Matrix Gla Protein and Alkaline Phosphatase Are Differently Modulated in Human Dermal Fibroblasts from PXE Patients and Controls

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Mineralization of elastic fibers in pseudoxanthoma elasticum (PXE) has been associated with low levels of carboxylated matrix gla protein (MGP), most likely as a consequence of reduced vitamin K (vit K) availability. Unexpectedly, vit K supplementation does not exert beneficial effects on soft connective tissue mineralization in the PXE animal model. To understand the effects of vit K supplementation and in the attempt to interfere with pathways leading to the accumulation of calcium and phosphate within PXE-mineralized soft connective tissues, we have conducted in vitro studies on dermal fibroblasts isolated from control subjects and from PXE patients. Cells were cultured in standard conditions and in calcifying medium (CM) in the presence of vit K1 and K2, or levamisole, an alkaline phosphatase (ALP) inhibitor. Control and PXE fibroblasts were characterized by a similar dose-dependent uptake of both vit K1 and vit K2, thus promoting a significant increase of total protein carboxylation in all cell lines. Nevertheless, MGP carboxylation remained much less in PXE fibroblasts. Interestingly, PXE fibroblasts exhibited a significantly higher ALP activity. Consistently, the mineralization process induced in vitro by a long-term culture in CM appeared unaffected by vit K, whereas it was abolished by levamisole.

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INTRODUCTION

Pseudoxanthoma elasticum (PXE) is a genetic disorder associated with mutations in the ABCC6 gene (Le Saux et al., 2000) and characterized by progressive mineralization of elastic fibers affecting skin, eyes, and the cardiovascular system (Quaglino et al., 2011). Compared with controls, fibroblasts from PXE patients exhibit similar in vitro and in vivo matrix gla protein (MGP) expression, but with a significant reduction in carboxylated MGP (cMGP; Gheduzzi et al., 2007), a well-known endogenous inhibitor of soft tissue calcification (Schurgers et al., 2008). Furthermore, PXE fibroblasts are characterized by downregulation of protein disulfide isomerase and upregulation of calumenin, two proteins of the endoplasmic reticulum (ER) that are involved in the vitamin K (vit K) cycle (Boraldi et al., 2009). Vit K is a group of fat-soluble vitamins, which, in their reduced form, are needed for the posttranslational γ-carboxylation of glutamic acid residues in proteins involved in blood coagulation and in the mineralization process (Booth, 2009). Interestingly, the low vit K concentrations measured in the circulation of PXE patients (Vanakker et al., 2010) were not associated by an impairment of their blood coagulation system, indicating that the vitamin can reach the liver to be metabolized and used for the activation of coagulation factors, whereas it seems to be extruded or transported less efficiently from hepatocytes to the periphery (Borst et al., 2008; Brampton et al., 2011), thus affecting connective tissues. In this scenario, it has been suggested that vit K supplementation might be capable of restoring MGP carboxylation and to inhibit ectopic calcifications (Borst et al., 2008). However, recent studies in the PXE animal model (ABCC6−/− mice) demonstrated that vit K supplementation does not counteract the mineralization of soft connective tissues (Brampton et al., 2011; Gorgels et al., 2011; Jiang et al., 2011). Key question is whether PXE fibroblasts are able to utilize vit K and restore an adequate MGP carboxylation.
As both vit K1 and vit K2 can be used as diet supplements (Cranenburg et al., 2007), the aim of the present study was to investigate the effects of vit K1 (phyloquinone) and vit K2 (menaquinone-4, MK-4) supplementation on control and on PXE dermal fibroblasts, for a better understanding of the role of vit K–dependent carboxylation of MGP in PXE pathogenesis, and in the negative outcome of treatments carried out so far in the animal model.

Moreover, as ectopic calcifications are mainly due to calcium phosphate precipitation, we also investigated the possibility to interfere with the mineralization process by modulating alkaline phosphatase (ALP) activity, which promotes hydroxyapatite formation by acting on phosphate-donor substrates (Orimo and Shimada, 2008).

**RESULTS AND DISCUSSION**

**Assessment of blood parameters related to ectopic calcification**

Calcification is subject to regulation on many different levels, both systemic and local. We have investigated in 79 PXE patients the levels of a number of circulating parameters known to be related to the development of ectopic calcifications, and compared the values with reference values in control individuals (Table 1).

Calcemia and phosphatemia are always within normal range, thus confirming that in PXE (1) deregulation of circulating levels of calcium and phosphate are only rarely observed, possibly as an additional unrelated clinical complication (Mallette and Mechanick, 1987); (2) ectopic calcification does not take place through the “calcification paradox” (Krueger et al., 2009), i.e., by increased circulating levels of calcium and phosphate because of decreased bone mineral density, as confirmed by the absence of altered bone metabolism in all patients investigated so far.

Similarly, activity of serum ALP activity never appeared upregulated in patients (Table 1), which is consistent with the absence of a generalized calcification process. As collagen fibrils never undergo mineralization in PXE, and normal as well as calcified elastic fibers coexist within the same tissues, it is conceivable to hypothesize a local deregulation of pro- and anti-calcifying factors.

Finally, we have measured the levels of dephospho uncarboxylated MGP and dephospho carboxylated MGP (Table 1); no significant differences were observed between control and PXE samples.

Although it cannot be excluded that altered, nevertheless, unknown serum factor/s could be present in the circulation of patients, control subjects and PXE patients, or PXE patients, with different extent/severity of clinical manifestations cannot be discriminated on the basis of these parameters.

**Vit K uptake by dermal fibroblasts and cell viability**

Few data are available in the literature concerning the ability of fibroblasts to respond to in vitro vit K treatments (Canfield et al., 1987; Ross et al., 1991), and no data are available on human dermal fibroblasts in primary culture. In addition, the demonstration that vit K supplementation does not counteract soft connective tissue mineralization in the PXE animal model (Brampton et al., 2011; Gorgels et al., 2011; Jiang et al., 2011) poses the question whether PXE fibroblasts are unable to utilize the vitamin (Boraldi et al., 2009).

Despite a certain heterogeneity observed between cells from different individuals, a similar dose-dependent vit K uptake is clearly detectable in both control and PXE fibroblasts (Figure 1), although the uptake of phylloquinone (Figure 1a) is higher than that of menaquinone (Figure 1b) at comparable doses in the culture medium. These data clearly demonstrate that fibroblasts can take up vit K, and that this trait is not affected by the pathologic phenotype (Figure 1).

In light of these results, further experiments were conducted with the lowest (0.1 μM) and the highest (100 μM) vitamin concentrations. The highest concentration was chosen to guarantee the possibility to quantify the effects of vitamin supplementation, even in short-term cultures (48 hours); the lowest concentration was chosen to follow the intracellular uptake and to look for eventual cumulative effects in long-term cultures (>20 days).

Evaluation of cell viability demonstrates that, in standard culture conditions (i.e., DMEM), both vitamins, even at very high concentrations and for long-term cultures (>20 days), never appeared to inhibit cell growth or to induce cell death (data not shown), as demonstrated for K2 in different tumor cell lines (Ogawa et al., 2007), where the pro-apoptotic effect of the vitamin could be the consequence of metabolic pathways associated with the neoplastic phenotype. Consistently, vit K has been safely used in many long-term in vivo treatments (Iwamoto et al., 2009), supporting the feasibility to use these dietary compounds for therapeutic strategies (McCann and Ames, 2009).

As far as the effects of vit K on the cellular redox balance, in contrast to the menadione-induced generation of reactive oxygen species (Loor et al., 2010), vit K1 and K2 supplementation never induced oxidative stress. In particular, vit K1 never modified the intracellular accumulation of O$_{2}^{−}$ and H$_{2}$O$_{2}$ (data not shown), whereas vit K2, at the highest dose (100 μM), significantly (P≤0.05) reduced the level of reactive oxygen species.

**Table 1. Plasma levels of selected ectopic calcification-related parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls$^{1}$</th>
<th>PXE</th>
</tr>
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<tbody>
<tr>
<td>Calcium (mg dl$^{-1}$)</td>
<td>8.6–10.3</td>
<td>9.53 ± 0.4</td>
</tr>
<tr>
<td>Phosphate (mg dl$^{-1}$)</td>
<td>2.4–4.8</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>ALP activity (U l$^{-1}$)</td>
<td>40–129</td>
<td>66.5 ± 37.7</td>
</tr>
<tr>
<td>dp-ucMGP (pmol l$^{-1}$)</td>
<td>426 ± 235</td>
<td>300 ± 221</td>
</tr>
<tr>
<td>dp-cMGP (pmol l$^{-1}$)</td>
<td>1,865 ± 597</td>
<td>1,169 ± 603</td>
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$^{1}$Normal reference range or values.
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To exclude that vit K supplementation may be associated with ER stress, having a negative effect on vit K–dependent carboxylation, and favoring the calcification process (Liberman et al., 2011), we have investigated the expression of GRP-78 (glucose-regulated ER stress response protein-78), a well-known marker of ER stress (Zhang and Kaufman, 2006). Data indicate that GRP-78 expression (Figure 2a and b) was very similar in control and in PXE fibroblasts, and remained unmodified after treatments, clearly demonstrating that intracellular accumulation of vit K is not associated with ER stress.

Moreover, as PXE fibroblasts are characterized by chronic oxidative stress (Pasquali-Ronchetti et al., 2006) and the redox environment within the ER is known to affect the carboxylation process, we investigated whether vit K supplementation can modify the expression of other molecules that, in the ER, are involved in the vit K cycle, such as protein disulfide isomerase (Figure 2a and c) and calumenin (Figure 2a and d), which are downregulated and upregulated, respectively, in PXE (Boraldi et al., 2009). Both vit K1 and K2, independently from concentration, similarly downregulated the expression of calumenin in all cell strains (Figure 2a and d), whereas changes were negligible in the case of protein disulfide isomerase (Figure 2a and c), indicating that the effect of vit K supplementation on the expression of ER proteins involved in the vit K cycle was not pathway-specific.

Protein carboxylation and MGP expression

Gamma carboxyglutamil residues were evaluated on total protein extracts as markers of protein carboxylation. Data reveal that control and PXE fibroblasts have a comparable number of bands of similar intensity (Figure 3a and b), suggesting that the whole carboxylation process in PXE fibroblasts is equivalent to that of controls.

Possibly because of the low sensibility of this parameter, low doses of vit K1 and K2 are not associated with significant differences in total densitometry of bands. By contrast, high doses of vit K1 and K2 markedly upregulated band intensity in all cell strains, indicating that the carboxylation process is similarly modulated in both control and PXE fibroblasts (Figure 3a and b).

Moreover, by comparing data on the intracellular vit K content (Figure 1) and the amount of carboxylated proteins (Figure 3a and b), it appears that vit K2, although present inside cells at lower levels compared with phylloquinone, has a similar effect on the carboxylation process, in agreement with previous findings that menaquinone is more efficient than phylloquinone in extra-hepatic tissues (Wallin et al., 2008).

On the basis of the positive effects of vit K on the carboxylation process, we have looked for changes in MGP expression and maturation. Unexpectedly, vit K supplementation never induced a significant MGP upregulation. In particular, at high doses vit K1 significantly reduced MGP protein expression in PXE fibroblasts (Figure 3f), whereas both in control and PXE fibroblasts vit K2 downregulated MGP at gene and protein levels (Figure 3c–f). Moreover, treatment with vit K2, at all doses, did not increase cMGP (Figure 3g and h), whereas vit K1 appeared to upregulate cMGP, although only in control fibroblasts (Figure 3g).

All these data are in agreement and help understand the observations by Brampton et al. (2011) that MGP remained

Figure 1. Amount of vitamins K1 and K2 taken up by control and pseudoxanthoma elasticum (PXE) dermal fibroblasts. Intracellular determinations of phylloquinone (vitamin K1, a) and menaquinone (vitamin K2, b) content in fibroblasts from control and PXE patients grown in vitro for 48 h after confluence in the presence of the vitamin (0.1, 1, 10, and 100 μM). As controls, cells were cultured in the presence of comparable amounts of dimethylformamide (DMF) without vitamin K. A dose-dependent uptake can be measured in both control and PXE cells (a linear regression analysis is shown in small graphs in the upper-left side of each panel).

of O$_2^{•-}$ in both control and PXE fibroblasts. In particular, values measured as fluorescence arbitrary units were $0.70 \pm 0.07$ in controls, $0.55 \pm 0.07$ in controls + K2, $1.11 \pm 0.06$ in PXE, and $0.66 \pm 0.07$ in PXE + K2, in agreement with previous observations that menaquinone can act as a positive modulator of oxidative stress (Li et al., 2009).
undercarboxylated in the muzzle tissue of ABCC6−/− mice, despite the increase in circulating levels of vit K. In particular, results from the present study demonstrate that in PXE, in a general setting of vit K availability and adequate carboxylation process, MGP is not sufficiently carboxylated. It could be suggested that the defect may specifically involve MGP and that the extent of gamma carboxylation could be related not only on the bioavailability of cofactors, but also on the binding affinity between proteins and gamma glutamylcarboxylase (Berkner, 2008).

Surprisingly, in the absence of any effect on cell counts and/or cell viability, K2 negatively modulated MGP expression, apparently in contradiction with the statement that in vivo vit K2 is specifically active in modulating the calcification process (Spronk et al., 2003; Beulens et al., 2009), although in most cases positive effects, observed either in vitro and/or in vivo, have to be related to supplementation with menaquinones with longer chains or by the simultaneous presence of other dietary components and/or to healthier lifestyle behavior (Rees et al., 2010). Moreover, it cannot be excluded that these results could be because of a different behavior of fibroblasts compared with smooth muscle cells or, such as those that have been described in other cell types (Goritz et al., 2007; Mertsch et al., 2009), with the association of low MGP expression with low proliferation/migration of cells as an additional effect of vit K2 supplementation.

**ALP activity**

ALP is considered a marker of calcification as it degrades phosphate donors, releasing inorganic phosphate and decreasing the concentration of inhibitors, such as pyrophosphate (Orimo and Shimada, 2008). Evaluation of ALP activity in fibroblasts cultured in standard medium clearly shows that values are elevated in PXE fibroblasts and remain unmodified by vit K, whereas a significant inhibition can be measured after levamisole supplementation (Table 2).

**Calcification assay**

Independent from their ability to induce mineralization in vivo, cells in normal culture conditions are not able to mineralize because of the presence in the culture medium of bovine serum factors, such as fetuin, which acts as a potent calcification inhibitor. Therefore, to induce the calcification process in vitro, a complex environment is necessary such as that provided by addition of β-glycerophosphate (a source of organic phosphate), dexamethasone (a glucocorticoid that upregulates and downregulates osteogenic or inhibitor molecules, respectively), and ascorbic acid (an essential cofactor for collagen synthesis as a fundamental substrate for mineral deposition) (Buranasinsup et al., 2006). Starting from 10 days of culture in this specific calcifying medium, areas of mineralization can be visualized in all fibroblast strains, progressively increasing with time (Figure 4). The heterogeneous distribution of calcifications on the cell surface indicates that this phenomenon is not a passive diffuse precipitation, but it is the result of specific cell-dependent processes. Treatment with levamisole inhibited the mineralization process, whereas vit K supplementation was always ineffective in all cell strains (Figure 4). The observation that menaquinone, at the lowest concentration, actually favored the accumulation of mineral deposits, could be because of the fact that, as observed in osteoblastic cell lines, vit K2, differently from vit K1, has a transcriptionally regulatory function on extracellular matrix genes (Koshihara et al., 2003; Ichikawa et al., 2006), consistent with the use of vit K2 as a potential anti-osteoporotic agent (Iwamoto et al., 2009).
Evaluation of ALP activity (Figure 4) in these long-term culture conditions indicates that enzyme activity (1) is higher in PXE compared with control fibroblasts; (2) is not significantly modified by vit K supplementation; and (3) is influenced by the extracellular environment, because of the progressive accumulation of matrix molecules as in the case of cells cultured in calcifying medium (CM). It is noteworthy that CM favors extracellular matrix deposition (Figure 1 and Supplementary Material online), leading to a thick layer of matrix covering and partially embedding the cell monolayer; and for this reason, at 30 days the matrix is so dense that it negatively interferes with protein solubilization, unable appropriate ALP activity determination. Surprisingly, for long periods of culture in CM (≥20 days) the highest dose of vit K2 was associated with changes in cellular morphology, leading to areas of cell detachment in both control and PXE fibroblasts (Figure 4). However, it is worth mentioning that no toxic effects were noticed on cells grown in standard medium supplemented with vit K2 (at all doses and for long times of culture; Figure 2 and Supplementary Material online), nor in CM alone or in the presence of phylloquinone (data not shown). Therefore, it could be hypothesized that menaquinone may interact with some CM component(s), thus negatively interfering with cell viability.

**Table 2. ALP activity in control and PXE fibroblasts treated for 48 hours after confluence**

<table>
<thead>
<tr>
<th></th>
<th>DMEM</th>
<th>DMEM + vitamin K1</th>
<th>DMEM + vitamin K2</th>
<th>DMEM + levamisole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 μM</td>
<td>100 μM</td>
<td>0.1 μM</td>
<td>100 μM</td>
</tr>
<tr>
<td>Control</td>
<td>1 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>1.2 ± 0.05</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>PXE²</td>
<td>1.5 ± 0.3³</td>
<td>1.3 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.3</td>
</tr>
</tbody>
</table>

Abbreviations: ALP, alkaline phosphatase; PXE, pseudoxanthoma elasticum.

1 Enzyme activity is measured in confluent fibroblasts. Values are normalized to those of control fibroblasts cultured in DMEM, set as one-fold, and expressed as mean values ± SD.

2 P<0.01 Levamisole treatment versus DMEM.

3 P<0.05 PXE versus control fibroblasts.
Conclusions

Despite the well-known limits provided by the simplifications usually associated with in vitro models, nevertheless, studies on cultured human dermal fibroblasts that, isolated from healthy subjects as well as from PXE patient's, maintain in vitro several characteristics related to their pathologic
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**MATERIALS AND METHODS**

**Materials**

Depending on the requirements of the study, other factors, including the upregulation of extracellular matrix, may be decisive in the question whether a tissue will start to calcify. Therefore, in addition to vit K–dependent molecules, other factors, including the unbalanced ratio between many different factors locally controlling calcium and phosphate homeostasis, as well as the characteristics of the extracellular environment in mineral deposit formation and in regulating cell behavior. In the development of ectopic calcifications, not a single component is prevalent or unique, but the unbalanced ratio between many different factors locally controlling calcium and phosphate homeostasis, as well as the characteristics of the extracellular matrix, may be decisive in the question whether a tissue will start to calcify. Therefore, in addition to vit K–dependent molecules, other factors, including the upregulation of ALP, must be taken into account for future therapeutic options.

**Methods**

**Supplementary Materials and Methods online.**

More detailed information on materials and methods is available at Supplementary Materials and Methods online.

**Patients**

PXE patients were diagnosed on the basis of clinical manifestations, histopathological findings, and identification of ABCC6 gene mutations (Gheduzzi et al., 2004).

Laboratory parameters (calcemia, phosphatemia, and ALP activity) were measured in the plasma of 79 patients, according to the Clinical Pathology Laboratory routine procedures.

Plasma levels of dephospho ucMGP and dephospho cMGP were measured in the same patients by sandwich ELISA techniques, already developed at VitaK BV (Maastricht, The Netherlands) (Cranenburg et al., 2010). The different sensitivity of the two antibodies does not allow a direct comparison of the amount of two forms of MGP, and evaluations have to be carried out between controls and PXE within the same parameter.

**Cells and treatments**

Human dermal fibroblast cultures, in accordance with the Declaration of Helsinki protocol and the guidelines of the ethical committee of the Modena University Faculty of Medicine after written informed consent, were obtained from five clinically healthy females (38 ± 7 years), who did not exhibit any sign of genetic, metabolic, or connective tissue disorders, and from six PXE patients (40 ± 10 years).

Confluent fibroblasts were treated with 0.1, 1, 10, and 100 μM phylloquinone (vit K1; Sigma, St Louis, MO) or menaquinone (MK-4; vit K2; Sigma) or with 100 μM levamisole (Sigma). Cells grown in the presence of dimethylfumaride (Sigma) alone were used as control of treatments.

Unless otherwise specified, cells from each individual were kept separate during all experiments.

Fibroblast’s morphology was evaluated by phase-contrast microscopy, and cell viability was assessed by cell count.

**HPLC chromatography**

Confluent fibroblasts were treated with vitamins for 48 hours, washed in buffer, trypsinized, and centrifuged. Cell pellets were stored at −80°C and sent to VitaK for measuring on cell extracts, vit K1 and K2 intracellular concentrations by HPLC, using a reversed-phase column with online zinc reduction and fluorescence detection (Schrugers et al., 2007).

**Flow cytometry**

Intracellular levels of O$_2^\bullet^-$ and H$_2$O$_2$ were estimated by FACS after incubation of fibroblasts with 1 μM DH$_2$ or with 2 μM H$_2$DCF-DA (Molecular Probes, Eugene, OR), respectively (Boraldi et al., 2009). Experiments were conducted three times in duplicate.

**Protein extraction and western blot**

Cells were homogenized in RIPA buffer with protease inhibitors (Sigma), centrifuged, and supernatants collected and stored at −80°C until analysis. After measurement of protein concentration (Bradford, 1976), proteins were separated by one- or two-dimensional PAGE (Gheduzzi et al., 2007; Boraldi et al., 2009). After separation, proteins were transferred to nitrocellulose and incubated with anti-GRP-78, anti-protein disulfide isomerase (Abcam, Cambridge, UK); anti-calumenin, anti-MGP (Santa Cruz Biotechnology, Santa Cruz, CA); anti-gamma carboxylglutamil residues (American Diagnostica, Stamford, CT); anti-cMGP (Vascular Products, Maastricht, The Netherlands); and thereafter with appropriate horseradish peroxidase–conjugated secondary antibodies (Abcam).

One-dimensional PAGE experiments were conducted at least two times with all different cell lines. In case of cMGP, owing to the low abundance of the protein, analyses by two-dimensional PAGE were performed on proteins extracted from a pool of fibroblasts from six patients and from five controls, and data are expressed as mean values ± SD of replicates.

**Quantitative real-time reverse transcriptase–PCR**

Total RNA was isolated from cells using the RNeasy Protect cells Mini kit (Qiagen, Milano, Italy) and reversely transcribed using Superscript III (Invitrogen, Monza, Italy) and Oligo dT$_{18}$ primers (Invitrogen). Complementary DNAs were amplified on an iCycler (BioRad, Hercules, CA) using SYBR GreenER qPCR SuperMix (Invitrogen). The MGP gene expression in each sample was normalized against CLK2 and quantified (Piafli, 2001). Experiments were conducted two times in replicate.
ALP activity
ALP activity was measured spectrophotometrically at 405 nm on control and PXE fibroblasts, and values of optical density were related to cellular protein concentration (Bradford, 1976). Experiments were conducted three times in triplicate.

In vitro calcification and von Kossa staining
Fibroblasts were routinely cultured in six-well plates (BD-Falcon, Franklin Lakes, NJ) up to confluence, then the standard medium (DMEM+10% fetal bovine serum, Gibco, Monza, Italy) was replaced by the same medium or by the CM (i.e., standard medium plus 10 μM β-glycerophosphate, 50 μg/ml-1 ascorbic acid and 10 mM dexamethasone, Sigma; Buranasinsup et al., 2006). Vit K1 and K2 (0.1 and 100 μM, respectively) and levamisole (100 μM) were added to each type of culture media. After 10–20–30 days, cells were stained with the von Kossa method, and areas of mineralization were quantified on digital images. Experiments were conducted three times.

Data analysis
Data are expressed as mean values ± SD of all measurements and compared using Mann–Whitney test or analysis of variance test using GraphPad software (San Diego, CA).

CONFLICT OF INTEREST
The authors state no conflict of interest.

ACKNOWLEDGMENTS
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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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