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¹A fast diagnostic test for familial Mediterranean fever based on a kinase inhibitor

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Short title: A fast diagnostic test for FMF

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1

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ABSTRACT

Background and Objective Familial Mediterranean fever (FMF) is the most frequent hereditary autoinflammatory disease. Its diagnosis relies on a set of clinical criteria and a genetic confirmation upon identification of bi-allelic pathogenic *MEFV* variants. *MEFV* encodes pyrin, an inflammasome sensor. Using a kinase inhibitor, UCN-01, we recently identified that dephosphorylation of FMF-associated pyrin mutants leads to inflammasome activation. The aim of this study was to assess whether quantifying UCN-01-mediated inflammasome activation could discriminate FMF patients from healthy donors (HD) and from patients with other inflammatory disorders (OID).

Methods Real time pyroptosis and IL-1 β secretion were monitored in response to UCN-01 in monocytes from FMF patients (n=67), HD (n=71), and OID patients (n=40). Sensitivity and specificity of the resulting diagnostic tests were determined by ROC curve analyses.

Results Inflammasome monitoring in response to UCN-01 discriminates FMF patients from other individuals. Pyroptosis assessment leads to a fast FMF diagnosis while combining pyroptosis and IL-1 β dosage renders UCN-01-based assays highly sensitive and specific. UCN-01-triggered monocytes responses were influenced by *MEFV* gene dosage and *MEFV* mutations in a similar way as clinical phenotypes are.

Conclusions UCN-01-based inflammasome assays could be used to rapidly diagnose FMF, with high sensitivity and specificity.

Keywords: Familial Mediterranean fever, pyrin, diagnosis, pyroptosis, interleukin-1

INTRODUCTION

Familial Mediterranean fever (FMF) is the most common inherited autoinflammatory syndrome.¹ FMF is present worldwide as a rare disease. A high prevalence (up to 1/500) is observed in the Mediterranean basin.² FMF is typically characterized by recurrent attacks of fever and serositis, associated with systemic inflammation. The major challenge of FMF is to establish a fast and definitive diagnosis, to avoid unnecessary and costly investigations, prolonged diagnostic wandering, or useless life-long treatment.³

FMF diagnosis results from a combination of clinical criteria⁴, and is confirmed when bi-allelic mutations in *MEFV*, the gene encoding pyrin, are observed.^{5,6} Yet, genetic tests may be inconclusive, about one-third of patients bearing only one mutated *MEFV* allele.⁷ Genetic analyses can also reveal variants of uncertain significance.⁸ Therefore, it is of great importance to develop a rapid diagnostic test.

Recently, using a kinase inhibitor, UCN-01, we demonstrated that pyrin dephosphorylation triggers full inflammasome activation in FMF patients' monocytes while it does not in healthy donor (HD) monocytes.⁹ We hypothesized that these differential responses could be the basis of a diagnostic test to quickly distinguish FMF patients from HD or patients suffering from other inflammatory disorders (OID).

METHODS

Methods are detailed in the supplementary material.

The study was approved by the French *Comité de Protection des Personnes* (#2018/95). Every participant (supplementary table 1) gave informed consent. Statistical analysis was performed with R software and P-values <0.05 were considered significant.

RESULTS

Cell death kinetics discriminates FMF patients from HD and patients with OID

Pyroptosis was monitored in real time in monocytes from FMF patients bearing biallelic p.M694V or p.M694I mutations, HD, and controls with OID. UCN-01 triggered a rapid cell death in FMF patients' monocytes while it was much delayed in monocytes from HD or OID (figure 1A). This difference in cell death kinetics was highly significant as determined both by quantifying the areas under the curve (AUC, $p < 0.0001$; figure 1B) and by comparing the UCN-01-incubation time leading to 20% of cell death ($p < 0.0001$; figure 1C). ROC curves were generated and regression analysis established that monitoring cell death during 60 minutes of UCN-01 treatment discriminated FMF patients from HD with a sensitivity of 95.7% and a specificity of 94.7% (figure 1D).

When applied to a cohort of patients with OID, the same analysis accurately classified 94.9% of the patients. Thus, monitoring UCN-01-triggered pyroptosis discriminates FMF patients from other patients with a sensitivity of 92.1% and a specificity of 97.5% (figure 1E).

Of note, this experiment does not require any LPS priming step, providing results within one hour post-monocyte isolation.

Biparametric analyses increase the sensitivity and specificity of the test

We then wondered whether a biparametric test, based also on the IL-1 β release quantification, could better discriminate the different patient groups.

Following LPS priming and pyrin activation, mean IL-1 β levels in monocyte supernatants were 15-fold higher in homozygous FMF patients (1521 ± 1168 pg/mL) than in HD (92.7 ± 111 pg/mL; $p < 0.0001$) or patients with OID (99.16 ± 108 pg/mL; $p < 0.0001$) (figure 2A). By comparing these FMF patients with HD, the discrimination

threshold was determined at 260.6 pg/mL, giving a sensitivity of 97.1% and a specificity of 91.2% (figure 2B).

Similar results were obtained when applying this analysis to a cohort of patients with OID (sensitivity of 100% and a specificity of 97.3%; figure 2C). Thus, IL-1 β dosage following UCN-01 treatment discriminates FMF patients from HD and other patients with OID.

Importantly, by combining the two parameters, homozygous FMF patients were fully segregated from HD ($p < 0.0001$), while only one patient in the OID group was classified as a false positive ($p < 0.0001$) (figure 2D). Of note, the *MEFV* genotype of this patient with Behçet disease is unknown and we cannot exclude the possibility that he is carrying a pathogenic *MEFV* variant (more prevalent in patients with Behçet disease than in an ethnically-matched population).^{10,11}

Hence, monitoring both pyroptosis and IL-1 β strengthens the discriminating power of the assay (sensitivity of 100% and specificity of 99%; figure 2E-F).

Gene dosage determines UCN-01-mediated responses

MEFV-gene dosage plays an important role in the phenotype of FMF.^{12,13} We therefore wondered whether cellular responses to UCN-01 were influenced by gene dosage in the same way as the clinical phenotype. Patients with mono- (n=10) or bi-allelic (n=38) variants at the p.M694 residue were selected for this analysis. Real-time pyroptosis monitoring identified significant differences between heterozygous and homozygous patients ($p = 0.0015$; figure 3A, B). IL-1 β analysis revealed significantly lower cytokine levels in heterozygous patients than in homozygous patients ($p = 0.024$; figure 3C). Biparametric analyses confirmed the gene dosage effect by demonstrating a significant

difference between monocytes from carriers of mono- vs. biallelic *MEFV* variants (p=0.0044; figure 3D).

Importantly, heterozygous FMF patients were also discriminated from HD using UCN-01-based assays (p<0.001; figure 3A-C).

Together, these results demonstrate a gene dosage impact on the UCN-01-triggered responses and show that UCN-01-based functional assays segregate homozygous or heterozygous FMF patients from HD. Interestingly, these results are highly consistent with the impact of the *MEFV* gene dosage on clinical phenotypes.¹² Of note, colchicine treatment did not impact the results of the test (supplementary figure 1).

***MEFV* genotype influences monocyte responses to UCN-01**

374 *MEFV* variants are listed in the Infevers registry and the pathogenicity of most of them is unclear.^{8,14} We thus investigated the responses of monocytes from FMF patients with homozygous mutations not located at the p.M694 site. Three patients with homozygous mutations (p.M680I, p.V726A, and p.P369S) were analyzed (supplementary figure 2A-D). Although caution must be raised due to the inclusion of a single patient per genotype, a gradient of UCN-01-triggered responses was observed which is highly consistent with the described impact of the corresponding mutations on clinical phenotypes.¹⁵⁻¹⁷ The impact of genotypes on *in vitro* phenotypes could be classified as follows p.M694V/I>p.M680I>p.V726A>p.P369S which mirrors the clinical phenotype-genotype studies.¹⁵⁻¹⁸ The biparametric analysis segregated the homozygous p.M680I and p.V726A patients from the HD, but not the FMF patient harboring the p.P369S/P369S genotype (supplementary figure 2D). Interestingly, p.P369S is a variant of uncertain significance which does not behave like typical FMF-associated *MEFV* variants, as observed in *in vitro* experiments on monocyte cell lines¹¹, or in a recently

developed colchicine-resistance assay.¹⁹ These results suggest that the p.P369S variant is a non-pathogenic variant or that its pathogenicity is associated with another molecular mechanism.

Finally, we analyzed the ability of UCN-01-based tests to discriminate compound heterozygous FMF patients from HD (supplementary figure 2E-H). The *in vitro* responses of monocytes bearing two different *MEFV* variants were lower than *in vitro* responses of p.M694V/I homozygous monocytes. When combined with the clearly pathogenic p.M694 variants, mutations on the second *MEFV* allele impacted differentially the *in vitro* responses with a hierarchy (p.M680I, p.R761H > p.V726A, p.E148Q) largely mirroring the gradient of clinical phenotypes (from severe to mild) observed in FMF patients. Importantly, biparametric analyses discriminated also compound heterozygous FMF patients from HD with a sensitivity of 92.3% and a specificity of 100%.

DISCUSSION

Here, we demonstrate that a functional assay, based on kinase inhibition and monitoring of pyroptosis and IL-1 β release, accurately diagnoses FMF over a large number of *MEFV* genotypes. This test discriminates FMF patients from both HD and patients with OID, including patients with well-defined monogenic diseases (supplementary figure 3). Besides the usual quantification of IL-1 family cytokine release, the test takes advantage of the real-time analysis of pyroptosis, a hallmark of inflammasome activation.

Cell death test brings first results within 3h (2h for sample preparation + 1h for cell death analysis; 12h for IL-1 β ELISA) and costs less than 7.2€/sample (supplementary table 2). Thus, it could be used in routine to support clinical findings. Due to its good positive predictive value, the test detects true positive FMF patients, for whom

treatment could be initiated promptly, deep investigations re-evaluated, and genetic confirmation reached. On the other hand, its good negative predictive value indicates a robust way to identify true negative patients, who require further investigations, reasoned genetic testing, and postponed (or even no) colchicine initiation. Of note, bi-parametric analyses should be used whenever the test based on cell death leads to negative results in order to build confidence in the results.

Our test identifies FMF patients with both monoallelic and biallelic *MEFV* mutations. These results are in line with clinical findings of patients with full-blown clinical FMF bearing monoallelic *MEFV* mutations. Interestingly, and although the results need to be confirmed in larger cohorts, the *in vitro* monocyte responses to UCN-01 largely mimic clinical responses to colchicine^{11,15} and the genotype-phenotype results.^{10,14,16,17} However, one limitation of our study stems from the fact that the results are mainly based on genetically-confirmed pathogenic mutations, thus requiring confirmatory analyses on larger cohorts of patients with rarer genotypes.

Our test uses isolated monocytes, preventing its transfer to routine labs in its current design. Nevertheless, we and others¹⁹ have preliminary data indicating that functional assays in whole blood are feasible and reliable, at least for cytokine release assessment (supplementary figure 4).

Altogether, functional assays are promising approaches for rapid detection of FMF patients with clearly-pathogenic mutations.^{19,20} Yet, the evaluation of pre-test probability (i.e. epidemiological and clinical data) remains the key in early decisions, thus positioning functional assays at the crossroads of clinics and genetics.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

YJ, FM, AB, AM, and TH are co-inventors and owners of a patent “Methods and kits for diagnosis of familial Mediterranean fever” (WO/2019/048569).

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FIGURES LEGENDS

Figure 1. PKC inhibitors trigger fast cell death specifically in monocytes from FMF patients. A–C Monocytes from HD (n=71), FMF patients bearing biallelic p.M694I/V variants (n=38) or patients with OID (n=40) were treated with 12.5 μ M UCN-01. (A) Cell death was monitored in real time by measuring propidium iodide influx every 5 minutes. (B) The areas under the curve (AUC) were computed for each patient after 60 minutes of UCN-01 treatment. (C) The time required to reach 20% cell death was calculated for each HD, FMF and patients with OID. In the figure the values were normalized by subtracting the result obtained for the HD with the value obtained for the FMF/OID analyzed at the same time. (D-E) Receiver operating characteristic (ROC) curves were computed for the area under the cell death kinetics curve following UCN-01 treatment by comparing HD and FMF (D) and FMF and patients with OID (E). For each ROC curve, the AUC, specificity, sensitivity, as well as the positive (PPV) and the negative (NPV) predictive values are indicated.

Data information: (A) Each point of the curve corresponds to the average of the mean cell death values from three biological replicates of monocytes from the indicated patients. (B-C) Each dot represents the value from one patient. (A-B) The bar represents the 95% confidence interval. *** $p < 0.001$ by Mann-Whitney rank-sum test.

Figure 2. Biparametric analysis discriminates FMF patients from HD or OID. (A) Monocytes from HD (n=71), FMF bearing biallelic p.M694I/V variants (n=35), and patients with OID (n=39) were primed with LPS during 3 h and treated with 12.5 μ M

UCN-01 for 1.5 h. IL-1 β levels in monocyte supernatants were quantified by ELISA. (B-C) ROC curves were computed for the obtained IL-1 β values by comparing FMF and HD (B) and FMF and patients with OID (C). (D) Cell death data and IL-1 β data were combined by multiplying the mean area under the cell death kinetics curve by the concentrations of IL-1 β for each patient. The log value is represented in the figure. (E-F) ROC curves were computed for the biparametric analysis data by comparing FMF and HD (E) and FMF and patients with OID (F). For each ROC curve, the AUC, specificity, sensitivity, as well as the positive (PPV) and the negative (NPV) predictive values are indicated.

Data information: (A, D) Each dot represents the mean value from three biological replicates for one patient (A) or the calculated value from one patient (D). (A, D) The bar represents the 95% confidence interval. *** $p < 0.001$ by Mann-Whitney rank-sum test.

Figure 3. UCN-01 treatment discriminates FMF heterozygous patients who present a gene dosage response. Monocytes from HD (n=71), FMF homozygous (p.M694I-V/p.M694I-V) patients (Homo, n=38) and FMF heterozygous (p.M694I-V/0) patients (Het, n=10) were treated with 12.5 μ M UCN-01 either after LPS priming (C) or not (A). (A) Cell death was monitored in real time by measuring propidium iodide influx every 5 minutes. (B) The AUC were computed for each patient after 60 minutes of UCN-01 treatment. (C) IL-1 β levels in monocyte supernatants were quantified by ELISA. (D) The two parameters were combined by multiplying the mean AUC by the concentration of IL-1 β obtained for each patient.

Data information:

(A) Each point of the curve corresponds to the average of the mean cell death values from three biological replicates of monocytes from the indicated patients. (B-D) Each dot represents the value from one patient. (C) Each dot represents the mean value from three biological replicates for one patient. (A-D) The bar represents the 95% confidence interval. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Mann-Whitney rank-sum test. ns: not significant.

KEY MESSAGES:

What is already known about this subject?

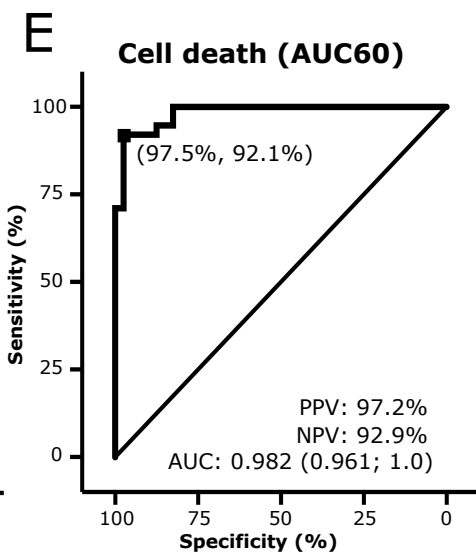
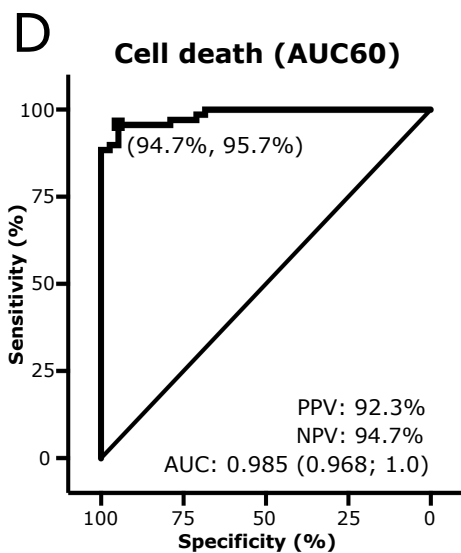
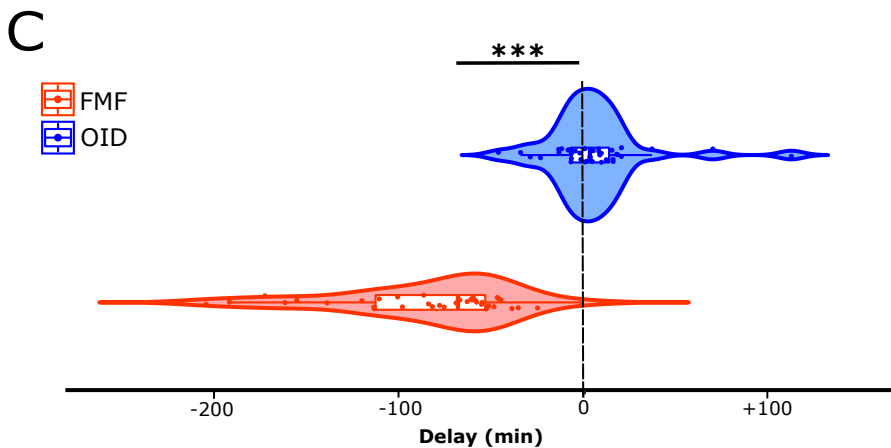
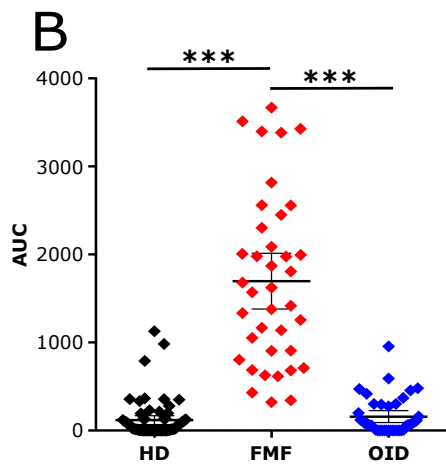
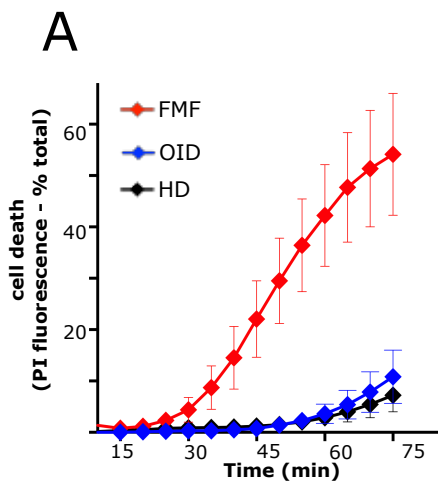
- Genetic analysis of the *MEFV* gene is often inconclusive due to the large number of variants of uncertain significance.

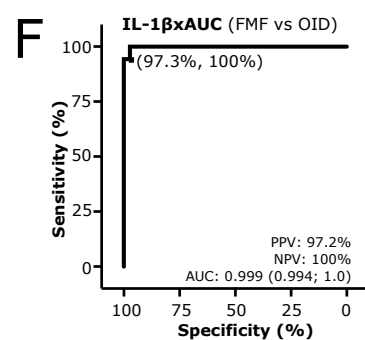
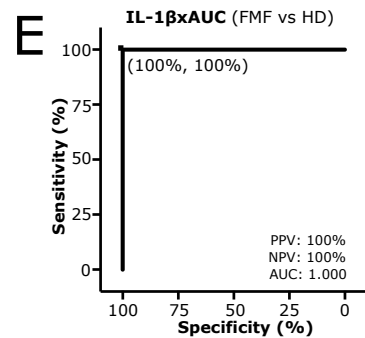
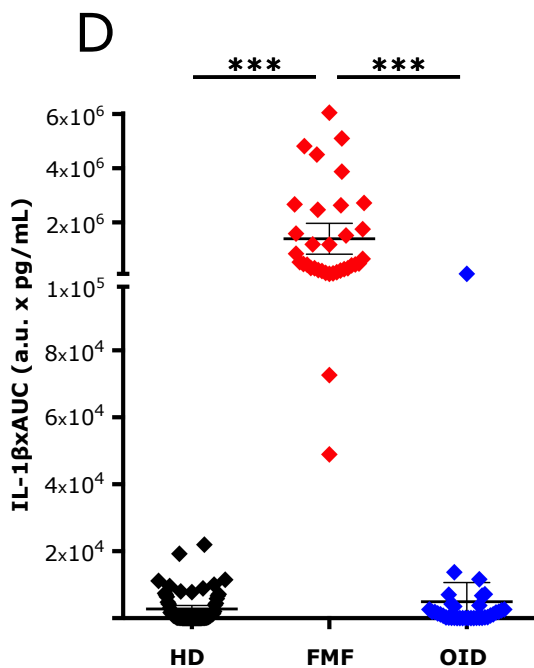
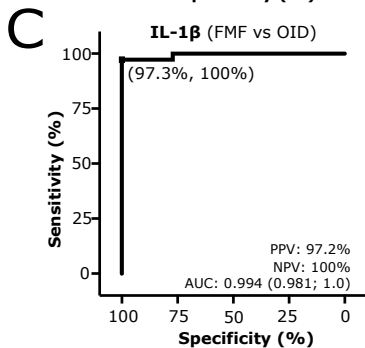
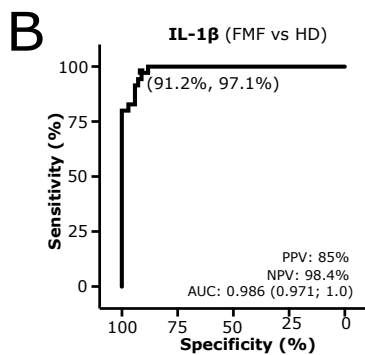
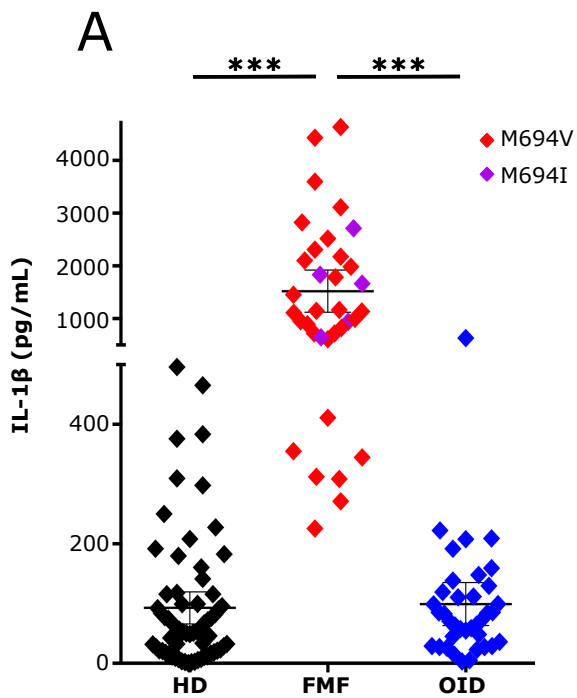
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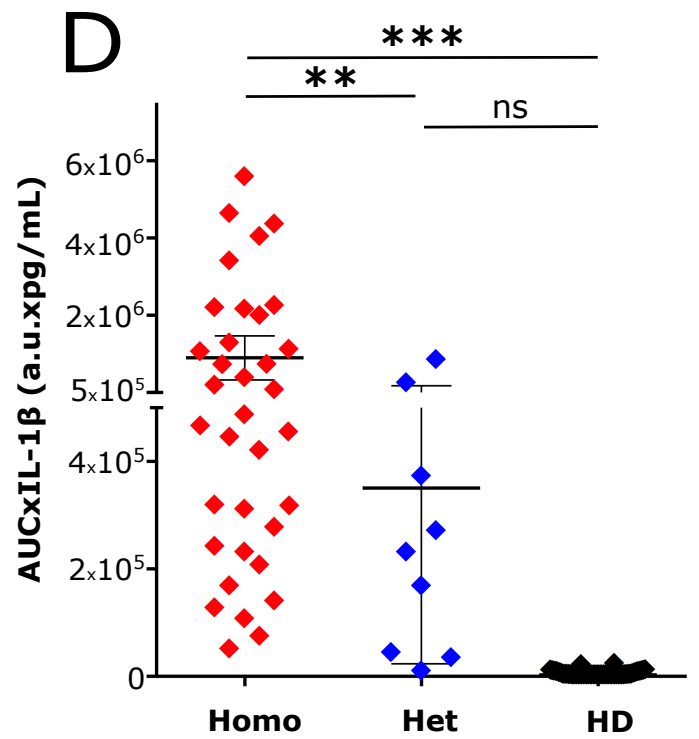
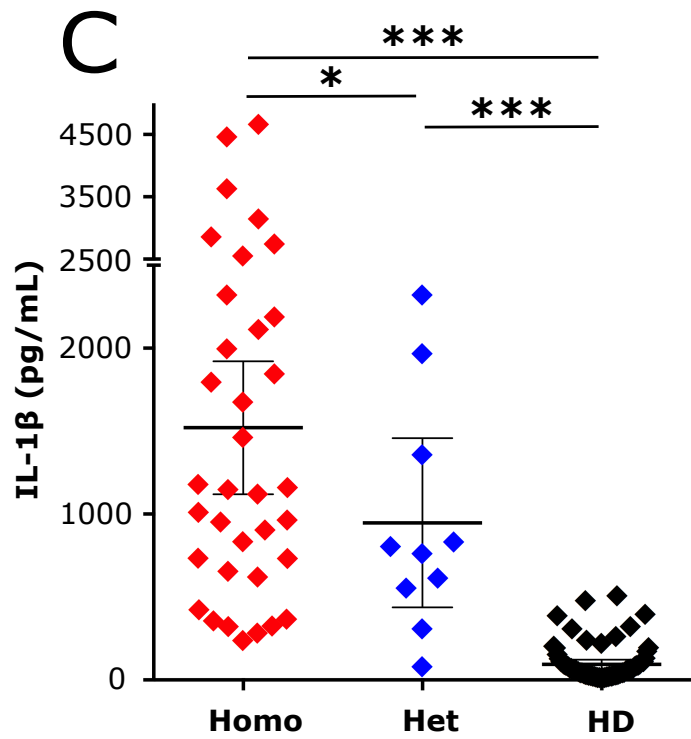
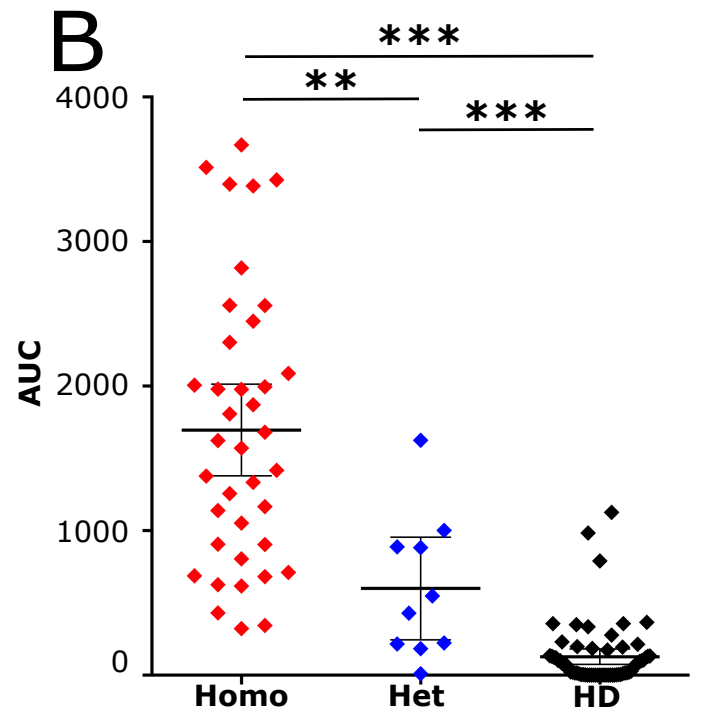
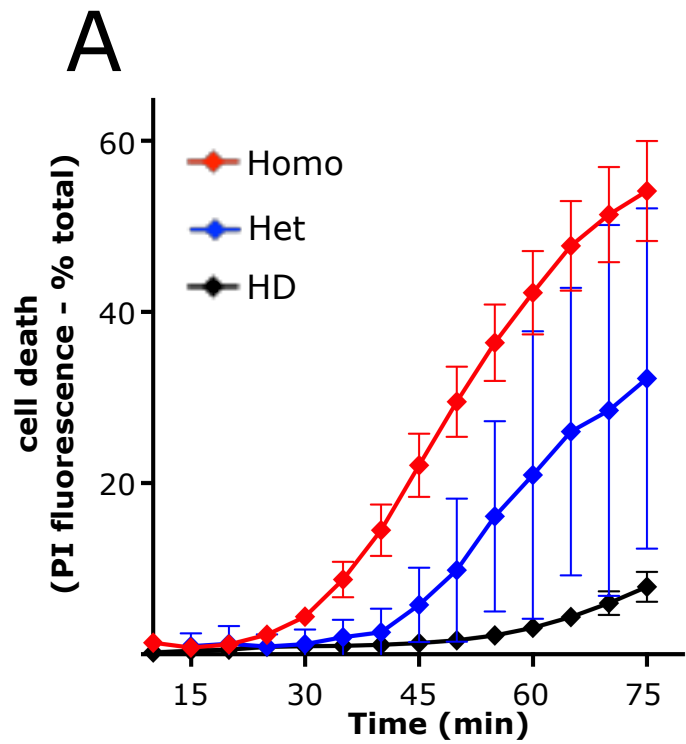
- Monitoring pyroptosis in real time allows a fast diagnosis of patients with familial Mediterranean fever.
- Monitoring both cell-death kinetics and IL-1 β release accurately discriminates patients with familial Mediterranean fever from other patients.
- The nature and the number of the *MEFV* variants influence the degree of *in vitro* activation of the pyrin inflammasome.

How might this impact on clinical practice or future developments?

- The test could guide early clinical decisions and management by identifying FMF patients who will require colchicine and genetic analyses while others (the negative ones) will need deeper investigations including the search for alternative diagnosis.







1 **- SUPPLEMENTARY MATERIAL -**

2 related to

3 **A fast diagnostic test for familial Mediterranean fever based on a kinase**
4 **inhibitor**

5 by Flora Magnotti, Tiphaine Malsot, Sophie Georgin-Lavialle, Fatima Abbas, Amandine Martin, Alexandre
6 Belot, Maxime Fauter, Muriel Rabilloud, Mathieu Gerfaud-Valentin, Pascal Sève, Agnès Duquesne,
7 Arnaud Hot, Stéphane Durupt, Gilles Grateau, Thomas Henry, and Yvan Jamilloux

8
9
10 **MATERIALS AND METHODS**

11 **Patients and controls**

12 67 patients with symptomatic FMF and bearing at least one *MEFV* mutation were
13 prospectively included in the study (homozygous, $n=41$; compound heterozygous, $n=16$;
14 heterozygous, $n=10$). The study was designed to include patients with symptomatic FMF
15 (with past or current symptoms), which diagnosis was based on international criteria.
16 Asymptomatic carriers of clearly pathogenic *MEFV* mutations were no included. 71 healthy
17 donors (HD) along with 40 patients suffering from other inflammatory disorders [i.e. adult-
18 onset Still's disease (AOSD, $n = 6$); systemic juvenile idiopathic arthritis (sJIA, $n = 2$);
19 Behçet's disease ($n = 4$); Periodic Fever, Aphthous stomatitis, Pharyngitis, Adenitis (PFAPA,
20 $n = 2$); inflammatory bowel disease (IBD, $n = 4$); sepsis ($n = 3$); Mevalonate Kinase
21 Deficiency (MKD, $n = 5$); TNF-Receptor-Associated Periodic Syndrome (TRAPS, $n = 2$);
22 Cryopyrin-Associated Periodoc Syndrome ($n = 2$); systemic lupus erythematosus (SLE,
23 $n = 2$); (non-systemic) polyarticular juvenile idiopathic arthritis (pJIA, $n = 1$); H-syndrome

24 ($n = 1$); haploinsufficiency of A20 (HA20, $n = 1$); fever of unknown origin (FUO, $n = 4$);
25 sarcoidosis ($n = 1$)); were used as controls (*see patient description in supplementary table*
26 *1*). Except those with a genetically-determined disease (i.e. MKD, TRAPS, CAPS, HA20, H-
27 syndrome), patients with OID did not undergo systematic genetic investigation.

28

29 **Monocyte isolation**

30 Blood was drawn in heparin-coated tubes and kept at room temperature overnight. Blood
31 samples from HD were drawn on the same day as patients. Peripheral blood mononuclear
32 cells (PBMCs) were isolated by density-gradient centrifugation using Lymphocyte
33 Separation Medium (Eurobio). Monocytes were isolated from PBMCs by magnetic selection
34 using CD14 MicroBeads (Miltenyi Biotec) and the AutoMACS Pro Separator (Miltenyi
35 Biotec) following the manufacturer's instructions. Monocytes were enumerated in the
36 presence of a viability marker (propidium iodide (PI), 10 $\mu\text{g}/\text{ml}$, Sigma) by flow cytometry
37 (BD Accuri C6 Flow Cytometer).

38

39 **Pyrimin inflammasome activation and cell death / IL-1 β secretion analyses**

40 Monocytes were seeded in Roswell Park Memorial Institute (RPMI) 1640, GlutaMAX
41 medium (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (Lonza). Cell
42 death was monitored by incubating 2×10^4 monocytes per well in black 96-well plates
43 (Costar, Corning) in the presence of PI at 5 $\mu\text{g}/\text{ml}$. Three technical replicates per condition
44 were done. UCN-01 was added at 12.5 μM in the absence of any priming signal. After UCN-
45 01 addition, real-time PI incorporation was measured every 5 min from 15 to 75 min post-
46 UCN intoxication, using a fluorimeter (Tecan) at the following wavelengths: excitation 535

47 nm (bandwidth 15 nm) and emission 635 nm (bandwidth 15 nm). Cell death was
48 normalized using PI incorporation in monocytes treated with Triton X-100 for 15 min
49 (corresponding to the 100% cell death).

50 For the IL-1 β secretion analysis, monocytes were seeded onto 96-well plates at 5×10^3
51 cells/well, incubated for 3 h in the presence of lipopolysaccharide (LPS, 10 ng/ml,
52 InvivoGen) and then treated with UCN-01 (12.5 μ M, Sigma) during 1.5 h. Following the
53 incubation, plates were centrifuged and supernatants were collected. IL-1 β levels were
54 quantified by ELISA (R&D Systems) following the manufacturer's instructions.

55

56 **Whole blood test analysis**

57 1 mL of blood from heparin-coated tubes was diluted with 1 mL Phosphate Buffered Saline
58 (PBS, ThermoFisher Scientific). The diluted blood was transferred into heparin-coated
59 tubes with plasma-separating gel (BD368497) and incubated for 1 h at 37°C in the
60 presence of LPS (10 ng/mL). UCN-01 was then added at 12.5 μ M final. Following a 1.5 h
61 incubation, the tube was centrifuged (10 min - 1500g) and the plasma was collected for IL-
62 1 β dosage by ELISA (R&D Systems) following the manufacturer's instructions.

63

64 **Ethical approval information**

65 The study was approved by the French *Comité de Protection des Personnes* (CPP,#L16-189)
66 and by the French *Comité Consultatif sur le Traitement de l'Information en matière de*
67 *Recherche dans le domaine de la Santé* (CCTIRS, #16.864). The experiments conformed to
68 the principles set out in the WMA Declaration of Helsinki and the Department of Health and
69 Human Services' Belmont Report. The *Etablissement Français du Sang* provided HD blood

70 in the framework of the convention #14-1820. Informed consent was received from each
71 participant prior to inclusion in the study.

72 All data are available within the manuscript, supplementary material, or on request to the
73 authors. DEPIST-FMF study is registered on www.clinicaltrials.gov under the unique id
74 #NCT03747315.

75

76 **Patient and Public Involvement Statement**

77 Patients were not involved in the design, recruitment or in the conduct of the study. Results
78 will be disseminated through communications to the French FMF patient association
79 (AFFMF) and through the FMF and AID Global Association website.

80

81 **Statistical analyses**

82 Patients and HD data normal distribution was verified using D'Agostino–Pearson omnibus
83 normality test. Quantitative data are presented as means (\pm standard deviation in the text,
84 or with the 95% confidence interval in the figures, as indicated). Triplicate reliability was
85 verified by calculating intraclass correlation coefficients.

86 The ability of the test to discriminate patients with FMF from HD or subjects with OID was
87 quantified by the construction of ROC curves and the estimation of areas under the curve
88 (AUC) with their associated 95%-confidence intervals (95%-CI). Differences in mean values
89 between groups were also tested using an analysis of variance (ANOVA and repeated
90 ANOVA). Differences with p-values <0.05 were considered statistically significant. p-value
91 <0.05 , <0.01 and, <0.001 are marked by *, ** and, ***, respectively.

92 ROC curves and AUC estimates were made using the non-parametric method of DeLong.
93 The threshold was estimated by maximizing the Youden index (Youden Index: $Se + Sp - 1$).
94 Sensitivity, specificity, positive and negative predictive values were calculated using this
95 threshold.

96 For cell death kinetic analysis, the best time point for discriminating FMF patients from HD
97 was searched by modelling ROC curve according to time (Parametric Distribution Free
98 [PDF] approach). Using the estimated best time point, the optimal threshold (Youden
99 Index) and the associated sensitivity, specificity, positive and negative predictive values
100 were assessed.

101 To calculate the time leading to 20% cell death, a non-linear regression analysis was used
102 to fit a sixth-order polynomial curve to the normalized cell death kinetics using the least
103 squares fit as the fitting method. The obtained curve was used to interpolate the time
104 corresponding to 20% cell death. The subtractions of (FMF or OID) patients - HD were then
105 plotted on violin-plots using R software. The biparametric test is based on multiplication of
106 the AUC at 60 minutes (AUC60) value obtained from the cell death kinetic modelisation by
107 the concentration of IL-1 β . The result is expressed as arbitrary unit x picogram / millilitre
108 (a.u. x pg / mL).

109 Statistical analyses were carried out with Prism (GraphPad, v6) and R software (R
110 Foundation for Statistical Computing, v4.0, <http://www.R-project.org>).

111

112 **References for statistical analyses**

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114 curves: a nonparametric approach. *Biometrics*. 1988; 44(3):837-45

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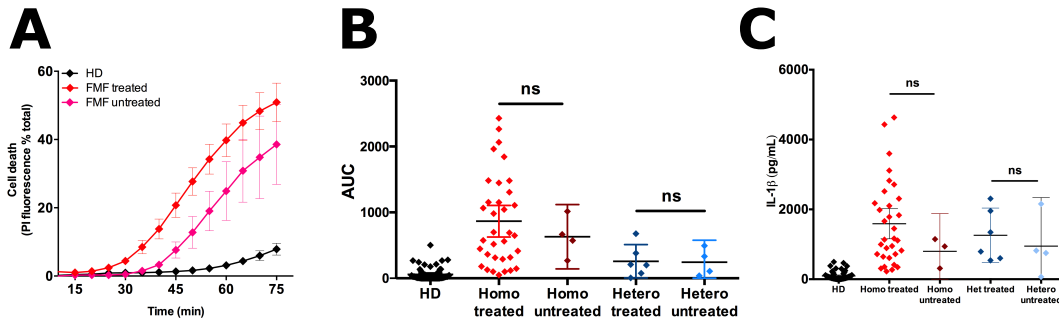
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- 123

124 **SUPPLEMENTARY FIGURES**

125

126 supplementary figure 1

127

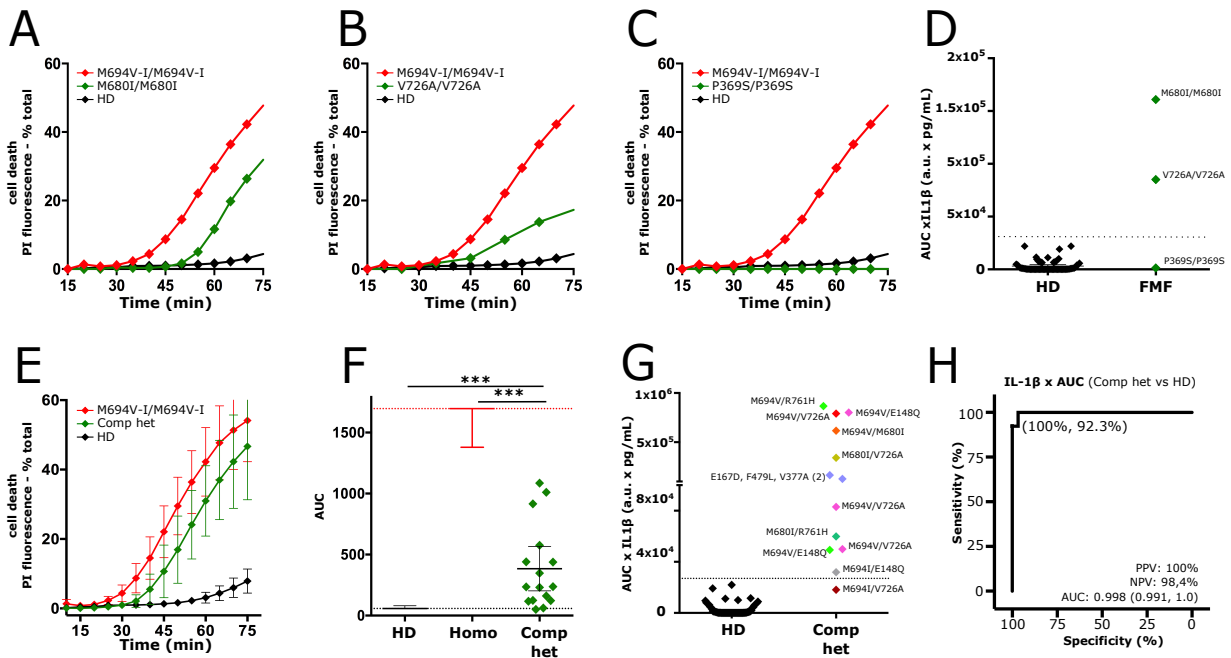


128

129 **Supplementary figure 1: impact of colchicine treatment on UCN-based test.**

130

Supplementary figure 2



131

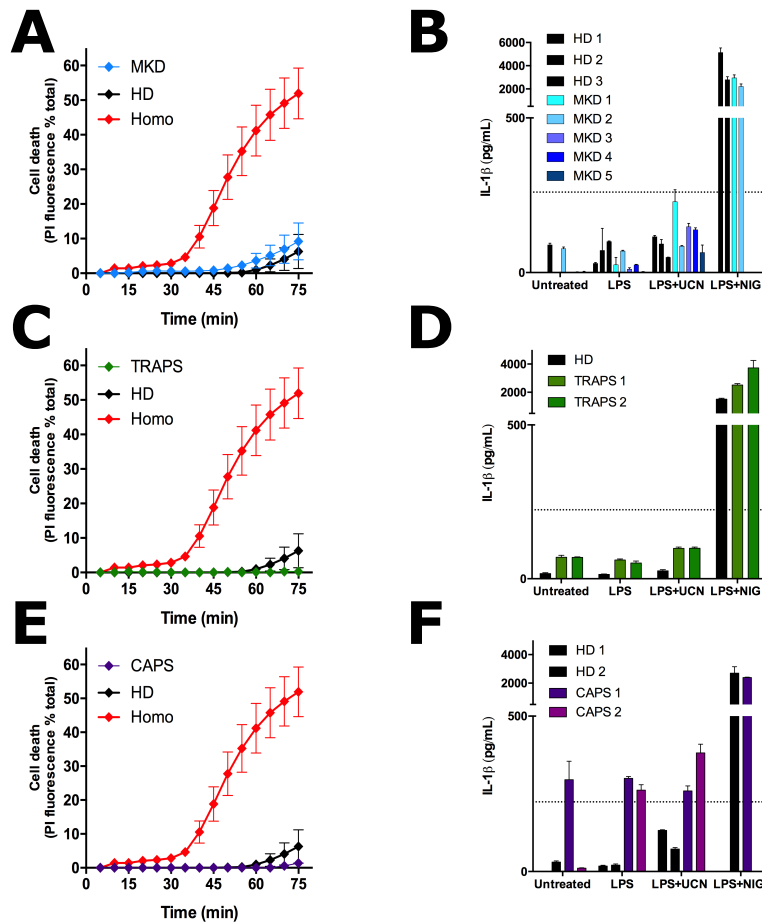
132 **Supplementary figure 2: *MEFV* genotype influences monocyte responses to UCN-01.** (A-C) Cell death
 133 kinetics was obtained by monitoring the propidium iodide influx every 5 minutes in monocytes from HD
 134 (n=71), FMF homozygous patients (carrying the p.M694I-V/p.M694I-V, n=38), one patient with the
 135 p.M680I/p.M680I mutation (A), one patient with the p.V726A/ p.V726A mutation (B) and one patient with
 136 the p.P369S/ p.P369S mutation (C). (D) The mean AUC value for each experiment was combined with the
 137 corresponding concentration of IL-1β for each patient to perform the biparametric analysis. (E) Cell death
 138 kinetics curves were obtained from monocytes from HD (n=71), FMF homozygous patients (p. M694I-V,
 139 n=38) and compound heterozygous FMF patients (Comp het, n=16). (F) AUC values for each patient were
 140 computed after 60 minutes of UCN-01 treatment. (G) Mean AUC and IL-1β release of 13 patients were
 141 combined for the biparametric analysis. (H) ROC curves were calculated for the biparametric analysis data by
 142 comparing Comp het FMF patients and HD.

143 Data information: (A-C, E) Each point of the curve corresponds to the average of the mean cell death values
 144 from three biological replicates of monocytes from the indicated patients. (D, F-G) Each dot represents the
 145 value from one patient. The bar represents the 95% confidence interval. ***p<0.001 by Mann-Whitney rank-
 146 sum test.

147

148

supplementary figure 3



149

150 **Supplementary figure 3: UCN-based test combined with ELISA discriminates FMF patients from**
 151 **patients with monogenic diseases.** Cell death kinetics was obtained by monitoring the propidium iodide
 152 influx every 5 minutes in monocytes from HD (n=8), patients with MKD (n=5) (A), TRAPS (n=2) (C) or CAPS
 153 (n=2) (E). (B,D,F) IL-1 β concentrations were analyzed in patients monocytes treated or not with LPS alone
 154 (3h), LPS+UCN-01 (UCN, 1.5h) or LPS+nigericin (NIG, 1.5h).

155 Data information: (A,C,E) Each point of the curve corresponds to the average of the mean cell death values
 156 from three biological replicates of monocytes from the indicated patients. (B,D,F) Each histogram represents
 157 the mean value of a triplicate from one patient. The bar represents the SEM; the dotted line is set at the
 158 threshold determined in figure 1, discriminating homozygous (M694V/I) FMF patients and HD.

159 As previously described, CAPS patients monocytes present constitutive IL-1 β secretion in the presence of LPS
 160 that does not significantly increase in the presence of UCN-01. Importantly, in the absence of LPS, neither
 161 constitutive pyroptosis nor UCN-01-induced pyroptosis is detected in CAPS patients monocytes (E). Patients

162 with very rare monogenic diseases linked to pyrin inflammasome or cytoskeleton disorders (e.g. PAPA, PFIT
163 and PAAND patients) could not be included in this study.

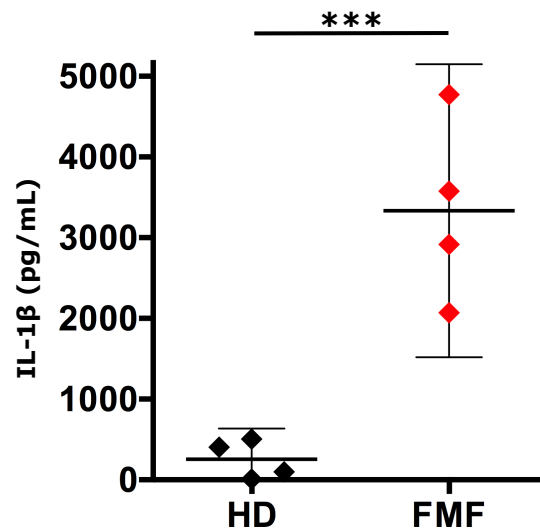
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173 **Supplementary figure 4: UCN-01-based inflammasome test in whole blood.** Whole blood samples from
174 HD (n=4) and homozygous FMF patients (p.M694I-V, n=4) were primed with LPS and treated with UCN-01 in
175 plasma-separating gel tubes. After centrifugation, plasma IL-1β concentrations were determined by ELISA.
176 Each dot represents the mean value from three biological replicates for one patient. The bar represents the
177 95% confidence interval. *** p<0.001 by Mann-Whitney rank-sum test.

178

Supplementary table 1: Description of patients and controls included in the study

#	Sex (Male/Female)	Age (years)	Disease	<i>MEFV</i> genotype	Colchicine at time of sampling	Symptoms at time of sampling
1	F	43	FMF	M694V/M694V	yes	no
2	M	34	FMF	M694V/M694V	yes	no
3	M	5	FMF	M694V/M694V	yes	no
4	F	11	FMF	M694V/M694V	yes	no
5	F	13	FMF	M694V/M694V	yes	no
6	F	9	FMF	M694V/M694V	yes	no
7	F	5	FMF	M694V/M694V	yes	no
8	F	8	FMF	M694V/M694V	no	no
9	F	7	FMF	M694V/M694V	yes	no
10	F	15	FMF	M694V/M694V	yes	no
11	M	4	FMF	M694V/M694V	yes	no
12	F	17	FMF	M694V/M694V	yes	no
13	M	63	FMF	M694V/M694V	yes	no
14	M	9	FMF	M694V/M694V	yes	no
15	M	4	FMF	M694V/M694V	yes	no
16	M	21	FMF	M694V/M694V	no	no
17	M	17	FMF	M694V/M694V	yes	no
18	F	44	FMF	M694V/M694V	yes	no
19	F	72	FMF	M694V/M694V	yes	no
20	M	44	FMF	M694V/M694V	yes	no
21	M	63	FMF	M694V/M694V	yes	no
22	M	35	FMF	M694V/M694V	yes	no
23	F	74	FMF	M694V/M694V	yes	no
24	M	71	FMF	M694V/M694V	yes	no
25	M	33	FMF	M694V/M694V	yes	no
26	F	43	FMF	M694V/M694V	yes	no
27	F	40	FMF	M694V/M694V	yes	no
28	M	73	FMF	M694V/M694V	yes	no
29	M	19	FMF	M694V/M694V	yes	no
30	M	32	FMF	M694V/M694V	yes	no
31	F	10	FMF	M694V/M694V	yes	no
32	M	22	FMF	M694V/M694V	yes	yes
33	M	30	FMF	M694I/M694I	yes	no
34	M	16	FMF	M694I/M694I	yes	no
35	F	37	FMF	M694I/M694I	yes	no
36	M	40	FMF	M694I/M694I	no	no
37	F	54	FMF	M694I/M694I	yes	yes
38	M	11	FMF	M694I/M694I	no	no

39	F	32	FMF	M680I/M680I	yes	no
40	F	35	FMF	V726A/V726A	yes	no
41	F	13	FMF	P369S/P369S	yes	no
42	M	15	FMF	M694V/0	yes	no
43	M	5	FMF	M694V/0	no	no
44	M	43	FMF	M694V/0	no	no
45	F	44	FMF	M694V/0	no	no
46	F	25	FMF	M694V/0	no	no
47	M	5	FMF	M694V/0	yes	no
48	M	40	FMF	M694V/0	yes	no
49	M	41	FMF	M694I/0	yes	no
50	F	7	FMF	M694I/0	yes	no
51	M	56	FMF	M694I/0	yes	no
52	F	10	FMF	M694V/M680I	yes	no
53	F	8	FMF	M680I/V726A	yes	no
54	F	36	FMF	M680I/R761H	yes	no
55	F	8	FMF	M694V/R761H	yes	no
56	M	5	FMF	E167D,F479L;V37 7A	yes	no
57	M	13	FMF	E167D,F479L;V37 7A	yes	no
58	M	8	FMF	E148Q/M694V	yes	no
59	F	30	FMF	M694I/V726A	yes	yes
60	M	12	FMF	M694I/E148Q	yes	no
61	F	22	FMF	M694V/M680I	yes	no
62	M	51	FMF	M694V/V726A	yes	no
63	M	55	FMF	M694V/V726A	yes	no
64	F	41	FMF	M694V/R761H	yes	no
65	F	30	FMF	M694V/R761H	yes	no
66	H	38	FMF	M694V/E148Q	yes	no
67	M	40	FMF	M694V/V726A	yes	no
1	M	17	AOSD	NA	no	no
2	M	53	AOSD	NA	yes	no
3	F	53	AOSD	NA	no	no
4	F	19	AOSD	NA	no	no
5	F	19	AOSD	NA	no	no
6	F	19	AOSD	NA	no	yes
7	F	32	Behçet	NA	yes	no
8	M	34	Behçet	NA	yes	no
9	F	28	Behçet	NA	yes	no
10	F	32	Behçet	NA	no	no
11	F	17	IBD	NA	no	no
12	F	17	IBD	NA	no	no

13	M	17	IBD	NA	no	no
14	M	13	IBD	NA	no	no
15	M	43	SLE	NA	no	no
16	M	47	SLE	NA	no	no
17	F	6	pJIA	NA	no	no
18	F	28	Sepsis	NA	no	no
19	F	27	Sepsis	NA	no	no
20	M	89	Sepsis	NA	no	no
21	F	13	sJIA	NA	no	no
22	F	16	sJIA	NA	no	no
23	M	3	MKD	NA	no	no
24	F	11	MKD	NA	no	no
25	M	5	MKD	NA	no	no
26	M	4	MKD	NA	no	yes (fever, abdominal pain, elevated CRP)
27	M	20	MKD	NA	no	yes (stomatitis, elevated CRP)
28	M	26	HA20	NA	no	no
29	F	4	H-syndrome	NA	no	no
30	F	36	TRAPS	NA	no	no
31	F	38	TRAPS	NA	no	no
32	F	19	CAPS	NA	no	no
33	M	49	CAPS	NA	no	no
34	F	17	FUO	NA	no	yes
35	F	22	PFAPA	NA	yes	no
36	M	41	PFAPA	NA	yes	no
37	M	44	FUO	NA	no	no
38	M	37	FUO	NA	no	no
39	M	48	FUO	NA	no	no
40	M	42	Sarcoidosis	NA	no	no
1	M	26	HD	NA	no	no
2	M	58	HD	NA	no	no
3	F	30	HD	NA	no	no
4	M	22	HD	NA	no	no
5	M	25	HD	NA	no	no
6	M	28	HD	NA	no	no
7	F	51	HD	NA	no	no
8	M	53	HD	NA	no	no
9	M	55	HD	NA	no	no
10	M	32	HD	NA	no	no
11	M	51	HD	NA	no	no
12	F	32	HD	NA	no	no
13	M	61	HD	NA	no	no

14	M	63	HD	NA	no	no
15	M	18	HD	NA	no	no
16	F	30	HD	NA	no	no
17	F	21	HD	NA	no	no
18	F	31	HD	NA	no	no
19	M	38	HD	NA	no	no
20	M	34	HD	NA	no	no
21	M	31	HD	NA	no	no
22	M	34	HD	NA	no	no
23	M	41	HD	NA	no	no
24	F	53	HD	NA	no	no
25	M	40	HD	NA	no	no
26	M	33	HD	NA	no	no
27	F	25	HD	NA	no	no
28	F	41	HD	NA	no	no
29	M	38	HD	NA	no	no
30	M	40	HD	NA	no	no
31	M	47	HD	NA	no	no
32	M	54	HD	NA	no	no
33	M	48	HD	NA	no	no
34	M	45	HD	NA	no	no
35	M	51	HD	NA	no	no
36	F	63	HD	NA	no	no
37	M	58	HD	NA	no	no
38	M	60	HD	NA	no	no
39	M	40	HD	NA	no	no
40	M	62	HD	NA	no	no
41	M	58	HD	NA	no	no
42	F	51	HD	NA	no	no
43	M	45	HD	NA	no	no
44	M	18	HD	NA	no	no
45	M	41	HD	NA	no	no
46	M	53	HD	NA	no	no
47	M	43	HD	NA	no	no
48	M	26	HD	NA	no	no
49	F	64	HD	NA	no	no
50	F	29	HD	NA	no	no
51	M	61	HD	NA	no	no
52	M	24	HD	NA	no	no
53	F	43	HD	NA	no	no
54	M	31	HD	NA	no	no
55	F	21	HD	NA	no	no

56	M	63	HD	NA	no	no
57	M	31	HD	NA	no	no
58	M	59	HD	NA	no	no
59	M	64	HD	NA	no	no
60	M	62	HD	NA	no	no
61	F	43	HD	NA	no	no
62	M	60	HD	NA	no	no
63	F	40	HD	NA	no	no
64	M	37	HD	NA	no	no
65	M	62	HD	N/A	no	no
66	M	55	HD	N/A	no	no
67	F	27	HD	N/A	no	no
68	M	40	HD	N/A	no	no
69	M	53	HD	N/A	no	no
70	F	42	HD	N/A	no	no
71	M	38	HD	N/A	no	no

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187 **Supplementary Table 2: Estimate of the costs of the functional assays**

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PRODUCT	STOCK		PER PATIENT	
	Quantity	Price	Quantity	Price
Ficoll	500 ml	47 €	10 ml	0.94 €
CD14 beads	2 ml	560 €	20 µl	5.60 €
AutoMACS columns	10	499 €	-	0.49 €
LPS	5 mg	146 €	10ng/ml (0.5µl/experiment)	0.07 €
UCN-01	5 mg	724 €	12.5µM (1µl/experiment)	0.07 €
Propidium Iodide	100 mg	129 €	5µg/ml (7.5µl/experiment)	0,09 €
IL-1β ELISA KIT	25 plates	521 €	12 wells	2.17 €
Total for cell death assay				7.19 €
Total with ELISA				<u>9.43 €</u>

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