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Natural Isoprenoids are Able to Reduce Inflammation in a Mouse Model of Mevalonate Kinase Deficiency

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ABSTRACT: Mevalonate kinase deficiency (MKD) is a rare disorder characterized by recurrent inflammatory episodes and, in most severe cases, by psychomotor delay. Defective synthesis of isoprenoids has been associated with the inflammatory phenotype in these patients, but the molecular mechanisms involved are still poorly understood, and, so far, no specific therapy is available for this disorder. Drugs like aminobisphosphonates, which inhibit the mevalonate pathway causing a relative defect in isoprenoids synthesis, have been also associated to an inflammatory phenotype. Recent data asserted that cell inflammation could be reversed by the addition of some isoprenoids, such as geranylgeraniol and farnesyl pyrophosphate. In this study, a mouse model for typical MKD inflammatory episode was obtained treating BALB/c mice with aminobisphosphonate alendronate and bacterial muramyldipeptide. The effect of exogenous isoprenoids—geraniol, farnesol, and geranylgeraniol—was therefore evaluated in this model. All these compounds were effective in preventing the inflammation induced by alendronate-muramyldipeptide, suggesting a possible role for these compounds in the treatment of MKD in humans. **(*Pediatr Res* 64: 177–182, 2008)**

Mevalonate kinase deficiency (MKD) is an autosomal recessive inborn disorder of cholesterol biosynthesis, due to mutations in the mevalonate kinase gene (*MVK*) coding for mevalonate kinase (MK), the second enzyme of the mevalonate pathway for the biosynthesis of cholesterol and nonsterol isoprenes. Different degrees of disease severity were observed, being linked with the residual activity of MK, ranging from autoinflammatory hyper immunoglobulinemia D and periodic fever syndrome (HIDS, OMIM 260920), with a 1% to 8% residual MK activity, to mevalonic aciduria (MA, OMIM 610377) in which MK activity is below the level of detection. Patients with the HIDS phenotype typically present only recurrent episodes of fever and associated inflammatory symptoms, whereas patients with MA show, in addition to these episodes, developmental delay, dysmorphic features, ataxia, cerebellar atrophy, psychomotor retardation, and may die in early childhood (1).

HIDS patients usually are treated with anti-inflammatory drugs and in particular corticosteroids; thalidomide is also used but its effect is limited (2). In most severe cases, patients may benefit from treatment with biologic agents such as etanercept and anakinra (1,3–5). No treatment has been proven effective in curing the neurological symptoms in severe cases of MKD. Statins, inhibitors of 3-hydroxy-3-methylglutaryl-Coenzime A (HMG-CoA) reductase, (the first enzyme of the mevalonate pathway), were paradoxically useful in selected cases of MKD, probably due to an indirect effect on MK induction (6). However, the same treatment was previously reported to severely worsen the disease in MA patients (7) and therefore has to be used with great care.

Although the genetic defect has been known for a decade, the molecular mechanisms underlying the inflammatory phenotype are still unclear, and thus an etiologic treatment for MKD is still unavailable. Frenkel *et al.* (8) demonstrated that the disease is not due to the accumulation of mevalonate, but rather to the lack of mevalonate-derived isoprenoids. This hypothesis is in agreement with clinical and biological data showing that drugs like statins and aminobisphosphonates, which produce some defect in mevalonate metabolism blocking geranylgeranyl-pyrophosphatase, could lead to inflammatory reactions both "*in vitro*" and "*in vivo*" (8–14). Moreover, it was suggested that *in vitro* inflammation could be reversed by the addition of some isoprenoids, such as geranylgeraniol (GGOH) and farnesyl pyrophosphate (8). Starting from these observations, we studied the effect of exogenous isoprenoids, namely geraniol (GOH), farnesol (FOH), and GGOH, in BALB/c mice with an MKD-like inflammatory disorder induced by aminobisphosphonates and muramyldipeptide (MDP). This pro-inflammatory bacterial compound was administered together with the aminobisphosphonate alendronate, to trigger a more severe systemic inflammatory response. This animal model seems to reproduce quite well the vacci-

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Abbreviations: ALD, alendronate; **FOH,** farnesol; **GGOH,** geranylgeraniol; **GOH,** geraniol; **HIDS,** hyper immunoglobulinemia D and periodic fever syndrome; **LPS,** lipopolysaccharide; **MDP,** muramyldipeptide; **MK,** mevalonate kinase (EC 2.7.1.36); **MKD,** mevalonate kinase deficiency; **MVK,** mevalonate kinase gene (NM_000431)

nation-induced inflammatory response typical of MKD (6). Isoprenoids, in particular GOH and GGOH, were able to inhibit the inflammatory reaction in these mice. The possibility of rescuing the inflammatory phenotype by the addition of exogenous isoprenoids suggests a possible use of these compounds in the treatment of patients affected by MKD.

MATERIALS AND METHODS

Animals. BALB/c male mice (Harlan, Udine, Italy) aged 6 ± 8 wk and weighting between 25 and 30 g were used. The mice had free access to tap water and pelleted food and were housed in standard cages with a 12 h light/dark cycle. Environmental temperature was constantly maintained at 21°C and the mice were kept under pathogen-free conditions. All experiments were carried out in accordance with Italians laws (Ministry of Health registration n 62/2000-B, October 6, 2000) and complied with the Guidelines for Care and Use of Laboratory Animals at the Trieste University.

Chemicals. Alendronate (ALD), *N*-acetylmuramyl-L-alanyl-D-isoglutamine hydrate (MDP), and GGOH were obtained from Sigma Chemical Co. Aldrich (St. Louis, MO); GOH and FOH were from Euphar group s.r.l. (Piacenza, Italy). The substances were dissolved in sterile saline (pH 7.0) and injected intraperitoneally in a volume of 0.1 mL per 10 g body weight.

Alendronate induced inflammation. Animals were randomly selected and divided in four groups: group 1, controls (saline); group 2, alendronate 40 μmol/kg on day 0; group 3, alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3; group 4, MDP 100 μg/kg on day 3. Animals were killed by decapitation on day 3, 2 h after MDP administration.

Isoprenoid treatment. In different experiments, three isoprenoids were tested: GOH, FOH, and GGOH. GOH and FOH were administered at 250 mg/kg or at 500 mg/kg in a single dose before alendronate injection (day −1), together (day 0), and the day after (day 1), or in a dose for 2 d in different combinations (day −1/0; day 0/1; day −1/1). GGOH was administered at 250 mg/kg in a dose at days 0 and 1.

Determination of serum amyloid-A (SAA). Blood was collected directly into test tubes following decapitation. Serum was recovered by centrifugation at 2000 × g at 4°C, and then stored at −80°C until used. The SAA was assayed using ELISA kits (Biosource, Camarillo, CA), the experimental procedures being performed according to the instruction of the manufacturer, and the amount of SAA expressed as microgram per milliliter serum.

Determination of the number of cells in the peritoneal exudate. Peritoneal exudate cells (PEC) were obtained as follows. Immediately after decapitation, 2 mL of PBS with BSA (0.1%) were injected into the peritoneal cavity, and the cavity was massaged for 4 min. The fluid (about 1.5 mL) was recovered using a syringe and the number of cells was counted after appropriate dilution using a Bürker chamber.

Spleen histological analysis. Spleens were embedded in OCT compound (Embedding Matrix, Kaltek srl, Italy), cut at 8 μm in a cryostat (Slee Cryostat, Emme 3 Bioteclonogie, Italy) and sections were stained with hematoxylin and eosin. Spleen leukocyte infiltration was evaluated using a Leica DC100 microscope.

Data analysis. SAA and PEC values are given as mean ± SE. The statistical significance of differences was analyzed using an unpaired *t* test.

RESULTS

Alendronate induced inflammation in BALB/c mice. Saline solution or alendronate (40 μmol/kg) were given to BALB/c mice at the first day of our experimental plan (day 0). Evaluation of inflammatory markers, SAA and PEC, was done 3 d later (on day 3), considering that this was the time required to obtain the maximum increase of inflammation as reported elsewhere (15,16). Alendronate induced a marked increase in SAA mean level (53.2 μg/mL compared with 0.8 μg/mL with saline) (Fig. 1*A*) and PEC number (487.5 × 10^4 cells compared with 79.0 × 10^4 cells in controls) (Fig. 1*B*).

The inflammatory response to MDP was studied in alendronate treated mice compared with controls. MDP showed a strong inflammatory effect in alendronate-treated mice, both as SAA levels (123.4 μg/mL compared with 53.2 μg/mL in ALD-treated mice) (Fig. 1*A*), and PEC number (777.5 × 10^4 cells compared with 487.5 × 10^4 cells in ALD-treated mice) (Fig. 1*B*). MDP alone induced only a mild, yet significant, increase in SAA (7.5 μg/mL compared with 0.8 μg/mL in controls) (Fig. 1*A*).

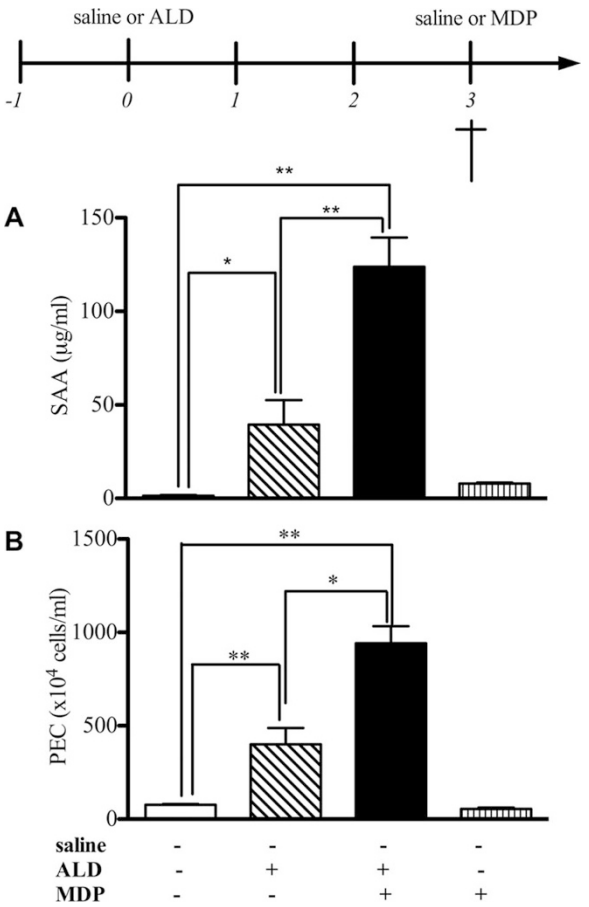

Figure 1. SAA levels (*A*) and PEC number (*B*) in controls (saline, $n = 9$), in mice treated with alendronate 40 μmol/kg on day 0 (ALD, $n = 16$), alendronate 40 μmol/kg on day 0, and MDP 100 μg/kg on day 3 (ALD/MDP, $n = 16$) or MDP 100 μg/kg alone on day 3 (MDP, $n = 9$). Data are reported as mean ± SE. Statistical significance was evaluated using a one-tailed *t* test for unpaired data. $*p < 0.05$; $**p < 0.001$.

These data were consistent with the hypothesis that the inhibition of mevalonate pathway could overdraw the physiologic activation of inflammatory process.

GOH reduces the MDP-induced inflammatory response in alendronate/MDP-treated mice. Hypothesizing that exogenous isoprenoids could rescue the deregulation of mevalonate pathway induced by amino-bisphosphonate, and the related inflammatory response, a natural monoterpene compound, GOH, was implied in our MKD mouse model.

GOH 250 mg/kg, given in two doses at day 0 and 1 significantly reduced SAA level and PEC number in alendronate-MDP-treated mice (SAA: 34.4 μg/mL in alendronate-MDP plus GOH compared with 100.0 μg/mL in alendronate-MDP; PEC: 115.2 × 10^4 cells in alendronate-MDP plus GOH compared with 744.9 × 10^4 cells in alendronate-MDP), but not in alendronate-treated ones (Fig. 2). In the same series of experiments, GOH was not able to significantly reduce MDP-induced inflammation.

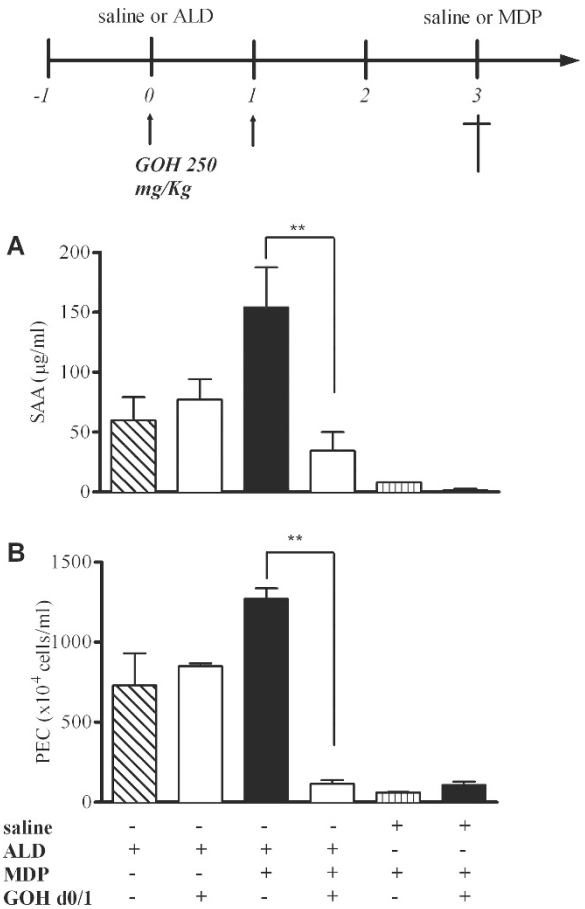

Figure 2. SAA levels (*A*) and PEC number (*B*) in mice treated with alendronate 40 μmol/kg on day 0 (ALD, $n = 11$); alendronate 40 μmol/kg on day 0 plus geraniol 250 mg/kg on days 0 and 1 (ALD + GOH, $n = 11$); alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3 (ALD/MDP, $n = 8$); alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3, plus geraniol 250 mg/kg on days 0 and 1 (ALD/MDP + GOH, $n = 9$); saline on day 0 and MDP 100 μg/kg on day 3 (MDP, $n = 6$); saline on day 0 and MDP 100 μg/kg on day 3, plus geraniol 250 mg/kg on days 0 and 1 (MDP + GOH, $n = 3$). Data are reported as mean ± SE. Statistical significance was evaluated using a one-tailed t test for unpaired data. *$p < 0.05$; **$p < 0.001$.

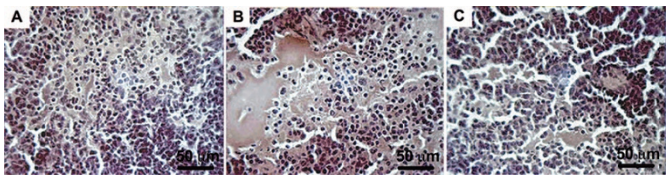

Figure 3. Hematoxylin and eosin-stained spleen from a control animal (*A*), a mouse treated with 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3 (*B*), and a mouse treated with alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3, plus geraniol 250 mg/kg on days 0 and 1 (*C*). The infiltration of lymphomonocytic and polymorphonuclear cells is evident in panel *B*. Treatment of animals with geraniol almost completely prevented the alendronate-MDP-induced leukocyte infiltration. Light microscope magnification: ×40.

The anti-inflammatory effect of GOH in ALD/MDP-mice was also evident in histological analysis of the animal spleens (Fig. 3). The leukocyte infiltration was present in the spleen from alendronate-MDP-treated mice (Fig. 3*B*), while it was clearly reduced in animals treated with GOH (Fig. 3*C*).

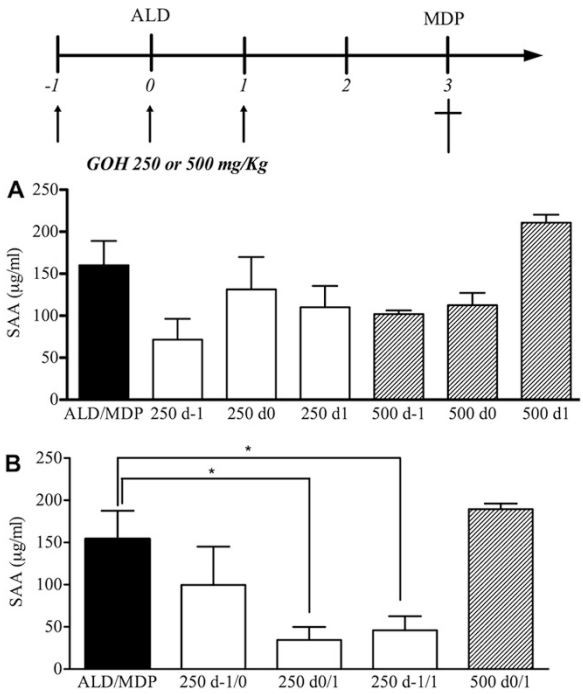

Figure 4. (*A*) SAA levels in mice treated with alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3 (ALD/MDP, $n = 14$); alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3, plus geraniol 250 mg/kg on day −1 (250 d-1, $n = 5$), on day 0 (250 d0, $n = 4$), on day 1 (250 d1, $n = 9$); alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3, plus geraniol 500 mg/kg on day −1 (500 d−1, $n = 4$), on day 0 (500 d0, $n = 4$), on day 1 (500 d1, $n = 4$). (*B*) SAA levels in mice treated with alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3 (ALD/MDP, $n = 12$); alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3, plus geraniol 250 mg/kg on day −1 and 0 (250 d−1/0, $n = 4$), on day 0 and 1 (250 d0/1, $n = 9$), on day −1 and 1 (250 d−1/1, $n = 6$), and plus geraniol 500 mg/kg on day 0 and 1 (500 d0/1, $n = 4$). Data are reported as mean ± SE. Statistical significance was evaluated using a one-tailed t test for unpaired data. *$p < 0.05$; (3*B*) geraniol 250 d0/1 *vs* ALD/MDP $p = 0.0046$; geraniol d-1/1 *vs* ALD/MDP $p = 0.0223$.

This effective dose schedule of administration of the GOH was chosen after some preliminary experiments. ALD/MDP-mice were treated with single dose of GOH at two concentrations (250 mg/kg or 500 mg/kg). It was administered together with (day 0), after (day 1), and before (day −1) alendronate. As described in Fig. 4*A*, no dose response was seen, whereas an inhibitory effect linked to the day of administration: GOH 250 mg/kg given at day −1 seems to have the best reducing effect on SAA level, but not in a statistical significant way. A second point was investigated: the administration of more than one dose of isoprenoid during the experimental plan. Established that 250 mg/Kg was the minor dose with maximum effect, GOH was given in different daily combination as reported in Fig. 4*B*. We obtained different inhibitory effect on SAA levels: day −1/0 > day −1/1 > day 0/1. As outlined above, the combination of daily doses seems to be important for the inhibitory effect of GOH. All these data were also reproduced with the other acute response marker, PEC number (data not shown).

Other isoprenoids are differently effective on alendronate-MDP-induced inflammation. We studied the effect of two other isoprenoid compounds, GGOH and FOH, previously

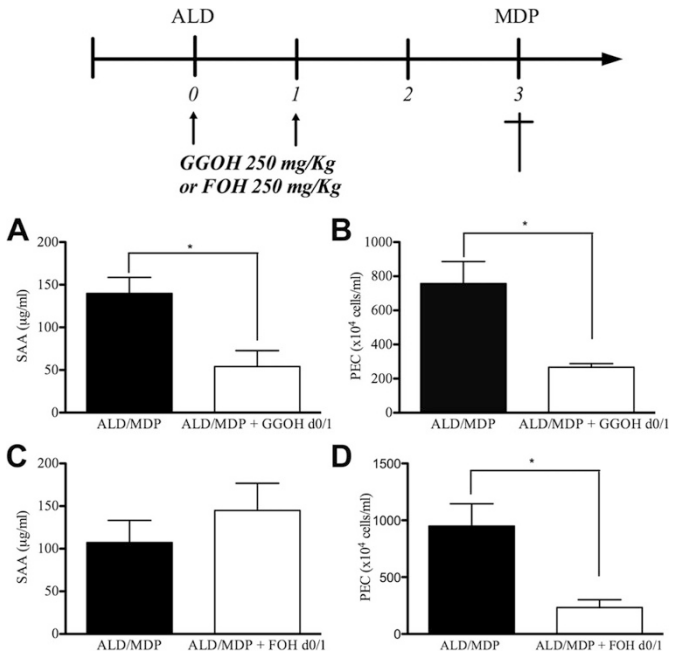

Figure 5. SAA levels (*A*) and PEC number (*B*) in mice treated with alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3 (ALD/MDP, $n = 4$); alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3, plus geranylgeraniol 250 mg/kg on days 0 and 1 (ALD/MDP + GGOH d0/1, $n = 4$). SAA levels (*C*) and PEC number (*D*) in mice treated with alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3 (ALD/MDP, $n = 4$); alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3, plus farnesol 250 mg/kg on days 0 and 1 (ALD/MDP + FOH d0/1, $n = 4$). Data are reported as mean ± SE. Statistical significance was evaluated using a one-tailed *t* test for unpaired data. $*p < 0.05$.

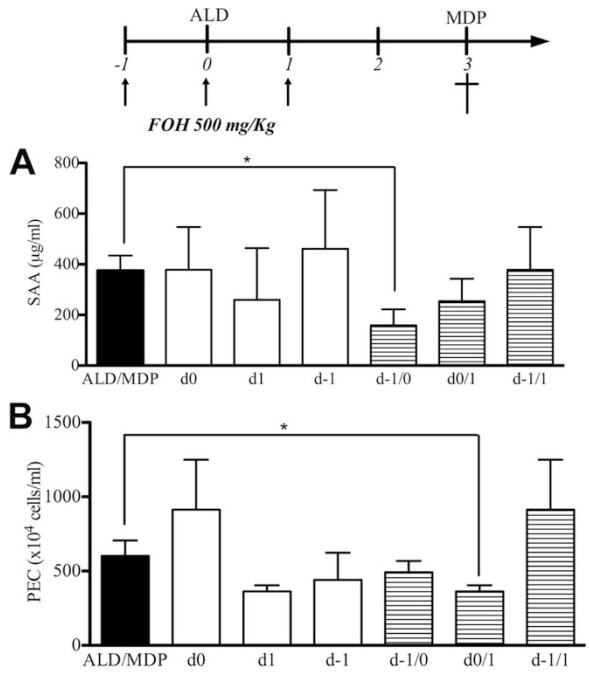

Figure 6. SAA levels (*A*) and PEC (*B*) number in mice treated with alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3 (ALD/MDP, $n = 7$), alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3, plus farnesol 500 mg/kg on day 0 (d0, $n = 4$), 1 (d1, $n = 4$) and -1 (d$-$1, $n = 4$). Alendronate 40 μmol/kg on day 0 and MDP 100 μg/kg on day 3, plus farnesol 500 mg/kg on days -1 and 0 (d$-$1/0, $n = 4$), 0 and 1 (d0/1, $n = 4$), -1 and 1 (d$-$1/1, $n = 4$). Data are reported as mean ± SE. Statistical significance was evaluated using a one-tailed *t* test for unpaired data. $*p < 0.05$.

used to rescue the mevalonate pathway inhibition in cellular models (8,17). The compounds were first used in ALD/MDP-treated mice, at the same concentration (250 mg/kg) and timing (days 0 and 1) chosen for GOH (Fig. 5).

GGOH significantly reduced both SAA (Fig. 5*A*) and PEC levels (Fig. 5*B*) (SAA: 57.3 μg/mL in alendronate-MDP plus GGOH compared with 165.6 μg/mL in alendronate-MDP; PEC: 270×10^4 cells in alendronate-MDP plus GGOH compared with 886.2×10^4 cells in alendronate-MDP).

On the other side, inflammation was not totally rescued by FOH, which was able only to significantly lower peritoneal infiltration (Fig. 5*D*) (PEC: 233.767×10^4 cells in alendronate-MDP plus FOH compared with 949.2×10^4 cells in alendronate-MDP) and not SAA (Fig. 5*C*).

To exclude a dose-dependent failure of FOH in reducing SAA levels, we repeated the experiments doubling the dose of the compound (500 mg/kg) and considering single dose administration (at days 0, 1, and -1), and 2 d combinations (at days 0/1, $\times$1/0, and $\times$1/1) on ALD/MDP-mice.

A substantial inhibition both of SAA (Fig. 6*A*) and PEC numbers (Fig. 6*B*) was obtained when FOH was given together (day 0) and the day after (day 1) or together (day 0) and the day before (day -1) the alendronate stimulus. A statistical significant decrease was observed for SAA at day $-1/0$ and for PEC at day 0/1. We hypothesized that increasing number of mice series could give significant values also for the other daily combination (day 0/1 for SAA, and day $-1/0$ for PEC).

Discussion and conclusions. The pathogenesis of the inflammatory phenotype associated to MKD is still not known. It has been hypothesized that inflammatory disorder could be due to a defect in isoprenoid intermediates downstream MK (8). Many studies (1,18,19) were done to investigate the pathogenesis of the disease and to find out an etiologic treatment both for inflammatory symptoms, and especially for severe complications such as amyloidosis and neurological impairment.

Recent findings suggest a pro-inflammatory role of statins and aminobisphosphonates, two classes of molecules involved in the inhibition of cholesterol metabolism, respectively, upstream (HMGCoA reductase) (20) or downstream MK (geranylgeranyl-pyrophosphatase) (8–14).

Starting from these data, we treated BALB/c mice with the aminobisphosphonate alendronate. According to previous research (15,16), alendronate leads to a statistical significant increase of acute phase markers (Fig. 1). To optimize this MKD model and to reproduce, at least in some aspects, the typical periodic inflammatory episode, often induced in patients by mild stimuli, such as a vaccination (6), bacterial MDP was administered to mice as second boost. As expected, the coadministration alendronate plus MDP triggered an acute condition significantly higher than the alendronate alone (Fig. 1). Other authors (15,16) used LPS instead of MDP in alendronate-treated mice, with similar results. In our study, MDP was preferred because it was much better tolerated than LPS by the animals, and because it better represents the "mild stimulus" capable to induce a MKD episode.

Recently, the effect of some natural isoprenoid compounds was tested *in vitro* on peripheral blood cells of HIDS patients, and a partial rescue of inflammatory phenotype was obtained (21). We decided to test the anti-inflammatory effect of these compounds in our MKD-like mice, hypothesizing a possible future use in the human condition.

Exogenous isoprenoid intermediates, GOH, FOH, and GGOH, were administered in alendronate-MDP-treated mice with different results. GOH 250 mg/kg—given together and the day after alendronate—was able to reduce inflammation in alendronate-MDP-treated mice, counteracting the aminobisphosphonate effect (Fig. 2). These data were supported also by the diminished leukocyte infiltration in the spleen from alendronate-MDP mouse treated with GOH (Fig. 3).

These data were partially reproduced using the other two isoprenoids. GGOH—at the same dose and timing of GOH—reduced inflammation in alendronate/MDP-mice, as demonstrated by SAA and PEC levels (Fig. 5). FOH was able to inhibit both SAA levels and PEC values, but only where it was administered in a double dose (500 mg/kg) in two daily injections (Fig. 6).

Our data strengthened previous observations about an important role of isoprenoids in inflammation (8,21–25), even if they do not explain how isoprenoids mediate this event. These compounds play an important role in different biological function, including protein prenylation and production of steroid hormones and biologically active terpenes. It seems unlikely that the alteration in prenylation might have a role in the inflammatory phenotype, as it was proven that prenylation of target proteins Ras and RhoA is normal in fibroblasts of MKD patients (10) and that proteins such as Rho have a positive role in inflammation (26). Thus, the inflammatory phenotype associated with the mevalonate pathway deficiency could be due to the lack of other biological actions of isoprenoids. It is of interest that presqualene-diphosphate has been shown to be an important counter-regulatory lipid in inflammatory activation of neutrophils (27).

Furthermore, farnesylation seems to play an important role in innate immune response. It was recently reported that a farnesyl-transferase inhibitor (Tipifarnib) was able to reduce the expression of many LPS-induced genes, probably targeting a protein of the TLR4 signaling pathway (28).

The different anti-inflammatory effective dose observed in this study for the three isoprenoids could be explained considering that FOH probably enters the pathway of farneslyation rather than geranylgeranylation, as we suppose for GOH and GGOH. Differences in intracellular concentration, phosphorylation to the pyrophosphate-form, interference with enzymatic activity, or still unknown collateral biochemical pathways, are more likely to account for the difference in anti-inflammatory effect observed between different compounds.

It will be interesting to use GOH and other isoprenoids in the recent published heterozygous *MVK* deficient mouse (29). $MVK^{\pm}$ mouse represent the first animal model of MKD. Even if, in this mouse, the enzyme activity is reduced to about a half compared with the 1 to 8% residual MK activity in MKD patients, the authors reported that the loss of a single *MVK* allele in the mouse results in an immunologic disorder very similar to the human MKD phenotype.

In conclusion, we hypothesize that the inflammatory phenotype observed in MKD and in aminobisphosphonate-treated mice is due to the lack of mevalonate-derived isoprenoids. Although further research will be necessary to identify which of these molecules is mainly involved in the observed anti-inflammatory activity, our data support the idea of developing and testing isoprenoid-based treatment for MKD.

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