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Mechanisms underlying genetic susceptibility to multisystem inflammatory syndrome in children (MIS-C)

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GRAPHICAL ABSTRACT

Genetic susceptibility to Multisystem Inflammatory Syndrome in Children (MIS-C)

Monogenic variants in:

- SOCS1
- XIAP
- CYBB

No genetic risk factors

Asymptomatic

SARS-CoV-2

inflammation

MIS-C

XIAP, X-linked inhibitor of apoptosis
CYBB, Cytochrome b(-245), beta subunit
SOCS1, Suppressor of cytokine signaling 1

From the Division of Immunology, the Division of Critical Care Medicine, Department of Anesthesiology, Critical Care and Pain Medicine, The TransLab, the Institutional Centers for Clinical and Translational Research, the Computational Health Informatics Program, The Manton Center for Orphan Disease Research, the Division of Hematology/Oncology, Boston Children’s Hospital, Harvard Medical School, Boston; the Department of Pediatric Oncology, the Dana-Farber Cancer Institute, Harvard Medical School, Boston; and the Dana-Farber/Boston Children’s Cancer and Blood Disorders Center, Boston Children’s Hospital, Harvard Medical School, Boston.

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Background: Multisystem inflammatory syndrome in children (MIS-C) is a pediatric complication of severe acute respiratory syndrome coronavirus 2 that is characterized by multiorgan inflammation and frequently by cardiovascular dysfunction. It occurs predominantly in otherwise healthy children. We previously reported haploinsufficiency of suppressor of cytokine signaling 1 (SOCS1), a negative regulator of type I and II interferons, as a genetic risk factor for MIS-C.

Objectives: We aimed to identify additional genetic mechanisms underlying susceptibility to severe acute respiratory syndrome coronavirus 2–associated MIS-C.

Methods: In a single-center, prospective cohort study, whole exome sequencing was performed on patients with MIS-C. The impact of candidate variants was tested by using patients’ PBMCs obtained at least 7 months after recovery.

Results: We enrolled 18 patients with MIS-C (median age = 8 years; interquartile range = 5-12.25 years), of whom 89% had no conditions other than obesity. In 2 boys with no significant infection history, we identified and validated hemizygous deleterious defects in XIAP, encoding X-linked inhibitor of apoptosis, and CYBB, encoding cytochrome b-245, beta subunit. Including the previously reported SOCS1 haploinsufficiency, a genetic diagnosis was identified in 3 of 18 patients (17%). In contrast to patients with mild COVID-19, patients with defects in SOCS1, XIAP, or CYBB exhibit an inflammatory immune cell transcriptome with enrichment of differentially expressed genes in pathways downstream of IL-18, oncostatin M, and nuclear factor κB, even after recovery.

Conclusions: Although inflammatory disorders are rare in the general population, our cohort of patients with MIS-C was enriched for monogenic susceptibility to inflammation. Our results support the use of next-generation sequencing in previously healthy children who develop MIS-C. (J Allergy Clin Immunol 2021;148:732-8.)

**Key words:** Multisystem inflammatory syndrome in children, MIS-C, COVID-19, SARS-CoV-2, whole exome sequencing

INTRODUCTION

Multisystem inflammatory syndrome (MIS-C) is a life-threatening complication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exposure occurring in individuals younger than 21 years of age. As defined by the Centers for Disease Control and Prevention, the diagnostic criteria include fever, elevated inflammatory marker levels, multisystem organ involvement, and SARS-CoV-2 infection or exposure within 4 weeks of symptoms without an alternative diagnosis. Because most children with MIS-C have mild or no symptoms at the time of initial infection, MIS-C is thought to be a postinfectious syndrome. Nearly all patients with MIS-C have detectable antibodies to SARS-CoV-2, and many have detectable SARS-CoV-2 virus by RT-PCR testing. The clinical features of MIS-C overlap with those of acute coronavirus disease 2019 (COVID-19) and those of the pediatric vasculitic disease Kawasaki disease. Symptoms include fever, rash, gastrointestinal symptoms, coagulopathy, cardiac dysfunction, and/or shock. Studies of hospitalized adults with severe COVID-19 have identified deleterious genetic variants impairing type I interferon signaling in up to 3.5% of patients. In contrast, MIS-C is not associated with preexisting cardiopulmonary, autoimmune and/or immune, or hematologic diseases, and its genetic basis is largely unknown.

We previously reported haploinsufficiency of suppressor of cytokine signaling 1 (SOCS1), a negative regulator of type I and II interferons, as a genetic risk factor for MIS-C. Here, we present findings from our prospective cohort sequencing study of children and adolescents with MIS-C.

RESULTS AND DISCUSSION

Whole exome sequencing (WES) was performed on 18 patients with a diagnosis of MIS-C (Table I). Nearly all of them (89% [n = 16]) had no preexisting medical conditions (other than obesity in 44%). Nine patients required critical care during their hospital stay. This cohort’s median age, predominance of Hispanic ethnicity, lack of other comorbidities, and clinical characteristics are concordant with those of previously published cohorts of patients with MIS-C. In addition to SOCS1 haploinsufficiency, we identified defects in X-linked inhibitor of apoptosis (XIAP) and CYBB, amounting to a genetic diagnosis in 3 of 18 patients in our cohort (17%).

Patient 1 is an 11-year-old boy with no history of disease other than obesity and resolved pityriasis lichenoides chronica. He developed fever, vomiting, diarrhea, rash, and conjunctivitis. The result of RT-PCR testing for SARS-CoV-2 was negative on the fourth day of illness but positive on the seventh. The patient had neutrophilia; T-cell lymphopenia; increased levels of the T-cell activation marker soluble CD25; and elevated levels of IL-18, IL-6, IL-10, and C-X-C motif chemokine ligand 9 (CXCL9) indicative of type I and II interferon signaling (Table II). He developed warm cardiogenic shock in the setting of severely depressed systolic cardiac function and third-degree heart block. He was treated with remdesivir, anakinra, methylprednisolone (2 mg/kg twice daily), intravenous immunoglobulin (IVIG) (1 g/kg), and vasopressors. While taking methylprednisolone, he was found to have EBV viremia (4.3 log10 copies/mL). He had undetectable IgM to capsid and early D antigen, consistent with EBV reactivation. His EBV viremia peaked at 6 log10 copies/mL while he was taking prednisone, and it persisted at 3.9 log10 copies/mL for 9 months until it was cleared by 1 dose of rituximab. After he recovered from MIS-C, his natural killer cytotoxicity function was found to be normal.

**Abbreviations used**

CGD: Chronic granulomatous disease  
COVID-19: Coronavirus disease 2019  
CYBB: Cytochrome b-245, beta subunit  
HLH: Hemophagocytic lymphohistiocytosis  
IVIG: Intravenous immunoglobulin  
MIS-C: Multisystem inflammatory syndrome in children  
NOD2: Nucleotide-binding oligomerization domain-containing 2  
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2  
SOCS1: Suppressor of cytokine signaling 1  
TLR: Toll-like receptor  
WES: Whole exome sequencing  
XIAP: X-linked inhibitor of apoptosis
WEIS identified a novel hemizygous missense variant in XIAP (NP_001158.2: p.Ser421Asn). XIAP is a widely expressed protein that contributes to cellular survival, activation, and negative regulation of the NLRP3 inflammasome. This variant has a minor allelic frequency of $1.1 \times 10^{-5}$ in the Genome Aggregation Database and was predicted to be benign with a Combined Annotation Dependent Depletion score of 4.9. However, structural modeling predicts that the variant creates a hydrogen bond with alanine 417 within the ubiquitin-associated domain, thereby potentially disrupting the domain’s structure and/or capacity for binding ubiquitin (Fig 1, A). The patient exhibited intact XIAP protein expression (Fig 1, B). The ubiquitin-associated domain is important for TNF-α secretion following nucleotide-binding oligomerization domain–containing 2 (NOD2) activation. Compared with the controls, CD14$^+$HLA-DR$^+$ monocytes from patient 1 secreted less TNF-α in response to NOD2 activation with muramyl dipeptide (Fig 1, C), which is indicative of impaired XIAP function. TNF-α secretion after LPS stimulation, which is independent of NOD2 signaling, was intact in the patient (Fig 1, C). Patients with hemizygous loss-of-function variants in XIAP are at risk for virally triggered hemophagocytic lymphohistiocytosis (HLH) and cytokine storm syndromes. This has been attributed to loss of XIAP-mediated negative regulation of the NLRP3 inflammasome, which relies on the protein’s ubiquitylation function. Mice lacking either XIAP or XIAP-mediated ubiquitinylination secrete increased IL-1β downstream of Toll-like receptor 3 (TLR3)- or TLR4-mediated activation of the NLRP3 inflammasome. In contrast, XIAP is not required for IL-6 or TNF-α secretion after TLR stimulation. TLR3 binds to double-stranded viral RNA intermediates, whereas TLR4 binds to SARS-CoV-2 spike protein. Compared with the controls, PBMCs from patient 1 secreted increased IL-1β after TLR3 and TLR4 stimulation (Fig 1, D). As anticipated, TNF-α secretion after TLR3 or TLR4 stimulation was comparable between patient 1 and the controls (Fig 1, E). These findings show the impaired function of XIAP-pSer421Asn, leading to inflammatory signaling that likely predisposed this patient to MIS-C.

Patient 2 is a 16-year-old male whose clinical course has been recently described. He was healthy until 3 weeks before admission, when he developed hematochezia. He had neutrophilia, CD4$^+$ and CD8$^+$ T-cell lymphopenia, procalcitonin and C-reactive protein levels exceeding the upper normal limits by 100- and 30-fold, a mild coagulopathy, and a positive result of IgG testing for SARS-CoV-2 (Table I). Gastrointestinal biopsies revealed duodenitis, patchy colitis, and crypt abscesses consistent with Crohn disease; notably, he also had duodenal submucosal vascularitis atypical for inflammatory bowel disease. Despite treatment with broad-spectrum antibiotics, methylprednisolone (0.3 mg/kg twice daily), vitamin K, and bowel rest, he had persistent episodes of fever, elevated inflammatory marker levels, and worsening hematochezia. After 7 days of hospitalization, he became febrile to 40°C and severely hypotensive. He had persistent T-cell lymphopenia with a predominance of naive CD4$^+$ and CD8$^+$ T cells and an elevated soluble CD25 level (Table I). No secondary infections were identified. As his duodenal vasculitis, coagulopathy, and compensated shock were more consistent with MIS-C than with inflammatory bowel disease, he was given methyprednisolone (increased to 0.5 mg/kg twice daily) and high-dose IVIG. He had rapid improvement of his inflammatory marker levels, hematochezia, and diarrhea. He is currently taking infliximab and is clinically well.

In patient 2, WEIS identified a novel hemizygous missense variant in CYBB (p.Arg229Thr), which encodes the p91phox subunit of the NADPH oxidase that is essential for the phagocytic oxidative burst. Deleterious variants in CYBB cause chronic granulomatous disease (CGD). Although the crystal structure of the extracellular domain harboring this variant has not yet been identified (Fig 1, F), this variant is predicted to be pathogenic, with a Combined Annotation Dependent Depletion score of 31, and it has a minor allelic frequency of $3.9 \times 10^{-4}$ in the Genome Aggregation Database. The patient’s neutrophil oxidative burst was impaired but not absent (Fig 1, G). This contrasts with the minimal neutrophil oxidative burst typical of CYBB variants that cause classical CGD, thereby indicating the variant’s hypomorphic effect on p91phox function. Unlike patients with typical inflammatory bowel disease, the majority of patients with CGD experience inflammatory sequelae, including inflammation-associated cytokine storm syndromes and granulomatous lesions. Loss of NADPH oxidase function impairs the generation of reactive oxidant species that inhibit type I interferon signaling, resulting in a proinflammatory macrophage phenotype.

We previously showed that SOCS1 haploinsufficiency in a patient with a history of MIS-C leads to increased tonic type I and/or II interferon signaling in unstimulated PBMCs on account of reduced inhibition of the Janus-activated kinases. Transcriptomic analysis of unstimulated PBMCs obtained at least 7 months after recovery from SARS-CoV-2 infection identified 712 genes with more than a 1.5-fold difference between the 3 patients in this cohort with likely genetic susceptibility to MIS-C and 4 otherwise healthy children with a history of mild COVID-19. Differentially

### TABLE I. Summary features of enrolled patients

| Feature | MIS-C, no. (%) |
|---------|---------------|
| Age (y) | Median 8      |
| Age range | 5–2.25       |
| Sex, no. (%) | Male 8 (44) |
|         | Female 10 (56) |
| Race and ethnicity, no. (%) | White, non-Hispanic 2 (11) |
|         | Black, non-Hispanic 1 (5.5) |
|         | Asian, non-Hispanic 1 (5.5) |
|         | Other, non-Hispanic 1 (5.5) |
|         | White, Hispanic 5 (28) |
|         | Black, Hispanic 1 (5.5) |
|         | Other, Hispanic 7 (39) |
| Prior medical diagnoses, no. (%) | None (excluding overweight and obesity)* 16 (89) |
|         | Body mass index, no. (%) | Normal (<50th percentile for age and sex) 7 (39) |
|         | Obesity (>95th percentile for age and sex) 3 (17) |
| SARS-CoV-2 testing result, no. (%) | Positive for SARS-CoV-2 RT-PCR 8 (44) |
|         | Positive for SARS-CoV-2 serology‡ 18 (100) |
| Hospital care required, no. (%) | Required critical care 9 (50) |

*Of the 2 patients with preexisting medical conditions, 1 had sickle cell anemia and the other had Evans syndrome.

‡Positive SARS-CoV-2 serology was obtained by using either the Roche Elecsys or Viracor assay.

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expressed genes were enriched in signaling pathways downstream of the inflammatory response, nuclear factor κB, IL-18, oncostatin M, and type I interferon signaling (Fig 2, A). Increased level of IL-18, a marker of inflammasome activation, is a known finding in individuals with defects in XIAP, CYBB, or SOCS1.14,16,17 Oncostatin M, a member of the IL-6 family of cytokines, induces type I interferon–stimulated genes.18 Type I interferon signaling triggers activation of the NLRP3 inflammasome in tissues such as the gastrointestinal and respiratory epithelium and coronary artery endothelium, as well as in myeloid cells.19,20 However, recovered patients lacking a genetic diagnosis for MIS-C did not exhibit significantly increased interferon or inflammatory gene expression in their unstimulated PBMCs (Fig 2, A). This contrasts with the robust immune cell activation characteristic of active MIS-C highlighted here by single-cell RNA sequencing of PBMCs from a patient with ongoing MIS-C despite the lack of any identifiable genetic risk factors. Compared with the control cells, the cells of the patient with active MIS-C exhibited enrichment of differentially expressed genes promoting interferon signaling in CD4+ T cells, CD8+ T cells, CD14+CD16low classical monocytes, and natural killer cells (Fig 2, B). Thus, the pathways upregulated in PBMCs

| Indicator                          | Patient 1 | Patient 2 | Reference value |
|------------------------------------|-----------|-----------|-----------------|
| **Hemogram results**               |           |           |                 |
| White blood cells (10^3 cells/μL)  | 18.0      | 21.7      | 29.4            | 5.52-9.29 |
| Neutrophils (10^3 cells/μL)        | 15.4      | 25.6      | 24.3            | 3.04-6.06 |
| Lymphocytes (10^3 cells/μL)        | 1.27      | 0.69      | 1.12            | 1.17-2.30 |
| Monocytes (10^3 cells/μL)          | 0.60      | 0.24      | 1.55            | 0.19-0.72 |
| Platelets (10^3 cells/μL)          | 173       | 395       | 621             | 189-342  |
| **Inflammatory markers**           |           |           |                 |
| C-reactive protein (mg/dL)         | 21.9      | 12.8      | 10.8            | <0.5     |
| Fibrinogen (mg/dL)                 | 551       | 528       | 623             | 200-400  |
| Ferritin (ng/mL)                   | 1138      | 231       | 1116            | 10-80    |
| D-dimer (μg/mL)                    | 3.1       | 9.1       | 4.2             | <0.5     |
| Soluble CD25 (pg/mL)               | 14,800    | nd        | 1550            | <1033    |
| **Lymphocyte subsets**             |           |           |                 |
| CD3+ (cells/μL)                    | 516       | 475       | 883             | 1000-2600 |
| CD3+CD4+ (cells/μL)                | 357       | 262       | 443             | 530-1500 |
| Naive (% CD3+)                     | 64.8      | nd        | 62.6            | 21-61.4  |
| Central memory (% CD4+)            | 16.7      | nd        | 20.6            | 26.8-62.1 |
| Effector memory (% CD4+)           | 13.1      | nd        | 16.1            | 7.6-25.1 |
| TEMRA (% CD4+)                     | 5.4       | nd        | 0.8             | 0.1-4.0  |
| CD3+CD8+ (cells/μL)                | 145       | 196       | 196             | 330-1100 |
| Naive (% CD8+)                     | 60.3      | nd        | 79.6            | 11.4-66.5 |
| Central memory (% CD8+)            | 16.7      | nd        | 2.4             | 3.7-23.2 |
| Effector memory (% CD8+)           | 13.1      | nd        | 13.6            | 16.8-54.6 |
| TEMRA (% CD8+)                     | 18.6      | nd        | 4.4             | 5.6-43.9 |
| CD19+ (cells/μL)                   | 421       | 197       | 387             | 110-570  |
| Naive (% CD19+)                    | 65.6      | nd        | 72.1            | 48.4-79.7 |
| Unswitched memory (% CD19+)        | 8.10      | nd        | 8.8             | 7.0-23.80|
| Switched memory (% CD19+)          | 21.1      | nd        | 14.3            | 8.30-27.8 |
| Plasmablast (% CD19+)              | 9.7       | nd        | 2.7             | 0.1-2.4  |
| CD3+CD56+ (cells/μL)               | 73        | 60        | 81              | 70-480   |
| **Immunoglobulin levels**          |           |           |                 |
| IgG (mg/dL)                        | 1147      | 1423      | 1522            | 639-1344 |
| IgM (mg/dL)                        | 320       | 90        | 148             | 40-240   |
| IgA (mg/dL)                        | 169       | nd        | 97              | 70-312   |
| Positive titers to pneumococcal subtypes (out of 23 subtypes) | 17 | nd | 8 | >14 |
| Tetanus (IU/mL)                    | 0.1       | nd        | 3.62            | >0.15    |
| **Cytokines (pg/mL)**              |           |           |                 |
| IL-2                               | 7         | nd        | <5              | ≤12      |
| IL-12                              | <5        | nd        | <5              | ≤6       |
| IL-10                              | 33        | nd        | 11              | ≤18      |
| IL-6                               | 44        | nd        | 8               | <5       |
| IL-18                              | 1427      | nd        | 89-540          |         |
| CXCL9 (induced by type I and type II interferons) | 1575 | nd | nd | <121 |

Bolded values are outside the normal range. At the time of this blood draw, patient 1 had not received any immunomodulatory medications, whereas patient 2 had received methylprednisolone (0.3 mg/kg) treatment for 6 days. IL-12, IFN-γ, IL-4, IL-5, IL-13, IL-1β, IL-8, and TNF-α levels were normal in both patients. Neither patient had received IVIG before testing. Naive T cells, CD45RA+CCR7+, Central memory T cells, CD45RA+CCR7+, effector memory T cells, CD45RA+CCR7+, TEMRA, CD45RA+CCR7+. Naive B cells, CD27+IgD−, unswitched memory B cells, CD27+IgD−, switched memory B cells, CD27+IgD−, plasmablasts CD24hi+CD38hi+. nd. Not detected; TEMRA, terminally differentiated effector cell.
vides proof of principle for recent perspectives proposing from those of SARS-CoV-2 infection. Our report thus pro-
nologic impairment may result in clinical outcomes distinct
SARS-CoV-2 infection. Therefore, varying degrees of immu-
variants with mild to no immunologic sequelae before
those also increased during active MIS-C.

Patients previously known to have primary immunodefi-
ciencies or autoinflammatory disorders are at increased risk
of development of life-threatening COVID-19 rather than
MIS-C.21 The cohort that we have presented is unique from
inborn errors of immunity in patients converge with
that genetic risk factors for MIS-C may be incompletely
penetrant.22 Future studies with larger cohorts are needed
to determine additional genetic risk factors for MIS-C,
because the risk of MIS-C may vary among different genetic
causes of autoinflammation.

In identifying a genetic variant that affects immunity in 17%
of patients, our study suggests that MIS-C may indicate an
underlying disorder of immune dysregulation. As clinical-
grade WES has become increasingly accessible as a diagnostic
tool, this study has clinically relevant implications for the use
of WES in identifying inborn errors of immunity in patients
with MIS-C.
For detailed methods, please see the Methods section of this article’s Online Repository materials (available at www.jacionline.org).

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**Key messages**

- In this prospective study of 18 patients with MIS-C, 17% of patients were found to have a genetic variant impairing negative regulation of interferon and inflammatory signaling.
- A history of MIS-C should prompt consideration of WES for the identification of variants affecting host immunity in affected children.
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METHODS

Study design
This prospective observational study was approved by the Boston Children’s Hospital Institutional Review Board. Informed assent and/or consent was obtained from participants and/or their legal guardians.

WES
WES was performed by GeneDx using IDT xGen probes with an average coverage across the WES of 100× and more than 95% of targets covered at 20×, as previously described. Variant calling and candidate variant analysis were completed by utilizing the Boston Children’s Hospital Genomic Learning System, as previously described. Minor allelic frequencies for the specified variants were identified by using the Genome Aggregation Database.

Single-cell RNA sequencing
For each sample, approximately 17,000 PBMCs at a concentration of 1000 cells/μL were input into a 10× Genomics Chromium Controller. Chromium Next GEM Single Cell 3′ kits (version 3.1) were used to generate single-cell gene expression libraries, which we subsequently sequenced by using an Illumina NextSeq 500 system with 150-bp paired-end sequencing. Libraries were processed by using CellRanger version 3.1 (10× Genomics, Pleasanton, Calif) and GrCh38 as the reference. Partek Flow was used to analyze the data output from CellRanger (data filtering, log normalization, integration, scaling, dimensionality reduction, and cluster identification). We excluded genes expressed in fewer than 5 cells, as well as those encoding ribosomal structural proteins and noncoding ribosomal RNA. Low-quality cells with more than a 10% mitochondrial gene content or fewer than 200 features were also excluded. Principal component analysis was performed by using the elbow heuristics method to determine the 15 top principal components for subsequent clustering analysis using the Louvain clustering algorithm, followed by Uniform Manifold Approximation and Projection visualization. Pathway analysis of differentially expressed genes within cell types was performed by using Ingenuity Pathway Analysis (Qiagen Bioinformatics, Redwood City, Calif).

Flow cytometry
Protocols for staining of XIAP were performed as previously described. For quantification of intracellular TNF-α, PBMCs were rested overnight in a 48-well plate and nonadherent cells were stimulated the following day. Adherent PBMCs, consisting primarily of monocytes, were stimulated with lipopolysaccharide muramyl dipeptide (200 ng/mL, InvivoGen, San Diego) or LPS (200 ng/mL, InvivoGen) for 25 hours with brefeldin A, followed by flow cytometric staining for HLA-DR+CD14+TNF-α+ monocytes as previously described. IL-1β secretion downstream was measured by using cytometric bead array (BD Biosciences, Piscataway, NJ) per the manufacturer’s protocol after PBMCs were stimulated with Poly(I:C) (10 μg/mL; InvivoGen, San Diego, Calif) or LPS (100 ng/mL; InvivoGen) for 24 hours.

Transcriptome analysis
mRNA was isolated from PBMCs by using the RNeasy Mini Kit (Qiagen), followed by cDNA synthesis using the SuperScript VILO cDNA Synthesis Kit (ThermoFisher Scientific). The Ion AmpliSeq Transcriptome Human Gene Expression Kit was used to prepare bar-coded libraries and sequenced by using an Ion S5 next-generation sequencer. The AmpliSeqRNA plug-in (ThermoFisher Scientific) was used to calculate differential gene expression analysis. Pathway analysis was performed by using Ingenuity Pathway Analysis and Gene Set Enrichment Analysis (Broad Institute and University of California San Diego) on genes with at least a 1.5-fold difference between the controls and patients (P < .05). Final analyses were performed by using adjusted P values calculated with the Benjamini-Hochberg procedure.

Statistical analysis
All tests were 2 sided, and P values less than .05 were considered significant when using the indicated statistical tests. Statistical analyses were performed by using Prism 8.0 software (GraphPad Software, San Diego, Calif).

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