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# Genetic complexity of diagnostically unresolved Ehlers-Danlos syndrome

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## ABSTRACT

**Background** The Ehlers-Danlos syndromes (EDS) are heritable disorders of connective tissue (HDCT), reclassified in the 2017 nosology into 13 subtypes. The genetic basis for hypermobile Ehlers-Danlos syndrome (hEDS) remains unknown.

**Methods** Whole exome sequencing (WES) was undertaken on 174 EDS patients recruited from a national diagnostic service for complex EDS and a specialist clinic for hEDS. Patients had already undergone expert phenotyping, laboratory investigation and gene sequencing, but were without a genetic diagnosis. Filtered WES data were reviewed for genes underlying Mendelian disorders and loci reported in EDS linkage, transcriptome and genome-wide association studies (GWAS). A genetic burden analysis (Minor Allele Frequency (MAF) <0.05) incorporating 248 Avon Longitudinal Study of Parents and Children (ALSPAC) controls sequenced as part of the UK10K study was undertaken using TASER methodology.

**Results** Heterozygous pathogenic (P) or likely pathogenic (LP) variants were identified in known EDS and Loeys-Dietz (LDS) genes. Multiple variants of uncertain significance where segregation and functional analysis may enable reclassification were found in genes associated with EDS, LDS, heritable thoracic aortic disease (HTAD), Mendelian disorders with EDS symptomatology and syndromes with EDS-like features. Genetic burden analysis revealed a number of novel loci, although none reached the threshold for genome-wide significance. Variants with biological plausibility were found in genes and pathways not currently associated with EDS or HTAD.

**Conclusions** We demonstrate the clinical utility of large panel-based sequencing and WES for patients with complex EDS in distinguishing rare EDS subtypes, LDS and related syndromes. Although many of the P and LP variants reported in this cohort would be identified with current panel testing, they were not at the time of this study, highlighting the use of extended panels and WES as a clinical tool for complex EDS. Our results are consistent with the complex genetic architecture of EDS and suggest a number of novel hEDS and HTAD candidate genes and pathways.

## WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The genetic basis for hypermobile Ehlers-Danlos syndrome (EDS) remains unknown.

## WHAT THIS STUDY ADDS

⇒ We report the results of whole exome sequencing for 174 patients with complex, genetically undiagnosed EDS.  
⇒ Using rare variant and genetic burden analysis, we identified new clinical diagnoses, variants of uncertain significance close to likely pathogenic classification and multiple novel candidate loci.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The study demonstrates the diagnostic utility of whole exome sequencing in diagnostically unresolved, complex EDS and adds to present knowledge of the genetic architecture of the Ehlers-Danlos Syndromes.

## INTRODUCTION

The Ehlers-Danlos syndromes (EDS) are heritable disorders of connective tissue (HDCT) that share key clinical features of generalised joint hypermobility (GJH), skin hyperextensibility and tissue fragility. The 2017 EDS nosology classifies 13 subtypes including primary disorders of collagen structure, processing, folding and cross-linking, disorder of the myomatrix, glycosaminoglycan synthesis, complement pathway and other unknown intracellular processes.<sup>1</sup> There are several other syndromes with EDS-like features including Loeys-Dietz syndrome (LDS), Ehlers-Danlos syndrome classic-like-2 (MIM 618000), lysyl hydroxylase 3 deficiency (*PLOD3*, MIM 612394) and inborn errors of metabolism such as homocystinuria. Newly identified genes that are associated with EDS-like syndromes but awaiting confirmation include *ALDH18A1* and *EFEMP1*.<sup>2,3</sup> Diagnostic genetic testing has high clinical utility when a rare EDS type is suspected, differentiating EDS subtypes with varying risks of vascular involvement and inheritance patterns from other EDS-like conditions.



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The genetic basis for hypermobile EDS (hEDS) remains unknown, although heterozygous *TNXB* mutations have been reported in association with features of hEDS in female patients.<sup>4</sup> GJH is a common population trait: 5% of 14 year olds had a Beighton score  $\geq 6$  in the ALSPAC cohort.<sup>5</sup> A genome-wide association study (GWAS) using self-reported Beighton scores  $>5$  identified 18 loci with p values between  $8.7 \times 10^{-7}$  and  $1.1 \times 10^{-12}$ .<sup>6</sup> Here, we have used WES and genetic burden analysis to investigate the genetic basis of EDS in patients with diagnostically unresolved, complex EDS.

## MATERIALS AND METHODS

### Patient recruitment and ethics approval

One hundred seventy-four patients from the national EDS diagnostic service (LNWUH) and specialist EDS rheumatology clinic (UCLH) were recruited. Patients had complex or suspected monogenic EDS, with arterial aneurysm(s) in proband and/or family member(s) and complex and/or severe symptoms. Patients consented to exome sequencing under approved protocols for Mendelian Disease research (Ethics Protocol Reference 11/LO/0883 (West London Research Ethics Committee)) and the NIHR BioResource project (Cambridgeshire 2 Research Ethics Committee Reference 04/Q0108/44). Patients were clinically categorised using the Villefranche criteria prior to skin biopsy and/or molecular testing.<sup>7</sup> The cohort comprised classical EDS (cEDS) (4 male/16 female), vascular EDS (vEDS) (5 female), hEDS (22 male/ 87 female), kyphoscoliotic EDS (kEDS) (2 male), (online supplemental tables 1-4). Patients not fulfilling the Villefranche criteria for a specific EDS subtype were categorised as HDCT (16 male/22 female; online supplemental table 5). At the time of recruitment, diagnostic gene sequencing for EDS-associated genes was available (LNWUH clinic); however, *B3GALT6*, *B4GALT7*, *C1R*, *C1S*, *COL6A1*, *COL6A2*, *COL6A3*, *COL12A1*, *DSE*, *PRDM5*, *ZNF469* and *LDS* genes were not offered. Patients with confirmed molecular diagnoses of monogenic HTAD or EDS were excluded. Patients reported previously by our research group, who had undergone non-diagnostic panel gene sequencing for EDS and HTAD, were enrolled.<sup>8</sup>

### DNA sequencing

DNA extraction was carried out as reported previously.<sup>8</sup> WES was performed in the Edinburgh Genomics and Cambridge sequencing laboratories.

### Variant analysis

WES data were filtered for variants with population frequency  $<0.1\%$  (rare variants) and Combined Annotation Dependent Depletion (CADD) score  $>15$  for further analysis using Varsome and Franklin, and were classified using the ACMG criteria and the Association for Clinical Genomic Science (ACGS) Best Practice Guidelines.<sup>9,10</sup> WES data were also analysed with the exomiser tool using HPO terms in the 2017 EDS nosology.<sup>1</sup>

### Genetic burden analysis

WES data ( $\sim 100$ -fold coverage) were analysed from 128 unrelated EDS cases of Caucasian ancestry together with whole-genome sequence data (2-fold to 20-fold coverage) from 248 ALSPAC controls<sup>11</sup> sequenced as part of the UK10K study.<sup>12</sup> The software package TASER<sup>13</sup> was used for burden analysis. This recalls variants in both cases and controls and constructs a test statistic while allowing for systematic differences in read depth (online supplemental method). WES data from 46 individuals of non-Caucasian or unknown ethnicity were excluded from this analysis.

## RESULTS

Variants in known EDS, HTAD, GJH associated syndromes and known Mendelian entities with EDS symptomatology were correlated with phenotypic data for each patient. We identified a small number of clearly pathogenic (P) and likely pathogenic (LP) variants.

### New diagnoses of EDS and HTAD

We identified 10 diagnostic P or LP variants in genes that are known causes of EDS and HTAD (table 1, online supplemental table 6). Two novel heterozygous pathogenic *COL12A1* variants

**Table 1** Diagnostic variants meeting the American College of Medical Genetics (ACMG) criteria for pathogenic and likely pathogenic classification

Patient ID	Variant ID	Age (years)	Gender	Clinical diagnosis	Gene/NM	Protein	ACMG classification
33	1	40–49	F	HDCT	<i>TGFβ3</i> NM_003239.4 c.463C>T	p.Arg155Trp	LP
34	2	30–39	F	HDCT	<i>COL5A1</i> NM_000093.4 c.4068G>A	Splice	LP
402	4	30–39	M	hEDS	<i>COL12A1</i> NM_004370.6 c.5097+1G>A	Splice	LP
479	8	20–29	F	HDCT	<i>SMAD2</i> NM_001003652.3 c.842A>T	p.Glu281Val	LP
564	9	20–29	M	HDCT	<i>TGFβ2</i> NM_001135599.3 c.989G>A	p.Arg330His	P
755	10	40–49	F	hEDS	<i>COL12A1</i> NM_004370.6 c.8321G>A	p.Gly2774Glu	P
814	14	30–39	F	HDCT	<i>TGFβR2</i> NM_001024847.2 c.1613T>C	p.Val538Ala	LP
1420	17	0–9	M	HDCT	<i>ALPL</i> NM_000478.6 c.394G>A	p.Ala132Thr	P
1484	18	50–59	F	hEDS	<i>COMP</i> NM_000095.3 c.2048G>T	p.Arg683Leu	LP
1528	19	30–39	M	cEDS	<i>COL5A1</i> NM_001278074.1 c.3397C>T	p.Arg1133Ter	P

Additional variant annotation is given in online supplemental table 6.

cEDS, classical Ehlers-Danlos syndrome; HDCT, heritable disorders of connective tissue; hEDS, hypermobile Ehlers-Danlos syndrome; LP, likely pathogenic; P, pathogenic.

were considered diagnostic. Splice site variant 4 was identified in patient 402 (bilateral congenital hip dislocation): the variant was found in one other individual in gnomAD and had high in silico prediction of pathogenicity (ADA score 0.999). *COL12A1* variant 10 resulted in a helical glycine substitution in patient 755 with multiple features suggestive for myopathic EDS (mEDS), including neonatal hypotonia and kyphoscoliosis.

Variant 19 resulted in loss of function in *COL5A1* in patient 1528, who had previously declined clinical diagnostic testing (ClinVar ID 280931). Patient 34 with hyperextensible skin, distal joint hypermobility and a carotid artery dissection had an overlapping HDCT/cEDS phenotype and carried the synonymous variant 2 in *COL5A1*. We had previously classified this as a variant of uncertain significance (VUS).<sup>8,14</sup> The variant impacts the last nucleotide of exon 51, with high in silico pathogenicity, and we now consider this likely pathogenic (ClinVar ID 212971). This patient also carried a pathogenic variant in *ITGB3* (variant 3) (autosomal recessive Lanzmann thrombasthenia MIM 173470), a gene that has been found to be abnormally expressed in skin fibroblasts from patients with hEDS,<sup>15</sup> and a novel variant in candidate gene *PGTFR4* (see below).

HDCT patient 814 carried novel LP *TGFBR2* variant 14 in the Ser/Thr kinase domain, without known vascular involvement. A recent report of this variant and accompanying functional data support LP classification.<sup>16</sup> HDCT patient 564, with pectus carinatum and aortic root dilatation, carried a *TGFBR2* pathogenic variant 9 (CADD=34). A different variant at the same nucleotide was reported as LP in association with syndromic aortic aneurysm (ClinVar ID 440982). Two patients (patient 33 and patient 479) had complex HDCT phenotypes and LP variants in *TGFBR3* (variant 1) and *SMAD2* (variant 8). hEDS patient 1484 had LP variant 18 in *COMP* (multiple epiphyseal dysplasia type 1, MIM 600310). HDCT patient 1420 had LP variant 17 in *ALPL* causative for hypophosphatasia (MIM 171760).

### VUS in EDS, LDS, HTAD and other syndromic genes with potential for pathogenicity reclassification

Thirty variants met the ACGS criteria where further segregation/functional work may enable reclassification as pathogenic or LP (online supplemental table 7).<sup>17</sup> Two patients with a clear cEDS phenotype harboured variants in *COL5A1* exon/intron 64, which encodes two transcripts in the C-propeptide domain, with alternate splicing in different tissue.<sup>18</sup> Patient 583 with *COL5A1* LoF variant 29 had cEDS major features: skin hyperextensibility, widened atrophic scars, generalised and small joint hypermobility with additional features of hEDS. cEDS patient 806 has a novel variant 35 at position +6 of intron 64. While a single multi-exon deletion including exon 64 (exons 63i-65i) has been reported as pathogenic, other exon 64 variants remain VUS (<https://databases.lovd.nl/shared/genes/COL5A1>).

cEDS patient 595 with missense *TGFBR3* variant 31 (CADD=25) had Mitral Valve Prolapse (MVP) and a high arched palate. hEDS patient 107, with a second-degree relative with an aneurysm, carried an *ULK4* splice variant 23. Loss of Function (LoF) variants in *ULK4* have been reported to increase the risk of aortic thoracic dissection in a single small study.<sup>19</sup> In syndromes with EDS-like features, patient 1530 (female) had splice variant 45, a VUS\* in the *UPF3B* gene, Lujan syndrome (MIM 309520, intellectual development disorder X linked, associated with Marfanoid habitus).<sup>20</sup> hEDS patient 107 carried variant 22, a VUS\* in *KCNH1* (MIM 135500, Zimmerman-Laband syndrome), which may have cartilage abnormalities and gingival hyperplasia as associated features. hEDS patient 967

carried variant 36, a VUS\* in *FLCN1* (MIM 607273, Birt-Hogg-Dube syndrome), associated with recurrent pneumothoraces and an increased risk of renal carcinoma.

We identified variants in genes associated with a skeletal dysplasia phenotype. cEDS patient 1451 had *COL9A3* variant 40, a glycine substitution in the triple helical domain (MIM 120270, AD multiple epiphyseal dysplasia type 3 with and without proximal myopathy) and also carried two VUS in *COL5A1* (online supplemental table 9). cEDS patient 1002 carried a novel cysteine substitution (variant 37) in *MAP3K7* (cardiospondylocarpofacial syndrome, MIM 157800) within the protein kinase domain.

We interrogated our data for Mendelian causes of symptomatology associated with EDS. Erythromelalgia is a *SCN9A* channelopathy associated with abnormal pain sensation and small fibre neuropathy (MIM 133020). We identified a novel *SCN9A* variant 27, at a transmembrane domain mutation hotspot, in patient 482 with a vEDS-like phenotype with thin skin and tissue fragility.

We identified patients with two or more rare/novel variants, for example, HDCT patient 72, with terminal digital and nail anomalies and a family history of HTAD had missense variant in *WNT10A* (variant 21, CADD=30, odontonychodermal dysplasia/tooth agenesis MIM 606268) and a VUS in *ROBO4* (aortic valve disease 3 MIM 618496) (online supplemental table 10). Multiple patients in the cohort had complex symptoms, signs and/or family histories, suggesting possible enrichment for patients with more than one rare Mendelian disorder.

### Variants of uncertain significance in genes associated with risk of ICA

We identified multiple variants in genes previously reported as associated with risk of intracranial aneurysm (ICA) (online supplemental tables 7; 8). hEDS patient 65 with a femoral artery aneurysm and family history of ICA carried *ROBO4* VUS and a second VUS in the fibrinogen-like domain of *ANGPTL6*. Rare variants in this domain have been reported as associated with familial ICA risk.<sup>21</sup> Variant 42 (VUS\*) in *PCNT* was found in hEDS patient 1495 who was not known to have a personal or family history of ICA; this variant has been previously reported in familial ICA.<sup>22</sup>

### Autosomal recessive disorders

A further eight heterozygous LP/P variants were identified in autosomal recessive EDS genes and other autosomal recessive genes overlapping with EDS symptomatology, *ZNF469*, *LAMA2*, *ITGB3*, *ELP1*, *ADAM22*, *C1QC* and *PRSS56* (table 1, online supplemental tables 6; 7; 9–11). Seven heterozygous VUS\* were identified in *LAMA2*, *TNFSF11*, *TONSL*, *RYR3*, *SLC2A10* and *CANT1*. Multiple VUS in *ZNF469*, *PRDM5*, *DSE*, *CHST14*, *ELP1*, *AEBP1*, *CCN6*, *RYR3*, *DYSF* and *LAMA2* (data not shown). HDCT patient 620 with an occipital horn syndrome phenotype, and consanguineous parents, was homozygous for a VUS in *SDSL* (NM\_138432.3 c.626C>T, p.Ala209Val) (MIM 618752, severe congenital neutropenia type 8). Phenotypic review did not show haematological abnormalities: these variants were therefore considered unlikely to be causative.

### VUS in EDS, HTAD, myopathy and inborn errors of metabolism genes

Additional VUS were identified in genes associated with EDS, HTAD, myopathy and inborn errors of metabolism (online supplemental tables 7; 9–11). A VUS in *BGN* was identified in

hEDS patient 1393 (female) with increased arm span to height ratio and talipes, and aortic root dilatation; loss of function mutations in this gene have been reported to result in Meester-Loeys.<sup>23</sup> A number of patients carried ultrarare variants in genes associated with non-syndromic HTAD (*ROBO4*, *PRKG1*, *SMAD6*, *ULK4*, *MAT2A*, *SMAD2*, *MFAP5*). HDCT patient 453 with carotid dissection had a 64 bp insertion predicted to result in out of frame/loss of function transcript in *PRKG1* (pLi=1). hEDS patient 1629 without known cardiovascular involvement had a novel *SMAD6* VUS in the MH1 domain. hEDS patient 1443 had a family history of abdominal aortic aneurysm in maternal relatives and ICA in a paternal relative carried novel VUS in *SMAD6*. Patient 526 had MVP and a family history of multiple individuals with cardiac valvular disease, with novel VUS in *IFIH* (CADD=31), in the helicase domain (MIM 606951, Singleton-Merten syndrome, acroosteolysis and aortic valve calcification).<sup>24</sup> HDCT patient 79 carried *EMILIN1* VUS at amino acid residue 28, close to residue 22, thought to affect N terminal signal peptide cleavage.<sup>25</sup> HDCT patient 422, with camptodactyly and Asperger's syndrome, carried a novel VUS, resulting in an in-frame deletion mutation in *MED12*.

We found a single VUS\* variant 43, and multiple VUSs in EDS and Bethlem myopathy genes (online supplemental table 9), HTAD (online supplemental table 10), myopathy, inborn errors of metabolism and dysautonomia genes (online supplemental table 11), many of which are similarly classified in ClinVar. These patients did not have specific clinical features (eg, contractures for Bethlem myopathy, cauliflower ears for Beals syndrome or aggressive periodontal disease for pEDS) which might contribute to ACMG criteria PP4.

#### EDS gene candidates based on linkage and skin fibroblast gene expression studies

We reviewed our data for germline variants in loci previously reported in a linkage study of a large family with hEDS, which identified *LZST1* as a candidate gene (online supplemental tables 12–16).<sup>26</sup> A single patient with hEDS in our cohort (patient 703) had a *LZST1* missense variant, with limited in silico evidence of pathogenicity (CADD=23). We also identified multiple rare variants (CADD >15) in genes within the reported region of linkage (online supplemental table 12). These included *SORBS3* (vinculin binding domain) reported to regulate extracellular matrix (ECM) stiffness in vitro,<sup>27</sup> *ADAM7*, *ADAM27* (variants in protease domains), multiple variants in the *CCAR1* gene (a regulator of cell division) and *DOCK5* (mouse model has reduced skeletal muscle, zebrafish has abnormal fast muscle).<sup>28</sup> In addition, we identified multiple rare variants in genes previously reported in a linkage study of Pelvic Organ Prolapse,<sup>29</sup> for example, *LAMC1*, *ROBO2* (online supplemental table 13, online supplemental methods).

Gene expression data from skin fibroblasts for patients with hEDS, cEDS and vEDS have been published, suggesting candidacy for several dysregulated genes.<sup>15 30 31</sup> We identified multiple rare germline variants with CADD >15, in several of these genes (online supplemental methods and online supplemental tables 14–16). These included integrin signalling, innate immune system function, TRAIL and TRAIL receptor genes, reported to affect integrin signalling in the ECM, controlling vascular remodelling.<sup>32</sup> We identified multiple rare heterozygous variants in *HSPG2* (Perlecan) (online supplemental table 15). Homozygous variants in *HSPG2* cause AR Schwartz-Jampel syndrome (MIM 142461) via disordered cartilage maintenance, osteonecrosis and endomysial dysfunction via a channelopathy mechanism.

A knock-in *HSPG2* mouse model demonstrated disordered acetylcholinesterase endplate morphology with abnormal patch clamp and a fatigability phenotype.<sup>33</sup> Two *POSTN* variants were found in FAS1 domains (online supplemental table 16): perlecan is reported as contributing to tissue repair after injury via upregulating collagen (I) and multiple other ECM component proteins.<sup>34</sup>

#### Rare variants in loci associated with GJH/self-reported Beighton score, rotator cuff injury and knee pain GWASs

We identified multiple rare variants with CADD >15 in genes associated ( $p < 5 \times 10^{-8}$ ) with self-measured Beighton score >5 in a published GWAS<sup>6</sup>: These included the PIEZO Type Mechanosensitive Ion Channel Component 1 (*PIEZO1*) and NEDD4 E3 ubiquitin protein ligase (*NEDD4*) (online supplemental table 17). *PIEZO1* is a mechanotransducer protein, important in the cellular responses to shear stress, maintenance of the vascular endothelium and mechanosensation in chondrocytes and epithelium.<sup>35</sup> *NEDD4* is a mediator of abnormal fibroblast proliferation in keloid scarring.<sup>36</sup>

#### HTAD candidate genes

Multiple patients in this cohort had a personal or family history of HTAD, carotid, intracranial and other aneurysmal disease. Careful review of all novel variants with CADD >15 in non-annotated genes revealed a small number of variants with high CADD scores (>20) in candidate genes with published data supporting a role in vascular disease and remodelling (online supplemental table 18). HDCT patient 1625 with a dilated aortic root and megacolon had a novel missense variant 63, in transforming growth factor beta 1-induced transcript 1 gene (*TGFB1/1*). This gene is regulated by TGF beta signalling; mice lacking its homologue, *hic5*, show deficient smooth muscle cell response to vascular injury (MIM 602353).<sup>37</sup> This variant at *TGFB1/1* Arg 67, neighbours phosphoserine 68, hence may disturb signal transduction. kEDS patient 1396 carried variant 59, a nonsense mutation *INO80D* (MIM 610169). Homozygous missense variants in *INO80D* were reported in a single family with aortic hypoplasia, aggressive atherosclerotic disease and periodontal disease,<sup>38</sup> pLi=1. Patient 34, with HDCT and carotid artery dissection, harboured variant 50 in prostaglandin E receptor 4 (*PTGER4*) (MIM 601586). Dysregulated expression of *PTGER4* has been reported in abnormal wound healing, regulation of vascular tone and blood pressure, in abdominal and thoracic aortic aneurysm and the regulation of cerebral blood flow.<sup>39</sup>

Reviewing murine and functional studies reported for Marfan syndrome, we identified germline variants in *TMBIM1* (MIM 610364), *SCUBE3*, *IRF7*, *IGFBP2* and *TMEM176B* and *MMP2*.<sup>40</sup> hEDS patient 1491 with kyphosis and a high arched palate carried *FBN3* variant 61 in the TGFbeta binding domain, disruption of the equivalent domain in *FBN1* cause Marfan syndrome. hEDS patient 1695 had a loss of function variant 64 in *NOTCH4*, (LOEUF=0.32), with livedo reticularis and a maternal aunt with pulmonary artery atresia. This gene is known to affect vascular morphogenesis in mice, but has not been associated with disease in humans.<sup>41</sup> HDCT patient 446 with carotid dissection carried four variants, including novel variant 54 in *NFAT5* (MIM 604708). Osmoregulatory stimulus has previously been found to upregulate *NFAT5* expression, resulting in abdominal aortic aneurysm and dysregulated immune function.<sup>42</sup> Two other *NFAT5* variants were also identified, in hEDS patients 1595 and 922 without aneurysms (online supplemental table

19). We identified an hEDS patient 566 with Marfanoid habitus, arterial rupture and collagen fibril irregularity, who carried a novel loss of function variant in the *SYAP1* gene (variant 56); a knockout mouse model for this gene has a highly distinctive motor deficit phenotype<sup>43</sup> (the pLi score is 0.94).

### Matrisome genes

We searched for rare variants with CADD >15 in genes known to interact with fibrillar collagen biosynthesis and signalling, chondroitin synthesis and modification (<https://reactome.org/PathwayBrowser>) (online supplemental table 19). Collagenases I/II/III (MMP1, 8, 13 and 4) are known regulators of the fibrillar collagens in the ECM. Variant 60 substituted a histidine residue of Zinc binding site in MMP8, which was previously reported in GWAS as associated with premature rupture of the membranes (MIM 120355). The patient had hEDS with a family history of recurrent miscarriage. Heterozygous missense variant 51 in MMP25 (608482) (online supplemental table 18) was identified in a patient with hEDS: this gene is functional in the innate immune system and abnormal expression has been associated with tendinopathy in a mouse model.<sup>44,45</sup> We also noted multiple heterozygous VUS in autosomal recessive skeletal dysplasia genes, *CANT1*, *TONSL*, *OSTM1* (data not shown).

Biallelic pathogenic variants in *ADAMTS2* cause dermatosparaxis type EDS. We identified a patient with HDCT (patient 446) with heterozygous Variant 52 in *ADAMTS5* and variant 53 in *ADAMTS16*. Both variants were in the spacer domains, known to regulate aggrecanase activity. Heterozygous missense variants were also identified in *ADAMTS20*, *ADAMTS22*, *ADAMTS23*, *ADAMTS28*. Pathogenic variants in *C1R/C1S* cause pEDS, by gain of function on as-yet unidentified targets,<sup>46</sup> we found multiple rare variants in other (non-annotated) serine proteases (online supplemental table 19).

### Integrins, ephrin, ciliopathy, *TSPANs*, *DOCK*, circadian rhythm pathways

Within the entire cohort, we noted clusters of variants in genes not currently associated with EDS and in novel genes and pathways with biologically plausible links to EDS, including integrins (*ITGA3*, *ITGB4*, *ITGA8*, *ITGAV* and *ITGB1BP1*) (online supplemental table 19). Integrin-collagen interactions are integral to wound healing, inflammation, innate immunity and via TGFβ signalling and other pathways.<sup>47</sup> We identified multiple rare variants in ephrins and their receptors (data shown for *EPHA8*, *EFNA1*), known to regulate vascular endothelial and corneal proliferation, tissue fibrosis, wound healing and catecholamine

synthesis.<sup>48</sup> Ciliopathies are generally associated with complex phenotypes; however, variants in *IFT88* and *NFATC3* were recently reported with bicuspid aortic valve.<sup>49</sup> We identified two novel variants in these genes. Wound healing is known to be under circadian rhythm control through local and central mechanisms.<sup>50</sup> We identified a small number of variants in *PER1* (MIM 602260), *PER2* (MIM 603426) and *ZFHX3* (MIM 104155). It is possible that abnormal wound healing seen in patients with EDS is due to the disruption of these control mechanisms. We identified multiple variants in *DOCK5* (MIM 616904), in the linked region for hEDS. While it has not yet been annotated as causative of disease in humans, a mouse model has a reduced skeletal muscle phenotype and a zebrafish model has abnormal fast muscle.<sup>28</sup> We also identified multiple variants in various *TSPANs*. *TSPAN2* regulates TGFβ1/SMAD expression in vascular endothelium (MIM 613133).

### Genetic burden analysis

In view of the large number of rare variants identified in multiple pathways, a formal burden analysis was carried out to seek statistically significant associations. Burden analysis was carried out using the TASER software<sup>13</sup> (table 2). While *LOC283685* was close to meeting the criteria for significance ( $p=2.34e-6$ , adjusted  $p=7.41e-6$ ), we identified that the coding sequence of the final exon of *GOLGA6L2* transcript ENST00000312015 (Glu308-Ter415), annotated separately in USC GRCh38, probably overlaps the C-terminal sequence of *LOC283685* (Glu61-Ter168). The overall burden of rare variants in *GOLGA6L2* including this terminal region did not meet significance ( $p=2.67e-3$ , adjusted  $p=4.36e-3$ ). The lack of statistically significant results of this analysis is likely related to the small sample size. A number of the top scoring loci, however, had biological plausibility. The LRTM4-HSPG (heparan sulfate proteoglycane) complex has been proposed a tetrapartite model for synaptic plasticity involving interactions with the ECM and HSPG has been noted in the vEDS transcriptome. *GOLGA6L2* is of unknown function; golgins are a large group of vesicle tethering proteins with tissue-specific effects, other golgins are known to result in reduced bone mineral density and neuromuscular phenotypes (*GOLGA2* MIM 602580). *ANKFY1* is involved in transport to the Golgi apparatus. *ADCY1* (MIM 103072) causes autosomal recessive deafness with abnormalities of circadian rhythm.<sup>50</sup>

### DISCUSSION

In this study, we generated WES in 174 patients with several EDS clinical subtypes: cEDS (n=20), vEDS (n=5), kEDS (n=2),

**Table 2** Results of genetic burden analysis using TASER methodology, with 128 cases and 248 controls

Gene	Chr (position)	L	M_s	M_st	M_p	New.SB_p	New.STB_p	Adjusted p value
LOC283685	15 (23684612–23685207)	21	7	7	7	2.34E-06	2.34E-06	7.41E-06
OR4C45	11 (48366903–48373999)	14	9	9	9	7.72E-06	7.72E-06	2.18E-05
KCNJ12	17 (21279699–21323179)	178	36	36	35.5	9.63E-06	9.63E-06	2.67E-05
PSMD2	3 (184017022–184026675)	74	6	6	6	5.65E-05	5.65E-05	1.32E-04
BX648489	20 (25825303–25834657)	18	10	10	10	6.34E-05	6.34E-05	1.47E-04
ANKFY1	17 (4066665–4167025)	71	8	8	8	6.79E-05	8.15E-05	1.84E-04
FRG1B	20 (29612306–29631629)	50	14	14	14	9.94E-05	9.94E-05	2.21E-04
LRRTM4	2 (76974850–77749502)	47	5	5	5	1.06E-04	1.06E-04	2.34E-04
MLLT10P1	20 (29637584–29638138)	21	20	20	20	1.41E-04	1.41E-04	3.03E-04
ADCY1	7 (45613739–45703971)	30	1	1	1	1.81E-04	1.81E-04	3.80E-04

Adjusted p value, p value after applying genomic control correction (inflation factor  $\lambda=1.11$ ) to the New.STP\_p  $\chi^2$  test statistic; L, number of variant sites that are considered 'rare' (alternate allele read count frequency AACF <0.05); M\_p, estimated number of SNVs in the dataset; M\_s, number of variant sites screened in; M\_st, number of variant sites screened in and passing threshold AACF >1/(2n), where n=128+248 (the cohort size); New.SB\_p, p value of the 'New-SB' test (based on M\_s); New.STP\_p, p value of the 'New-STB' test (based on M\_st).

(hEDS n=109) and HDCT (n=38) from two specialised clinical EDS services. Patients underwent extensive clinical diagnostic and research testing for known EDS/HTAD genes prior to being recruited into this study. Those with a confirmed genetic diagnosis in the clinical laboratory or in our previous research study were excluded.<sup>8</sup> Ten patients previously without a genetic diagnosis were given a new diagnosis: two patients were diagnosed with mEDS, two with cEDS and four with LDS. The pathogenic and LP variants in these patients were subsequently confirmed in the clinical diagnostic laboratory. A molecular diagnosis may be important for clinical management and may facilitate assessment of vascular risk. Although many of the pathogenic (P) and likely pathogenic (LP) variants reported in this cohort would be identified with current panel testing, they were not at the time of this study, highlighting the use of extended panels and WES as a clinical tool for complex EDS.

We also identified a number of high priority VUS in genes for EDS (n=3), LDS/ HTAD (n=3), Lujan syndrome (n=1), Birt-Hogg-Dube syndrome (n=1), skeletal dysplasia and bone metabolism (n=4), erythromyalgia (n=1) with compelling supporting clinical and in silico criteria for pathogenicity, according to ACGS criteria, segregation and functional work may enable reclassification to LP. These findings reflect the overlap between the clinical features of EDS, LDS, HTAD and Mendelian disorders associated with EDS symptomatology. Further, a small number of patients were identified as carrying more than one such variant, suggestive of two separate Mendelian disorders, which may explain the complex phenotypes observed in these patients.

We identified single patients with novel variants with CADD >15 in genes not previously reported as associated with a Mendelian phenotype (*PGTER4*, *TGFB1/1*, *INO8D*, *SYAP1*), with biological plausibility based on published in vitro and animal models of vascular disease and EDS phenotypes. A large number of rare variants with CADD >15 were identified in genes previously identified in EDS GWAS and transcriptome studies (eg, *HSPG2*, *PIEZO1*, *COL27A1*). We note that these included a number of genes reported as causes of autosomal recessive skeletal dysplasia and other pathways implicated in the repair and maintenance of the ECM: Integrins, Ephrins and DOCK genes.

While a formal burden analysis did not identify any genome-wide statistically significant associations, several plausible candidate loci were identified that will benefit from further investigation.

One limitation of this study was the inability to identify chromosomal CNVs, which are implicated in HTAD, *TNXB* and familial mast cell disorders, leading to potential under-ascertainment of these abnormalities in this cohort.<sup>4</sup> Finally, the occurrence of GJH as a normal trait and unknown prevalence of symptomatic hypermobility/hypermobility spectrum disorders (HSD) and hEDS presents a challenge to assessment of the expected prevalence of rare variants in relation to disease.<sup>5</sup>

## CONCLUSIONS

We report WES analysis for a large cohort of patients with complex and unresolved EDS phenotypes to have undergone deep phenotyping and WES. This study suggests that large panel-based sequencing and WES will have clinical utility in patients with complex presentations that are unresolved by clinical examination and EDS panel gene sequencing, by making new molecular diagnoses for rare Mendelian disorders that had not been previously suspected in earlier detailed investigation. In addition, multiple heterozygous variants were identified in

genes associated with skeletal dysplasia, myopathy and integrins, although these are not as yet proven to be causative for EDS. A smaller number of variants in non-annotated genes with biological plausibility were also identified. Our results are consistent with the complex genetic architecture of EDS and have suggested a number of novel hEDS and HTAD candidate genes and pathways that are worthy of further investigation.

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