First Description of Late-Onset Autoinflammatory Disease Due to Somatic NLRC4 Mosaicism

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Objective. Autoinflammatory diseases are inherited disorders of innate immunity that usually start during childhood. However, several recent reports have described an increasing number of patients with autoinflammatory disease starting in adulthood. This study was undertaken to investigate the underlying cause of a case of late-onset uncharacterized autoinflammatory disease.

Methods. Genetics studies were performed using Sanger sequencing and next-generation sequencing (NGS) methods. In silico, in vitro, and ex vivo analyses were performed to determine the functional consequences of the detected variant.

Results. We studied a 57-year-old woman who at the age of 47 years began to have recurrent episodes of fever, myalgias, arthralgias, diffuse abdominal pain, diarrhea, adenopathies, and systemic inflammation, which were relatively well controlled with anti–interleukin-1 (anti–IL-1) drugs. NGS analyses did not detect germline variants in any of the known autoinflammatory disease–associated genes, but they identified the p.Ser171Phe NLRC4 variant in unfractionated blood, with an allele fraction (2–4%) compatible with gene mosaicism. Structural modeling analyses suggested that this missense variant might favor the open, active conformation of the NLRC4 protein, and in vitro and ex vivo analyses confirmed its propensity to oligomerize and activate the NLRC4 inflammasome, with subsequent overproduction of IL-18.

Conclusion. Our findings indicate that the postzygototic p.Ser171Phe NLRC4 variant is a plausible cause of the disease in the enrolled patient. Functional and structural studies clearly support, for the first time, its gain-of-function behavior, consistent with previously reported NLRC4 pathogenic variants. These novel findings should be considered in the diagnostic evaluation of patients with adult-onset uncharacterized autoinflammatory disease.

INTRODUCTION

Autoinflammatory diseases encompass a group of immune disorders characterized by recurrent episodes of sterile inflammation. The genetic basis of ~40 monogenic autoinflammatory diseases has been identified, with inflammasomopathies representing the largest subgroup. They are a consequence of gene defects affecting the sensing proteins of the inflammasome (1). Cryopyrin-associated periodic syndromes (CAPS) and NLRC4-associated autoinflammatory disease are 2 inflammasomopathies that are a consequence of gain-of-function mutations in the NLRP3 and NLRC4 genes, respectively. The 2 diseases are different but share some features, such as onset during childhood, cutaneous lesions, fever, and systemic inflammation (1–3). Both germline and postzygototic variants in the respective genes have been identified as causes of disease. Postzygototic variants in the NLRC4 gene were first identified in

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children with severe CAPS (4) and subsequently in adult patients with late-onset CAPS (5–7). In contrast, only 2 children carrying postzygotic NLRC4 variants have been reported, and there are no known patients whose disease began during adulthood (8–9).

In this study, we evaluated a patient with a late-onset uncharacterized autoinflammatory disease partially controlled with interleukin-1 (IL-1) blockade. Genetics investigations detected the p.Ser171Phe NLRC4 variant with a mutant allele fraction of 2–4%. To demonstrate the functional consequences of this variant, different in silico, in vitro, and ex vivo analyses were performed, which showed NLRC4 inflammasome hyperactivation comparable with that provoked by previously reported NLRC4 mutations.

PATIENTS AND METHODS

Patients. Data were collected through individual interviews and medical chart review. Samples from the patient, healthy controls, and disease controls were collected after obtaining written informed consent and approval of the Ethical Review Boards of Hospital San Pedro de Alcántara and Hospital Clinic. All investigations were performed in accordance with the 1964 Declaration of Helsinki and its later amendments.

Genetics and functional analysis. Analyses of autoinflammatory disease–associated genes were performed by next-generation sequencing (NGS) methods (see the Supplementary Methods, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999, for a detailed explanation). In silico structural modeling was performed with the NLRC4 protein in the UniProt database and different programs (MODELLER, Chimera, Prosa2003, and SPserver). Functional studies of the NLRC4 inflammasome were performed both in vitro with transfection of cells with mutant or wild-type NLRC4 gene, and ex vivo with fresh blood samples from the patient, healthy controls, and CAPS patients carrying the p.Ala439Thr NLRP3 variant. Circulating cytokines were measured using a bead-based Luminex immunoassay (Supplementary Table 1, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999). See the Supplementary Methods, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999, for a detailed description of the methods used for all of these procedures.

RESULTS

Clinical description. The patient, a 57-year-old woman, was born to a nonconsanguineous Spanish couple, with no relevant family history of disease (Figure 1A). Her medical history included left renal agenesis with normal renal function, late-onset sensorineural hearing loss, and hypothyroidism. At the age of 47 years, she began to experience episodes of fever (39–40°C) and systemic inflammation (Figure 1B and Supplementary Figure 1, Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999), which lasted 2–3 days and recurred every 6–8 weeks. Infectious, autoimmune, and malignant causes were ruled out. Colchicine (1 mg once daily) and ibuprofen (600 mg every 8 hours) were ineffective in controlling the disease. At the age of 53 years, the frequency of the episodes increased, with associated sweating and shivering. At that time, prednisone (1 mg/kg once daily) was started, but the fever did not resolve. Anakinra (100 mg subcutaneously once daily) was then started, which led to clinical improvement and a decrease in, but not normalization of, inflammatory parameters (Figure 1B and Supplementary Figure 1).

At the age of 55 years, despite anakinra treatment, the patient’s clinical status worsened, presenting with high fever, sweating, shivering, weakness, myalgias, arthralgias, asthenia, diffuse abdominal pain, diarrhea, and mesenteric adenopathies. Laboratory tests performed at that time revealed increased levels of acute-phase reactants and anemia, but no hyperinflammatory episodes resembling macrophage activation syndrome (MAS) have ever been detected. An endoscopic study revealed gastritis and amyloid deposits in biopsy specimens from the colon, stomach, and duodenum. To characterize the concrete type of amyloidosis, serum and urine proteinogram and immunofixation, serum free light chain quantification, TTR gene analyses, cardiac magnetic resonance imaging, and immunohistochemistry of amyloid deposits were performed. All of these tests yielded normal or negative results, with the exception of immunohistochemistry studies, which revealed the presence of AA-type amyloid deposits (Supplementary Figure 2, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999). At that time, anakinra was up-titrated (200 mg subcutaneously once daily), leading to a normalization of inflammatory markers as well as the disappearance of amyloid deposits.

Disclosure form.pdf.

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mesenteric adenopathies. At the end of 2019, canakinumab (150 mg subcutaneously every 4 weeks) was started due to pain and subcutaneous nodules at sites of anakinra injections, with a good clinical and analytical control of the disease, with the only exception being hearing loss, which did not improve.

Genetics. Based on clinical history, a genetics study for autoinflammatory disease was performed using targeted gene panel sequencing (Supplementary Table 2, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999). Analyses for germline variants did not detect any variant in any gene. In contrast, when the sequence reads were specifically filtered for postzygotic variants, the c.512C>T transition in the \( \text{NLRC4} \) gene was identified with a mutant allele fraction of \(~3\%\) in peripheral blood (PB), with a \( 2,865 \times \) deep coverage. To confirm the presence of the postzygotic variant, amplicon-based deep sequencing (ADS) studies were performed in PB samples collected during the last 3 years. These studies detected the variant in all samples with a relatively stable mutant allele fraction (2–4\%) (Supplementary Table 3, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999).

The c.512C>T \( \text{NLRC4} \) variant is predicted to lead to the p.Ser171Phe variant, which may be classified as pathogenic by its absence from public databases, the results of bioinformatics analyses (Supplementary Table 4, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999), and its location in an
evolutionarily conserved amino acid residue (Supplementary Figure 3, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999), as well as according to a previous study (9). Sanger chromatograms revealed a very small peak at position c.512 corresponding to the variant allele (Figure 1C). ADS studies also detected the NLRC4 variant in cells of both hematopoietic and nonhematopoietic lineages, suggesting an early occurrence of the mutational event. Genetics investigations performed in the patient’s relatives did not detect the p.Ser171Phe NLRC4 variant (Figure 1A). When the p.Ser171Phe NLRC4 variant was first described, no data about its functional consequences were available (9). Consequently, we performed additional experiments to address this issue.

Structural modeling. The in silico structural studies were performed using murine NLRC4 without the caspase recruitment domain (UniProt code Q9NPP4), which is highly homologous to human NLRC4. These studies showed that the location of p.Ser171 was completely buried in both the closed (monomeric) and open (oligomeric) conformations (Supplementary Figure 4, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999). However, the environment of residue side chains around p.Ser171 is markedly

Figure 2. Structural analysis of the environment of Ser171 in the closed and open conformations of NLRC4 without the caspase recruitment domain. A, Ribbon plot of the closed conformation of NLRC4, highlighting the side chains of Ser171 (red) and the side chains of the closest residues in contact (yellow), Glu280, Thr282, Ser445, and Glu448, all of which have a polar character. B and C, Energy score profiles of the difference between the mutant form S171F and the wild-type form of NLRC4 in the closed conformation, calculated using Prosa2003 (B) and SPserver (C). D, Ribbon plot of the open conformation of NLRC4, highlighting the side chains of Ser171 (red) and the side chains of the closest residues in contact, Cys428 (yellow) and Phe442 (green). E and F, Energy score profiles of the difference between the mutant form S171F and the wild-type form of NLRC4 in the open conformation, calculated using Prosa2003 (E) and SPserver (F).
different between the 2 conformations. While the environment of p.Ser171 in the closed conformation is composed of polar and charged residues (p.Thr280, p.Glu282, p.Ser445 and p.Glu448) (Figure 2A), the environment in the open conformation is less polar (p.Cys428 and p.Phe442) (Figure 2D).

The p.Ser171Phe substitution largely destabilizes the contacts of the side chain in the closed conformation, as is shown by the difference in Prosa2003 and SPserver scores between the mutant and the wild-type forms (>4 with Prosa2003 and >1 with SPserver) (Figures 2B and C). In contrast, this substitution stabilizes the local contacts of the open conformation according to the SPserver scores. The Prosa2003 score shows that the p.Ser171Phe substitution in the open conformation is less stable than the native form (Figures 2E and F), with the overall effect being less stressful than for the closed conformation. Consequently, these models strongly suggest that the p.Ser171Phe variant might favor the open conformation of NLRC4 that is found in active oligomers, which either stabilizes or preserves the stability of the oligomerization, yielding the inflammasome complex.

**Functional analyses.** In vitro expression of the p.Ser171Phe NLRC4 variant in HEK 293T cells resulted in a

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**Figure 3.** The p.Ser171Phe NLRC4 variant induces increased inflammasome activation. **A,** Percentage of cells with NLRC4 oligomers among HEK 293T cells expressing wild-type NLRC4 or the p.Ser171Phe NLRC4 variant. **B,** Percentage of cells with ASC oligomers among HEK 293T cells expressing ASC alone, ASC and wild-type NLRC4, or ASC and the p.Ser171Phe NLRC4 variant. **C,** Percentage of ASC-specking monocytes in healthy controls, the patient with the p.Ser171Phe NLRC4 variant, and patients with cryopyrin-associated periodic syndromes (CAPS) with the p.Ala439Thr NLRP3 variant, determined by time-of-flight assay. Samples were left untreated, treated with lipopolysaccharide (LPS) alone, or primed with LPS and stimulated with ATP. **D** and **E,** Interleukin-18 (IL-18) **(D)** and IL-1β **(E)** release in cell-free supernatants from peripheral blood mononuclear cells from healthy controls without autoinflammatory disease, the patient with the p.Ser171Phe NLRC4 variant, and CAPS patients with the p.Ala439Thr NLRP3 variant. Samples were left untreated, treated with LPS alone, or primed with LPS and stimulated with ATP. Circles represent individual samples; bars show the mean ± SD. * = P < 0.05; ** = P < 0.005; *** = P < 0.0005; **** = P < 0.00005. NS = not significant.
higher number of cells with NLRC4 oligomers when compared to cells expressing wild-type NLRC4 (Figure 3A and Supplementary Figures 5A and B, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999). To gain insight into whether these oligomers were functional, both wild-type and mutant NLRC4 were expressed together with ASC, which showed that the p.Ser171Phe variant induces a higher number of cells with ASC specks (Figure 3B and Supplementary Figure 5C).

Similarly, the proportion of monocytes with ASC specks was constitutively increased in a single sample from the patient when compared to healthy controls, similar to the increase seen in CAPS patients (Figure 3C). Lipopolysaccharide (LPS) treatment increased the percentage of monocytes with ASC specks from the patient compared to healthy controls (Figure 3C). These differences disappeared when the canonical NLRP3 inflammasome was activated with LPS and ATP (Figure 3C). The patient’s peripheral blood mononuclear cells (PBMCs) released higher amounts of IL-18 when compared to healthy controls (Figure 3D), but they failed to release IL-1β, even after canonical NLRP3 inflammasome activation (Figure 3E). The similar release of LPS-induced tumor necrosis factor (TNF) and IL-6 seen in both the patient and healthy controls ruled out a potential defect in LPS priming (Supplementary Figure 6A, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999). However, we found that PBMCs from the patient with the p.Ser171Phe NLRC4 variant failed to increase IL1B messenger RNA after LPS stimulation, but presented an induction of IL6 (Supplementary Figure 6B). The deficiency in IL-18 production was not dependent on a deficient number of monocytes in the patient’s PBMC samples (Supplementary Figure 6C). Finally, NLRC4 activation in the patient’s PBMCs did not result in a significant increase in the percentage of ASC-specking monocytes, IL-18, or IL-1β release (Supplementary Figure 6D), suggesting that mutant NLRC4 is not potentiated with specific NLRC4 stimuli.

The results of these ex vivo assays were consistent with the pattern of cytokines quantified in different serum samples from the patient. Despite these samples being obtained at different times during treatment with anti-IL-1 agents, an increased serum level of IL-18 was persistently detected. These IL-18 levels were similar to those detected in patients carrying the p.Ser445Pro NLRC4 pathogenic variant, and significantly higher than those detected in healthy controls and CAPS patients (Figure 1D).

In contrast, no differences were detected in the remaining cytokines quantified (Supplementary Figure 7, available on the Arthritis & Rheumatology website at https://onlinelibrary.wiley.com/doi/10.1002/art.41999 and data not shown).

**DISCUSSION**

NLRC4 is a cytosolic nucleotide-binding oligomerization domain–like receptor that cooperates with neuronal apoptosis inhibitor protein to detect flagellin and bacterial type 3 secretion system components (10). Once detected, NLRC4 oligomerizes, forms an inflammasome complex, activates caspase 1, and promotes the production of inflammatory cytokines of the IL-1 family. Gain-of-function NLRC4 pathogenic variants have been described as the cause of a dominantly inherited autoinflammatory disease with variable phenotype presentations. The first described phenotype was characterized by early-onset cutaneous lesions, enterocolitis, arthritis, and recurrent MAS episodes (2,3). Subsequently, novel phenotypes were reported, including a familial form of cold-induced autoinflammatory syndrome and painful erythematous nodules (11,12). Increasing evidence suggests a major role of IL-18, and not IL-1β, in disease pathogenesis, including persistently elevated serum levels of total and free IL-18, and clinical efficacy of IL-18 blockade (13). From a genetics point of view, most reported cases are a consequence of germline NLRC4 variants, with only 2 cases with postzygotic variants and starting during childhood described to date (8,9).

In the patient described herein, recurrent fever and systemic inflammation were the main features detected at disease onset, with no cutaneous lesions. As the disease progressed, additional manifestations appeared, including gastrointestinal and musculoskeletal features as well as changes in the frequency of episodes. Interestingly, all of these manifestations improved, but did not normalize, with IL-1 blockade. Overall, the clinical picture strongly suggested an uncharacterized late-onset autoinflammatory disease, but the concrete features did not fit well with any of the monogenic autoinflammatory diseases (14). Despite the low probability of finding a genetic defect, the genetics study performed identified the p.Ser171Phe NLRC4 variant, with a mutant allele fraction compatible with that expected for postzygotic variants causing mosaicism (15). Interestingly, this NLRC4 variant has previously been reported as mosaic in a 2-month-old infant with a fatal disease with laboratory features of MAS (9). The differences in clinical manifestations, results of laboratory tests, and outcomes between these 2 cases are enormous, and we speculate that this may be mainly attributable to the differences in the degree of mosaicism. In the patient described herein, the mutant allele fraction remained stable at 2–4% during the most recent years, whereas a mutant allele fraction of 25% was reported in the previously described fatal case (9).

The lack of investigations addressing the functional consequences of the p.Ser171Phe NLRC4 variant in a previous study was the major difficulty in unequivocally establishing its pathogenicity (9). To address this, different in silico, in vitro, and ex vivo analyses were performed. The results of structural modeling analyses strongly suggested that a Ser-to-Phe amino acid exchange at residue 171 might favor an open conformation of the NLRC4, either stabilizing or preserving the stability of the polymerization yielding the assembly of the NLRC4 inflammasome complex. These results are consistent with the data obtained in both in vitro and ex vivo studies. In vitro analyses clearly showed a
higher degree of NLRC4 oligomerization and ASC speck formation when mutant NLRC4 was expressed in HEK 293T cells in comparison with cells transfected with wild-type NLRC4, a behavior expected for a gain-of-function variant.

The data obtained by ex vivo assays were also consistent with gain-of-function for the NLRC4 variant and support hyperactivation of the NLRC4 inflammasome as the mechanism of disease, with increased ASC specks and IL-18 overproduction, similar to previous studies of NLRC4-associated MAS (3). However, IL-1β release from the patient’s PBMCs was decreased when compared to healthy controls and to patients with CAPS, contrasting with previous data on NLRC4-associated MAS that indicated exacerbated IL-1β production (3). The low IL-1β production by the patient’s cells could not be attributed to a decrease in monocytes in the PBMCs or impaired LPS priming and NF-κB activation, since the percentage of monocytes or TNF and IL-6 release was similar among the patients and healthy controls, which indicates a normal priming of the cells by LPS. However, LPS failed to increase IL1B gene expression, but not IL6 gene expression, suggesting that this failure could be responsible for the lack of IL-1β release from the patient’s cells.

Therefore, this patient is differentiated from patients in other studies where enhanced release of IL-1β and IL-6 was found from LPS-treated monocytes from patients with NLRC4-associated MAS (3). These differences could be due to the use of positively isolated monocytes versus whole PBMCs used for ex vivo stimulation in this study, the duration of LPS stimulation (4 hours less in our study), or different phenotype responses due to distinct NLRC4 variants. The results of our ex vivo functional experiments, as well as the pattern of circulating cytokines, support the treatment of our patient, and potentially other autoinflammatory disease patients with NLRC4 gain-of-function mutations, with IL-18-blocking therapies, some of which have already been successfully employed in a patient with severe NLRC4-associated MAS (13).

In conclusion, the evidence here summarized clearly supports a gain-of-function behavior of the p.Ser171Phe variant in a manner similar to other NLRC4 variants previously reported as disease-causing mutations. To the best of our knowledge, this patient represents the first case of late-onset NLRC4-associated autoinflammatory disease as a consequence of somatic NLRC4 mosaicism, raising the question of whether inflammatory manifestations starting during adulthood in other patients may be a consequence of a similar genetic defect. In this sense, it is appropriate to consider using NGS methods with high coverage depths and adequate filtering strategies in the reads analyses during the search for postzygotic variants. Despite the partial control of inflammatory manifestations with IL-1 blockade, the high serum levels of IL-18 detected and the overproduction of IL-18 from the patient’s cells strongly suggest that she may benefit from treatment with novel anti-IL-18 drugs. Additional studies addressing this particular point are without doubt warranted.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Aróstegui had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Fernández-Pereira, Pelegrín, Aróstegui.

Acquisition of data. Ionescu, Peñín-Franch, Mensa-Vilaro, Castillo, Hurtado-Navarro, Molina-López, Romero-Chala, Plaza, Fabregat, Buñáñ, Marques, Casals, Yagüe, Oliva, Fernández-Pereira, Pelegrín, Aróstegui.

Analysis and interpretation of data. Ionescu, Peñín-Franch, Mensa-Vilaro, Oliva, Fernández-Pereira, Pelegrín, Aróstegui.

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