BRIEF CONCLUSIVE REPORT

Neutrophil dysfunction triggers inflammatory bowel disease in G6PC3 deficiency

Anu Goenka1,2,3 | John A. Doherty1 | Tariq Al-Farsi2 | Christopher Jagger1 | Siddharth Banka4,5 | Edmund Cheesman6 | Andrew Fagbemi7 | Stephen M. Hughes1,2 | Robert F. Wynn8 | Tracy Hussell1 | Peter D Arkwright1,2

Abstract

The glucose-6-phosphatase catalytic subunit 3 (G6PC3) encodes a ubiquitously expressed enzyme that regulates cytoplasmic glucose availability. Loss-of-function biallelic G6PC3 mutations cause severe congenital neutropenia and a diverse spectrum of extra-hematological manifestations, among which inflammatory bowel disease (IBD) has been anecdotally reported. Neutrophil function and clinical response to granulocyte colony-stimulating factor (G-CSF) and hematopoietic stem cell transplantation (HSCT) were investigated in 4 children with G6PC3 deficiency-associated IBD. G6PC3 deficiency was associated with early-onset IBD refractory to treatment with steroids and infliximab. The symptoms of IBD progressed despite G-CSF treatment. In vitro studies on the patients' blood showed that neutrophils displayed higher levels of activation markers (CD11b, CD66b, and CD14), excessive IL-8 and reactive oxygen species, and increased apoptosis and secondary necrosis. Secondary necrosis was exaggerated after stimulation with Escherichia coli and could be partially rescued with supplemental exogenous glucose. HSCT led to normalization of neutrophil function and remission of gastrointestinal symptoms. We conclude that neutrophils in G6PC3 deficiency release pro-inflammatory mediators when exposed to gut bacteria, associated with intestinal inflammation, despite treatment with G-CSF. HSCT is an effective therapeutic option in patients with G6PC3 deficiency-associated IBD refractory to immune suppressants.

KEYWORDS
neutrophil, inflammatory bowel disease, HSCT, transplant, cell death

1 INTRODUCTION

Autoinflammation is a recognized feature of primary immunodeficiencies.1 One example is inflammatory bowel disease (IBD) associated with severe congenital neutropenia (SCN) caused by glucose-6-phosphatase catalytic subunit 3 (G6PC3) deficiency.2-11 Case reports suggest that G6PC3 deficiency-associated IBD can be complicated by progressive intestinal inflammation refractory
to medical treatment and often requires surgery. Improving clinical outcomes for affected patients depends on understanding the underlying disease mechanisms, which are currently unknown.

G6PC3 deficiency is caused by biallelic loss-of-function variants in G6PC3, which encodes glucose-6-phosphatase-β (G6Pase-β), a ubiquitously expressed enzyme that regulates cytoplasmic glucose availability through hydrolysis of glucose-6-phosphate during glycolysis and glycogenolysis. As well as SCN, G6PC3 is associated with a diverse spectrum of extra-hematological manifestations, including cardiac and urogenital abnormalities. G6PC3 deficiency results in neutropenia because neutrophils are highly dependent on glycolysis as an energy source. Studies in humans and mice with G6PC3 deficiency have demonstrated neutrophil dysfunction characterized by accelerated apoptosis. However, IBD is not a feature of other SCNs that also demonstrate accelerated neutrophil apoptosis. This suggests a mechanism independent of apoptosis underpins IBD in G6PC3 deficiency.

Treatments with G-CSF normalizes peripheral neutrophil count and reduces the risk of neutropenic infections, but it is not known if it promotes G6PC3 deficiency-associated IBD.

Here, we describe 4 children with G6PC3 deficiency-associated IBD and neutrophil dysfunction characterized by excessive production of pro-inflammatory mediators and increased necrosis. Hematopoietic stem cell transplant (HSCT) was performed in 3 children and led to normalization of neutrophil function and remission of IBD, and thus should be considered as a therapeutic option for refractory G6PC3 deficiency-associated IBD.

2 | MATERIALS AND METHODS

2.1 | Participants

The research protocol was approved by the NHS Research Ethics Committee (Reference 14/SW/0100). Written informed consent was obtained from healthy volunteer adult controls and the parents/legal guardians of children with G6PC3 deficiency. Venipuncture was deferred if there was history of febrile illness or active IBD flare within the preceding 14 days.

2.2 | Cell culture

Cells were isolated by negative immunomagnetic selection within 1 h of venipuncture by EasySep Neutrophil or Monocyte Direct Isolation kits (STEMCELL Technologies). Neutrophils were incubated at 37°C/5% CO2 with gentle rocking in triplicate sterile 5 mL polystyrene tubes at 0.5 × 10^6 cells/ml in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) containing 10% autologous serum. Monocytes were incubated in triplicate tissue culture wells at 37°C/5% CO2 at 0.5 × 10^6 cells/ml in RPMI medium/10% FCS.

2.3 | Bacteria

Escherichia coli American Type Culture Collection (ATCC) 25922 (gift from Professor Ian Roberts, University of Manchester) were grown in lysogeny broth medium (Thermo Fisher) at 37°C and harvested in mid-log phase.

2.4 | Flow cytometry

Neutrophils were incubated with Fc receptor blocking solution (TruStain FcX, BioLegend) and then the appropriate Ab cocktail at 4°C for 20 min. In cell death assays, TO-PRO 3 (Thermo Fisher) was added for the final 5 min of incubation. After washing, at least 100,000 cells were acquired on a BD Fortessa cytometer (immunophenotyping) or BD FACS Canto-II cytometer (cell death and reactive oxygen species). Single stain controls were prepared using compensation beads (OneComp, Thermo Fisher). Samples were analyzed after compensation was set using FlowJo (Version 10.3, FlowJo LLC) and gating determined using the fluorescence-minus-one principle.

2.5 | Soluble mediator measurement

After thawing culture supernatants from -80°C, cytokines were quantified by multiplex bead array (Soluble Protein Human Flex Set, BD Biosciences) and acquired on a BD FACSVerse system, with analysis by FCAP Array (Version 3.0, Soft Flow Inc.). Neutrophil elastase and MMP-9 were quantified by sandwich ELISA (Abcam). Lactate dehydrogenase was measured by the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega).

2.6 | Measurement of RNA expression

Cells were disrupted by buffer RLT (QIAGEN) and ribonucleic acid (RNA) isolated using RNeasy Micro Kit (QIAGEN). RNA yield and purity were measured on the Nanodrop 2000c system (Thermo Fisher), and reverse transcribed (high-capacity RNA-to-cDNA kit, Applied Biosystems). Quantitative real-time polymerase chain reaction (PCR) was performed on duplicate cDNA samples using Taqman primers and Master Mix on a Quantstudio 12K System (Life Technologies). Relative RNA expression was calculated to the mean expression of 2 housekeeping genes (RPLP0 and ACTB) and fold-change calculated relative to an unstimulated sample.

2.7 | Statistical analysis

Statistical analysis was undertaken using Prism (Version 7.0, GraphPad Software). Variability between technical replicates was analyzed by calculation of the coefficient of variation (CVV). Parametric distribution of the data was confirmed by the Shapiro-Wilk normality test. Comparisons were made using the Student’s t-test with Welch’s correction for unpaired data, with control of False Discovery Rate (FDR) by the Benjamini-Hochberg method where appropriate. When more than 2 groups were compared, significance was determined by a 1-way ANOVA with Bonferroni’s correction for multiple comparisons. Dependent variables that were measured more than once were analyzed by a 1-way repeated measures ANOVA with Dunnett’s multiple comparisons test. The significance levels were set at P < 0.05 and FDR < 0.05.
FIGURE 1  Clinical phenotype of IBD in G6PC3 deficiency. (A) Daily stool frequency of P3 for days following weekly pegylated G-CSF injections. Line represents median of observations taken over a 6-week period. Statistical significance was determined by 1-way ANOVA with Dunnett’s multiple comparisons test. **P < 0.01. (B) Colonoscopy image from P1 demonstrating severe mucosal ulceration and sloughing. (C) Intestinal biopsies (H&E, 400×) demonstrating marked cryptitis with neutrophilic inflammatory infiltrate in the lamina propria in P1 (right) and idiopathic Crohn’s disease (center). Images presented in comparison with biopsy from a healthy individual (left)

3 | RESULTS AND DISCUSSION

Four patients (P1-P4) with G6PC3 deficiency aged 7–21 years were studied. All suffered from chronic debilitating diarrhea beginning in early infancy, as well as severe recurrent lower respiratory tract infections. Following the diagnosis of constitutional neutropenia at the age of 2 days–10 years old, all patients were commenced on subcutaneous G-CSF treatment. Bone marrow aspirate and trephine demonstrated a paucity of mature neutrophils, or normal cellularity with no maturation arrest. One patient (P1) exhibited other immunophenotypic abnormalities including CD4+ T cell lymphopenia and a low serum IgG level (Supplemental Table 1). Homozygous variants in G6PC3 were identified in all 4 patients (Supplemental Fig. 1). In 2 patients, mutations were truncating (P1: c.282delA and P2: c.882_903dup) and predicted to be more deleterious and were associated with a more severe pattern of extra-hematological manifestations (Supplemental Table 1). Stools were described as watery, with episodic exacerbations characterized by bloody diarrhea, abdominal pain, and malaise. Diarrhea impacted the patients’ quality of life, causing waking from sleep and school absences. No pathogens were detected by microscopy, bacterial culture, or PCR. Gut histology from P1 and P2 showed no granulomata. However, there were diffuse inflammatory changes in the stomach, duodenum, terminal ileum, and particularly colon that was most severe, with cryptitis, crypt abscesses, and focal ulceration (Fig. 1B and C). In some biopsies, foamy Mϕs were observed, which can be seen in chronic granulomatous disease (CGD). Pediatric Crohn’s Disease Activity Index (PDCAI) suggested moderate-severe disease, except P4 who had mild disease (Supplemental Table 2). P3 and P4 did not require endoscopic evaluation. The colitis was poorly responsive to prednisolone in patients P1-P3 and azathioprine, sulfasalazine, and infliximab in patients P1 and P2.

Both amelioration5,7 and deterioration2,8 of enteritis has been described following the commencement of G-CSF therapy in patients with G6PC3 deficiency. In all our patients, severity of the diarrhea increased following the initiation of G-CSF therapy, except P1 who had commenced treatment in the neonatal period. Absolute neutrophil count (ANC) was maintained on a daily G-CSF dose of 3–10 mcg/kg/day in all patients except P3 who received weekly pegylated G-CSF injections at 120 mcg/kg/week (Supplemental Table 1). In P3, ANC trough was 1.7×10⁹/L and peak was 9.7×10⁹/L 2 days after pegylated G-CSF injection, which coincided with a significant increase in stool frequency (Fig. 1A).

HSCT can cure neutropenia and has been recently reported to ameliorate IBD in an adult with G6PC3 deficiency.11,20 We first performed HSCT in P4 primarily due to poorly tolerated local and systemic side effects of G-CSF injections, and the wishes of the patient and her family, as well as her enteritis (Table 1). We then performed HSCT on P2 and P3 for treatment-refractory enteritis (Table 1). P1 is currently scheduled for HSCT. Conditioning consisted of a standard reduced toxicity regimen including in vivo T cell depletion with alemtuzumab for prevention of graft versus host disease (GvHD; Table 1). Grafts were from a matched unrelated donor in 1 patient, and sibling donors in the other 2 patients (Table 1). All patients had high levels of donor engraftment. Post-HSCT, all 3
|                  | P1       | P2       | P3       | P4       |
|------------------|----------|----------|----------|----------|
| Current age (years) | 7        | 14       | 21       | 18       |
| Age at HSCT (years) | Currently scheduled for HSCT | 11 | 17 | 14 |
| Indication for HSCT | IBD     | IBD     | IBD & poorly tolerated G-CSF |
| Conditioning regimen | TT/Treo/Flu/Alem | TT/Treo/Flu/Alem | TT/Treo/Flu/Alem |
| GvHD prophylaxis   | Ciclo   | Ciclo   | Ciclo   |
| CMV Recipient/Donor | +/+  | +/+  | +/+  |
| EBV Recipient/Donor | +/+  | +/+  | −/−  |
| Graft (donor)      | MUD     | MSD     | MSD     |
| Graft (type, HLA match) | bmsc 10/10 | bmsc, 10/10 | bmsc, 10/10 |
| Platelet engraftment | 33     | 41       | 20       |
| Neutrophil engraftment | 12  | 21       | 13       |
| Lymphocyte engraftment | 42  | 42       | 23       |
| GvHD Grade         | Nil     | Nil     | Nil     |
| VOD                | Nil     | Nil     | Nil     |
| Other significant post-HSCT complications | Nil | AIHA | Nil |
| Systemic viral infection after day 0 | CMV | CMV | CMV |
| Latest chimerism (as of end 2019) | 100% donor | 98% donor | 100% donor |

A Platelet count >20 x 10^9 for 7 consecutive days without need for transfusion; B Neutrophil count >0.5 x 10^9 for 3 consecutive days; C Lymphocyte count >0.3 x 10^9.

Abbreviations: IBD, inflammatory bowel disease; GvHD, graft versus host disease; VOD, veno-occlusive disease; TT, thiotepa; Treo, treosulfan (42 g/m^2 as a once daily dose for 3 days); Flu, fludarabine (160 mg/m^2 as a once daily dose for 4 days); Alem, alemtuzumab (total 0.3 mg/kg given over 3 days in MSD, and 1 mg/kg given over 5 days in MUD, as a once daily dose and completing on day −5); Ciclo, ciclosporin; MUD, matched unrelated donor; MSD, matched sibling donor; bmsc, bone marrow stem cells; AIHA, autoimmune hemolytic anemia; CMV, cytomegalovirus.

Given that G-CSF injections were associated with increased stool frequency and HSCT was associated with a remission of gastrointestinal symptoms evidenced by the dramatic and sustained reduction in PDCAI scores (Supplemental Table 2). In addition, all are now attending school or university 2–3 years post-HSCT, with the only residual medical problems being bronchiectasis requiring prophylactic antibiotics (P2 and P4), and gonadal dysfunction requiring hormone replacement (P2 and P3; Supplemental Table 2).

Given that G-CSF injections were associated with increased stool frequency and HSCT was associated with remission of gastrointestinal symptoms, we considered whether G6PC3 deficiency-associated IBD may be driven by phagocyte dysfunction. As previous studies have described accelerated neutrophil cell death from patients with G6PC3 deficiency,10,15,16 we hypothesized that this might drive intestinal inflammation, particularly in the gut where neutrophils are exposed to commensal microbes. We therefore assessed neutrophil cell death following incubation with live Escherichia coli. Neutrophils from patients with G6PC3 deficiency underwent significantly more apoptosis and secondary necrosis, particularly when incubated with E. coli, compared with equivalents from healthy control volunteers (Fig. 2A–C). The pattern of neutrophil death in 1 patient (P4) who had undergone HSCT was in keeping with the healthy controls.

Accelerated neutrophil death in G6PC3 deficiency has been attributed to ER and oxidative stress driven by a lack of cytoplasmic G6P.21 It is therefore possible that increasing the exogenous glucose to supra-physiological levels might rescue neutrophils from death. We therefore compared cell death of neutrophils from a patient with G6PC3 deficiency (P2) and a healthy control, following culture with increasing concentrations of D-glucose (Fig. 2D). In the complete absence of exogenous glucose, the median proportion of neutrophils undergoing secondary necrosis was similar in the patient with G6PC3 deficiency (14%) and the healthy control (16%; Fig. 2D). Increasing the exogenous glucose concentrations to 1 mM or more led to almost complete rescue of neutrophils of the healthy donor from necrosis (2–3% necrotic neutrophils), while only partial rescue (7–8% necrotic neutrophils) in the patient with G6PC3 deficiency (Fig. 2D). This observation is consistent with previous studies in neutrophils from G6PC3−/− mice demonstrating: (i) impaired glucose uptake associated with impaired GLUT1 translocation to the plasma membrane17; and (ii) impaired glycolysis caused by failure to destroy an analogue of phosphorylated glucose (1,5-anhydroglucitol-6-phosphate) that inhibits hexokinases.22 Glucose within the subepithelial space may thus prevent neutrophil necrosis in healthy individuals but not in patients with G6PC3 deficiency. This may help explain why patients with other genetically defined causes of SCN without...
Increased neutrophil necrosis in G6PC3 deficiency is exacerbated by stimulation with live *E. coli*. (A) Representative flow cytometry of neutrophils cultured with *E. coli* (ATCC #25922) at MOI 1:1 for 1 h and stained with Annexin V and a cell impermeant dye TO-PRO 3. Left panel representative of healthy controls and right panel representative of patients with G6PC3 deficiency. (B) Cell death as ascertained by flow cytometric measurement of Annexin V and TO-PRO 3; (C) LDH release as assessed by colorimetric assay of culture supernatant; (D) Cell death assessed by staining with Annexin V and TO-PRO 3 from a patient with G6PC3 deficiency (P2) and a healthy control, following an 8 h incubation of neutrophils in glucose-free RPMI 1640 medium with supplemental exogenous D-glucone at the indicated concentrations in glucose-free RPMI 1640 (results representative of 2 independent experiments). Data points in (B) and (C) each represent median of 3 separate measurements all with CVV < 12% performed on single individuals, denoted by circles (patients with G6PC3 deficiency receiving G-CSF therapy, P1 ●; P2 ○; P3 □) or squares (P4 ■ 2 years post-HSCT not receiving G-CSF therapy; and □ healthy adult controls). Significance determined by Student’s t-test with Welch’s correction for unpaired data where **” and “***” denote *P* < 0.01 and < 0.001, respectively (A-C), and 1-way ANOVA with Bonferroni’s correction for multiple comparisons where *, **, ***, and **** denote *P* < 0.05, < 0.01, < 0.001, and < 0.0001, respectively (D).

Defective glucose metabolism (e.g., HAX1 deficiency) do not suffer from IBD.

Increased necrosis implies that neutrophils in patients with G6PC3 deficiency have an altered inflammatory activation status, or a lower proportion that are immature. We tested this theory by measuring expression of neutrophil surface markers. There was significantly higher expression of CD14, CD66b, and CD11b on freshly isolated live neutrophils from patients with G6PC3 deficiency compared with healthy controls (Supplemental Fig. 2A). There was however no significant difference in the expression of CD64, CD16, TREM-1, and CD62L (Supplemental Fig. 2A) nor the proportion of CD11bhigh/CD16low/immature neutrophils (Supplemental Fig. 2B). These observations suggest that neutrophils from patients with G6PC3 deficiency mature, but constitutively activated.

Constitutively activated neutrophils in patients with G6PC3 deficiency could contribute to IBD through excessive release of soluble inflammatory mediators. We therefore measured soluble inflammatory mediators and found significantly higher IL-8 release, but not TNF-α, IL-6, and IL-1β, from the unstimulated neutrophils of patients with G6PC3 deficiency, compared with healthy controls (Fig. 3A). Colonic IL-8 levels correlate with IBD severity, as excessive IL-8 drives tissue inflammation by its action as a potent neutrophil chemoattract and activation ligand. We also found significantly lower release of neutrophil elastase and MMP-9 in *E. coli*-stimulated neutrophils from G6PC3 patients compared with healthy controls (Fig. 3A).

Dysregulated reactive oxygen species (ROS) production by neutrophils is associated with mucosal inflammation, as observed in colonic resection specimens of IBD patients in which ROS is increased, and IBD caused by CGD in which ROS is reduced. We therefore measured neutrophil ROS production by its oxidation of dihydrorhodamine (DHR) to rhodamine-123. Phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophils from patients with G6PC3 deficiency...
FIGURE 3  Dysregulated production of soluble pro-inflammatory mediators in G6PC3 deficiency. (A) Measurement of soluble mediators in the culture supernatant of neutrophils incubated in media alone or with *E. coli* (ATCC #25922) at MOI 1:1 for 1 h. TNF-α, IL-8, IL-6, and IL-1β were measured by cytokine bead array. Neutrophil elastase and MMP-9 were measured by ELISA. (B and C) Assessment of reactive oxygen species (ROS) production by measurement rhodamine-123 (R-123) fluorescence in neutrophils by flow cytometry after loading with dihydrorhodamine (25 ng/ml) for 15 min, and incubation (B) PMA (100 ng/ml) for 45 min; or (C) fMLP (1 μM) for 20 min or with (D) culture medium alone. Dihydrorhodamine (DHR) index calculated as: PMA-stimulated MFI/unstimulated MFI (MFI: geometric mean fluorescence intensity). Data points each represent median of 3 separate measurements with CVV < 16% performed on single individuals, denoted by circles (patients with G6PC3 deficiency receiving G-CSF therapy, P1●; P2○; P3□) or squares (P4■ 2 years post-HSCT not receiving G-CSF therapy; and □ healthy adult controls). Fluorescence minus one sample denoted by unfilled histogram. Significance determined by Student’s t-test with Welch’s correction for unpaired data where *** denoted P < 0.05, and where appropriate control of False Discovery Rate (FDR) by the Benjamini-Hochberg method was applied in which *** denotes FDR < 0.05.

had a significantly lower DHR index compared with healthy controls (Fig. 3B). Where cellular yield was sufficient, stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP) was also performed and similar results were observed (Fig. 3C). In contrast, unstimulated neutrophils from patients with G6PC3 deficiency demonstrated a significantly higher mean fluorescence intensity of rhodamine-123 compared with healthy controls (Fig. 3D). The pattern of inflammatory mediator production of the patient with G6PC3 deficiency who had undergone successful HSCT 2 years earlier (P4) closely aligned with the healthy controls. Collectively these observations suggest that constitutively activated neutrophils in G6PC3 deficiency produce excessive pro-inflammatory mediators in steady state conditions but have a reduced capacity to do so upon stimulation.

Given previous reports of mononuclear phagocyte dysfunction in G6PC3 deficiency, we considered whether monocyte dysfunction might also contribute to intestinal inflammation.9,32 We therefore stimulated freshly isolated peripheral monocytes with LPS and assessed cytokine production and cell death. Under both media and LPS conditions, there were no significant differences in innate cytokine production by monocytes from patients with G6PC3 deficiency compared with healthy controls (Supplemental Figure 3). There were also no significant differences in the RNA expression of genes involved in regulating apoptosis or LDH production from the monocytes of patients compared with healthy controls (Supplemental Figure 3). Therefore, defective function of peripheral phagocytes in G6PC3 deficiency appears restricted to neutrophils.

For the first time, we show that neutrophils from patients with G6PC3 deficiency exhibit elevated constitutive expression of inflammatory cell surface markers (CD11b, CD66b, and CD14), cytokines (IL-8), and ROS reflecting an intrinsically higher inflammatory
activation state of neutrophils in G6PC3 deficiency, possibly because neutrophils are particularly dependent on glycolysis as an energy source.\textsuperscript{13,14} Reduced release of neutrophil elastase and MMP-9 from G6PC3 deficient neutrophils suggests a defect in degranulation, which could be tested in future studies. It is unlikely that the observed differences were caused by G-CSF treatment rather than G6PC3 deficiency, as previous studies have demonstrated that G-CSF treatment is associated with reduced apoptosis, normal ROS production and changes in surface marker expression that return to normal after a few days treatment.\textsuperscript{33–35}

An important limitation of our study was the small number of participants and therefore it would be useful if our findings were confirmed in larger independent studies. We were unable to recruit more patients with G6PC3 due to the rarity of the condition, and the necessity to perform functional neutrophil studies on freshly drawn blood. Nonetheless, our data highlight that early-onset IBD can be a major complication of G6PC3 deficiency associated with significant morbidity. Gastrointestinal symptoms were more severe in patients with genotypes predicted to be more deleterious. Three patients successfully underwent HSCT, associated with complete remission of their IBD. Post-HSCT, neutrophil responses closely aligned with those of healthy controls. We therefore conclude that G6Pase-\(β\) (and therefore cytoplasmic glucose availability in neutrophils) has an important role in mucosal immunity and increased neutrophil inflammatory output may drive enteritis in patients with G6PC3 deficiency. Future studies should seek to test whether G6PC3 deficiency-associated IBD can be ameliorated by rescuing neutrophils from death, by lowering 1,5-anhydroglucitol-6-phosphate levels with therapeutic inhibitors of the kidney glucose transporter SGLT2.\textsuperscript{22} In the interim, we propose that clinicians check for IBD in patients with G6PC3 deficiency and consider HSCT when IBD is difficult to control.

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AUTHORSHIP

A.G., S.M.H., R.F.W., and P.D.A. conceived the idea for the study. A.G., T.H., and P.D.A. designed the experiments. A.G. compiled the clinical information incorporating clinical data provided by S.B., A.F., E.C., and R.F.W. A.G. obtained the clinical samples and performed all the experiments, and received assistance from J.A.D., T.A., and C.J. A.G. wrote the manuscript and incorporated comments from R.F.W., T.H., and P.D.A.

DISCLOSURE

The authors declare no conflict of interest.

ORCID

Anu Goenka https://orcid.org/0000-0002-8152-2155

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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