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► **To cite this version:**

Alessandra Pontillo, Elisa Paoluzzi, Sergio Crovella. The inhibition of mevalonate pathway induces up-regulation of NALP3 expression: new insight in the pathogenesis of mevalonate kinase deficiency.. European Journal of Human Genetics, 2010, n/a (n/a), pp.n/a-n/a. 10.1038/ejhg.2010.9. hal-00511183

HAL Id: hal-00511183

<https://hal.science/hal-00511183>

Submitted on 24 Aug 2010

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1 **Title page.**

2

3 **The inhibition of mevalonate pathway induces up-regulation of *NALP3* expression:**
4 **new insight in the pathogenesis of mevalonate kinase deficiency.**

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10

11 **Running title:** NALP3 and MKD.

12

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1 **ABSTRACT.**

2 Mevalonate Kinase Deficiency (MKD) is a rare hereditary auto-inflammatory syndrome
3 due to mutations in mevalonate kinase, the second enzyme of mevalonate pathway of
4 cholesterol and non sterol-isoprenoids biosynthesis. The shortage of mevalonate-derived
5 intermediates, and in particular of geranylgeranyl pyrophosphate (GGPP), has been
6 linked to the activation of caspase-1 and thereby to the production of IL-1 β , but the true
7 concatenation of these two events has not been clarified yet.

8 We hypothesized that inflammasomes could mediate the activation of caspase-1 due to
9 the shortage of GGPP.

10 We monitored the expression of the principal proteins (*NALP1*, *NALP3*, *IPAF*) of the
11 three known inflammasomes, first in a cellular model of MKD, then in two MKD
12 patients, after bacterial lipopolysaccharide (LPS) stimulation.

13 In healthy subjects, alendronate alone induced the expression of *NALP1* and *NALP3*,
14 when together with LPS it induced a dramatic increase in *NALP3* expression. In MKD
15 patients, *NALP3* expression was higher than in untreated healthy controls.

16 Our results, even though preliminary, demonstrated that the inhibition of the mevalonate
17 pathway lead to a hyper-expression of *NALP3*, suggesting a possible involvement of
18 *NALP3*-inflammasome in the activation of caspase-1 consequent to GGPP decrement.

19 This is the first time that the involvement of the inflammasome complexes was
20 demonstrated in MKD pathogenesis.

21

22 **Key words:** Mevalonate kinase deficiency, inflammasome, *NALP3*, caspase-1.

23

1 INTRODUCTION

2 Mevalonate Kinase Deficiency (MKD) is a rare autosomal recessive disease (MIM
3 #610377) due to mutation in MVK gene (12q24) and to the consequent reduced
4 enzymatic activity of mevalonate kinase (MK), the second enzyme of mevalonate
5 pathway.

6 MKD belongs to auto-inflammatory syndromes and it is characterized by periodic
7 fevers variably associated to lympho-adenopathy, abdominal involvement, arthralgia,
8 rash, and in most severe cases, to neurological impairment.

9 The pathogenesis of MKD is not fully understood. Recently it was reported that the
10 reduced level of geranylgeranyl pyrophosphate (GGPP), a downstream mevalonate-
11 derived intermediate, lead to the activation of caspase-1 and to the secretion of the pro-
12 inflammatory cytokine IL-1 β (1;2) explaining, almost in part, the link between the
13 metabolic pathway impairment and the inflammatory phenotype.

14 The NLR (nucleotide-binding domain leucine-rich repeat containing) family of
15 intracellular sensors is a crucial component of the innate immune system. Three
16 members of NLR family (NALP1/*NLRP1*, NALP3/*NLRP3*, IPAF/*NLRC4*) form
17 multiprotein complexes, called inflammasomes, and are capable of activating the
18 caspase-1 in response to a wide range of stimuli including microbial and danger/stress.

19 (3). Mutations within *NLRP1* and *NLRP3* genes have been associated with several
20 human diseases; sequence variants in the *NLRP1* gene have been linked to auto-immune
21 and auto-inflammatory diseases associated with vitiligo (4), and mutations in *NLRP3*
22 are responsible for the auto-inflammatory syndromes, Muckle-Wells syndrome, familial
23 cold auto-inflammatory syndrome, and neonatal-onset multisystem inflammatory
24 disease known collectively as cryopyrin associated periodic syndrome (CAPS) (5).

1 In the pathogenesis of MKD, it is not clear how the reduction of geranyl-geranylation
2 leads to the activation of caspase-1; therefore, we hypothesized that this event could be
3 mediated by the inflammasome, because of the key role of this protein complex in the
4 inflammatory process and particularly in IL-1 β secretion (3).

5 Recently we described a mouse model of MKD obtained through the inhibition of
6 mevalonate pathway with alendronate combined with the induction of acute
7 inflammation with a bacterial compound (6). Similarly we treated human monocytes
8 with alendronate and bacterial lipopolysaccharide (LPS) to simulate a cellular model for
9 a MKD typical episode.

10 Since NALP1/*NLRP1*, NALP3/*NLRP3* and IPAF/*NLRC4* are the principal and
11 characteristic molecules of the respective inflammasome, we monitored their expression
12 firstly in our experimental cellular model of MKD pathology, then in 2 MKD patients,
13 in order to verify their role as specific markers for the involvement of each
14 inflammasome.

15

16 MATERIAL AND METHODS.

17 **Cells isolation and culture.** After approval by the ethics review board of the IRCCS
18 “Burlo Garofolo” and after written informed consent, blood was drawn from 8 healthy
19 volunteers and from 2 patients with MKD. Human peripheral blood mononuclear cells
20 (HPBM) were isolated by density gradient centrifugation using Ficoll-Paque (*GE*
21 *Healthcare, USA*) and cultured in RPMI-1640 (*Euroclone, Italy*) containing 2 mM
22 glutamine, 100 U/ml penicillin-streptomycin and 10% Fetal Calf Serum (FCS,
23 *Euroclone*). 2x10⁶ HPBM from healthy donors or MKD patients were incubated with or
24 - in the case of patients - without alendronate 100 μ M for 24h and with LPS 1 μ g/ml for

1 4h. Alendronate (*Sigma Aldrich*) and LPS (*Sigma Aldrich*) were dissolved in distilled
2 water (respectively at 10 mM and 1mg/ml).

3 **Patients.** Two patients affected by MKD were enrolled in this study (P1 male, 12 years
4 old; P2 female, 20 years old). They both showed characteristic clinical features of the
5 disease, such as fever (3-5 days) every 2-3 weeks, always accompanied by lympho-
6 adenopathy, skin rash, abdominal pain, arthralgy. Laboratory exams showed increased
7 inflammatory markers (CRP, ESR) and leucocytosis during fever episodes. Compound
8 heterozygosity for common mutations in *MVK* gene were identified in both patients. P1
9 carries the V377I (c.1129G>A) and the I268T (c.803T>C) substitution; P2 the V377I
10 and the deletion c.16-34 del.

11 **IL-1 β measurement.** IL-1 β measurement was performed using the Endogen IL-1 β
12 ELISA kit (*Pierce Biotechnology Inc., USA*). Results were expressed as pg/ml.

13 **RNA isolation and expression analysis.** Total RNA was extracted using the
14 Purescript® kit (*Gentra Systems, USA*) and retro-transcribed with the GeneAmp RNA
15 PCR kit (*Applied Biosystems, USA*). *NLRP1*, *NLRP3*, *NLRC4*, *CASP1* were amplified
16 with specific TaqMan® Gene Expression Assays (Hs00248187_m1, Hs00366465_m1,
17 Hs00368367_m1, Hs00354838_m1) using the ABI 7900 HT SDS platform (*Applied*
18 *Biosystems*). *HPRT1* was the housekeeping gene (4333768F, *Applied Biosystems*) used
19 for normalization. Relative quantitative expression was calculated as 2^{-DDCT} following
20 the indications of Livak et al. [7].

21

22 **RESULTS.**

23 HPBM were cultured for 24 hours in the presence or absence of alendronate and
24 stimulated for an additional 4 hours with LPS. The inhibition of mevalonate pathway

1 with alendronate is able to induce in monocytes the secretion of IL-1 β in the presence of
2 a second stimulus (Figure 1) as previously reported for RAW 264 murine macrophage
3 cells and J774.1 cells (8; 9).

4 Expression data obtained in ALD-, LPS- and ALD/LPS- treated HPBM were compared
5 to those acquired using untreated cells (R), and the results obtained in a representative
6 experiment are reported in Figure 1.

7 In healthy subjects alendronate induced about a 2 fold increase in the expression of
8 *NLRP1* and *NLRP3*. LPS induced a 4 fold increase in *NLRP3* expression. ALD/LPS
9 induced a major increase in *NLRP3* expression (10.55 fold). *NLRP4* was not detectable
10 in HPBM. The ALD/LPS induction of *NLRP3* was always accompanied by the
11 induction of *CASP1* and by the relevant secretion of IL-1 β (Figure 1).

12 When HPBM isolated from healthy controls were compared with HPBM from MKD
13 subjects, we found an increase in the basal expression of *NLRP3* in the patients with the
14 disease. In particular, as showed for patient P1 in Figure 2A, in the untreated patient
15 *NLRP3* was 3.41 fold more expressed in comparison to untreated healthy control.

16 MKD HPBM stimulated with LPS showed increased levels of *NLRP1* and *NLRP4*
17 mRNA when compared to untreated ones (R), whereas *NLRP3* expression was the same
18 in R and LPS-treated cells. In patient P1 *NLRP1* and *NLRP4* were induced by LPS
19 treatment showing expression values of 2.80 fold and 2.65 fold with respect to the
20 untreated cells (Figure 2B). A similar expression pattern was observed also in the other
21 patient even if the induction rates varied (*NLRP1* = 0.82 fold; *NLRP3* = 1.27 fold;
22 *NLRP4* = 0.74 fold; *CASP1* = 1.95 fold).

23

24 **DISCUSSION.**

1 MKD is an auto-inflammatory syndrome, caused by a genetic defect in mevalonate
2 pathway and isoprenoid biosynthesis. Recent studies have established that patients have
3 hyperactive caspase-1 due to a shortage of geranyl-geranylated proteins and an
4 increased IL-1 β secretion [1-2]. When PAMPs or DAMPs (i.e. vaccine, microbial
5 infection, stress, fatigue) stimulate innate immune system of MKD patients, it results in
6 typical inflammatory attacks.

7 In this study, we considered the hypothesis that the activation of caspase-1 - due to the
8 inhibition of mevalonate pathway - could be mediated by one of the three known
9 inflammasomes. Indeed the principal role demonstrated for NALP1-, NALP3- and
10 IPAF-inflammasome is the cleavage of pro-caspase-1 in its active form.

11 Because of the limited number of patients, we used a cellular model based on the
12 treatment of human monocytes with the aminobisphosphonate alendronate (inhibitor of
13 mevalonate pathway) and a bacterial compound, to substantiate the inflammasome
14 involvement in the pathogenesis of MKD, through the study of the NALP1/*NLRP1*,
15 NALP3/*NLRP3*, IPAF/*NLRC4* expression.

16 In this model, we have demonstrated that alendronate alone and even more together
17 with LPS, induces a great IL-1 β secretion and also up-regulates the transcription of
18 *CASP1* and of the three inflammasome' genes. In particular, NALP3/*NLRP3* seems to
19 be the most susceptible to inhibition of the mevalonate pathway. In physiologic
20 condition *NLRP3* expression could be induced by common pro-inflammatory
21 compounds, such as LPS, TNF and IL-1 β [10]; this is the first time to our knowledge
22 that the induction of *NLRP3* through a metabolic impairment was reported.

23 When the same experiments were conducted in monocytes isolated from the only two
24 MKD patients available at the moment of the study, we observed that NALP3/*NLRP3* is

1 hyper-expressed already in untreated cells and probably it was the reason why it could
2 not be further inducible by LPS. This result suggests that MKD patients are
3 characterized by basal high levels of *NLRP3*, independently of any kind of stimulation.
4 The association between the genetic defect in mevalonate pathway and *NLRP3*
5 transcription needs to be replicated in a greater number of MKD patients.
6 Differently to what expected on the basis of the results obtained in our cellular model,
7 the induction of *NLRP1* and *NLRC4* by LPS treatment was observed only in patients
8 and not in the control samples, leading us to hypothesize that being *NLRP3* transcription
9 dysregulated, other inflammasomes (i.e. *NLRP1* and *NLRC4*) can play a role in the
10 regulation of inflammation.
11 Of course we cannot exclude that a more general consequence of the mevalonate
12 pathway metabolic block involves other genes besides *NLRP3* and inflammasome. How
13 the metabolic dysregulation could affect the gene transcription is currently under
14 investigation and we cannot exclude that an epigenetic effect occurs.
15 Further investigations are needed to confirm the involvement of NALP3-inflammasome
16 deregulation in the pathogenesis of MKD. In chronic inflammatory disorders, such as
17 rheumatoid arthritis, *NLRP3* has been reported to be up-regulated [11] but no role for
18 NALP3-inflammasome has been demonstrated so far for in the pathogenesis of this
19 disease. Considering that the constitutive activation of NALP3 inflammasome is
20 associated to several auto-inflammatory syndromes such as Familial Cold Urticaria,
21 Muckle Wells syndrome, CINCA syndrome and Familial Mediterranean Fever, it was
22 conceivable that, at least indirectly, its deregulation could be involved also in the
23 pathogenesis of MKD. Furthermore the constitutive activation of NALP3

1 inflammasome leads to decreased apoptosis [13], an event that was observed also in
2 leucocytes from MKD subjects [14].

3 It could be interesting to monitor *NLRP3* expression in our previously reported animal
4 model of MKD after treatment with alendronate [6]. Moreover the role of NALP3-
5 inflammasome in the inflammation induced by the mevalonate pathway inhibition could
6 be substantiated in mice KO for *NLRP3* or other inflammasome genes.

7 A proteomic approach nowadays is difficult because of the lack of easy technical
8 methods to discriminate the active form of NALP3 from its inactive one. All the
9 research in this field has been made using recombinant mutated proteins. [12]

10 The results reported in this study, although preliminary, support our hypothesis about
11 the involvement inflammasomes, in particular NALP3 inflammasome, in the activation
12 of caspase-1 within the pathogenetic events that starting from the impairment of
13 mevalonate-derived intermediates lead to the inflammatory systemic effects observed in
14 MKD.

15

16 **Competing interests**

17 The authors declare that they have no competing interests.

18

19 **Acknowledgements**

20 We acknowledge the participation of the patients, control subjects and family members.

21 This work was funded by the Institute for Maternal and Child Health - IRCCS "Burlo
22 Garofolo", Trieste, Italy (RC 13/08).

23

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1 **Titles and legends to figures.**

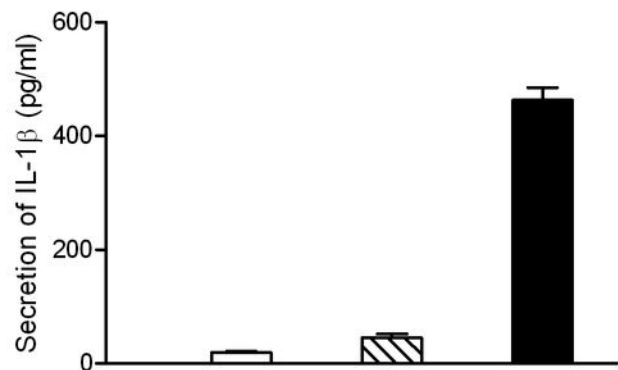
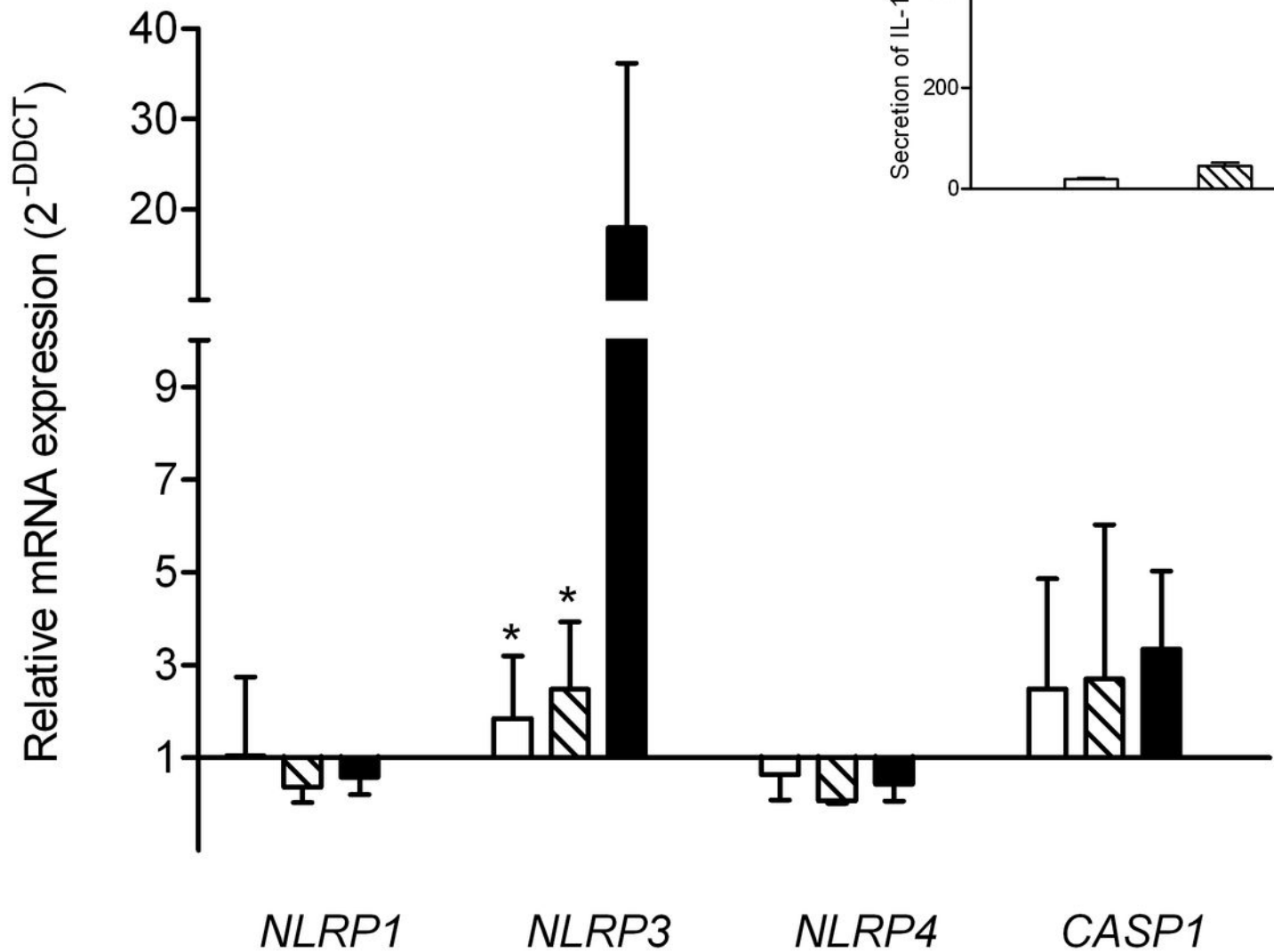
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3 **Figure 1. ALD and ALD/LPS treatment alter inflammasome genes expression in**
4 **HPBM.** HPBM from healthy blood donors were treated with alendronate (ALD),
5 bacterial lipopolysaccharide (LPS), or ALD/LPS. Expression of *NLRP1/NALP1*,
6 *NLRP3/NALP3*, *NLRC4/IPAF*, *CASP1/ICE1* was measured by Real Time quantitative
7 PCR, and results normalized to *HPRT1* expression. Expression of untreated cells was
8 normalized to 1 (not reported in the graph). Expression data for the 8 experiments are
9 reported as 2^{-DDCt} average \pm standard deviation., where $DDCt = DCt_{stimulated_{HC}} -$
10 $DCt_{R_{HC}}$, **are relative to one of the experiments as exemplificative.** The small panel
11 above shows average \pm standard deviation (for the 8 experiments performed) of **the** IL-
12 1β secretion of **relative to the same experiment.** Results of t-test were expressed with *
13 $p < 0.05$; ** $p < 0.01$.

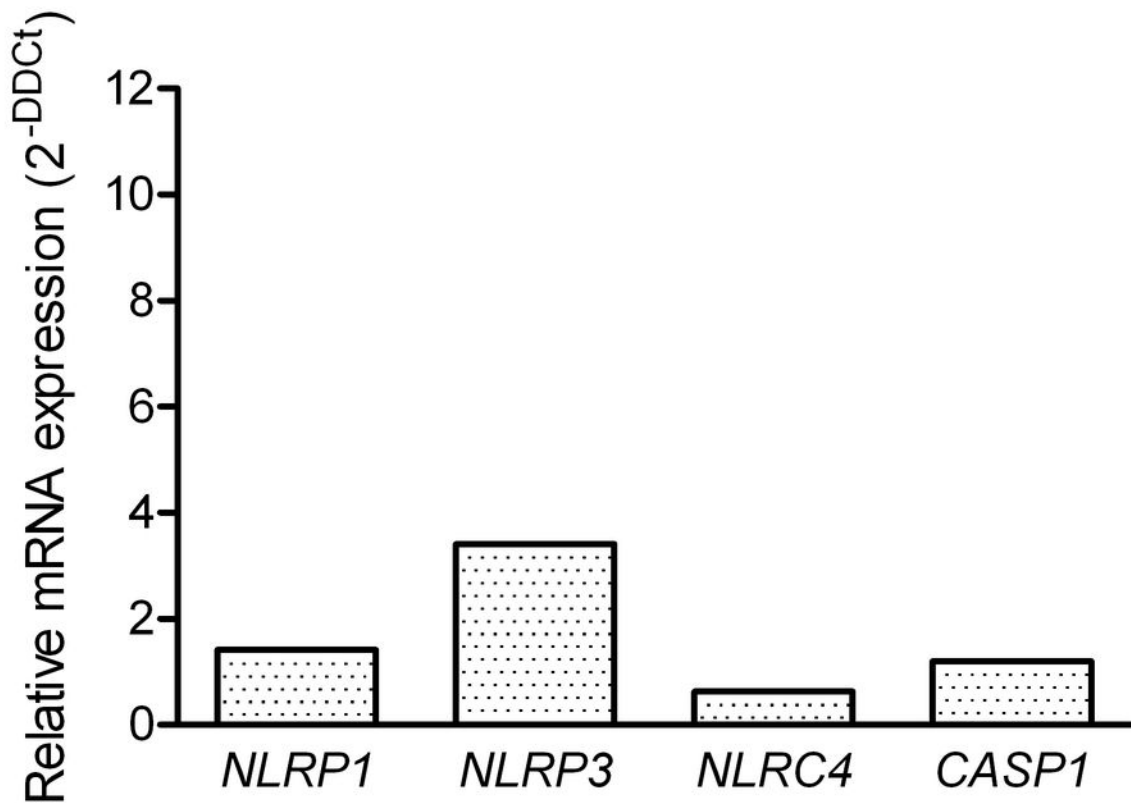
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15 **Figure 2. Inflammasome genes expression in MKD patients.** Expression of
16 *NLRP1/NALP1*, *NLRP3/NALP3*, *NLRC4/IPAF*, *CASP1/ICE1* in one subjects affected
17 by MKD was measured by Real Time quantitative PCR, and results normalized to
18 *HPRT1* expression. **(2A)** Basal (resting, R) mRNA level of *NLRP1*, *NLRP3*, *NLRC4*,
19 *CASP1* were compared in HPMB isolated from the MKD patient and from one healthy
20 donor and reported as 2^{-DDCt} , where $DDCt = DCt_{R_{MKD}} - DCt_{R_{HC}}$. **(2B)** HPBM from
21 the MKD patient were treated with LPS and the mRNA level of *NLRP1*, *NLRP3*,
22 *NLRC4*, *CASP1* were evaluated by comparing patient expression, after LPS induction,
23 with patient expression in basal and untreated condition (R). Expression results are
24 reported as 2^{-DDCt} , where $DDCt = DCt_{LPS_{MKD}} - DCt_{R_{MKD}}$. Expression of untreated

- 1 cells is normalized to 1 (not reported in the graph). The LPS-induced secretion of IL-1 β
- 2 relative to the same experiment is reported on the right of the graph.
- 3 Data are referring to one of the two patients studied.



2A



2B

