A new autoinflammatory and autoimmune syndrome associated with NLRP1 mutations: NAIAD (*NLRP1*-associated Auto-Inflammation with Arthritis and Dyskeratosis)

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Supplementary Methods

Biological analyses

CRP, Vitamin A and Immunoglobulin levels were measured with standard techniques (hospital laboratories CHRU Montpellier for patients 1 and 2; and UMC St Radhoub Nijmegen for patient 3). The normal ranges indicated in the figure legends correspond to the limits values of each laboratory. Patient 1 and 2 CRP levels were measured during the systematic evaluation of arthritis in a clinical rheumatology consultation, whereas the patient 3 CRP levels were assayed during its clinical follow-up or during his hospitalization following symptomatic periods.

Histopathological analysis

Skin biopsies were collected on lesions located on feet, left arm, and left knee for patient 1 as well as from left leg for patient 2 and arm for patient 3. We also obtained excisions of lesions of the left vocal cord and the inter-arythenoid space for patient 1. All samples were fixed in Bouin, paraffin embedded, and 4 micrometers sections were cut and counterstained with hematoxylin and eosin.

Flow cytometric immunophenotyping

Cell labelling was performed on fresh whole EDTA-treated blood samples using conjugated-antibodies specific for cell surface markers (CD3, CD4, CD19, CD20, CD24, CD25, CD27, IgD, CD38, CD45RO, CD45RA, CD56, CD127, HLA-DR) from BD Biosciences or Beckman Coulter according to the manufacturer recommendations. Following incubation with antibodies, red blood cells were lyzed with either IMMUNOPREP Reagent System (Beckman Coulter) or the FACS-Lysing solution (BD Biosciences) and cells were acquired on a Navios

(Beckman-Coulter) or a Canto II (BD Biosciences) analyser. Results were analysed by using KALUSA or DIVA software.

SNP array and homozygosity mapping

DNA was extracted from whole blood using the QIAamp DNA Blood Midi kit (Qiagen, France) from the patients 1 and 2, their four parents and three unaffected relatives of the family 1. Homozygosity mapping was performed using Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, USA). Data were used for multipoint LOD score analysis using easyLINKAGE Plus software[1].

Exome sequencing

Exome sequencing was performed in both patients from family 1 and one unaffected relative. Library preparation, capture, sequencing, and variant detection were performed by IntegraGen (Evry, France) using an Illumina HiSeq 2000 platform with an average coverage of 50-fold.

Genomic DNA was captured using Agilent in-solution enrichment methodology with their biotinylated oligonucleotides probes library (Human All Exon v3 – 50 Mb, Agilent), followed by paired-end 75 bases massively parallel sequencing on Illumina HiSEQ 2000. Sequence capture, enrichment and elution were performed according to manufacturer's instruction and protocols (SureSelect, Agilent). Briefly, 3 µg of each genomic DNA were fragmented by sonication and purified to yield fragments of 150-200 bp. Paired-end adaptor oligonucleotides from Illumina were ligated on repaired, A tailed, DNA fragments, then purified and enriched by 4 to 6 PCR cycles. 500ng of these purified Libraries were hybridized to the SureSelect oligo probe capture library for 24 hr. After hybridization, washing, and elution, the eluted fraction was PCR-amplified with 10 to 12 cycles, purified and quantified by QPCR to obtain sufficient DNA template for downstream applications. Each eluted-enriched DNA sample was

then sequenced on an Illumina HiSEQ 2000 as paired-end 75b reads. Image analysis and base calling is performed using Illumina Real Time Analysis (RTA) Pipeline version RTA 1.13.48.0 with default parameters.

The bioinfomatic analysis of sequencing data was based on the Illumina pipeline (CASAVA1.8.0). CASAVA performs alignment of the reads to a reference genome (hg19) with the alignment algorithm ELANDv2 (performs multiseed and gapped alignments), then calls the SNPs based on the allele calls and read depth, and detects variants (SNPs & Indels). Only the positions included in the oligonucleotide probe coordinates +/- 20bp were conserved. Genetic variation annotation was performed using the IntegraGen in-house pipeline, which consists on gene annotation (RefSeq), detection of known polymorphisms (dbSNP 132, 1000Genomes) followed by a mutation characterization (e.g. exonic, intronic, silent, nonsense). For each position, the homozygous and heterozygous exomic frequencies were determined from IntegraGen exome database and the exome results provided by HapMap. IntegraGen provides results per sample upon tabulated text files, and performed coverage/depth statistical analysis for the whole exome and exon per exon.

After filtering for rare variants and recessive inheritance, we identified only 6 genes with a homozygous unknown variation: *MICALL2* (NM_182924 on chr7), *NLRP1* (NM_033004.3 on chr17), *DSG3* (NM_001944 on chr18), *DSG2* (NM_001943 on chr18), *ZNF674* (NM_0011904, chrX) and *ZNF157* (NM_003446, chrX). We considered only the *NLRP1* gene in the homozygous candidate region of chromosome 17.

Next generation sequencing

Next generation sequencing (NGS) including a panel of 55 confirmed and candidate autoinflammatory genes (list available upon request) was also undertaken for the patient 3 from family 2 to detect possible pathogenic mutations in other genes. The exons of the genes

were captured using a SureSelectXT Custom kit (Agilent), sequenced on a MiSeq (Illumina, France) equipment, and reads were interpreted using the SeqNext (JSI medical system, France) and Alamut (Interactive Biosoftware) softwares.

Sanger sequencing

Blood from 95 healthy volunteers from Algeria was collected for DNA extraction for sequence validation. For *NLRP1* mutations scanning, 17 additional patients with similar phenotype to the patients from family 1 and negatives for the *MEFV*, *MVK*, *TNFRSF1A* and *NLRP3* genes were recruited with the help of physicians of the EUROFEVER consortium[2]. For all the candidate variants, sequence validation and segregation analyses, as well as mutation scanning of the entire *NLRP1* (NM_033004.3) coding sequence (exons 1–17), were performed on genomic DNA using PCR and primers described in the online supplementary Table 1. PCR products were directly sequenced in both orientations using the BigDye 1.1 and 3.1 chemistry and a 3130XL Genetic Analyser, and analysed with the SeqScape v2.6 software (Applied Biosystems).

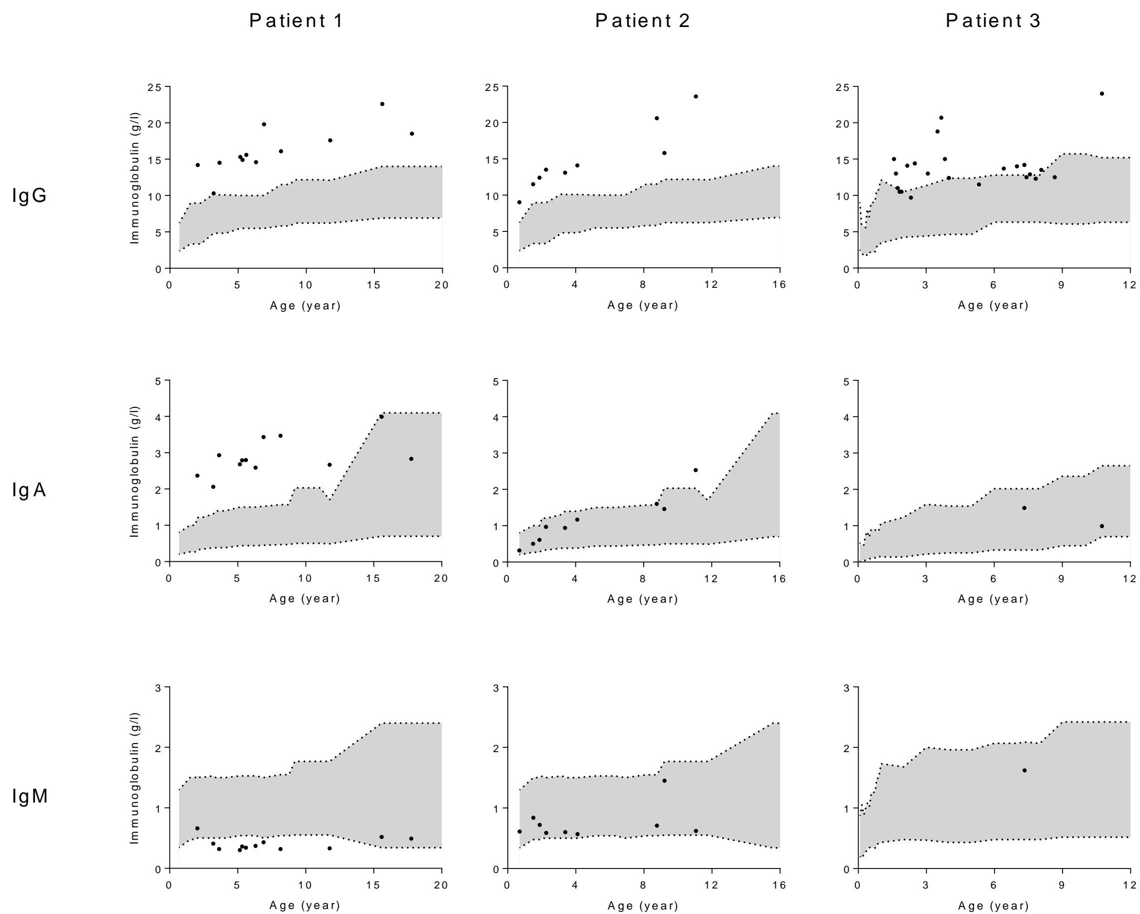
Quantification of inflammasome components

Blood samples from healthy controls (n=11), two heterozygous parents and the three patients were obtained in silicone-coated Vacutainer® tube. After centrifugation for 5 minutes at 300 g, serum samples were collected and stored at -80°C until analysis. Serum levels of caspase-1 and IL-1β were quantified by Quantikine® and DuoSet® ELISA kits, respectively (R&D Systems). IL-18 was measured using commercial kits from e-biosciences according to manufacturer's instructions. Concentrations below the detection limit were considered as not detectable (ND).

SUPPLEMENTARY FIGURES

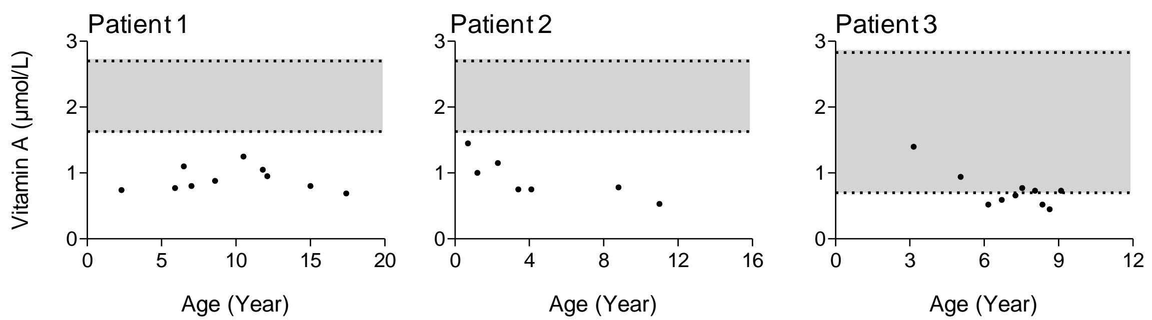
Supplementary Figure S1. Levels of Immunoglobulin G, A and M in the three patients.

The Y-axes indicate the serum levels of immunoglobulin G (IgG, top), A (IgA, middle) and M (IgM, bottom) in g/L measured in the three patients with their corresponding normal values (gray range) at different ages (X-axis in decimal age).



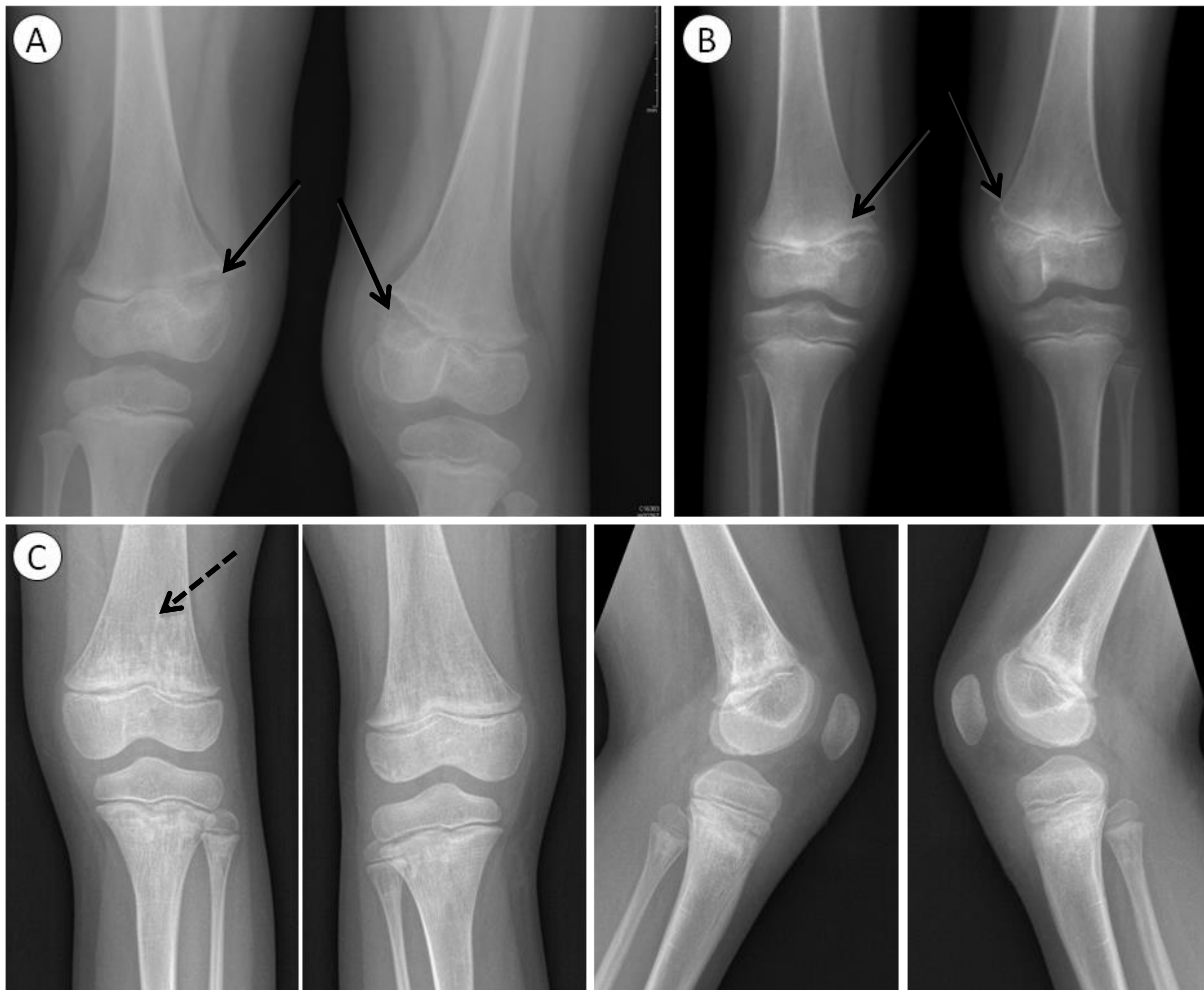
Supplementary Figure S2 Vitamin A deficiency in the three patients.

The Y-axes indicate the serum levels of Vitamin A in µmol/L measured in the three patients at different ages. The gray range indicates the corresponding normal limit values (from 1.63 to 2.7 µmol/L for patients 1 and 2; or from 0.7 to 2.83 µmol/L for patient 3).



Supplementary Figure S3

Skeletal X-rays showed abnormal metaphyses of knee in patients 2 and 3. Knee of patient 2 at 6 years (A) and 9 years (B). Note bilateral interne metaphyseal femoral lesions (arrows). Front and lateral view of the knee of patient 3 (C), note bilateral abnormal striation of the lower metaphyses of the femur (doted arrow) as well as dense and irregular condensation of the femoral inferior and tibila superior metaphyseal plate.



Supplementary Figure S4 Homozygosity mapping

A Linkage analysis in family 1

Homozygosity mapping was performed using Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, USA). The analysis were carried out using easyLINKAGE program[1]. The recessive inheritance model chosen for filtering (red rectangle) and the five best markers are depicted in the upper panel. The output window of results sorted by the pLOD score of all chromosomes for the family 1 (the 2 affected individuals, as well as all the parents and 3 unaffected sibs) are shown in the lower panel. The two homozygous regions with a pLod score > 2 on chromosomes 8 (pLod score 2.18) and 17 (pLod score 2.77) are pointed by the red arrows.

B Homozygous region on chromosome 17pter from the family 1

The output window of results for the chromosome 17pter is shown as described in A.

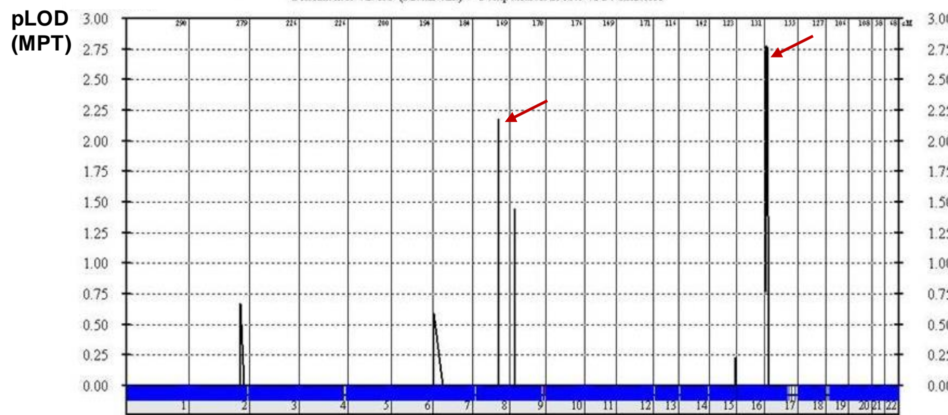
A

Project:		Inheritance:	Recessive
Family name:	TOTALS	Common allele:	99.90 %
Used map:	AFFY 250kNSP Marshfield v3 (	Disease allele:	0.10 %
Marker positions:	256487 ok / 5827 ? / 0 outside	LC1 PCOPY rate:	0.00 %
Allele frequencies:	All individuals from marker file	LC1 PENET wt/mt:	0.00 %
HAP algo / SCFct:	Off / All	LC1 PENET mt/mt:	99.00 %

Marker	CHR	cM	pLOD	NPL	npLOD	P val	INFO	Alpha	hLOD
1. rs9890496	17	10.70	2.7712	4.7752	4.9515	0.0039	0.8848	1.0000	2.7712
2. rs7207359 - rs9890496	17	10.69	2.7712	4.7752	4.9515	0.0039	0.8847	1.0000	2.7712
3. rs4790618 - rs4239046	17	10.70	2.7712	4.7752	4.9514	0.0039	0.8848	1.0000	2.7712
4. rs7207359	17	10.68	2.7712	4.7752	4.9514	0.0039	0.8846	1.0000	2.7712
5. rs4790618	17	10.70	2.7712	4.7751	4.9513	0.0039	0.8848	1.0000	2.7712

Pair setting: All pairs of affected / phenotyped sibs

GeneHunter v2.1r5 (MultiPoint) - Computation in sets of 50 markers



Marker coverage and chromosomes

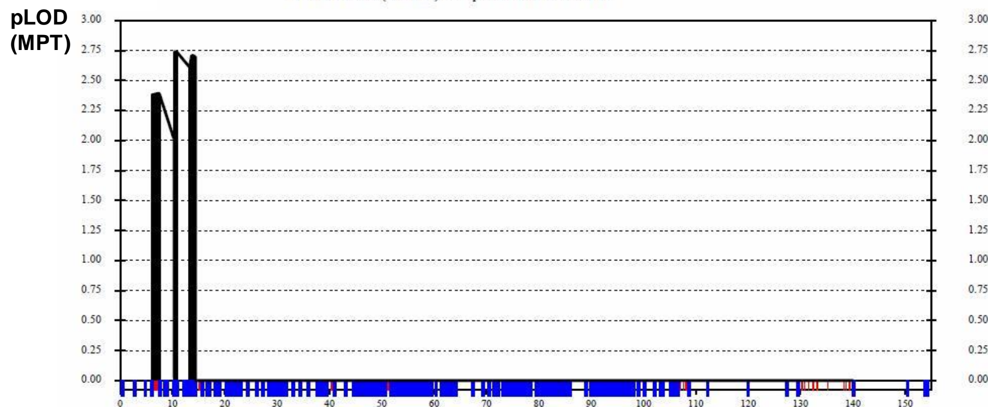
B

Project:		Inheritance:	Recessive
Family name:	TOTALS	Common allele:	99.90 %
Used map:	AFFY 250kNSP Marshfield v3 (sex-aver	Disease allele:	0.10 %
Marker positions:	4854 ok / 5827 ? / 0 outside	LC1 PCOPY rate:	0.00 %
Allele frequencies:	All individuals from marker file	LC1 PENET wt/mt:	0.00 %
HAP algo / SCFct:	Off / All	LC1 PENET mt/mt:	99.00 %

Marker	CHR	cM	pLOD	NPL	npLOD	P val	INFO	Alpha	hLOD
1. rs9890496	17	10.70	2.7403	4.5745	4.5441	0.0039	0.8506	1.0000	2.7403
2. rs4790618 – rs4239046	17	10.70	2.7403	4.5745	4.5440	0.0039	0.8506	1.0000	2.7403
3. rs7207359 – rs9890496	17	10.69	2.7403	4.5745	4.5440	0.0039	0.8504	1.0000	2.7403
4. rs4790618	17	10.70	2.7403	4.5744	4.5439	0.0039	0.8506	1.0000	2.7403
5. rs7207359	17	10.68	2.7403	4.5744	4.5438	0.0039	0.8503	1.0000	2.7403

Pair setting: All pairs of affected / phenotyped sibs

GeneHunter v2.1r5 (MultiPoint) – Computation in sets of 25 markers



Marker coverage, genetic position from 17pter [cM]

Supplementary Figure S5 Alignment of NLRP1, NLRP3 and NLRP12

NLRP1 (NP_12749.1), NLRP3 (NP_001230062.1) and NLRP12 (NP_653288.1) were aligned using CLUSTAL Omega. The conserved regions around the p.R726W mutation of NLRP1 are depicted. Amino acids (aa) modified by mutations are represented by red bolded letters and the protein nomenclature mutations were noted below the protein sequence. Homology of human NLRP1 to NLRP3 and NLRP12 is 34.44% and 36.31% respectively. The consensus symbols “ * ” , “ : ” or “ . ” mean that the amino acids are identical, conserved or semi-conserved in all sequences, respectively. More information on the NLRP mutations can be found on infevers, a dedicated online registry for autoinflammatory mutations (http://fmf.igh.cnrs.fr/ISSAID/infevers/).

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NLRP1     R--NLMQWVPS----LQLLLQPHSLESLHCLYETRNKTFLTQVMAHFEEMG-MCVETDME   750
                                          p.R726W
NLRP3     IRLELLKWIEVKAKAKKLQIQPSQLELFYCLYEMQEEDFVQRAMDYFPKIE-INLSTRMD   660
                        p.E627G/p.E627D p.L632F     p.D646Y       p.M659K
NLRP12    IKMDLLQWIQSKAQSDGSTLQQGSLEFFSCLYEIQEEEFIQQALSHFQVIVVSNIASKME   646
                                                  p.A629D
             :*::*:          :*  .** : **** ::: *: :.: :*  :    : : *:


NLRP1     LLVCTFCIKFSRHVKKLQLIEGRQH--------RSTWSPTM-VVLFRWVPVTDAYW----   797
NLRP3     HMVSSFCIENCHRVESLSLGFLHNMPK-EEEEEEKEGRH-------------------LD   700
        p.M662T
NLRP12    HMVSSFCLKRCRSAQVLHLYGATYSADGEDRARCSAGAHTLLVQLPERTVLLDAYSEHLA   706
                                            p.G683R
           :*.:**:: .: .: * *               .
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Supplementary Table 1 *NLRP1* primer pairs for Sanger sequencing

Name of primers	**Sequence** *
NLRP1-E1-F	GTAAGAGCCAAGGCAAAGGA
NLRP1-E1-R	CATAGTCTGGGGCCTGGAT
NLRP1-E2-F	GGTGTATCCTGACCTCTCTTGG
NLRP1-E2-R	TCCCCTATCCTTCCTCTGCT
NLRP1-E3-F	GCCCCTCTACTTCAACATGG
NLRP1-E3-R	TCCAGAACCTCTGCTTAGCC
NLRP1-E4A-F	GCATGGCAAGAACATACCAGT
NLRP1-E4A-R	GCCAGTGTTGACTTCCCAAT
NLRP1-E4B-F	AAGCTGGCCTGATTATGTGG
NLRP1-E4B-R	AGGTTCTGCAGAGCTGTGGT
NLRP1-E4C-F	CTGAGCTCTGTCTGCACTGG
NLRP1-E4C-R	CCCCATCTAACCCATGCTT
NLRP1-E4D-F	AGCGGAAGGAAAAACTCACA
NLRP1-E4D-R	ACTGCATCAGGTTCCTCCC
NLRP1-E4E-F	GGAGGATGAGAAGGGGAGAG
NLRP1-E4E-R	TCACTGGAGACCCTGATCCT
NLRP1-E5-F	ATGGAGGAGAGGGACCTAGC
NLRP1-E5-R	CAGACCTCCCTCAAACCTCA
NLRP1-E5seq-F**	CAGACAGGCAGGGAGGG
NLRP1-E5seq-R**	AAATCCCTCAGCCCAGACTC
NLRP1-E6-F	AGTACCATGTGCATTTCTGGG
NLRP1-E6-R	CTACTGCCTGGCTGAGATCC
NLRP1-E7-F	CTCAAAAGGCCACAGATGCT
NLRP1-E7-R	GAAACTTGGGTTGGCTATGC
NLRP1-E8-F	GCTGCCTCAGAAGGTGACA
NLRP1-E8-R	GCTGTGGTGTCTGGGTTTCT
NLRP1-E9-F	ACAGACATGAGCCACTGCAC
NLRP1-E9-R	TAACGGAAATCCAGCCAGTT
NLRP1-E10-F	CTAGTGTTCAGGCTGGAGTGC
NLRP1-E10-R	GCTTCTGCTCCTGCTCCA
NLRP1-E11-F	CATGGAGCAGGAGCAGAAG
NLRP1-E11-R	GAGTGTGAGTTGGGGGTAGG
NLRP1-E12-F	TGAAGCCACTGTACCGTATCC
NLRP1-E12-R	CAGAGAAGAGTGGAATGCAGG
NLRP1-E13-F	TGCAGTGAGCCGAGATTGTA
NLRP1-E13-R	TACCTTGGAGAGGCAGGAGA
NLRP1-E14-F	TCTCTGTAGCAGTGCATGGG
NLRP1-E14-R	TTGGAAGCATTCCCACTTTC
NLRP1-E15-F	GTGTCCTTATGGAGCCAAGC
NLRP1-E15-R	AGGCTGGTCTTGAACTCCTG
NLRP1-E16-t2-F	CCAGAATTTGTCTGTGGCTTT
NLRP1-E16-t2-R	GGCCTGACTCTTTGTGAGGT
NLRP1-E17-t2-F	GACACAGGTGCATTTCTGGA
NLRP1-E17-t2-R	GGCAAACCAGATGGCAAC
NLRP1-E16-F	CTGGTTACAGCGTTTCAGCA
NLRP1-E16-R	TTCAAAGACCTGCCTCACCT

* All primers were purchased from MWG (Biotech, Germany)
** Primer pairs used to sequence the Exon 5 (E5) amplicon

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