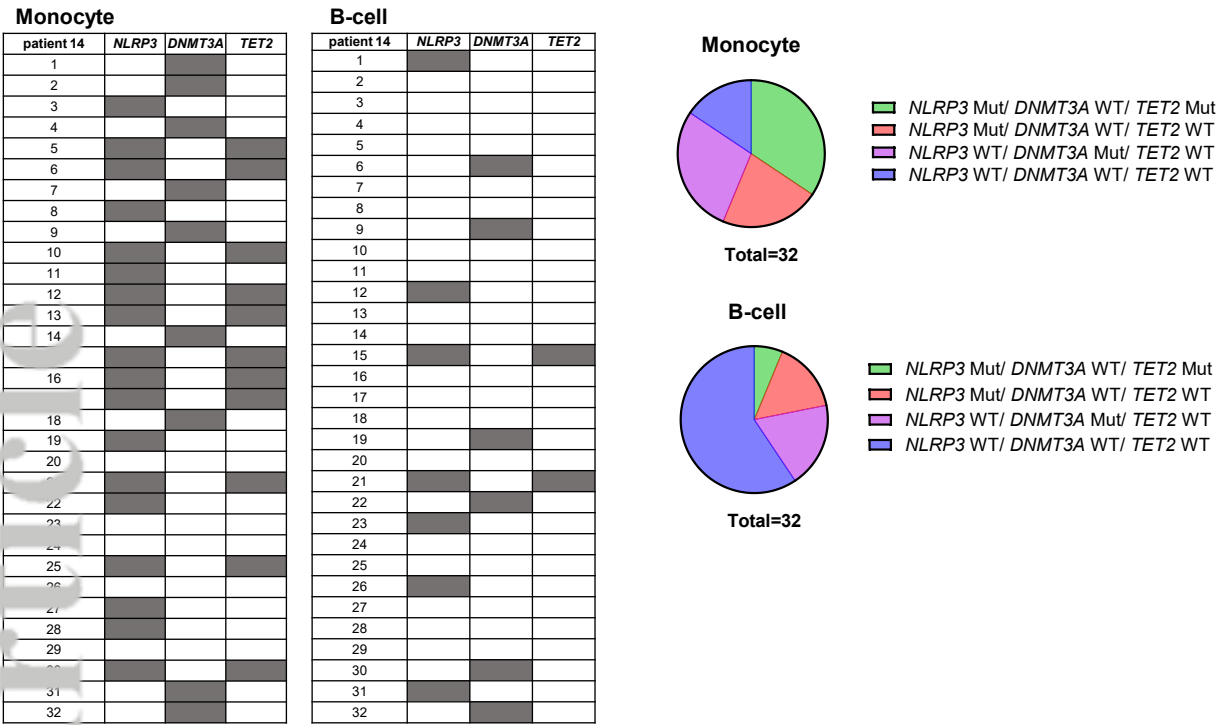


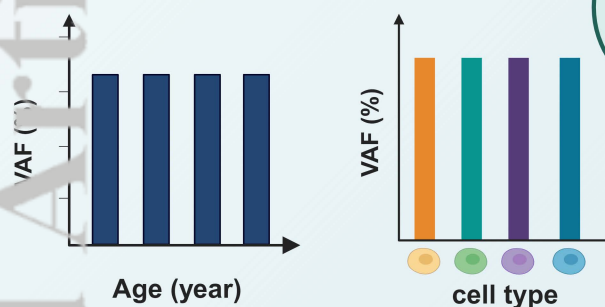
Figure 3. (B)



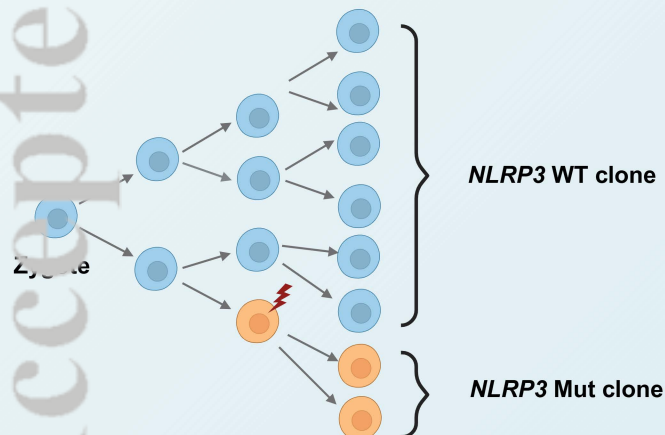
Accepted Article

Longitudinal Analysis of Somatic Mosaicism in Cryopyrin-Associated Periodic Syndrome

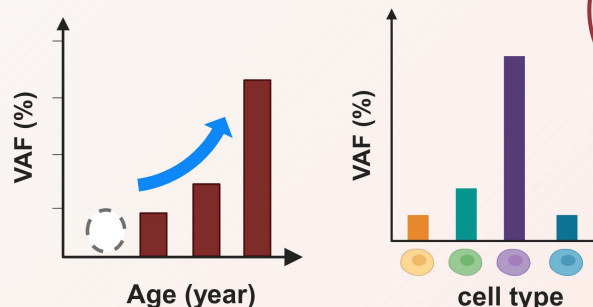
Early-onset Type



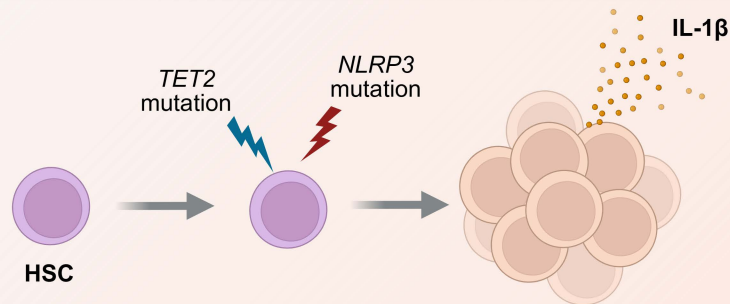
✓ *NLRP3* Mut clones are stable and equally distributed.



Late-onset Type



✓ *NLRP3* Mut clones are enriched in myeloid cells and can increase.



Frequent co-occurrence of *NLRP3* & *TET2* mutations

⚡ *TET2* mutation (driver) — Myeloid expansion
 +
 ⚡ *NLRP3* mutation (passenger) — Inflammation

VAF: variant allele frequency, WT: wild type, Mut: mutant type, HSC: hematopoietic stem cell

Kato K, Honda Y, Kamatani T, et al. Longitudinal analysis of somatic mosaicism and clonal hematopoiesis in cryopyrin-associated periodic syndrome. *Arthritis Rheumatol* 2025.

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Supplementary Table 1. Digital PCR probe information.

assay_ID	Protein change	forward primer (5'→3')	reverse primer (5'→3')	WT probe	mutation probe
ANNK9PP	NLRP3 I334V	ACCGCTCTGCACTGACT	GCCTCGGGAAGCAGCTT	TGAGCAGCCTCATCAGAA	AGCAGCCTCGTCAGAA
ANPR3AM	NLRP3 K355T	GAGGCCTCTCTGCTCATCAC	CCGAGGATGGTCCAGCAA	TGCTGCAGTTTCTCCAG	CTGCAGTGTCTCCAG
ANRWWVJ	NLRP3 E567K	GACGTGACAGTCCTTCTGGAAAA	GCCAAAGAGGAAACGTACAACAAAA	CTATGGCAAATTCGAAAAG	TGGCAAATTCAAAAAG
ANU7JZE	NLRP3 I572S	AGTCCTTCTGGAAAACATATGGCAAA	GGTTTACCAGGCCAAAGAGGAA	ACGTACAACAAAAATCAAAT	ACGTACAACAAACTCAAAT
ANH6RYX	NLRP3 L264F	CTACCAAGACAGGTTTGACTATCTGTT	GGGCAGCAGCTCATGATCA	TGTCACAAGGCTCACC	TGTCACAAAGCTCACC
ANMGE4T	NLRP3 D303H	CCTCCAGAATCCTTCTCCTCATG	TGCTCGTCAAAGGCACCTT	ACGGCTTCGATGAGCT	ACGGCTTCCATGAGCT
ANKCKJV	NLRP3 G307S	CCTCATGGACGGCTTCGAT	TGCAGAGCGGTCTCTATGTG	AGCTGCAAGGTGCCTT	AGCTGCAAAGTGCCTT
ANMGDGX	NLRP3 Y570C	AGTCCTTCTGGAAAACATATGGCAAA	CCAGGCCAAAGAGGAAACGTA	AACAAAAATCAAATACCCC	ACAAAAATCAAACACCCC
ANRWW2C	NLRP3 G755R	GGACCTCAGTGACAATTCTCTGG	GCTGGAGCGTTTCACACAAC	CTCTCATCCCTGGGTCC	CTCTCATCCTTGGGTCC
ANKCKHH	NLRP3 G564D	CGAGACGTGACAGTCCTTCTG	GCCAAAGAGGAAACGTACAACAAAA	AAACTATGGCAAATTC	AAAACTATGACAAATTC
ANU7K74	NLRP3 I572F	AGTCCTTCTGGAAAACATATGGCAAA	CCAGGCCAAAGAGGAAACGTA	CAACAAAAATCAAATACC	AACAAAAAACAAATACC
ANT2RFG	NLRP3 Y563C	CGAGACGTGACAGTCCTTCTG	GCCAAAGAGGAAACGTACAACAAAA	AATTTGCCATAGTTTTC	TGCCACAGTTTTC
AN7D9PZ	NLRC4 T177A	AGAGCCCCTGCATCATTGAAG	CCCAGAGCATGGCAATTCTG	CAGCAGAGTGGACTTG	AGCAGAGCGGACTTG
ANPR3GE	NLRP3 R260W	CACTCTACCAAGACAGGTTTGACT	CAGGCTCCTCTGTGTCACAAG	CTCACCTCCCGACAGTG	CACCTCCCAACAGTG

Supplementary Table 2. Deep sequence primer information.

primer name	gene	protein change	forward primer (5'→3')	reverse primer (5'→3')	amplicon length (bp)
primer1	<i>NLRP3</i>	G564D, E567K, Y570C, I572S	ACGTTCCAGGGAGTCGTTTG	TGGTTTACCAGGCCAAAGAGG	124
primer2	<i>NLRP3</i>	D303H, G307S	CAAACCCACCCATCCACAAG	TCTGCCAGTCAGTGCAGAG	123
primer3	<i>NLRP3</i>	I334V	GAGCACATAGGACCGCTCTG	CCACAGGTCTCGTGGTGATG	124
primer4	<i>NLRP3</i>	G755R	TTCAGTTCTGAGCACCAGCC	CACGGGGACTCACCACAATC	139
primer5	<i>NLRC4</i>	T177A	GACCCTGAATGGCCTCCTG	ACTTGGTCAGAGCCTTGAC	134

Supplementary Table 3. Results for NLRP3 mutations in different tissue specimens over time.

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
1	DUC	0.0	39.2	deep seq	
1	DUC	0.0	36.7/34.0	dPCR	
1	Whole blood	13.1	37.4	deep seq	
1	Whole blood	13.1	39.1/37.4	dPCR	
1	Whole blood	22.3	37.0/36.9	dPCR	
1	Neutrophils	22.3	36.7/38.8	dPCR	
1	PBMC	22.3	34.3/32.7	dPCR	
1	B-cells	22.3	28.4/27.2	dPCR	
1	PBMC	24.2	37.2	dPCR	
1	T-cells	24.2	36.7	dPCR	
1	NK-cells	24.2	36.1	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
2	Whole blood	12.0	20.2	target seq (depth100)	
2	Whole blood	19.2	12.4/10.6	dPCR	
2	Neutrophils	19.2	10.4/10.7	dPCR	
2	PBMC	19.2	8.3/7.8	dPCR	
2	T-cells	19.2	8.3/2.4	dPCR	
2	B-cells	19.2	8.7/8.0	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
3	DUC	0.0	3.3	deep seq	
3	DUC	0.0	4.9/4.0	dPCR	
3	Whole blood	14.8	7.4	deep seq	
3	Whole blood	14.8	8.2/7.3	dPCR	
3	Whole blood	25.9	9.4/9.5	dPCR	
3	Neutrophils	25.9	10.7/9.6	dPCR	
3	PBMC	25.9	9.0/9.6	dPCR	
3	T-cells	25.9	6.1/6.4	dPCR	
3	B-cells	25.9	7.7/8.3	dPCR	
3	Monocytes	25.9	9.7/10.3	dPCR	
3	NK-cells	25.9	7.5/8.4	dPCR	
3	Buccal	25.9	8.1/6.7	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
4	Whole blood	15.4	9.3	deep seq	
4	Whole blood	15.4	6.8/6.8	dPCR	
4	Whole blood	25.0	6.9/6.6	dPCR	
4	Neutrophils	25.0	6.2/6.1	dPCR	
4	PBMC	25.0	5.6/5.5	dPCR	
4	T-cells	25.0	3.5/4.3	dPCR	
4	B-cells	25.0	5.8/4.6	dPCR	
4	Monocytes	25.0	3.7/4.1	dPCR	
4	NK-cells	25.0	3.7/3.4	dPCR	
4	Buccal	25.0	1.4/1.3	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
5	Whole blood	4.8	4.7/4.4	dPCR	cell sorting was conducted by MACS
5	Monocytes	4.8	4.3	dPCR	
5	Whole blood	9.8	3.6/4.0	dPCR	
5	Neutrophils	9.8	3.7/4.4	dPCR	
5	PBMC	9.8	2.6/3.1	dPCR	
5	T-cells	9.8	1.6/1.0	dPCR	
5	B-cells	9.8	2.7/3.0	dPCR	
5	Monocytes	9.8	1.8/1.7	dPCR	
5	NK-cells	9.8	2.8/2.4	dPCR	
5	Buccal	9.8	5.4/3.7	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
6	Whole blood	3.9	7.0/6.5	dPCR	cell sorting was conducted by MACS
6	Monocytes	5.1	3.7/5.5	dPCR	
6	PBMC	6.3	7	dPCR	
6	T-cells	6.3	7.5	dPCR	
6	B-cells	6.3	5.4	dPCR	
6	Whole blood	7.4	6.2	deep seq	
6	Whole blood	7.4	5.8/5.7	dPCR	
6	Neutrophils	7.4	6.5/5.6	dPCR	
6	PBMC	7.4	6.2/5.4	dPCR	
6	T-cells	7.4	7.2/6.0	dPCR	
6	B-cells	7.4	6.1/6.0	dPCR	
6	Monocytes	7.4	3.3/5.7	dPCR	
6	NK-cells	7.4	6.7/5.8	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
7	Whole blood	18.8	10.4/12.9	dPCR	
7	Whole blood	18.8	4.3	TA cloning	reference 6
7	Whole blood	32.3	11.2/11.7	dPCR	
7	Neutrophils	32.3	10.0/10.5	dPCR	
7	PBMC	32.3	11.0/12.1	dPCR	
7	T-cells	32.3	12.0/11.2	dPCR	
7	B-cells	32.3	11.2/10.4	dPCR	
7	Monocytes	32.3	6.7/3.0	dPCR	
7	NK-cells	32.3	5.8/4.6	dPCR	
7	Buccal	32.3	8.4/9.7	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
8	DUC	0	11.9/12.9	dPCR	
8	DUC	0	11.9	deep seq	
8	Whole blood	1.8	11.9	TA cloning	reference 6
8	Whole blood	1.8	15.7/13.4	dPCR	
8	Whole blood	1.8	16.9	deep seq	
8	Neutrophils	1.8	14.4	TA cloning	reference 6
8	Neutrophils	1.8	15.1/15.8	dPCR	
8	T-cells	1.8	7.7	TA cloning	reference 6
8	B-cells	1.8	8.5	TA cloning	reference 6
8	Monocytes	1.8	8.7	TA cloning	reference 6
8	Buccal	1.8	14.9/15.9	dPCR	
8	Whole blood	14.5	14.5/14.0	dPCR	
8	PBMC	14.5	8.6/8.3	dPCR	
8	T-cells	14.5	12.5/11.1	dPCR	
8	B-cells	14.5	14.1/14.7	dPCR	
8	Buccal	14.5	14.3/15.0	dPCR	
8	Neutrophils	15.8	13.3	dPCR	
8	PBMC	15.8	10.7	dPCR	
8	T-cells	15.8	11.4	dPCR	
8	B-cells	15.8	13.7	dPCR	
8	Monocytes	15.8	9.9	dPCR	
8	NK-cells	15.8	11.8	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
9	DUC	0	13.1/12.2	dPCR	
9	DUC	0	15.1	deep seq	
9	Whole blood	10.8	10.1/10.3	dPCR	
9	Whole blood	10.8	10.7	TA cloning	reference 6
9	Neutrophils	10.8	8.5/8.8	dPCR	
9	Neutrophils	10.8	9.1	TA cloning	reference 6
9	T-cells	10.8	11.5/8.1	dPCR	
9	T-cells	10.8	6.9	TA cloning	reference 6
9	B-cells	10.8	10.6	TA cloning	reference 6
9	Monocytes	10.8	10.8	TA cloning	reference 6
9	Buccal	10.8	9.1/8.1	dPCR	
9	Buccal	10.8	9	TA cloning	reference 6
9	Whole blood	26.7	3.5	deep seq	
9	Whole blood	26.7	3.3/3.7	dPCR	
9	Neutrophils	26.7	3.6/3.6	dPCR	
9	PBMC	26.7	5.6/4.5	dPCR	
9	T-cells	26.7	6.3/5.6	dPCR	
9	B-cells	26.7	3.4/3.5	dPCR	
9	Monocytes	26.7	2.9/2.2	dPCR	
9	NK-cells	26.7	3.8/3.0	dPCR	
9	Buccal	26.7	6.1/6.7	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
10	Whole blood	27.7	6.5	TA cloning	reference 6
10	Whole blood	27.7	8.1/8.2	dPCR	
10	Neutrophils	27.7	3.5	TA cloning	reference 6
10	Neutrophils	27.7	6.7/7.9	dPCR	
10	T-cells	27.7	3.7	TA cloning	reference 6
10	B-cells	27.7	3.4	TA cloning	reference 6
10	Monocytes	27.7	2.3	TA cloning	reference 6
10	Buccal	27.7	2.2	TA cloning	reference 6
10	Buccal	27.7	3.6/4.1	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
11	Whole blood	21.4	14.8	deep seq	
11	Whole blood	21.4	11.7/11.1	dPCR	
11	Whole blood	21.4	12.2	TA cloning	reference 6
11	Neutrophils	21.4	12.6	TA cloning	reference 6
11	PBMC	21.4	13.4	deep seq	
11	T-cells	21.4	12.2	deep seq	
11	T-cells	21.4	8	TA cloning	reference 6
11	B-cells	21.4	12	deep seq	
11	B-cells	21.4	9.5	TA cloning	reference 6
11	Monocytes	21.4	13.2	deep seq	
11	Monocytes	21.4	9.8	TA cloning	reference 6
11	NK-cells	21.4	6.3	deep seq	
11	Buccal	21.4	8.3	TA cloning	reference 6
11	Whole blood	31.8	17.6	deep seq	
11	Whole blood	31.8	12.1	dPCR	
11	PBMC	31.8	22.2	deep seq	
11	T-cells	31.8	10.6	deep seq	
11	T-cells	31.8	14.3	dPCR	
11	B-cells	31.8	11.6	deep seq	
11	B-cells	31.8	17.3	dPCR	
11	Monocytes	31.8	7.5	deep seq	
11	Monocytes	31.8	12.2	dPCR	
11	NK-cells	31.8	11.4	deep seq	
11	NK-cells	31.8	12.6	dPCR	
11	Buccal	31.8	9.2	deep seq	
11	Neutrophils	32.7	20	deep seq	
11	Neutrophils	32.7	13.5	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
12	DUC	0.0	38.7	deep seq	
12	DUC	0.0	40.0/37.4	dPCR	
12	DBS	0.0	34.8/34.6	dPCR	
12	Whole blood	1.0	30.9	deep seq	
12	Whole blood	1.0	33.4/32.9	dPCR	
12	PBMC	1.0	32.9/34.2	dPCR	
12	T-cells	1.0	33.4/34.1	dPCR	
12	B-cells	1.0	33.0/30.8	dPCR	
12	Monocytes	1.0	31.8/31.4	dPCR	
12	NK-cells	1.0	33.1/32.5	dPCR	
12	Buccal	1.0	35.5/37.2	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
13	DUC	0.0	0.1	deep seq	
13	DUC	0.0	0.0/0.1	dPCR	
13	Whole blood	41.9	3.0/2.7	dPCR	
13	Whole blood	43.9	7.2	deep seq	
13	Whole blood	43.9	5.7/4.9	dPCR	
13	Neutrophils	43.9	7.6	deep seq	
13	Neutrophils	43.9	6.1/6.3	dPCR	
13	PBMC	43.9	1.5/1.6	dPCR	
13	T-cells	43.9	0.6/1.1	dPCR	cell sorting was conducted by MACS
13	B-cells	43.9	0.5/1.3	dPCR	cell sorting was conducted by MACS
13	Monocytes	43.9	5.8/5.5	dPCR	cell sorting was conducted by MACS
13	Buccal	43.9	0.1	deep seq	
13	Buccal	43.9	0.3/0.7	dPCR	
13	Hair	43.9	0.1	deep seq	
13	Hiar	43.9	0.0/0.0	dPCR	
13	Nail	43.9	0.1	deep seq	
13	Nail	43.9	0.5/0.3	dPCR	
13	PBMC	46.2	5.2	deep seq	
13	T-cells	46.2	0.1	deep seq	
13	T-cells	46.2	0.2/0.1	dPCR	
13	B-cells	46.2	1.8	deep seq	
13	B-cells	46.2	3.0/2.3	dPCR	
13	Monocytes	46.2	11.2	deep seq	
13	Monocytes	46.2	7.4/8.5	dPCR	
13	NK-cells	46.2	0.7	deep seq	
13	NK-cells	46.2	0.7/0.7	dPCR	
13	Whole blood	47.8	12.2/13.7	dPCR	
13	Neutrophils	47.8	13.1/12.1	dPCR	
13	Whole blood	48.3	10.6	WES	
13	Neutrophils	48.3	16.4	WES	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
14	Whole blood	71.0	26.1/26.2	dPCR	
14	Neutrophils	71.0	35.3/33.2	dPCR	
14	PBMC	71.0	11.8	dPCR	
14	T-cells	71.0	0.2	dPCR	
14	B-cells	71.0	16.7	dPCR	
14	Monocytes	71.0	34.1	dPCR	
14	NK-cells	71.0	11.5	dPCR	
14	Buccal	71.0	0.6/2.0	dPCR	
14	Hair	71.0	0.5/0.0	dPCR	
14	Whole blood	74.0	37.0	deep seq	
14	Neutrophils	74.0	36.7	deep seq	
14	PBMC	74.0	19.7	deep seq	
14	T-cells	74.0	0.2	deep seq	
14	B-cells	74.0	16.5	deep seq	
14	Monocytes	74.0	41.1	deep seq	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
15	Whole blood	64.6	12.6/12.4	dPCR	
15	Neutrophils	64.6	13.9/13.9	dPCR	
15	PBMC	64.6	2.6	dPCR	
15	T-cells	64.6	1.4	dPCR	cell sorting was conducted by MACS
15	B-cells	64.6	2.9	dPCR	cell sorting was conducted by MACS
15	Monocytes	64.6	11.2	dPCR	cell sorting was conducted by MACS
15	Buccal	64.6	6.5/6.3	dPCR	
15	Hair	64.6	0.0/1.0	dPCR	
15	Nail	64.6	0.7/0.9	dPCR	
15	PBMC	66.8	10.3	dPCR	
15	T-cells	66.8	0.4/0.3	dPCR	
15	B-cells	66.8	0.4/0.5	dPCR	
15	Monocytes	66.8	5.0/5.2	dPCR	
15	NK-cells	66.8	5.6/5.2	dPCR	

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
16	Whole blood	13.2	23.8/24.9	dPCR	
16	Whole blood	29.7	28.9/28.1	dPCR	
16	Whole blood	29.7	34.7	deep seq	
16	Neutrophils	29.7	28.0/27.2	dPCR	
16	PBMC	29.7	29.8	dPCR	
16	T-cells	29.7	29.2	dPCR	
16	B-cells	29.7	29.4	dPCR	
16	Monocytes	29.7	29.6	dPCR	
16	NK-cells	29.7	32.6	dPCR	
16	Buccal	29.7	25.2/23.8	dPCR	

ID	Sample	Age at sampling (y)	VAF (%)	modality	comment
Healthy_1	Whole blood		0.00/0.08	dPCR	using G564D probe
Healthy_1	Hair		0.1/0.38	dPCR	using G564D probe
Healthy_2	DUC		0.0/0.0	dPCR	using G564D probe
Healthy_3	DUC		0	dPCR	using G564D probe

Patients No.	Sample	Age at sampling (y)	VAF (%)	modality	comment
Patient with NLRP3 R260W germline mutation	DUC	0.0	48.1/46.4	dPCR	

VAF, variant allele frequency; DUC, dried umbilical cord; PBMC, peripheral blood mononuclear cells; deep seq, PCR based deep amplicon sequence; dPCR, digital PCR; target seq, target sequence; WES, whole exome sequence; MACS, Magnetic activated cell sorting; y, year

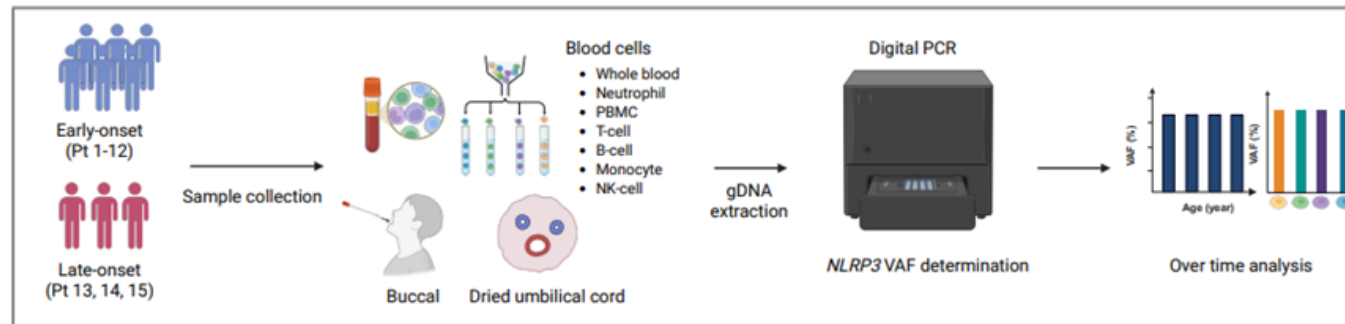
Supplementary Table 4. Single cell sequence primer information.

primer name	gene	protein change	forward primer (5'→3')	reverse primer (5'→3')	amplicon length (bp)
primer1	<i>DNMT3A</i>	P777fs	CTTTAAGGCTCGACCCCAGC	CCCAGGAGCTTTCACCAACC	188
primer2	<i>DNMT3A</i>	Q534fs	GTCCTGGTGGTTTCTGACCC	CTAAGGAGACCACTGGAGGC	184
primer3	<i>TET2</i>	C834X	ATGTCCAAATGGGACTGGAGG	GCATGTGATGCAAGTTTGGG	195
primer4	<i>NLRP3</i>	G564D, I572F	ACGTTCCAGGGAGTCGTTTG	TGGTTTACCAGGCCAAAGAGG	124

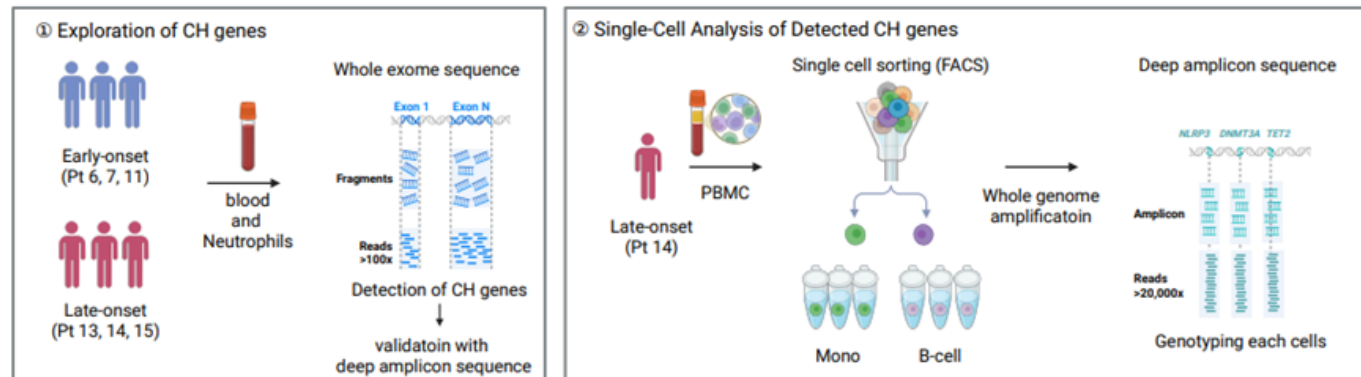
Supplementary Figure 1. Experimental Workflow

Experimental workflow

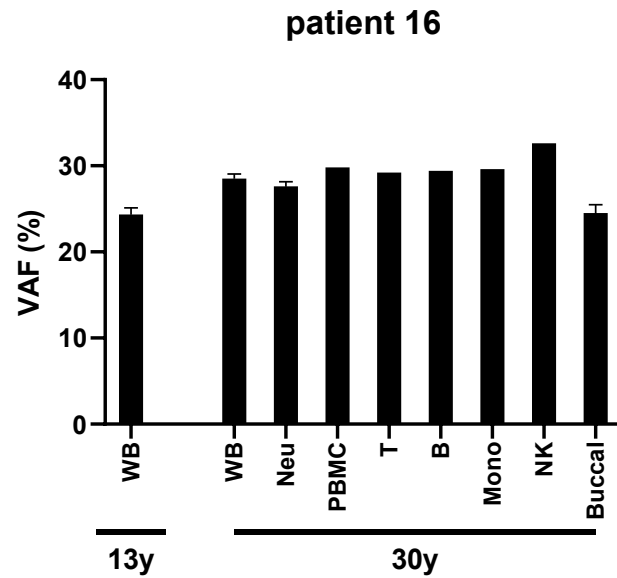
Temporal Changes in *NLRP3* VAF



Clonal Hematopoiesis in CAPS Mosaicism



Supplementary Figure 2. Result of patient 16



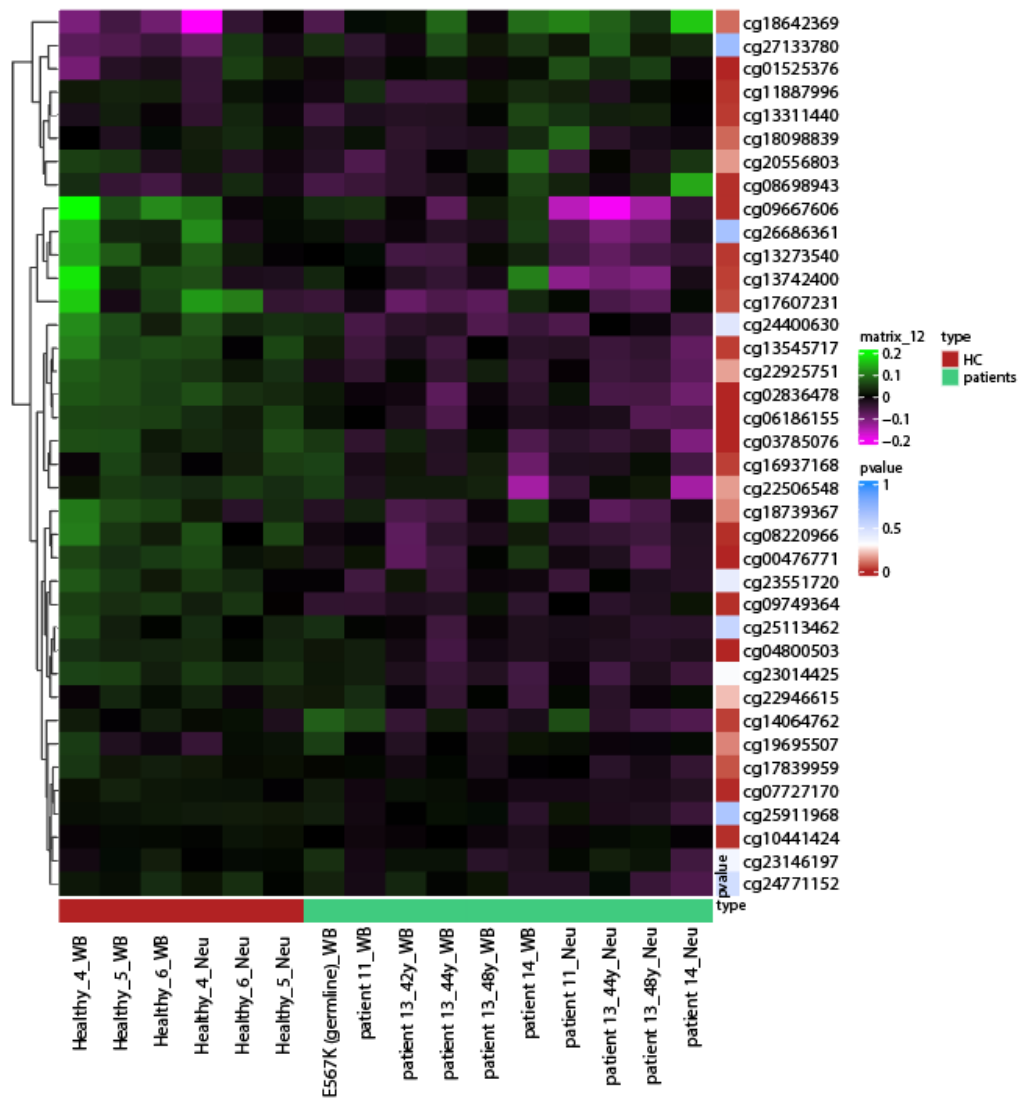
This figure shows the mean data obtained from two measurements using dPCR without any specific annotations (see Supplementary Table 3 for detailed information). The year below the graph indicates the age of the patients when each sample was collected.

VAF, variant allele frequency; DUC, dried umbilical cord; Neu, neutrophils; PBMC, peripheral blood mononuclear cells; T, T cells; B, B cells; Mono, monocytes; NK, NK cells; dPCR, digital PCR; y, year; WB, whole blood

Supplementary Figure 3. Heatmap

This heatmap depicts scaled beta values for 38 CpG sites previously linked to DNMT3A- and TET2-associated CHIP (PMID: 36097025), comparing Patients 13, 14, E567K germ line patient and healthy controls. Rows represent CpG sites; columns represent samples. Annotations include NLRP3 mutation status and adjusted p-values for each differentially methylated probe.

HC, Healthy Control; WB, Whole blood; Neu, neutrophils



Supplementary Materials

FULL CASE DESCRIPTIONS

Patient 13

The patient presented in 2014 at the age of 38 years with urticarial rashes on the legs and arms. In subsequent years, the rash worsened, and the patient experienced recurrent fever, arthralgia, muscle pain, headache, and conjunctivitis. In 2016, she was admitted to our hospital for the first time. Despite thorough investigation, a precise diagnosis has not yet been established. Based on the clinical presentation of urticarial vasculitis, she was prescribed colchicine 0.5 mg/day. However, her symptoms did not improve even after gradually increasing the dosage to 2.0 mg/day. Consequently, in 2017, we initiated treatment with prednisolone (PSL) 25 mg/day, which led to symptom improvement. However, she reported experiencing significant fatigue and psychological symptoms, including thoughts of self-harm, possibly because of the side effects of PSL. When the PSL dose was reduced, the patient's symptoms recurred. Sanger sequencing did not reveal any *NLRP3* mutations. To minimize the use of PSL, we explored the use of tacrolimus, methotrexate, and tocilizumab, but these medications had to be discontinued because of side effects such as nausea, fatigue, and rash. In 2018, the patient was diagnosed with an auditory impairment in the right ear. Despite the ongoing diagnostic challenges, we attempted treatment with azathioprine, salazosulfapyridine, etanercept, and iguratimod, all of which proved ineffective or had to be discontinued because of side effects. However, sarilumab and baricitinib became ineffective in 2019. We subsequently conducted genetic testing using next-generation sequencing, which revealed the presence of G564D somatic mosaicism at a 4.1% frequency. Canakinumab

300 mg/4 weeks was initiated, and PSL was discontinued without symptom recurrence. However, in 2021, the patient began experiencing fever and general fatigue during the canakinumab treatment period, leading us to increase the dose to 450 mg/4 weeks. Later that year, she presented with visual and auditory hallucinations along with abnormal behavior and disorientation, which required admission to the emergency room. Brain-enhanced MRI and cerebrospinal fluid and blood cultures did not reveal any abnormalities. Owing to elevated IL-6 levels in the cerebrospinal fluid (61.9 pg/mL; serum 10.5 pg/mL), autoimmune cerebritis was suspected, and treatment with pulsed methylprednisolone, intravenous immunoglobulin, and sodium valproate was initiated, resulting in improvement. In 2023, the patient tested positive for blood in her stool and experienced abdominal pain. Colonoscopy revealed mild ulceration in the terminal ileum and colon, without granulomas or evidence of CMV or amoeba infection. The symptoms were self-limiting and subsequent colonoscopy showed no worsening. At the most recent follow-up, most of her symptoms were under control, except for the headaches, and she continued receiving canakinumab (450 mg/4 weeks).

Patient 14

A 68-year-old man was referred for evaluation of long-standing systemic inflammation. Elevated CRP and leukocytosis had been present since his 40s, with subsequent development of sensorineural hearing loss, chronic urticaria, and arthritis in his late 50s. At age 67, renal biopsy revealed AA amyloidosis. Chronic aseptic meningitis was also suspected based on cerebrospinal fluid findings. A somatic I572F mutation in NLRP3 was detected (VAF 29.1%), confirming a diagnosis of CAPS mosaicism. Hematological evaluation prior to treatment showed no evidence of macrocytic anemia

or myelodysplastic features: hemoglobin 9.9 g/dL, mean corpuscular volume (MCV) 82.6 fL, mean corpuscular hemoglobin (MCH) 25.6 pg, and mean corpuscular hemoglobin concentration (MCHC) 31.0%. No cytopenias or morphological abnormalities were noted on peripheral smear.

Treatment with subcutaneous canakinumab, titrated to 600 mg every 8 weeks, led to a rapid and sustained improvement in inflammatory markers and renal function. The patient remains in clinical remission on maintenance therapy.

Patient 15

A 64-year-old man was admitted with abdominal pain, bloody stool, and pancytopenia. He had a long history of recurrent skin rash, periodic fever, and arthritis since his 40s, initially treated as unclassified arthritis. Multiple immunosuppressants, including methotrexate and tofacitinib, were used with limited benefit. Following persistent systemic inflammation and atypical skin lesions, genetic testing revealed a somatic mosaic NLRP3 mutation (p.Y563C), confirming a diagnosis of CAPS. At age 64.7, canakinumab therapy (300 mg every 8 weeks) was initiated, improving fever and arthralgia, though the rash persisted. AA amyloidosis was later diagnosed, and cardiac involvement led to complete atrioventricular block at age 65.7. The canakinumab dose was escalated owing to progressive inflammation. Despite improved inflammatory markers, he developed recurrent severe infections and died of septic shock at age 67.6. No autopsy was performed.

Supplementary Methods: Methylation Analysis

Differentially Methylated Probes (DMPs):

Raw data from Illumina EPIC arrays were imported and preprocessed to obtain methylation beta values using the minfi (v1.50.0) package in R v4.4.1. In cases where multiple probes targeted the same CpG site, the probe with the lowest detection p-value was retained. Detection p-values were calculated to assess probe reliability, and probes with p-values above 0.05 were excluded from further analyses as part of initial preprocessing. Quality control (QC) was conducted using the champ.filter function, with the following probes removed: those with a detection p-value above 0.01 in more than 5% of samples, non-CpG probes overlapping SNPs, multi-hit probes, and probes on sex chromosomes. Normalization was then performed using the champ.norm function from ChAMP (v2.34.0) package. Batch effects associated with chip ID were assessed using the champ.SVD function, and adjusted using the champ.runCombat function. DMPs between Patient 13 and a healthy control were identified using champ.DMP, with thresholds of adjusted p-value < 0.05 and $|\Delta\beta| > 0.20$.

Heatmap Visualization:

Heatmaps were generated using the ComplexHeatmap (v2.20.0) package in R to visualize scaled beta values for selected CpG sites previously associated with DNMT3A and TET2-related CHIP (cg04800503, cg23014425, cg25113462, cg07727170, cg23551720, cg03785076, cg16937168, cg06186155, cg24400630, cg23146197, cg09749364, cg02836478, cg22925751, cg17839959, cg01525376, cg22506548, cg25911968, cg24771152, cg22946615, cg13545717, cg13742400, cg17607231, cg19695507, cg26686361, cg12976883, cg13311440, cg10441424, cg11887996, cg18098839, cg14064762, cg00476771, cg18642369, cg05165553, cg27133780, cg20556803, cg08698943, cg08220966, cg09667606, cg13273540, cg18739367) . (PMID: 36097025).

1 Annotations for the heatmaps included the presence or absence of mutations in the
2 NLRP3 gene and p-values associated with differentially methylated probes (DMPs). The
3 corresponding figure appears as Supplementary Figure 3.

4

5 追記

6 DMPs に関する Supplementary Data File (Supple_DMP.xlsx) は下記リンクよりダウ

7 ンロードが可能

8 <https://acrjournals-onlinelibrary-wiley-com.kyoto-u.idm.oclc.org/doi/10.1002/art.43329>