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ORIGINAL ARTICLE

Cryptogenic multifocal ulcerating stenosing enteritis associated with homozygous deletion mutations in cytosolic phospholipase A2-α

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ABSTRACT

Objective Cryptogenic multifocal ulcerating stenosing enteritis (CMUSE) is an extremely rare, but devastating, disease of unknown aetiology. We investigated the genetic basis of this autosomal recessive condition in a pair of affected siblings who have 40-year histories of catastrophic gastrointestinal and extraintestinal disease.

Design Genome-wide single-nucleotide polymorphism homozygosity mapping in the two affected family members combined with whole-exome sequencing of one affected sibling. This was followed by confirmatory Sanger sequencing of the likely disease-causing sequence variant and functional studies in affected and unaffected family members.

Results Insertion/deletion variation analysis revealed the presence of a homozygous 4 bp deletion (g.155574_77delGTAA) in the *PLA2G4A* gene, located in the splice donor site directly after exon 17 (the penultimate exon) of the gene in both affected siblings. This introduces a frameshift of 10 amino acids before a premature stop codon (p.V707fsX10), which is predicted to result in the loss of 43 amino acids (residues 707–749) at the C-terminus of cytosolic phospholipase A2-α (cPLA$_2$α). cPLA$_2$α protein expression was undetectable in the gut of both siblings, with platelet aggregation and thromboxane A$_2$ production, as functional assays for cPLA$_2$α activity, grossly impaired.

Conclusions We have identified mutations in *PLA2G4A* as a cause of CMUSE in two affected siblings. Further studies are needed to determine if mutations in this gene are also responsible for disease of a similar phenotype in other cases.

Significance of this study

What is already known about this subject?
- Non-steroidal anti-inflammatory drug (NSAID) use leads to upper gastrointestinal ulceration.
- Cryptogenic multifocal ulcerating stenosing enteritis is a disease of unknown origin associated with severe intestinal ulceration.
- A previous study of a single case with onset of intestinal ulceration in early middle age was associated with compound heterozygote mutation in the gene encoding cytoplasmic phospholipase A2-α.

What are the new findings?
- We describe two siblings with severe, early-onset cryptogenic multifocal ulcerating stenosing enteritis.
- We found a homozygous 4 bp deletion (g.155574_77delGTAA) in the *PLA2G4A* gene, located in the splice donor site directly after exon 17 (the penultimate exon) of the gene in both siblings, which introduces a frameshift of 10 amino acids before a premature stop codon (p.V707fsX10).
- Functionally, platelet aggregation and thromboxane A$_2$ production were the same as in platelets from normal individuals treated with aspirin.

How might it impact on clinical practice in the foreseeable future?
- Patients with intestinal ulceration in the absence of NSAID use should be screened for mutations in cPLA$_2$α.

INTRODUCTION

There have been a number of genome-wide association studies (GWAS) linking common genetic variation with more prevalent gastrointestinal inflammatory conditions such as inflammatory bowel disease (IBD) and coeliac disease. Genetic studies on much rarer, highly penetrant conditions of unknown aetiology, usually presenting with gastrointestinal symptoms early in life have also been highly informative. For example, the disease formerly known as autoimmune enteropathy is now known to be caused by mutations in the *FOXP3* gene, which result in a defect in immune regulation.[1 2] Mutations in the *IL-10R* gene also result in a very severe IBD in children, which can, importantly, be cured by a bone marrow transplant,[3] while polymorphisms in this same gene and its receptor have been associated by GWAS with early-onset ulcerative colitis[4 5] and Crohn's disease.[6] We have also recently identified a pair of siblings, who presented as infants with diarrhoea and skin lesions, as knockouts for *ADAM17*, the tumour necrosis factor-α- converting enzyme.[7]

Recurrent small bowel ulceration and stenosis of unknown aetiology (cryptogenic multifocal ulcerous

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stenosing enteritis (CMUSE)) is an extremely rare condition, and has thus far eluded pathophysiological explanation.[8 9] The lesions in patients with CMUSE resemble those associated with use of non-steroidal anti-inflammatory drugs (NSAIDS), a well-known cause of gastric[10 11] and small bowel[12 13] ulcers, and in extreme cases small bowel strictures and multiple concentric stenoses of the intestinal lumen (so-called 'diaphragm disease').[14–17] NSAIDs are inhibitors of the cyclo-oxygenase (COX-1 and -2) enzymes, which function in the synthesis of prostaglandins, using free arachidonic acid (AA) as a substrate.[18] Reduction in prostaglandin production is considered primarily responsible for NSAID-associated enteritis, because of the role of prostaglandins in the regulation of gastrointestinal blood flow, cytoprotection and intestinal mucus secretion.[12 19]

Here, we describe two siblings of Serbian origin who have a 40-year history, beginning in early childhood, of severe peptic and upper small-intestinal ulceration, extensive small-intestinal stricturing, fibrosis and fistulae, and multiple severe extraintestinal complications. In each patient, we identified a homozygous 4 bp deletion in *PLA2G4A,* which maps in a large region of shared homozygosity on chromosome 1, which results in the loss of the C-terminal region of its protein product, cytosolic phospholipase A2-α (cPLA$_2$α), and, putatively, the abrogation of its enzymatic function.

CASES

The parents of the affected siblings are not known to be consanguineous, although they both come from a small, isolated community in Serbia.

The first affected sibling (male, born 1963) presented at the age of 4 years with severe peptic ulceration and bleeding. This was initially treated medically, but vagotomy and gastroenterostomy were required by the age of 7 years after the patient developed pyloric stenosis and duodenal ulceration. This was then followed by surgery for a gangrenous terminal ileum, secondary to volvulus and multiple adhesions. At the age of 13, the patient required surgery for a perforated gastric ulcer. In 1982, he underwent a partial gastrectomy for a large gastric ulcer, and in 1986 he had a fibrous polyp removed and was noted to have multiple gastric ulcers. In the 1990s, he was found to have a small gastric lumen, generalised gastritis, and Barrett's oesophagus with histological gastric metaplasia, but a normal duodenum. In 1993, he underwent laparotomy for revision of a retrocolic gastrojejunostomy, at which time multiple adhesions were seen. In 1996, he presented with a stenotic duodenum. By 2001, symptoms of dysphagia and abdominal pain caused by partial bowel obstruction were severe, and he was malnourished with low albumin, low vitamin B12 and peripheral oedema. In September 2008, he underwent endoscopic retrograde cholangiopancreatography and was noted to have a thickened, tortuous, dilated common bile duct, suggestive of fibrosis, and ulceration at the biliary sphincter. In 2011, he was admitted to hospital with a gastric and small bowel stricture and peritoneal adhesions and underwent gastroplasty, jejunojejunal anastomosis and adhesiolysis. One month after surgery, he was admitted with worsening malnutrition, hypoalbuminaemia and oedema. He had multiple liver abscesses and an enterocutaneous fistula. He has had iron deficiency anaemia since 1996, with haemoglobin levels as low as 5 g/dl. He was diagnosed with type 2 diabetes in 2010 and has developed severe peripheral neuropathy. He also has osteoporosis. Colonoscopy in 2010 showed a normal large intestine and terminal ileum.

The second affected sibling (female, born 1966) presented at the age of 2 years with peptic ulceration, bleeding and pyloric stenosis, which required pyloroplasty and selective vagotomy. In 1980, she underwent gastrojejunostomy for further peptic ulceration and duodenal stenosis, and in 1996 was diagnosed with pernicious anaemia. In 2000, gastroscopy showed oesophageal and gastric ulcers, a tight ulcerated pyloric canal, and a chronic duodenal ulcer. In the same year, she experienced *Salmonella enteriditis* infection and underwent surgery for ileal perforation. In addition in 2000, she experienced two episodes of severe *Campylobacter* enteritis and was diagnosed with xanthogranulomatous pyelonephritis on renal biopsy; she continues to have chronic renal failure. In 2001, she experienced another perforation of the ileum and an ileoileal fistula, complicated by *Candida* septicaemia, resistant staphylococcal chest infection, acute respiratory distress syndrome and acute renal failure requiring haemofiltration. Later that year, she required drainage of a pelvic collection. In 2009, she required a transverse colon resection for volvulus. In 2010, she was malnourished with hypoalbuminaemia and oedema, and started total parenteral nutrition. Also in 2010, she developed biliary reflux with severe oesophagitis and strictures. She required repeated oesophageal dilatation in 2010–2011, but, despite this, developed bronchiectasis secondary to recurrent aspiration pneumonia. She underwent a revision gastroenterostomy in 2001. Further complications include endometriosis (1990), gall stones (2006), left ventricular concentric hypertrophy with a small cavity, and a fibrotic, unstable bladder with carbapatite stones, requiring ureteric stents (both 2009). Furthermore, she is infertile.

Based on the clinical history and histology, we consider that the siblings have CMUSE.[5 6]

Symptoms in both siblings have progressed despite maximal medical therapy—most recently with high-dose proton pump inhibitors—including eradication of *Helicobacter pylori*. Short courses of moderate-dose corticosteroids were unhelpful in the male sibling, and have not been evaluated in the female.

The siblings' mother, father and brother (all heterozygous carriers of the mutation) are healthy and have no family history of similar symptoms, with the single exception of a peptic ulcer in the father's middle age, which resolved with a typical clinical course.

METHODS

Ethics and consent

Written informed consent was obtained from all family members. This study was approved by the South East NHS Research Ethics Committee and was performed according to the Declaration of Helsinki Principles.

Genetics

Whole-genome single-nucleotide polymorphism (SNP) array analysis of the two affected siblings was performed using the Human Omni2.5Quad V.1.0 to identify shared regions of homozygosity. This array provides more than 2.4 M SNP with a physical position genome-wide. At first, a linkage analysis was carried out using the program Merlin[20] with a reduced marker set of 64 983 SNPs, a hypothetical pedigree assuming consanguinity with a cousin marriage of 2nd degree, allele frequencies from a European population, a genetic map adjusted to Rutgers map v2,[21] and a recessive genetic model with complete penetrance (see online supplementary materials). To narrow down recombination events, we used all 2.4 M markers from the array. With a self-written Perl script, we identified all regions where both affected siblings were homozygous on the same allele (see online supplementary materials). In tandem, whole-exome sequencing was performed in the affected female using a

SureSelect Human all Exon 50 Mb kit (Agilent Technologies, Santa Clara, California, USA) and sequenced on an Illumina HiSeq. Sequencing reads were aligned to the hg19 build of the human reference genome using the software novoalign (http://www.novocraft.com). SNP and indel calling were performed using samtools V.0.18 and were annotated using the software ANNOVAR.[22] Candidate variants were filtered on the basis of function (as predicted by ANNOVAR), and 1000 Genomes (http://www.1000genomes.org) and NLHBI exome sequencing project (http://evs.gs.washington.edu/EVS/) frequencies. Rare variants mapping to the shared regions of homozygosity were investigated further. PCR and Sanger sequencing were performed in the two affected individuals, their unaffected sibling and both parents, to confirm the segregation of the mutation with disease, using an ABI PRISM 3130xl sequencer and the primer pair PLA2-F and PLA2-R, designed specifically against the mutation site. Sequence traces were analysed using Chromas Lite software and sequences were aligned using the Multalin multiple alignment tool (multalin.toulouse.inra.fr/multalin). Details of the cPLA$_2\alpha$ protein and active site domain sequences were obtained from the NCBI Protein (Accession Number CAB42689.2) and Conserved Domain (Accession Number cd07200) databases, respectively. Details of cPLA$_2\alpha$ protein sequences for non-human species were also obtained from the NCBI Protein database; these were aligned and compared using the ClustalW2 multiple sequence alignment tool (http://www.ebi.ac.uk/tools/msa/clustalw2) and visualised using the Jalview multiple alignment editor (http://www.jalview.org).

Immunofluorescence and western blotting

Immunofluorescence and western blotting studies were performed using antibodies against cPLA$_2\alpha$ (Ab58375; Abcam, Cambridge, UK), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control (Ab9485; Abcam). For cPLA$_2\alpha$ staining, paraffin-embedded small bowel sections from the two affected siblings and unaffected controls were dewaxed, antigen-retrieved using citrate buffer, and blocked with 3% serum in phosphate-buffered saline and avidin/biotin blocking solution (Vector Laboratories, Burlingame, California, USA). The sections were then incubated with rabbit cPLA$_2\alpha$ antibody (Abcam) in 1:100 dilutions overnight at 4°C. Sections were then further incubated with anti-rabbit IgG conjugated with fluorescein isothiocyanate for 1 h and mounted using mounting medium containing the nuclear stain 4′6-diamidino-2-phenylindole. Stained tissues were imaged using a Zeiss laser confocal microscopy 710 (Carl Zeiss, Welwyn Garden City, UK), and images were processed using Adobe Photoshop CS (Adobe Systems, San Jose, California, USA).

For western blotting studies, peripheral blood mononuclear cells (PBMCs) were isolated, following the manufacturer's instructions, from whole-blood samples taken from the two affected siblings and their unaffected father using Ficoll Paque premium isolation medium (GE Healthcare Life sciences, Little Chalfont, Buckinghamshire, UK). Isolated PBMCs were lysed, and western blotting was performed on the lysates using antibodies against cPLA$_2\alpha$ and GAPDH.

cPLA$_2\alpha$ activity studies

Platelet aggregation and release reactions were determined as reported previously.[23 24] Briefly, blood was collected by venepuncture into trisodium citrate (Vacutainer367 691; BD Diagnostics, UK). Platelet-rich plasma (PRP) was obtained by centrifugation at 175 *g* for 15 min at 25°C. Platelet-poor plasma was obtained by centrifugation of PRP at 15000 *g* for 2 min. All experiments were completed within 2 h of blood collection.

For light transmission aggregometry, responses to arachidonic acid (AA) (1 mM; Sigma, UK), collagen (0.3–3 μg/ml; Nycomed, Austria) or adenosine diphosphate (ADP) (5 μM; Chronolog, UK) were measured in a Bio/Data PAP-8E turbidometric aggregometer, with platelet aggregation determined as percentage change in absorbance. At the end of platelet aggregation monitoring, cyclooxygenase (COX) activity was halted by the addition of 1 mmol/l diclofenac (Sigma, UK), the samples were centrifuged at 1300 *g* for 10 min at 4°C, and the supernatants removed and frozen. Plasma thromboxane B_2 (TXB_2) levels, as a surrogate for thromboxane A_2 (TxA_2) production, were determined using a selective, competitive enzyme immunoassay (Cayman Chemical, USA).

For 96-well plate aggregometry (Optimul),[25 26] PRP was added to clear half-area 96-well microplates containing lyophilised platelet agonists: AA (0.03–1 mM), ADP (0.005–40 μM), collagen (0.01–40 μg/ml), epinephrine (0.0004–10 μM; Chronolog, UK), U46619 (aTxA_2, receptor agonist; 0.005–40 μM; Cayman Chemical) or ristocetin (0.14–4 mg/ml; Helena Bioscience, UK) (final concentration) or vehicle. Plates were then vigorously mixed (1200 rpm, 37°C; BioShake IQ, Q Instruments, Germany) for 5 min, and absorbance at 595 nm was measured using a standard absorbance microplate reader (Sunrise, Tecan, Switzerland). Platelet aggregation was calculated as percentage change in absorbance.

To measure adenosine triphosphate (ATP) secretion, PRP was activated with collagen (0.1–30 μg/ml) or ADP (5 μM) and vigorous mixing (1200 rpm, 37°C; BioShake IQ) in white 96-well microplates. After 2 min, Chrono-lume reagent (1:5, v/v; Chronolog, UK) was added, and plates were incubated for a further 2 min before the luminescence of each well was read in a multimode plate reader (Mithras LB940, Berthold Technologies, Germany). The luminescence of platelet-poor plasma was subtracted from all readings, and ATP concentrations determined by comparison with the luminescence of wells containing a known quantity of ATP (Chronolog, UK).

In all platelet studies, responses of sibling platelets were compared with those prepared from healthy volunteers, with and without in vitro treatment with aspirin (30 μM; Sigma, UK). ATP and TXB_2 release data were normalised to the platelet count in PRP. Statistical analyses were performed in Prism V.5.0 (GraphPad software).

RESULTS

SNP homozygosity mapping and exome sequencing reveals a homozygous deletion in *PLA2G4A* in affected individuals

To identify the genetic cause of this condition, a combination of whole-genome SNP array analysis and exome sequencing was performed. The linkage analysis with the less dense marker set revealed two long stretches with the maximal logarithm of the odds score of 2.38. On chromosome 1, the markers rs6425457 and rs4607826 border a region of 9.5 Mb, and on chromosome 10 the region length is 5.42 Mb between rs11201179 and rs12265445 (see online supplementary material). Homozygosity mapping with the full marker set identified the same two regions on the autosome as the longest stretches. The region on chromosome 1 contains 6765 homozygous SNPs and spans 8.6 Mb. The first heterozygote markers on each side defining the recombination events are kgp4929831 and kgp15471158 (178 785 646 Mb and 187 437 747 Mb, NCBI build 37). On chromosome 10, a set of 4169 adjacent markers were homozygous, spanning 5.07 Mb with restricting markers kgp7926438 and rs12782553 (86 578 120 Mb and 91 649 717 Mb). Insertion/deletion variation analysis of the exome data from the affected female subject revealed the presence of a homozygous 4 bp deletion

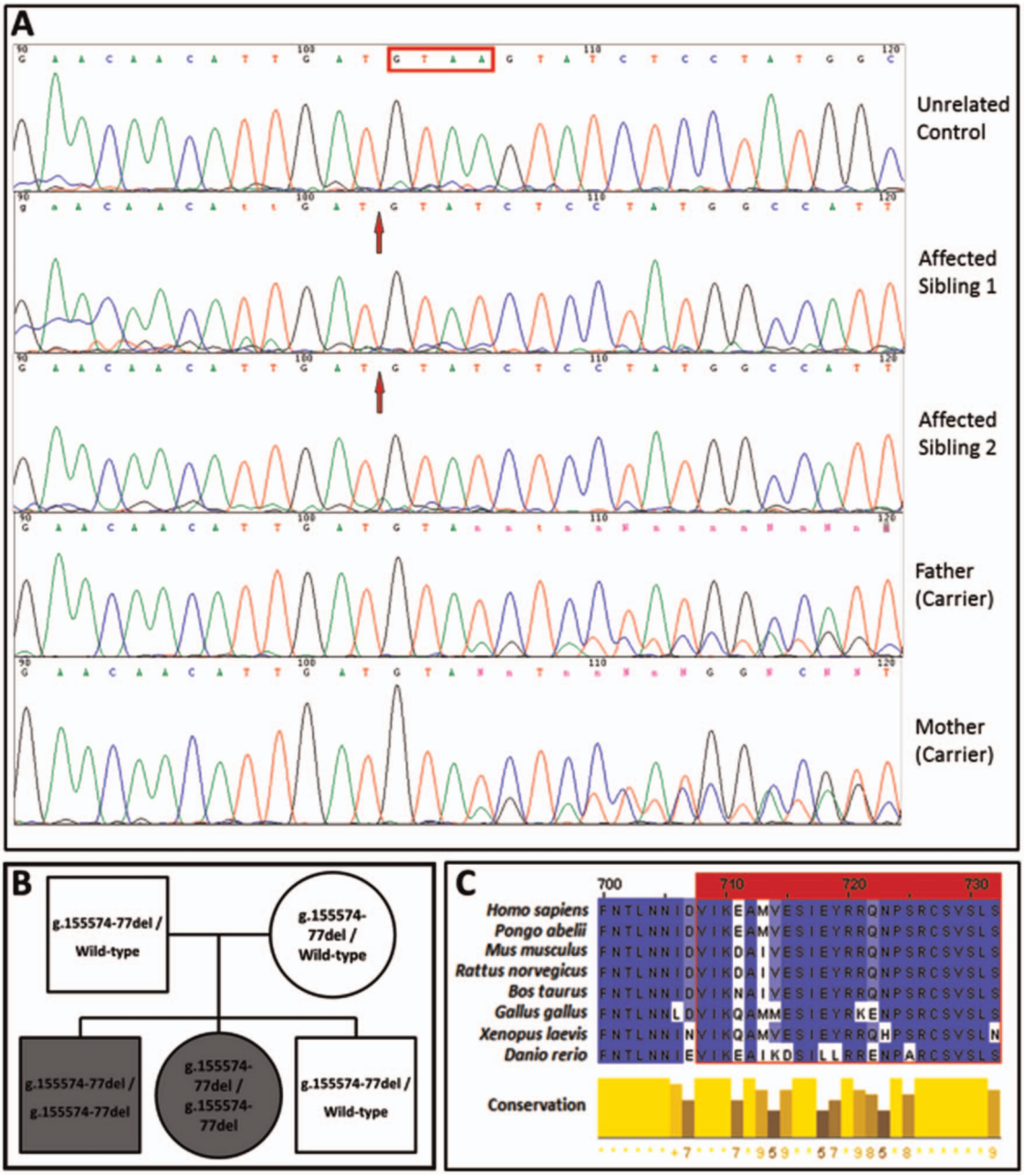

Figure 1 Mutation analysis. (A) Sanger sequence traces showing homozygous g.155574-77delGTAA mutations in *PLA2G4A* in two affected siblings. Both parents and an unaffected sibling (not shown) are heterozygous carriers. (B) Pedigree of the affected family illustrating segregation of the g.155574-77delGTAA mutation with disease. Grey and white symbols represent affected and unaffected family members respectively. (C) Conservation of amino acid residues in part of the cytosolic phospholipase A2-α catalytic domain across species. The region predicted to be deleted is highlighted in red. The lower yellow graph illustrates the degree of conservation of each residue, a full-height yellow bar indicating complete conservation between species.

(g.155574_77delGTAA) in the *PLA2G4A* gene, located in the splice donor site directly after exon 17 (the penultimate exon) of the gene. *PLA2G4A* (Accession Number NG_012203.1) maps within the common region of homozygosity on chromosome 1q25. The presence of this deletion was confirmed by Sanger sequencing, with the deletion found to be in homozygosity in both affected siblings. Both parents and their unaffected brother were heterozygous for the deletion (figure 1A, B). This deletion has not been described before, nor is it present in the dbSNP or 1000 Genome databases. Bioinformatic analysis predicts that this deletion results in the loss of the affected splice donor site, and introduces a frameshift of 10 amino acids before a premature stop codon (p.V707fsX10). This would result in the loss of 43 amino acids (residues 707–749) at the C-terminus of cPLA$_2$α (illustrated on cPLA$_2$α structure models in online supplementary figure S1). The deleted region contains residues highly conserved across species (figure 1C), with many of the deleted residues forming part of the cPLA$_2$α catalytic domain (which encompasses residues 144–730[27]). Furthermore, the deleted section contains a regulatory site (Ser-727) at which phosphorylation is required for cPLA$_2$α activity.[28] This analysis therefore suggested that the observed deletion would be expected to ablate cPLA$_2$α enzymatic

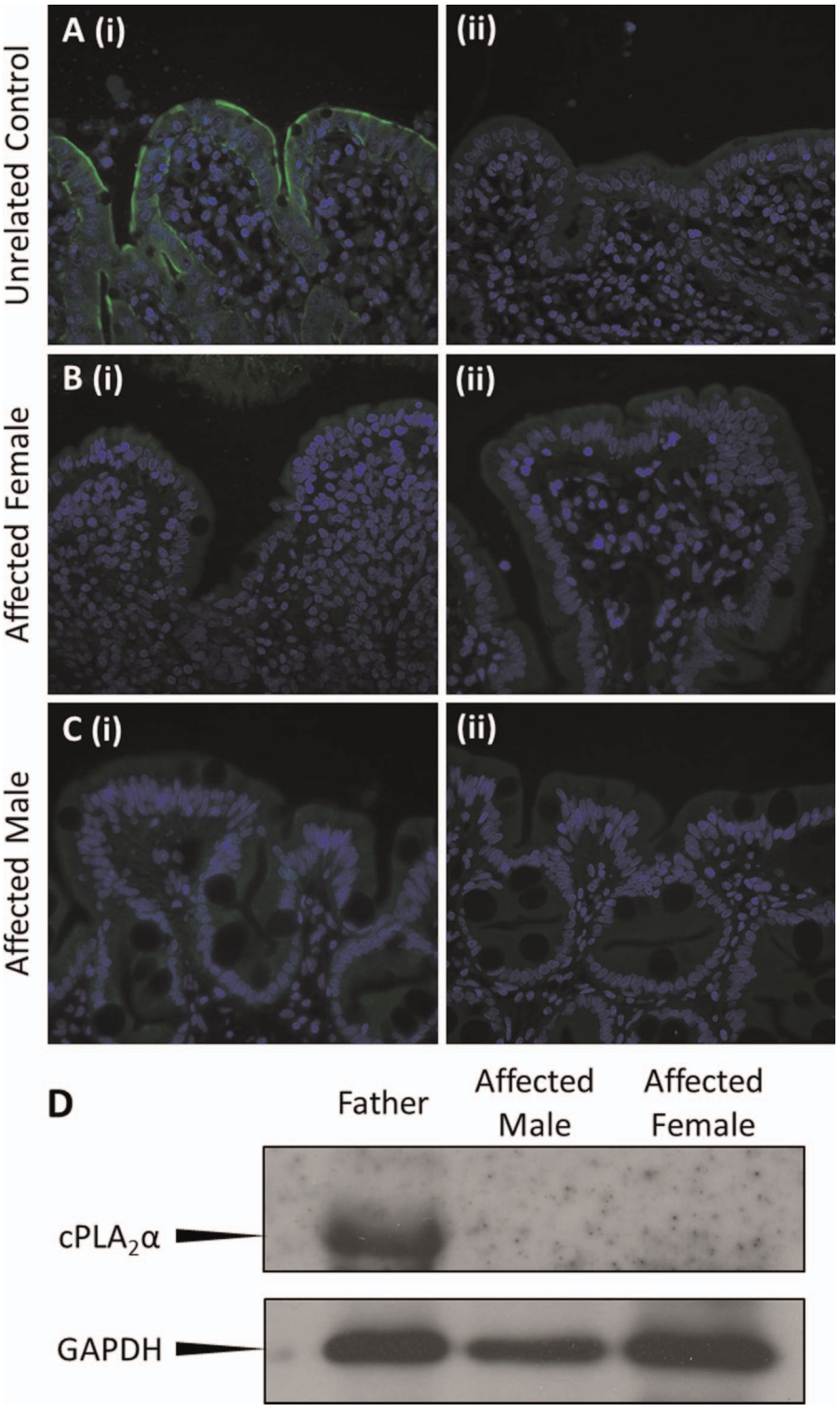

Figure 2 (A–C) Representative immunohistochemical analysis of cytosolic phospholipase A2-α (cPLA$_2$α) expression in small bowel biopsy specimens from an unaffected, unrelated control individual (A) and the two affected individuals (B and C). Immunohistochemical staining is shown in the presence (i) and absence (ii) of a cPLA$_2$α primary antibody. (D) Western blot of cPLA$_2$α expression in protein lysates of peripheral blood mononuclear cells, isolated from the unaffected father (left lane) and the two affected individuals (next two lanes).

function. With the exception of a single report of cPLA$_2$α loss-of-function associated with compound heterozygous point mutations in *PLA2G4A*—to be discussed below—mutations or common variations in *PLA2G4A* have not previously been associated with any pathological condition, in the gastrointestinal tract or elsewhere.

cPLA$_2$α protein cannot be detected in affected patients' gut or PBMCs

Immunohistochemical analysis of small bowel biopsy specimens from the two affected individuals and an unaffected, unrelated normal control individual, using an antibody against cPLA$_2$α, showed significant expression of cPLA$_2$α in the small bowel epithelium of the unaffected individual (figure 2A). However, cPLA$_2$α expression could not be detected in the epithelium of the two affected siblings (figure 2B, C), with the level of staining observed comparable to that seen in negative control specimens. Furthermore, western blotting analysis of PBMCs isolated from the two affected siblings and their unaffected father showed an absence of cPLA$_2$α expression in the two affected individuals, in contrast with their unaffected father (figure 2D).

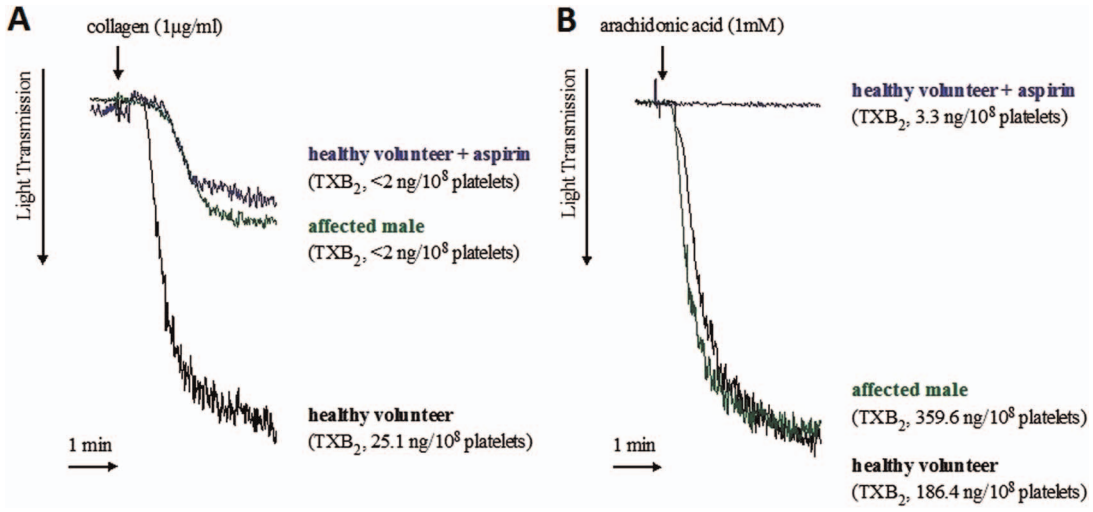

Figure 3 Light transmission aggregometry. (A) Platelet aggregation induced by collagen is greatly blunted in the affected male, to levels equivalent to that found in aspirin-treated platelet rich plasma (PRP) from healthy volunteers, and this is matched by loss of thromboxane A_2 (TXA_2) production (measured as thromboxane B_2 (TXB_2)) in PRP from the brother and from a healthy volunteer after treatment with aspirin. (B) Platelet aggregation induced by arachidonic acid (AA) (1 mM) in PRP from healthy volunteers is matched by that in PRP from the affected male, and both are associated with production of large amounts of TXA_2. Treatment of PRP from healthy volunteers with aspirin blocks aggregation in response to AA and associated production of TXA_2.

Platelet aggregation and TxA_2 production are significantly abrogated in affected patients

To assess the effect of the $cPLA_2\alpha$ mutations on its enzymatic function, aggregation of platelets in the plasma of the affected individuals—a process dependent on the production of the $cPLA_2\alpha$ downstream product TxA_2—was measured. Platelet aggregation in response to stimulation by collagen was significantly reduced in the two affected individuals compared with controls. Indeed, platelet aggregation in the affected individuals was comparable to that of aspirin-treated platelets from a healthy, unrelated volunteer (figure 3A). However, the addition of 1 mM AA to the affected patients' PRP resulted in a level of aggregation in the affected individuals equal to that of a healthy volunteer, a response that was not seen in aspirin-treated platelets from healthy volunteers (figure 3B). This illustrates that the TxA_2 synthesis pathway downstream of $cPLA_2\alpha$-dependent AA production remains unaffected in these individuals.

Platelet aggregometry further demonstrated significantly reduced platelet aggregation in the affected individuals in response to collagen or adrenaline (table 1 and figure 3), with a less marked reduction in aggregation observed in response to ADP. For collagen and adrenaline stimulation, the results were again comparable to those seen in aspirin-treated platelets from healthy volunteers. A synthetic TxA_2 analogue (U46619) almost completely abolished the reduction in platelet aggregation, illustrating that no dysfunction exists in either the thromboxane receptor itself or its downstream signalling pathway. Furthermore, platelet agglutination, stimulated in a TxA_2-independent manner by the antibiotic, ristocetin, was virtually identical with control levels in affected individuals. Once again, treatment with AA abolished the reduction in platelet aggregation seen in affected individuals, a response again not observed in aspirin-treated platelets from healthy volunteers.

TxA_2 produced by affected individuals' platelets was also measured, via levels of its inactive metabolite, TxB_2 (to which TxA_2 is converted with a half-life of ~30 s). Once more, this revealed significantly reduced TxA_2 production by affected individuals' platelets compared with controls in response to collagen, with the reduction entirely abolished by 1 mM AA (table 2).

As a final measure of platelet function, dense granule secretion was measured by means of ATP release from PRP. Again, the platelets of the two affected individuals showed significantly reduced function compared with controls in response to collagen (table 3), although there was no substantial difference in response to ADP (not shown).

DISCUSSION

We describe a pair of siblings with a catastrophic 40-year clinical history of intestinal and extraintestinal disease. A combination of genome-wide SNP homozygosity mapping and whole-exome sequencing revealed the siblings to be homozygous for a 4 bp deletion in the *PLA2G4A* gene, which encodes the enzyme $cPLA_2\alpha$,[29] which we predict will eliminate enzymatic activity. Functional studies revealed absent production of the eicosanoid, TxA_2, and severely impaired platelet aggregation in response to

Table 1 Changes in platelet aggregation, relative to healthy volunteers, in affected siblings or after exposure of platelets to aspirin

	Collagen (%)	Adrenaline (%)	ADP (%)	U46619 (%)	Ristocetin (%)	Arachidonic acid (1 mM) (%)
Healthy volunteers (N=4)	0	0	0	0	0	0
Healthy volunteers+aspirin (N=4)	−77	−49	1	−12	14	−86
Affected female	−91	−52	−36	−18	0	−8
Affected male	−60	−66	−15	−7	2	+10

Responses are calculated as changes in area under the curve from full concentration response curves.

Table 2 Thromboxane A_2 production, measured as thromboxane B_2 (ng/10^8 platelets), accompanying platelet aggregation determined by light transmission aggregometry

	Collagen (0.3 μg/ml)	Collagen (1 μg/ml)	Collagen (3 μg/ml)	Arachidonic acid (1 mM)
Healthy volunteers (range) N=4	10–11	20–22	22–81	150–406
Affected female	nd	nd	<2	297
Affected male	<0.5	<0.5	<2	360

nd, not determined.

collagen, which was restored by the addition of exogenous AA —the enzymatic product of cPLA$_2$α.

A single case of ileojejunal ulceration, accompanied by gastrointestinal blood loss, anaemia and impaired eicosanoid biosynthesis, has previously been reported to be associated with compound heterozygous single-base-pair mutations in *PLA2G4A*.[24] Interestingly, this patient only developed disease requiring surgery in the 4th decade of life, although milder symptoms of gastrointestinal ulceration had been 'lifelong'. In addition, unlike the siblings we describe here, who have severe duodenal, gastric and oesophageal disease, the compound heterozygote had a normal upper bowel. Although in the course of their disease, both affected siblings had ileal disease, this may have been secondary to volvulus in the affected male and *Salmonella* infection in the affected female. We have not yet carried out sufficient detailed analysis to determine the magnitude of the defect in production of all eicosanoids in our patients, although we would predict—on the basis of the nature of the deletion, the protein expression pattern observed, and the substantial defects in TxA_2 production and platelet aggregation—that there should be no cPLA$_2$α activity. However, the previously described compound heterozygote appeared to retain some degree of phospholipase activity, which may result from some residual enzymatic activity of his cPLA$_2$α—perhaps reflecting incomplete abolition of cPLA$_2$α activity by one or both heterozygous point mutations observed in this individual, in contrast with the complete loss of function observed here—or due to the activity of other phospholipases, and which may account for his milder phenotype compared with the siblings we describe here. It would, however, be of extreme interest to analyse other case series of patients with CMUSE,[6] to determine if they also have mutations in *PLA2G4A* and to accumulate sufficient cases to correlate disease type with mutation status.

cPLA$_2$α is a widely expressed, Ca^{2+}-dependent enzyme, which functions to specifically catalyse the release of AA from membrane phospholipids, via the hydrolysis of *sn-2* ester bonds.[30] Expressed in the vast majority of cell types, cPLA$_2$α activity, and therefore intracellular AA production, is tightly regulated by intracellular Ca^{2+} concentration and phosphorylation of the enzyme itself by mitogen-activated protein kinases and MNK1-related kinases[31] at a number of residues. AA serves as the substrate for a spectrum of enzymes involved in the synthesis of eicosanoids, including COX-1 and -2, lipoxygenases and cytochrome P450 expoxygenase. cPLA$_2$α-mediated AA release is thus a rate-limiting step in eicosanoid production, and cPLA$_2$α activity therefore plays an important role in the wide variety of physiological and pathological processes affected by eicosanoids (reviewed by Ghosh *et al*[32]). Mice deficient in cPLA$_2$α show normal development and lifespan, but defects in reproduction,[33] parturition,[34] renal function[29] and the allergic response,[30] alongside exaggerated heart and striated muscle growth[35] and the presence of numerous ulcerative lesions of the small intestine.[36] These effects can be associated with particular eicosanoid pathways. For instance, mice deficient in COX-2 showed impaired inflammatory responses, impaired resolution of inflammation, impaired renal development and impaired female reproduction.[37 38]

Table 3 ATP release from platelet-rich plasma (nmol/10^8 platelets)

	Collagen (0.3 μg/ml)	Collagen (1 μg/ml)	Collagen (3 μg/ml)	Collagen (10 μg/ml)
Healthy volunteers (range) (N=4)	0.1–0.5	0.9–3.1	0.7–5.7	0.9–6.8
Affected female	nd	nd	0.4	1.3
Affected male	0.1	0.1	0.6	1.5

nd, not determined.

As the siblings described here had undetectable cPLA$_2$α protein in their intestine, it is very likely that they have a global defect in their ability to produce AA from membrane phospholipids, and consequently have a systematic impairment in prostaglandin and leukotriene production. It is also highly likely that, in the absence of prostaglandins, the pathophysiology of the disease of these individuals mirrors that seen in individuals with enteropathy associated with long-term NSAID use, in that the oesophageal, gastric and duodenal mucosa becomes very sensitive to injury, perhaps mediated by acid, dietary components or bacteria. Furthermore, these individuals would be expected to be deficient in a number of COX-2 products, such as prostaglandin D_2[39] and 15-deoxy-$\Delta^{12\ 14}$-prostaglandin J_2[40]—as well as the lipoxygenase-produced lipoxins[41]—which have key anti-inflammatory and pro-resolution roles. Prostaglandin D_2, for example, has been shown to be specifically upregulated in the long-term remission of ulcerative colitis,[42] illustrating a gastrointestinal-protective effect, which would presumably be lacking in these individuals. In this way, mucosal injury can initiate a vicious cycle of ulceration and fibrosis as the lesions persist in the presence of impaired resolution.

It is difficult to determine whether the systemic disease seen in these patients was secondary to the problems observed in their gastrointestinal tract, or was a separate phenomenon. However, the male sibling has clear pathologies of his biliary tree, accompanied by liver abscesses and perhaps liver fibrosis. This damage is most likely mechanical in nature, reflecting the increased susceptibility of the bile duct and related tissues to stress in the absence of eicosanoid-mediated protection—although it may also be associated with the role of cPLA$_2$α in protecting the liver from injury.[43] The female sibling, meanwhile, has developed pernicious anaemia—perhaps as a result of intrinsic factor deficiency due to gastric damage, bladder problems and endometriosis, the pathogenesis of which is not known.

The female sibling in particular has also suffered repeatedly from infectious diseases, including infection by *Candida albicans, Campylobacter* and *Salmonella*, as well as repeated, severe *Staphylococcus* infections. This may be indicative of the role cPLA$_2$α is known to play in immunity—for example, cPLA$_2$α is necessary for efficient neutrophil-mediated bacterial killing,[44] which some reports have suggested may be related to a requirement for cPLA$_2$α in the activation of NADPH oxidase,

and thus the generation of reactive oxygen species.[45 46] Conditions related to neutrophil killing disorders in humans (such as chronic granulomatous disease) often present with abscesses of the skin, tissues and organs, suggesting an explanation for the presence of such lesions in this pair of siblings.[47 48] Furthermore, immunity to intracellular pathogens such as *Chlamydia trachomatis* requires $cPLA_2\alpha$, since, in its absence, production of type I interferon is deficient.[49] In addition, *Candida albicans* has been shown to rapidly upregulate $cPLA_2\alpha$ in macrophages, suggesting that its absence may compromise innate immunity to *Candida*.[50] Furthermore, $cPLA_2\alpha$ is also involved in the induction, by proinflammatory cytokines, of intercellular adhesion molecule 1 expression on the surface of endothelial cells, suggesting that $cPLA_2\alpha$-deficient patients may be affected by impaired mobilisation and transmigration of leucocytes from the bloodstream into tissues to deal with infectious agents.[51]

In conclusion, we have identified mutations in the *PLA2G4A* gene as the probable cause of CMUSE of over 40 years standing in two siblings. Further studies are needed to fully characterise the biochemical consequences of these mutations and to determine if mutations in the *PLA2G4A* gene are present in other cases with a similar phenotype.

Contributors MAB carried out the Sanger sequencing, immunofluorescence and western blotting and cowrote the paper with TTM and DPK. HJL referred the patients to TTM and provided the clinical histories. VP and FR carried out the bioinformatics of exome and SNP array data, respectively. NSK, JAM and TDW carried out all the platelet assays. DPK supervised the genetic and immunohistochemical aspects of the work. TTM cowrote the paper with MAB and DPK, and supported the exome sequencing.

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Cryptogenic multifocal ulcerating stenosing enteritis associated with homozygous deletion mutations in cytosolic phospholipase A2- α

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