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Single amino acid charge switch defines clinically distinct proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1)–associated inflammatory diseases

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Abstract

Background—Hyperzincemia and hypercalprotectinemia (Hz/Hc) is a distinct autoinflammatory entity involving extremely high serum concentrations of the proinflammatory alarmin myeloid-related protein (MRP) 8/14 (S100A8/S100A9 and calprotectin).

Objective—We sought to characterize the genetic cause and clinical spectrum of Hz/Hc.

Methods—Proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1) gene sequencing was performed in 14 patients with Hz/Hc, and their clinical phenotype was compared with that of 11 patients with pyogenic arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome. PSTPIP1-pyrim interactions were analyzed by means of immunoprecipitation and Western blotting. A structural model of the PSTPIP1 dimer was generated. Cytokine profiles were analyzed by using the multiplex immunoassay, and MRP8/14 serum concentrations were analyzed by using an ELISA.

Results—Thirteen patients were heterozygous for a missense mutation in the PSTPIP1 gene, resulting in a p.E250K mutation, and 1 carried a mutation resulting in p.E257K. Both mutations substantially alter the electrostatic potential of the PSTPIP1 dimer model in a region critical for protein-protein interaction. Patients with Hz/Hc have extremely high MRP8/14 concentrations ($2045 \pm 1300 \mu\text{g/mL}$) compared with those with PAPA syndrome ($116 \pm 74 \mu\text{g/mL}$) and have a distinct clinical phenotype. A specific cytokine profile is associated with Hz/Hc. Hz/Hc mutations

altered protein binding of PSTPIP1, increasing interaction with pyrin through phosphorylation of PSTPIP1.

Conclusion—Mutations resulting in charge reversal in the y-domain of PSTPIP1 (E→K) and increased interaction with pyrin cause a distinct autoinflammatory disorder defined by clinical and biochemical features not found in patients with PAPA syndrome, indicating a unique genotype-phenotype correlation for mutations in the PSTPIP1 gene. This is the first inborn autoinflammatory syndrome in which inflammation is driven by uncontrolled release of members of the alarmin family.

Keywords

Hyperzincemia and hypercalprotectinemia; myeloid-related protein 8/14; calprotectin; S100 proteins; zinc; proline-serine-threonine phosphatase-interacting protein 1; pyogenic arthritis; pyoderma gangrenosum; and acne syndrome; genotype; phenotype; autoinflammation

Myeloid-related protein (MRP) 8 (S100A8) and MRP14 (S100A9) are endogenous ligands of Toll-like receptor (TLR) 4¹ that are highly expressed in granulocytes, monocytes, and activated keratinocytes.² Both proteins belong to the family of alarmins, which are danger signals released during cellular stress or damage; have strong proinflammatory effects *in vitro*^{3–7}; and promote inflammation *in vivo*.^{8–10} Highly increased serum concentrations of MRP8/14 complexes (calprotectin) have been reported in patients with systemic juvenile idiopathic arthritis (SJIA) and familial Mediterranean fever (FMF; OMIM #249100).^{11,12}

Hypercalprotectinemia and hyperzincemia (Hz/Hc) is a rare autoinflammatory condition characterized by marked dysregulation of MRP8/14 metabolism associated with accumulation of zinc caused by the zinc-binding capacities of MRP8/14.² Together with extremely high MRP8/14 serum levels, the syndrome is characterized by severe systemic and cutaneous inflammation, hepatosplenomegaly, arthritis, pancytopenia, and failure to thrive.² The genetic cause of the disease has not been identified thus far.^{2,13–15}

Proline-serine-threonine phosphatase-interacting protein 1 (PSTPIP1; also known as CD2BP1) is a cytoskeleton-associated adaptor protein that modulates T-cell activation,¹⁶ cytoskeletal organization, and IL-1 β release.¹⁷ Heterozygous mutations in the *PSTPIP1* gene cause the dominantly inherited pyogenic arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome (OMIM #604410). PAPA syndrome is a rare autosomal dominant autoinflammatory disease typically caused by the missense mutations p.A230T (c.688G>A) and p.E250Q (c.748G>C) in PSTPIP1.¹⁸ Mutations causing PAPA syndrome are thought to disrupt the interaction of PSTPIP1 with a protein tyrosin phosphatase (PTP-PEST), a regulatory phosphatase, leading to uncontrolled secretion of IL-1 β , probably through binding to pyrin, which is mutated in patients with FMF.¹⁹ Patients with PAPA syndrome typically present with recurrent sterile pyogenic arthritis in childhood, occurring spontaneously or after minor trauma. In adolescence the propensity for cutaneous inflammation increases, encompassing severe cystic acne, and recurrent pyoderma gangrenosum-like ulcers, including the positive pathergy phenomenon.²⁰ In contrast, systemic inflammation, failure to thrive, and high mortality are not part of the clinical picture of PAPA syndrome.

In this study, for the first time, we identified *de novo* p.E250K and p.E257K mutations in the *PSTPIP1* gene as the cause of Hz/Hc disease and characterized this autoinflammatory entity in comparison with PAPA syndrome. We here describe the first inherited syndrome in which the inflammatory pathogenesis is largely driven by unrestrained release of proinflammatory danger signals of the alarmin family.

Methods

Patients and samples

This study was approved by the Medical Ethics Committee at the University of Muenster, Muenster, Germany, and performed in accordance to the Helsinki Declaration. All patients or relatives provided written informed consent. Fourteen patients with Hz/Hc were enrolled. Patients P1,² P2,¹³ P3,¹⁴ P4,¹⁵ and P11²¹ have been previously reported. Patients' clinical symptoms are summarized in Table E1 and the Methods section in this article's Online Repository at www.jacionline.org.

Patients with PAPA syndrome, SJIA, and FMF

As a control group, 11 patients with genetically confirmed PAPA syndrome (8 patients carrying the p.A230T mutation, 2 patients with the p.E250Q mutation, and 1 patient with the p.E256G mutation) were included (for patient's details, see Table E2 in this article's Online Repository at www.jacionline.org). Patients PI and PII have been reported previously.²² Furthermore, 20 patients with active SJIA (sex, 12 male/8 female; age range, 7-16 years), 20 patients with active FMF (9 male/11 female; age range, 8-17 years), and 20 healthy control subjects (11 male/9 female; age range, 7-24 years) were included for serologic studies.

Analysis of MRP8/MRP14 in serum and skin and zinc serum levels

Serum concentrations of MRP8/14 were determined by using a sandwich ELISA, as previously described. The readers of the laboratory assay were blinded for diagnosis and inflammatory activity of the patients. Skin sections of patient P1 were stained with monospecific affinity-purified rabbit antisera to MRP8 and MRP14. Immunostaining was performed with a standard 3-stage immunoperoxidase method, as previously described.³ Zinc serum levels were determined by using atomic absorption spectroscopy. Normal serum levels were defined as 11.5 to 21.4 $\mu\text{mol/L}$ (95% CI).

Analysis of anti-neutrophil antibodies

Neutrophil-reactive antibodies were detected by using the agglutination, microscopic immunofluorescence, and mAb-specific immobilization of neutrophil antigen assay, as previously described.²³

Genotype analysis

In patients with Hz/Hc, exons 1 to 15 of the *PSTPIP1* gene and all 10 exons of the Mediterranean fever gene were sequenced. PCR amplification products of exons were purified with the ExoSAP-IT reagent (USB, Cleveland, Ohio) and sequenced with the ABI

PRISM Big Dye Terminator v3.1 Ready Reaction Cycle Sequencing kit and the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, Calif).

Structural model and sequence alignment

The PSTPIP1 sequence (residues 3-285, UniProtKB code O43586) was defined as the target, and the F-bar domain of FCHO2 (PDB ID 2V0O, residues 2-271) with a sequence identity of 23% served as the template.²⁴ Homology models were based on an alignment using the Clustal Omega tool²⁵ and built with the MODELLER program.²⁶ One thousand models were calculated and clustered, and the model with the best ANOLEA score²⁷ belonging to the largest cluster was further refined. A dimeric structure was generated by aligning 2 copies of the resulting model with chains A and B of the x-ray structure 2V0O and minimizing them by using the CHARMM22 all-atom force field.²⁸ Structural models for the mutant proteins p.E250K and p.E257K, as well as for the PAPA syndrome mutant p.E250Q, were generated by mutating the respective amino acids of the dimer model and reminimizing the structure. For each of the 4 models, the electrostatic potential was calculated with the UHBD code.²⁹ The FoldX program was used to perform an *in silico* alanine scan by mutating each residue to an alanine and estimating the resulting change in folding energy.³⁰ Molecular visualization was done with CHIMERA.³¹

Immunoprecipitation and phosphorylation of PSTPIP1

HEK293 cells were transfected (PolyFect; Qiagen, Hilden, Germany) with wild-type (WT) enhanced green fluorescent protein–PSTPIP1 or p.E250Q or p.E250K mutated enhanced green fluorescent protein–PSTPIP1 and pyrin for coprecipitation or cAbl (31284;pSG5-ABL; Addgene, Cambridge, Mass) for detection of tyrosine phosphorylated proteins. Transfected HEK cells were incubated in lysis buffer (50 mmol/L Tris [pH 7.7], 300 mmol/L NaCl, 2 mmol/L MgCl, 0.1% Chaps, and protease inhibitor cocktail; Roche, Mannheim, Germany) for 20 minutes on ice and pushed through a 15-gauge cannula. For detection of phosphotyrosine, 0.4 mmol/L Na₃VO₄ was added to lysis buffer. After centrifugation, supernatants were incubated overnight at 4°C for coprecipitation of pyrin or for 3 hours at 4°C for detection of tyrosine phosphorylated proteins with antibody (mouse α-PSTPIP1 [ab88474]; Abcam, Cambridge, United Kingdom) linked to a/g agarose beads (Pierce Biotechnology, Rockford, Ill). After washing, samples were analyzed by means of SDS-PAGE and Western blotting with appropriate antibodies (rabbit α-PSTPIP1 [HPA 010600]; Atlas Antibodies, Stockholm, Sweden; mouse α-phosphotyrosine [4G10]; Merck Millipore, Darmstadt, Germany). Additionally, coimmunoprecipitation band intensities of blots from 3 independent experiments were densitometrically analyzed with ImageJ 1.46r (National Institutes of Health, Bethesda, Md). All values were normalized to the PSTPIP1 value.

Extracellular cytokine measurement

Cytokines and soluble receptors were measured in serum by using a multiplex immunoassay, as previously described.³² Acquisition of data was performed with the FlexMap3D system (Bio-Rad Laboratories, Hercules, Calif) and xPonent v4.2 software (Luminex, Austin, Tex). Data analyses were performed by using Bio-Plex Manager software version 6.1.1 with 5 parametric curve fitting (Bio-Rad Laboratories). Cytokines analyzed included IL-1α, IL-1β, IL-2, IL-4, IL-15, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, IL-21, IL-22, IL-33,

CCL2, CCL3, CCL11, CCL17, CCL18, CCL22, CXCL9, granulocyte colony-stimulating factor, GM-CSF, macrophage colony-stimulating factor, IFN- α , IFN- γ , macrophage migration inhibitory factor (MIF), intercellular adhesion molecule, vascular cell adhesion molecule, receptor activator of nuclear factor κ B ligand, IFN- γ -induced protein 10, and TNF- α . Serum samples for this analysis were immediately frozen and shipped on dry ice.

Statistical analysis

Statistical analyses were performed with Prism 5.0 software (GraphPad Software, La Jolla, Calif). Analyses between 2 groups were performed with an unpaired 2-tailed Student *t* test. Comparisons among 3 or more groups were performed by using 1-way ANOVA, followed by Bonferroni multiple means tests for comparing all pairs of columns. Differences in protein serum profiles were analyzed by using the Mann-Whitney *U* test. Differences were considered statistically significant at a *P* value of less than .05.

Results

Characterization of clinical phenotype in patients with Hz/Hc

Patients with Hz/Hc had a persistent early-onset (median age at disease onset, 13 months) inflammatory disease characterized by cutaneous inflammation (12/14), (hepato)splenomegaly (12/14), failure to thrive (9/14), lymphadenopathy (6/14), and joint involvement (arthritis in 7/14 and arthralgia/morning stiffness in 2/14). Most of these patients showed marked abnormalities in hematologic parameters, including neutropenia (14/14), anemia (hemoglobin, <100 g/L; *n* = 13/14), and thrombocytopenia (7/14, see Table E1). As a consequence of the detected pancytopenia, bone marrow biopsy specimens were analyzed in 8 patients showing dyserythropoiesis, fibrosis, and lymphocytosis. Additionally, the frequency of anti-neutrophil antibodies was screened in serum of 12 of 14 patients with Hz/Hc and 9 of 11 patients with PAPA syndrome. We found a constellation of autoimmune neutropenia (AIN) in 4 patients and a constellation consistent with AIN (without identification of antigen) in 4 other patients with Hz/Hc (66% together), whereas only in 3 patients with classical PAPA syndrome could such a constellation be found (33%, see Table E3 in this article's Online Repository at www.jacionline.org). Analysis of B-cell subpopulations (total [CD19⁺], transitional [CD24⁺⁺ and CD38⁺⁺], naive [CD27⁻, IgG⁻, and IgA⁻], memory [CD27⁺], IgM memory [CD27⁺, IgG⁻, and IgA⁻], and switched memory [CD27⁺, IgG⁺, and IgA⁺]) did not show relevant changes in 5 patients with Hz/Hc (E250K) or 5 patients with PAPA syndrome (3 E250Q and 2 E256G) when compared directly or with healthy control subjects.

Interestingly, in the cohort of patients with PAPA syndrome (*n* = 11) the most relevant clinical findings of anemia, neutropenia, thrombocytopenia, (hepato)splenomegaly, and failure to thrive were absent (see Table E2). Inversely, pyogenic arthritis, a prominent clinical feature of PAPA syndrome (*n* = 8/11) was present in only 1 patient with Hz/Hc. The only common clinical symptom in both groups was skin inflammation (PAPA syndrome, *n* = 9/11; Hz/Hc, *n* = 12/14), which appeared in a variety of manifestations. The significance of the differences in the clinical phenotype and laboratory findings is depicted in Fig 1, A (median age at disease onset in patients with PAPA syndrome, 4 years).

Among cutaneous findings, palpebral lesions occurred in 2 patients with Hz/Hc (Fig 1, *B*), whereas they were absent in patients with PAPA syndrome. Patient P1 had pyoderma gangrenosum (Fig 2, *C*) and several ulcers in the lower extremities (Fig 1, *D* and *E*). Here, biopsy samples from affected and unaffected skin showed a dramatic upregulation of MRP8 and MRP14 in hyperproliferative epidermis and marked leukocyte infiltration in inflamed dermis (Fig 1, *F* and *G*) in contrast to clinically unaffected skin from the same patient (Fig 1, *H* and *I*).

Cutaneous inflammation was characterized by an unspecific infiltrate composed of neutrophils and macrophages admixed with plasma cells, as well as perivascular lymphocellular infiltrations in 5 patients with Hz/Hc.

Genetic studies

Interestingly, genetic analyses of patients with Hz/Hc revealed in all of them an amino acid exchanges from the acidic glutamic acid to basic lysine. In patients P1 to P13, the heterozygous G>A transition at the c.748 position in exon 11 of the *PSTPIP1* gene, leading to the missense p.E250K variant, was found.²¹ In patient P14 the heterozygous G>A transition at c.769 position, also in exon 11 and leading to the missense p.E257K variant, was found. Multiple sets of evidence indicate that these 2 variants are very likely deleterious for the structure, function, or both of PSTPIP1: their absence in the database of genomic diversity (0% allele frequency for the 2 variants in the 1000 Genomes project), *in silico* analyses with the algorithms SIFT and Polyphen-2 (deleterious and probably damaging), and family-based cosegregation studies. Parental DNA was available from patients P2 to P8, P12, and P13, which confirmed a *de novo* mutation in 8 patients. Patients P9 (father) and P8 (son) shared the same *PSTPIP1* E250K mutation, and both were affected with a variable clinical expression.

In all patients carrying the mutations, we detected excessively increased MRP8/14 and zinc serum levels compatible with earlier findings reported in patients with Hz/Hc (see Table E1).

Alteration of electrostatic potential of PSTPIP1

Both 250 and 257 residues are located on the convex face of the dimeric PSTPIP1 model, close to the dimer interface but exposed to the solvent (Fig 2, *A*). Because α helices have a periodicity of 3.6, both mutations point to the same face of the helix and to the outside of the structure. Moreover, copies of E250 and, to a lesser degree, E257 from chains A and B are in proximity, suggesting that their influence could be potentiated in the dimer. E250 is located in a cluster of 4 negatively charged side chains formed by D246, E247, E250, and E251 (Fig 2, *B*). An *in silico* alanine scan suggests that neither E250 nor E257 is relevant for the structural integrity of the protein. The electrostatic potential projected on the molecular surface of the PSTPIP1 model (Fig 2, *C* and *D*) displays a large negative patch in the central part of the convex face that has been proposed to be important for pyrin and PTP-PEST binding. Both mutants, p.E250K and p.E257K, substantially affect this negative patch of WT PSTPIP1 (Fig 2, *E* and *F*), whereas the influence of the PAPA syndrome mutant p.E250Q on the electrostatic potential is much less pronounced (Fig 2, *G*).

Binding of pyrin and phosphorylation of PSTPIP1

The PAPA syndrome-associated p.E250Q mutation causes increased pyrin binding and hyperphosphorylation of PSTPIP1. Therefore we tested the effect of the p.E250K mutation on phosphorylation and pyrin binding. Relative to the E250Q mutant, the E250K mutant exhibits markedly increased binding to pyrin (Fig 3, *A*). The 2 mutant proteins were hyperphosphorylated relative to WT in the presence of the cAbl kinase (Fig 3, *B*). Although the increase in PSTPIP1-pyrin binding was significant, phosphorylation only showed a tendency toward stronger p.E250K hyperphosphorylation (Fig 3, *C*).

Serum concentration of MRP8/14 and zinc in patients with PSTPIP1 mutations

Serum samples were obtained at the time of clinical and serologic active disease. Serum concentrations of MRP8/14 in patients with Hz/Hc (mean \pm 95% CI, 2070 \pm 1190 $\mu\text{g/mL}$) were found to be significantly increased (up to 20-fold) compared with those in patients with PAPA syndrome (116 \pm 74 $\mu\text{g/mL}$, $P < .001$; Fig 4, *A*). Levels in healthy control subjects were 0.48 \pm 0.1 $\mu\text{g/mL}$.³³ We have previously shown that MRP8/14 levels are increased in patients with SJIA and FMF at the time of clinical and serologic active disease.^{11,12} However, the MRP8/14 levels in patients with Hz/Hc were more than 20 or 40 times higher than those in patients with FMF (110 \pm 82 $\mu\text{g/mL}$) or SJIA (45 \pm 13 $\mu\text{g/mL}$, both $P < .001$), respectively. There were no significant differences among patients with PAPA syndrome, FMF, and SJIA. Concomitantly, zinc levels were found to be significantly increased in patients with Hz/Hc (114.5 \pm 28.3 $\mu\text{mol/L}$) compared with those in patients with PAPA syndrome (21.9 \pm 7.8 $\mu\text{mol/L}$), FMF (14 \pm 8 $\mu\text{mol/L}$), and SJIA (12.1 \pm 1.9 $\mu\text{mol/L}$) or healthy control subjects (12.8 \pm 1.0 $\mu\text{mol/L}$, all $P < .001$; Fig 4, *B*). The degree of increase in MRP8/14 and zinc levels correlated closely in the entire patient cohort ($r = 0.838$, $P < .001$; Fig 4, *C*).

Serum cytokine profile of Hz/Hc and PAPA syndrome

In view of the increased MRP8/14 levels detected in patients with Hz/Hc, we investigated the profile of circulating cytokines to define an inflammatory signature using serum samples obtained during active disease from 8 patients with Hz/Hc (7 with p.E250K and 1 with p.E257K), 5 patients with PAPA syndrome (2 with p.A230T, 2 with p.E250Q, and 1 with p.E256G), and 22 healthy control subjects (female/male sex, 12/10; age range, 3-16 years). All samples were obtained at the time of clinical and serologic active disease. Expression levels were stratified into (1) cytokines that were only upregulated or downregulated in patients with Hz/Hc (ie, IL-2, IL-15, IL-22, and IFN- γ); (2) cytokines that were upregulated in patients with Hz/Hc and those with PAPA syndrome but significantly higher in patients with Hz/Hc (ie, IL-8, IL-18, and IL-33); and (3) cytokines that were significantly upregulated or downregulated in patients with Hz/Hc and those with PAPA syndrome compared with control subjects (ie, IL-1 β , IL-4, IL-6, IL-10, CCL2, CCL3, CCL17, CCL18, CCL22, CXCL9, intercellular adhesion molecule, vascular cell adhesion molecule, IFN- α , macrophage colony-stimulating factor, and MIF). Levels of IL-1 α , IL-5, IL-12, IL-13, IL-17, IL-21, CCL11, granulocyte colony-stimulating factor, GM-CSF, IFN- γ -induced protein 10, TNF- α , and receptor activator of nuclear factor κB ligand were not significantly

different from those in control subjects (Fig 5 and see Table E4 in this article's Online Repository at www.jacionline.org).

Response to treatment

Response to treatment was evaluated in all patients with Hz/Hc (see Table E5 in this article's Online Repository at www.jacionline.org). Interestingly, no consistently effective therapy was observed. Best responses were noted when using IL-1 inhibitors, either anakinra (n = 4) or canakinumab (n = 1), but there were a few patients who were unresponsive to this therapy (n = 5). Treatment with cyclosporine (n = 5) or prednisolone (n = 6) resulted in a partial response. Of note, cutaneous symptoms, arthritis, anemia, and systemic inflammation improved, but neutropenia persisted in all patients after treatment. In 3 patients (hepato)splenomegaly resolved with treatment.

Serum profiles of cytokines, MRP8/14, and zinc were investigated in paired samples of 4 patients with a partial response to IL-1 or TNF inhibition or GM-CSF and 4 patients with no response to IL-1 or TNF inhibition and a flare under continuous treatment with cyclosporine. Partial response to treatment was accompanied in all 4 treated patients by a decrease of IL-8, IL-18, IL-33, IL-6, CCL18, MIF, MRP8/14, and zinc serum concentrations (see Figs E1 and E2 in this article's Online Repository at www.jacionline.org).

Discussion

Hz/Hc is an autoinflammatory disorder characterized by chronic systemic inflammation, prominent skin inflammation, arthralgia/arthritis, hepatosplenomegaly, pancytopenia, and failure to thrive.² A hallmark of the disease is the extreme increase in serum concentrations of the proinflammatory alarmins MRP8 and MRP14 and concomitantly zinc. In this study we report the genetic cause for this disorder, namely a single amino acid substitution in the γ -domain of PSTPIP1 (E→K) either at position p.E250 or p.E257. Clinically, Hz/Hc is distinct from the other *PSTPIP1*-associated disease, PAPA syndrome, based on the presence of a severe course and early-onset disease, hepatosplenomegaly, and failure to thrive. Variable expressivity of disease was noted both in patients with Hz/Hc and those with PAPA syndrome, with patient P8 showing a weaker phenotype than his father (patient P9) in the cohort with Hz/Hc. Incomplete penetrance has been previously reported in families with PAPA syndrome²¹ and seems also to be a feature of the p.E250K mutation. Two patients with Hz/Hc died of postoperative complications after liver transplantation or sepsis.

Regarding laboratory findings, Hz/Hc is characterized by severe anemia and neutropenia, which is present in all patients and accompanied by thrombocytopenia in most patients. By contrast, anemia and neutropenia were not or only rarely observed in patients with PAPA syndrome. Bone marrow aspirates in patients with Hz/Hc showed evidence of dysgranulopoiesis and dyserythropoiesis, which is consistent with observed pancytopenia. It is yet unclear whether the hematologic findings are related to chronic inflammation or could be the result of altered *PSTPIP1* function. *PSTPIP1* is known to regulate neutrophil and macrophage functions,^{34,35} but its role in cell maturation has not been reported. Because of the severe neutropenia, we were not able to perform functional studies with purified neutrophils. In addition, patients with Hz/Hc could be clearly separated from patients with

PAPA syndrome based on extremely high MRP8/14 and zinc serum levels. In summary, these data suggest a unique genotype-phenotype correlation for mutations in the *PSTPIP1* gene.

MRP8/14 alarmins have been identified as endogenous ligands for TLR4-mediated expression of proinflammatory cytokines, chemokines, NADPH oxidase, or adherence molecules.^{1,6,7,11} The extremely high expression of this endogenous TLR4 ligand in patients with Hz/Hc suggests a role for MRP8/14 in the pathogenesis of severe PSTPIP1-mediated inflammation. In addition, patients with Hz/Hc could be clearly differentiated from those with PAPA syndrome based on a distinct cytokine profile (Table E3). Interestingly, many of these proteins play a role in positive inflammatory feedback mechanisms between innate immune cells and the epithelium.^{36–38} IL-22 and combination of IFN- γ and TNF- α are known inducers of MRP expression in epithelial cells.^{37–39}

Hyperzincemia is caused by the zinc-capturing properties of MRP8/14,² and zinc levels are increased in patients with Hz/Hc but not in patients with PAPA syndrome, FMF, or SJIA. Usually inflammation induces a reduction in plasma zinc concentrations because of redistribution in the cellular compartment.³⁹ In patients with sepsis, lower plasma zinc concentrations were associated with higher illness scores and increased cytokine production.⁴⁰ Therefore the unique high zinc levels in patients with Hz/Hc are interesting observations in an inflammatory context. Zinc deficiency is associated with higher IL-1 β production and lower IL-2 and IL-6 production.^{41–44} Conversely, in promonocytic cells zinc increased both IL-6 and IL-8 levels,⁴⁵ and under inflammatory conditions, IFN- γ gene expression seems to be reduced by zinc deficiency.^{46,47} Therefore hyperzincemia might contribute to the high IL-2, IL-6, IL-8, and IFN- γ levels found in patients with Hz/Hc.

Among the identified cytokines, some are characteristic for the T-cell compartment as IL-2 or IL-22, indicating a role of the adaptive immune system in patients with Hz/Hc. Additionally, in a murine model of systemic autoimmune disease, we have shown that MRP8 and MRP14 expression at the site of inflammation is essential for the development of autoreactive T cells and promotion of systemic autoimmunity.⁸ This mechanism might enhance inflammation in the chronic phase of this autoinflammatory syndrome. Although B-cell distribution was normal in patients with Hz/Hc and those with PAPA, we found serologic constellations of AIN as a result of anti-neutrophil antibody production in both phenotypes (66% vs 33% of samples). Because autoantibodies were not found in all neutropenic patients, this seems not to be the only responsible mechanism. However, AIN might contribute to neutropenia in patients with Hz/Hc and seems to be a result of the activation of the adaptive immune system within an autoinflammatory disease.

The p.E250K and p.E257K substitutions in PSTPIP1 are located within the coiled-coil region, which is important for pyrin and PTP-PEST interaction. The electrostatic modeling of PSTPIP1 displays a large negative patch in the critical region that is significantly affected by both mutations but not found for the classical PAPA-associated mutation E250Q. The PAPA-causing mutations have been shown to disrupt PSTPIP1-PEST phosphatase interactions, resulting in hyperphosphorylation of PSTPIP1 and increased pyrin avidity.¹⁹ This binding might release pyrin from its autoinhibition, triggering increased apoptosis-

associated speck-like protein containing a carboxyterminal CARD oligomerization and caspase-1 activation.⁴⁸ Furthermore, the increased PSTPIP1-pyrin avidity also results in altered PSTPIP1 cellular distribution and its greater colocalization with the apoptosis-associated speck-like protein containing a carboxy-terminal CARD inflammasome.¹⁹ We showed that p.E250K mutation increases PSTPIP1-pyrin interactions compared with p.E250Q. The pE250K-mutated PSTPIP1 protein shows comparable hyperphosphorylation with p.E250Q indicating that the increased PSTPIP1-pyrin binding is not just dependent on the phosphorylation status of the protein. These data are in accordance with earlier reports indicating a role of the coiled-coil domain of PSTPIP1 in addition to phosphorylation of tyrosine Y344 in the binding of pyrin.¹⁷ The fact that a single negative charge switch in PSTPIP1 causes major phenotypic changes with clinical significance is unique for this group of autoinflammatory diseases, and the analysis of the underlying molecular mechanism will be a challenging matter for future research. Because significantly increased MRP8/14 levels can be found in patients with FMF and those with PAPA syndrome and are extremely high in patients with Hz/Hc, it seems to be likely that the MRP secretion is regulated by the pyrin-PSTPIP1 axis. A link might be the involvement of tubulin in the pyrin-PSTPIP1 axis^{19,49} because MRPs are released in an alternative tubulin-dependent manner.⁴ Patients with cryopyrin-associated periodic syndromes with mutations in the NOD-like receptor family, pyrin domain, containing 3 gene have MRP8/14 levels within the range of systemic infections.⁵⁰ Therefore highly increased serum levels of MRP8/14 are the first specific biomarker for diseases affecting NOD-like receptor family, pyrin domain containing 3-independent inflammasome activation.^{51,52}

The efficacy of anti-TNF treatment and inconsistent efficacy of recombinant IL-1RA (anakinra) treatment of patients with PAPA syndrome have been reported.²¹ Reports about treatment of Hz/Hc are limited, with preliminary evidence for favorable treatment with cyclosporine¹⁵ and adalimumab.⁵³ In our cohort of patients with HZ/Hc, IL-1 blockade was partially effective in 5 of 10 patients; cyclosporine or prednisolone showed reasonable responses as well. Anti-TNF treatment was only effective in 1 of 3 patients. Interestingly, improvement in cutaneous symptoms, arthritis, anemia, systemic inflammation, and hepatosplenomegaly was noted, whereas neutropenia was still present in all 3 patients. Therefore our data indicate the necessity for an individual tailored treatment regimen. Response to treatment was accompanied by a decrease in levels of serum proteins, including IL-18 and MRP8/14.

This study reports the largest cohort of patients with *PSTPIP1* mutations. PSTPIP1-associated diseases show variable expressivity and are associated with a range of clinical phenotypes. To avoid confusion, we would like to suggest a novel nomenclature for this group of related diseases that is based on the causal genotypes, biochemical findings, and clinically distinct symptoms. In analogy to cryopyrin-associated periodic syndrome, we suggest the term PSTPIP1-associated inflammatory diseases (PAID) as a new class that encompasses PAPA syndrome (p.A230T, p.E250Q, and p.E256G), pyogenic arthritis, pyoderma gangrenosum, acne, and hidradenitis suppurativa syndrome (p.E277D)⁵⁴ and patients with Hz/Hc (p.E250K and p.E257K). We would like to suggest the term PSTPIP1-associated myeloid-related proteinemia inflammatory syndrome (PAMI) for the latter entity. We further propose that patients with undefined autoinflammatory syndromes and

significant skin inflammation (or pyoderma gangrenosum) should be tested for MRP8/14 serum levels to initiate targeted genetic testing.

In conclusion, an amino acid change in the y-domain of PSTPIP1 (E→K) causes a clinically and serologically distinct autoinflammatory disorder representing a novel and unique genotype-phenotype correlation. The PSTPIP1-associated myeloid-related proteinemia inflammatory syndrome (Hz/Hc) inflammatory phenotype is associated with a characteristic cytokine profile and characterized by extremely high levels of the proinflammatory alarmins MRP8 and MRP14.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AIN	Autoimmune neutropenia
FMF	Familial Mediterranean fever
Hz/Hc	Hyperzincemia and hypercalprotectinemia
MIF	Macrophage migration inhibitory factor
MRP	Myeloid-related protein
PAPA	Pyogenic arthritis, pyoderma gangrenosum, and acne
PSTPIP1	Proline-serine-threonine phosphatase-interacting protein 1
SJIA	Systemic juvenile idiopathic arthritis
TLR	Toll-like receptor
WT	Wild-type

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Key messages

- The novel heterozygous missense mutations p.E250K and p.E257K in the *PSTPIP1* gene are the cause for the autoinflammatory disorder Hz/Hc.
- Patients with Hz/Hc can be separated from patients with PAPA syndrome based on a distinct clinical phenotype, cytokine profile, and first of all, extremely high MRP8/MRP14 expression and serum concentrations.

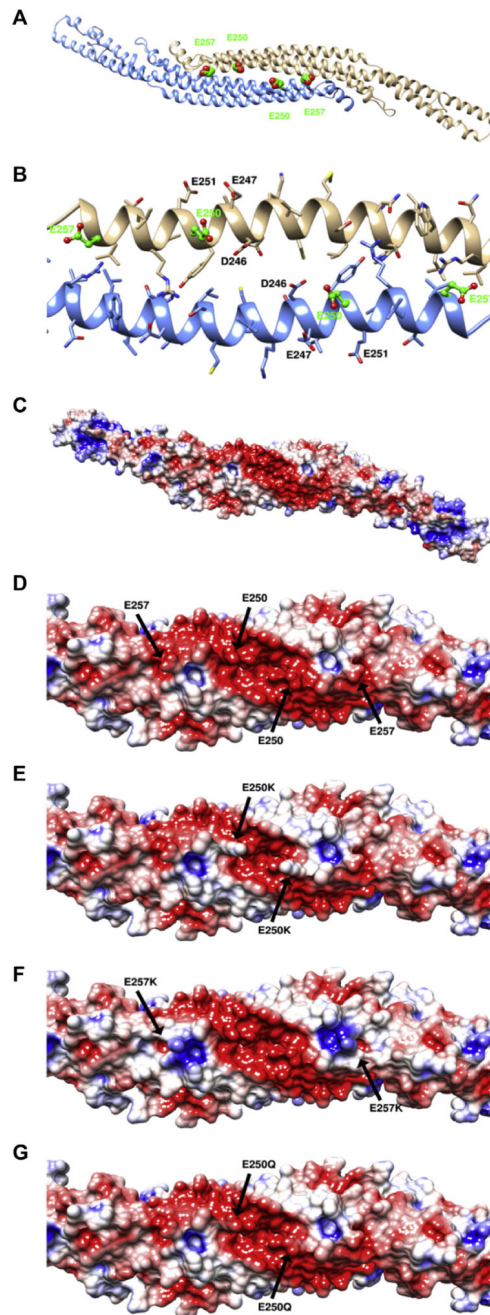


Fig 2. Mutations of patients with Hz/Hc alter the electrostatic potential of PSTPIP1. **A**, PSTPIP1 FCH domain in a ribbon diagram of PSTPIP1 dimer with Hz/Hc mutations (*green*). **B**, p.E250 in a cluster of 4 negatively charged side chains. **C-G**, Electrostatic potential in (*blue*, positive charge; *red*, negative charge; Fig 2, *C*) in WT (Fig 2, *D*), p.E250K (Fig 2, *E*), p.E257K (Fig 2, *F*), and p.E250Q mutants (Fig 2, *G*).

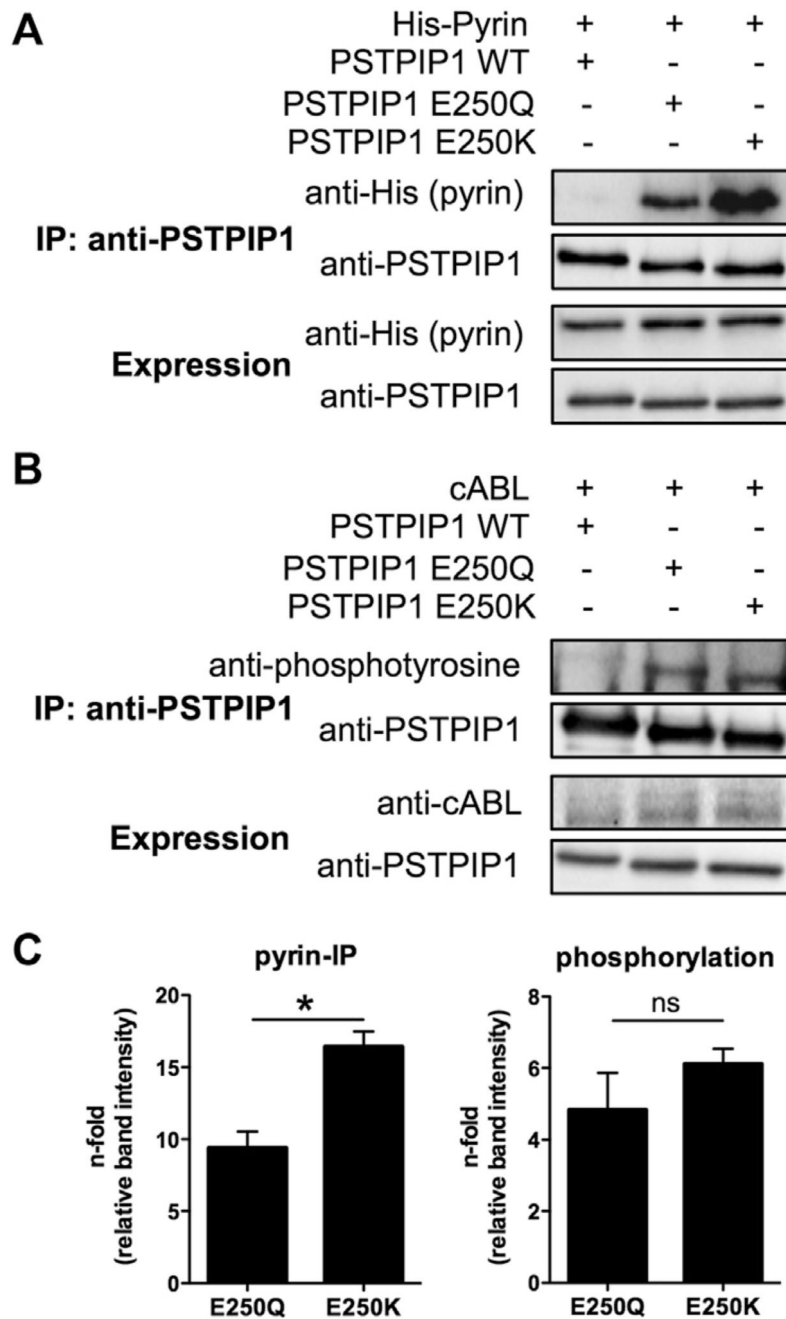


Fig 3. p.E250K mutation increases PSTPIP1-pyrin binding. **A**, Cotransfection with pyrin and PSTPIP1 WT or p.E250Q or p.E250K mutants. Immunoprecipitation (*IP*) was performed with anti-PSTPIP1 and immunoblotted with anti-pyrin and anti-PSTPIP1. **B**, Cotransfection with PSTPIP1 WT, p.E250Q or p.E250K mutants, and cAbl. Immunoprecipitation with anti-PSTPIP1, phosphorylation was detected with anti-phosphotyrosine. **C**, Relative band intensity was measured and expressed as n-fold to PSTPIP1 WT. * $P < .05$.

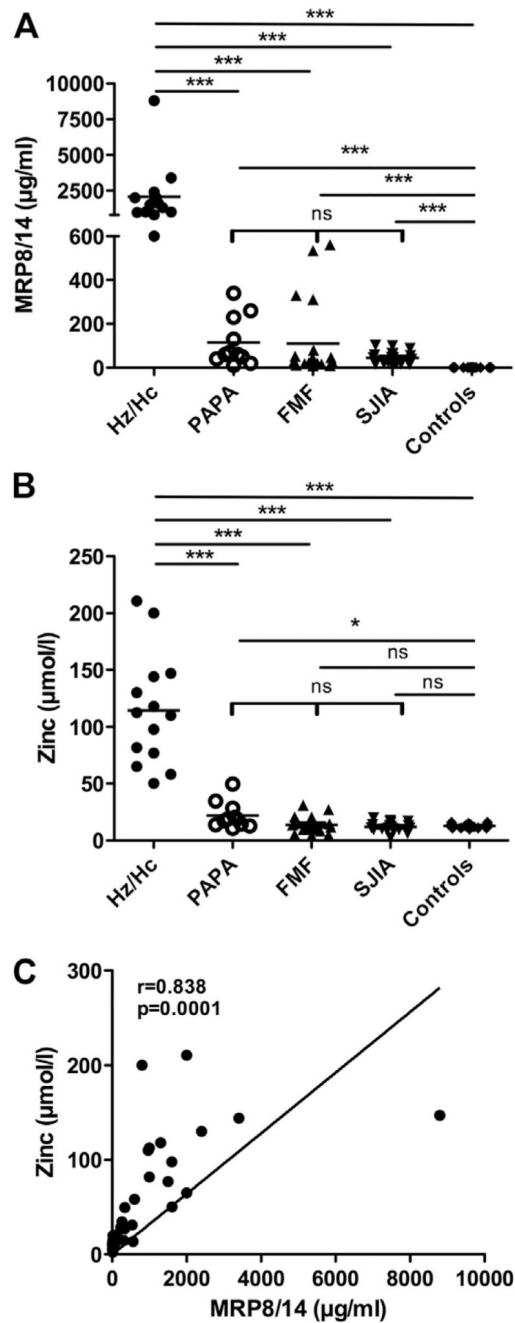


Fig 4. Patients with Hz/Hc can be distinguished from those with PAPA syndrome, FMF, or SJIA based on MRP8/14 and zinc serum levels. **A** and **B**, MRP8/14 (Fig 4, **A**) and zinc (Fig 4, **B**) serum levels in patients with active disease (patients with Hz/Hc, n = 13; patients with PAPA syndrome, n = 11), patients with FMF (n = 20), patients with SJIA (n = 20), or healthy control subjects (n = 20). **C**, Correlation of MRP8/14 and zinc levels in all patients. * $P < .05$ and *** $P < .001$.

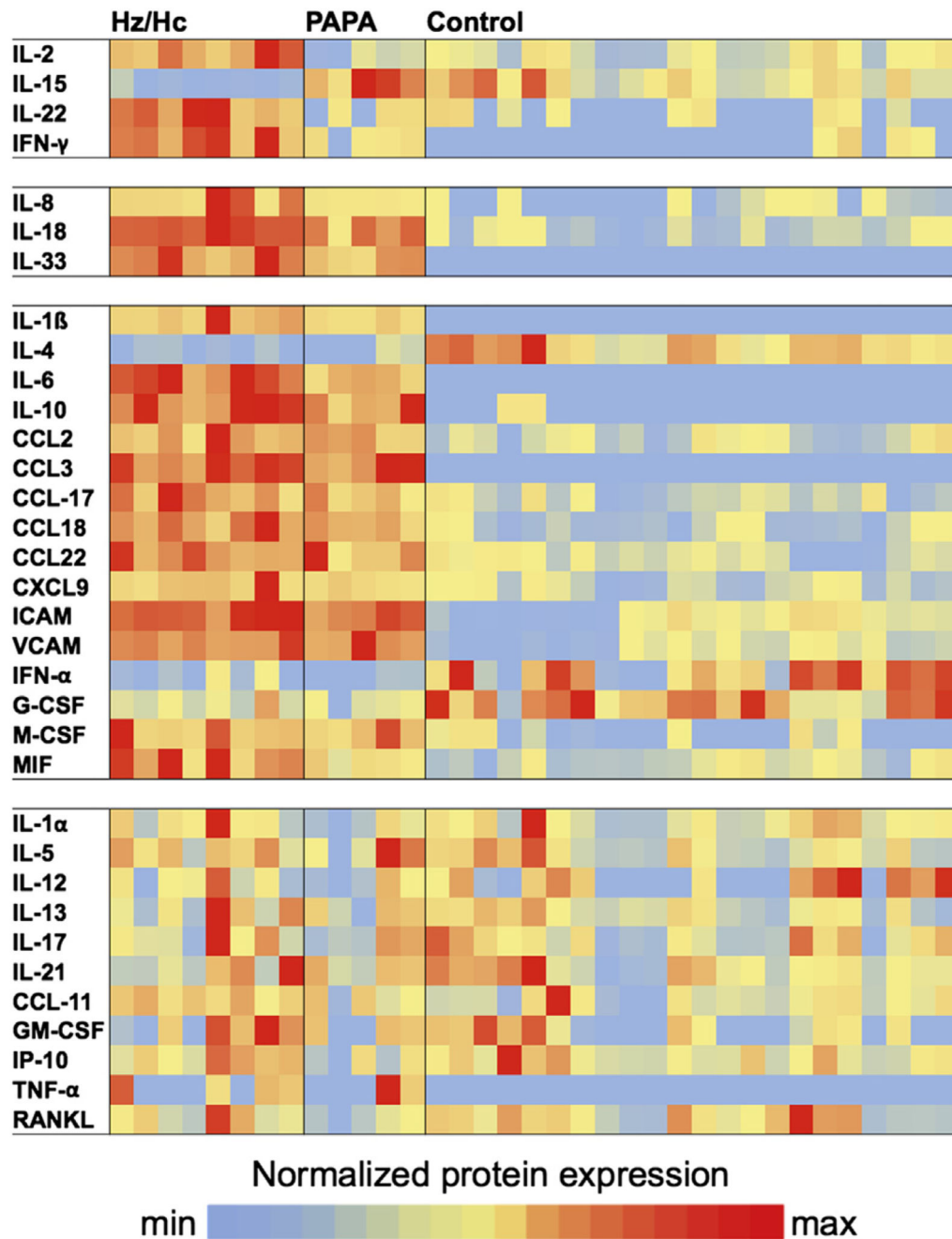


Fig 5. Patients with Hz/Hc can be distinguished from patients with PAPA syndrome and healthy control subjects based on serum protein expression levels. Protein serum concentration levels were classified by differential expression between patients with Hc/Hz, patients with PAPA syndrome, and control subjects. Expression values are normalized for each gene to the healthy group. Transformed expression levels are indicated by color scale with relatively high (*red*) and relatively low (*blue*) expression.