



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

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HAL Authorization

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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22

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 $\beta$ , 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 $\beta$  (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 $\beta$  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*). 2x10<sup>6</sup> HPBM from healthy donors or MKD patients were incubated with or  
24 - in the case of patients - without alendronate 100 $\mu$ M for 24h and with LPS 1 $\mu$ 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 $\beta$  measurement.** IL-1 $\beta$  measurement was performed using the Endogen IL-1 $\beta$   
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 $\beta$  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 $\beta$  (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).

## 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 $\beta$  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 $\beta$  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 $\beta$  [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

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**Titles and legends to figures.**

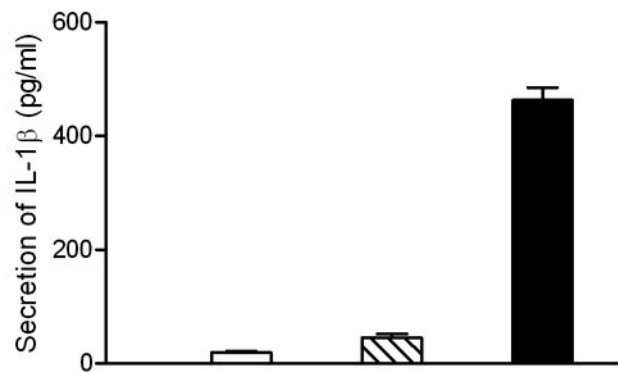
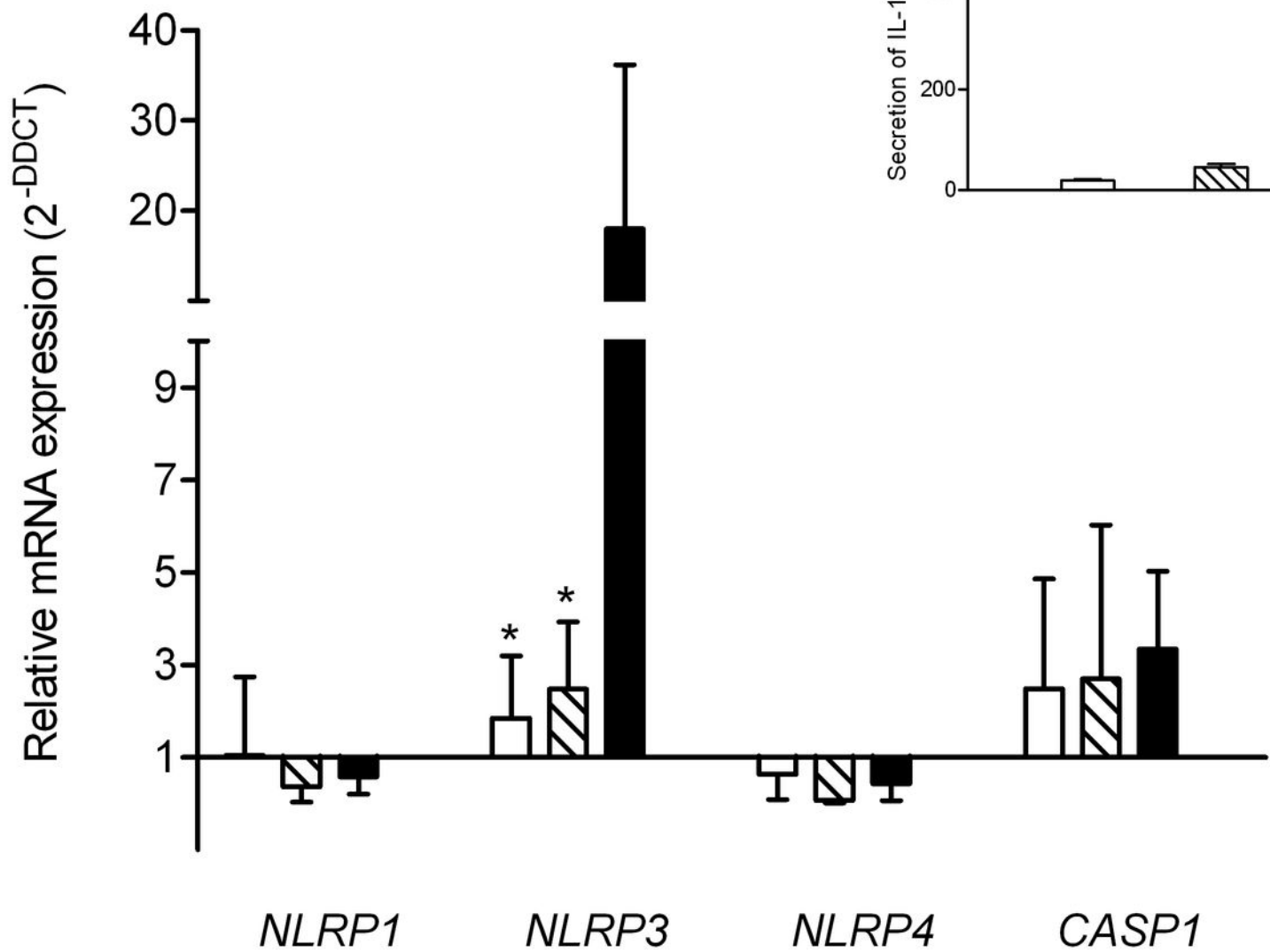
**Figure 1. ALD and ALD/LPS treatment alter inflammasome genes expression in**

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

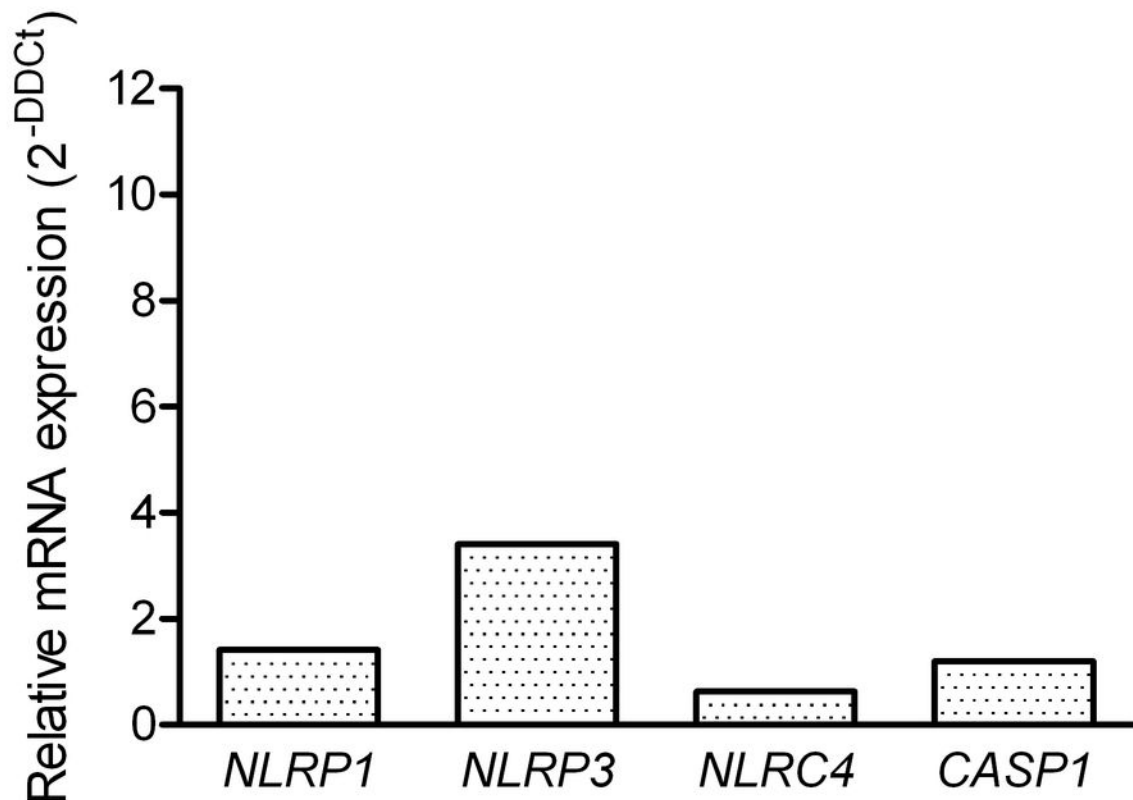
**Figure 2. Inflammasome genes expression in MKD patients.**

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

- 1 cells is normalized to 1 (not reported in the graph). The LPS-induced secretion of IL-1 $\beta$
- 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**

