

# COL3A1 haploinsufficiency results in a variety of Ehlers-Danlos syndrome type IV with delayed onset of complications and longer life expectancy

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**Purpose:** To characterize the clinical outcome of heterozygosity for *COL3A1* null mutations in Ehlers-Danlos syndrome type IV, the vascular type. **Methods:** We identified mutations that produced premature termination codons and resulted in nonsense-mediated messenger RNA decay in 19 families. We reviewed the clinical and family histories and medical complications in 54 individuals from these families with *COL3A1* null mutations. **Results:** Compared with individuals with missense or exon-skipping mutations, we found that life span was extended, the age of first complication was delayed by almost 15 years, and major complications were limited to vascular events. The families were ascertained after a complication in a single individual, but only 28% of relatives, some of whom had reached their seventies or eighties without incidents, had a complication and only 30% had minor clinical features of Ehlers-Danlos syndrome type IV. **Conclusion:** Null mutations have reduced penetrance compared with missense and splicing mutations, and the phenotype seems to be limited almost entirely to vascular events. *Genet Med* 2011;13(8):717–722.

**Key Words:** Ehlers-Danlos syndrome type IV, vascular EDS, *COL3A1* haploinsufficiency, arterial aneurysm or rupture, natural history

Ehlers-Danlos syndrome (EDS) type IV, known as the vascular type (OMIM# 130050), is a dominantly inherited disorder that results from mutations in the *COL3A1* gene (OMIM# 120180). EDS type IV is characterized by the major complications of arterial and bowel rupture, uterine rupture during pregnancy, and the clinical features of easy bruising, thin skin with visible veins, and characteristic facial features. Because of these dramatic complications, mean life expectancy is shortened to <50 years.<sup>1</sup>

We have identified heterozygous *COL3A1* mutations in 508 families, approximately 95% of which lead to the synthesis of abnormal type III procollagen molecules. Alterations in glycine codons that lead to substitutions for glycine residues in the triple helical domain of the pro $\alpha$ 1(III) chain of type III procollagen account for two thirds of identified mutations, and splicing mutations comprise most of the remainder.<sup>1,2</sup> The remaining mutations include small genomic deletions and duplications, multiexon deletions, and more complex alterations. A total of

19 of the 508 families have mutations that lead to premature termination codons expected to result in nonsense-mediated messenger RNA (mRNA) decay of the encoded product of the mutant allele. This distribution of mutations is similar to that reported in the Database of Collagen Mutations.<sup>3</sup> To date, no correlations have been identified between the nature of the missense mutations or splice-site alterations and the type or frequency of major complication,<sup>1</sup> perhaps because almost all reported individuals have been ascertained as a result of complications of the disorder.

In most other fibrillar collagenopathies, production of half the amount of the encoded protein as a result of haploinsufficiency usually results in a milder phenotype than seen with missense or splicing mutations. For example, haploinsufficiency mutations in *COL1A1* (OMIM# 120150) result in osteogenesis imperfecta (OI) type I, the mildest phenotype. Similar mutations in *COL2A1* result in Stickler syndrome, rather than the more severe phenotypes seen with missense and exon skipping mutations. Mice heterozygous for a *COL3A1* knockout do not develop arterial rupture<sup>4</sup> but have histologic and mechanical changes of the artery wall,<sup>5</sup> which suggests that in humans haploinsufficiency for *COL3A1* might be expected to have a milder clinical picture than in individuals with missense or other mutations that alter type III collagen structure. In the initial set of EDS type IV families identified with heterozygosity for *COL3A1* null mutations, the index patients had a clinical course similar to that seen in individuals with missense or splice-site mutations.<sup>2</sup> However, testing of relatives determined that many affected family members survived beyond age 50 years without major complications (see data presented herein). One teenager, who was homozygous for a *COL3A1* frameshift mutation that led to mRNA instability, died from complications similar to those seen in others with heterozygous missense mutations, but neither of her heterozygous parents had any evidence of such adverse effects, and there was no family history of similar complications.<sup>6</sup>

To understand the clinical consequences of heterozygosity for *COL3A1* null mutations, we reviewed the clinical and family histories and medical complications of 19 index patients with null mutations and 35 of their relatives who had the same mutation. Compared with individuals with missense or exon-skipping mutations, we found that in the cohort with *COL3A1* null mutations, life span was extended, the age of first complication was delayed, major complications were limited to vascular events, and penetrance was reduced.

## MATERIALS AND METHODS

### Study subjects

Eighteen of 19 index patients (95%) with identified *COL3A1* null mutations were referred for evaluation after a major arterial event. The remaining index patient was referred because a parent died at an early age from an arterial event. Testing was completed on 54 relatives because of the perceived risk for vascular complications in individuals with

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**Table 1** Mutations identified in the *COL3A1* gene that lead to mRNA instability and their effects

Family	<i>COL3A1</i> mutation (A of the initiator methionine codon = 1)	Reading frame shift?	Amino acid change (initiator methionine = 1)	Location of premature termination codon	Effect on splicing	Amount of structurally normal type III procollagen by SDS-PAGE	Demonstration of loss of expression of one <i>COL3A1</i> allele in RNA/ cDNA
1 <sup>a</sup>	c.413delC	Yes	p.Pro138Leufs*27	Exon 6		No detectable diminution	rs1800255 (c.2092): G/A in gDNA */A in cDNA
2 <sup>a</sup>	c.555delT	Yes	p.Gly186Valfs*36	Exon 9		No detectable diminution	rs35010692 (3'UTR): +/- in gDNA +/* in cDNA
3	c.766delA	Yes	p.Ile256Tyrfs*7	Exon 11		No detectable diminution	rs1800255 (c.2092): G/A in gDNA G/* in cDNA
4	c.798+1G>A (IVS11+1G>A)	Yes		Exon 12	Use of a cryptic splice donor site 17nt upstream in exon 11	Reduced amount	rs7579903 (c.1851): A/G in gDNA */G in cDNA
5	c.955_974delinsTTT ACATCGAGGGTT TTAAAGTTTACA	Yes	p.Ala319Phefs*13	Exon 15		Cells not available	Not done, presumed loss of expression
6	c.1410delT	Yes	p.Gly471Glufs*65	Exon 23		Reduced amount	rs1800255 (c.2092): G/A in gDNA G/* in cDNA
7	c.1773dupT	Yes	p.Lys592*	Exon 26		Cells not available	Not done, presumed loss of expression
8 <sup>a</sup>	c.1831_1832delAA	Yes	p.Asn611Trpfs*2	Exon 27		Reduced amount	rs1800255 (c.2092): G/A in gDNA G/* in cDNA
9	c.1808delG	Yes	p.Gly603Alafs*188	Exon 35		No detectable diminution	Sequencing of cDNA across the mutation site: only the normal allele was present
10	c.2023-1G>A (IVS30-1G>A)	Yes		Exon 35	Shift of the intron 30 splice acceptor site by 1nt into exon 31	Reduced amount	rs1800255 (c.2092): G/A in gDNA G/* in cDNA
11	c.2655_c.2661+7del insGACCTGAGAC	Yes		Within the 10nt insertion (exon 39- intron 39)	Use of a cryptic splice donor site 54nt downstream in intron 39	Reduced amount	rs1801184 (c.2244): T/C in gDNA */C in cDNA
12	c.2823+1G>A (IVS40+1G>A)	Yes		Exon 41	Use of a cryptic splice donor site 5nt downstream in intron 40	Slightly reduced amount	cytoplasmic RNA: no detectable abnormal splice product nuclear RNA: detectable abnormal mRNA that retains the first 4nt of intron 40
13	c.2935G>T	No	p.Glu979*	Exon 42		Cells not available	Not done, presumed mRNA instability
14	c.3070C>T	No	p.Arg1024*	Exon 43		Cells not available	Not done, presumed mRNA instability

(Continued)

Table 1 Continued

Family	COL3A1 mutation (A of the initiator methionine codon = 1)	Reading frame shift?	Amino acid change (initiator methionine = 1)	Location of premature termination codon	Effect on splicing	Amount of structurally normal type III procollagen by SDS-PAGE	Demonstration of loss of expression of one COL3A1 allele in RNA/ cDNA
15	c.3496C>T <sup>b</sup>	No	p.Arg1166*	Exon 48		≈ 10% of normal; overmodified due to sequence alteration in second allele <sup>b</sup>	c.3569: C/T in gDNA */T in cDNA
16	c.3575_3576ins AGGG	Yes	p.Pro1193Glyfs*20	Exon 49		Cells not available	Not done, presumed mRNA instability
17	c.2554-1G>A (IVS37-1G>A)	Yes		Exon 49	Shift of the intron 37 splice acceptor site by 1nt into exon 38	Cells not available	Not done, presumed mRNA instability
18	c.2824-1G>A (IVS40-1G>A)	Yes		Exon 49	Shift of the intron 40 splice acceptor site by 1nt into exon 41	Reduced amount	rs35795890 (c.1804): C/A in gDNA C/* in cDNA
19	c.3847C>T	No	p.Gln1283*	Exon 50		Cells not available	Not done, presumed mRNA instability

We have used the recommended Human Genome Variation nomenclature in this table in which \* indicates a termination codon.

<sup>a</sup>Previously published as P2, P3, and P1 in Schwarze et al.<sup>2</sup>

<sup>b</sup>Has second variant (significance unknown) on the stable COL3A1 allele: c.3569C>T, p.Pro1190Leu, Pro1023Leu in the triple helix.

these mutations.<sup>2</sup> All relatives in whom a COL3A1 mutation was identified in the Collagen Diagnostic Laboratory were referred because of a positive family history; none of these relatives was tested because of a vascular complication. Three of the index patients included in this study (1, 2, and 8) were reported by Schwarze et al.<sup>2</sup> (P1, P2, and P3 in that article). The remaining subject (P4) in that study is not included in this study because that mutation resulted in protein instability rather than mRNA instability, and it is not known whether mutations that interfere with chain association activate an unfolded protein response that may add an additional component to the response. Those with premature termination codons seem to activate only the nonsense-mediated mRNA decay pathway.

### Biochemical and DNA sequencing studies

Dermal fibroblasts were obtained and cultured from 12 of the 19 index patients (but none of the relatives) referred to the Collagen Diagnostic Laboratory for collagen screening studies, and the synthesis of type III procollagen was studied as described previously.<sup>7</sup> Blood samples were submitted from the remaining index patients and 54 of the relatives for clinical testing. DNA was extracted by standard procedures. The coding exons and flanking intron sequences of COL3A1 were amplified by PCR in 25 reactions using primer pairs based on the 2006 freeze of the Human Genome Sequence. The amplified fragments were sequenced by automated sequencing (primers and amplification and sequencing conditions are available on request). To test relatives, only the region of COL3A1 that contained the mutation in the family was amplified and sequenced, using the same amplification and sequencing primers used for mutation discovery.

### Analysis of mutation outcome

Total cellular RNA was isolated by RNeasy Mini Kit (Qiagen) from cultured dermal fibroblasts from 12 of the index patients. In addition, nuclear RNA was isolated for index patient 12 as described elsewhere.<sup>8</sup> Complementary DNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, San Diego, CA). A region of the COL3A1 coding sequence was amplified that included the mutation, a second unique heterozygous variant, or a polymorphic site found to be heterozygous during genomic DNA sequence determination and then sequenced to determine the relative abundance of the products of the two COL3A1 alleles (Table 1).

### Assignment of affected status

We identified 63 relatives with a 50% risk to have inherited or transmitted a null mutation found in the 19 families. We were able to test 54 of these relatives to determine whether they had their respective familial mutation. Of these, we found a mutation in 26 and did not find a mutation in 28. All those tested in whom the familial mutation was identified were designated as “affected” for the sake of this study. There were nine individuals among the 63 relatives who we could not test but to whom we assigned an affected status because they had a major arterial complication and a confirmed affected first-degree relative (6 subjects) or on the basis of obligate inheritance (3 subjects). Relatives were classified as unaffected if the results of testing excluded the diagnosis. We did not include in the analysis individuals for whom no clinical information was available, if clinical information was insufficient to make a clinical diagnosis, or if the individual was reported as healthy and not tested.

**Table 2** Characteristics of index patients and relatives with *COL3A1* null mutations

Characteristics	All subjects	Male subjects	Female subjects
All patients (index and relatives)	<i>N</i> = 54	<i>N</i> = 25	<i>N</i> = 29
Mean age at ascertainment, yr (age range)	44.4 (5–83)	41.5	46.8
Index patients, no. (%)	19	11 (57.9)	8 (42.1)
Mean age at ascertainment, yr (age range)	38.5 (17–59)	39.8	36.8
Mean age at first complication in index patients, <sup>a</sup> yr (age range)	37.8 (17–57)	38.9	36.1
No. of patients with data available	18 <sup>b</sup>	11	7
Relatives, no. (%)	35	14 (40.0)	21 (60.0)
Mean age at ascertainment, yr (age range)	47.5 (5–83)	42.9	50.6
Mean age at first complication in relatives, <sup>a</sup> yr (age range)	48.1 (28–61)	45.0	49.4
No. of patients with data available	10 <sup>c</sup>	3	7

<sup>a</sup>All complications were arterial dissection or rupture.

<sup>b</sup>One subject, age 18 years, had not had a complication at the age of ascertainment.

<sup>c</sup>25 subjects had not had a complication at the age of ascertainment.

From clinical histories available at the time of diagnostic testing, we recorded the number and type of medical complications, the ages at which they occurred, the cause of and age at death, and minor clinical findings of EDS type IV,<sup>9</sup> if noted. The age of testing (i.e., ascertainment) in the index patients was the age at which we confirmed the diagnosis (Table 2). For their relatives, the age of ascertainment was the age at which we identified the familial mutation in them (26 relatives), their last known age (three subjects), the age they were reported in the literature (one subject),<sup>10</sup> or their age of death as recorded in the family history (five subjects).

### Statistical analysis

We used Kaplan-Meier analysis (SPSS statistical software) to generate a survival curve and confidence intervals for the null cohort (Figure, Supplemental Digital Content 1, <http://links.lww.com/GIM/A176>); we included the age of death or the last known living age of each living subject. For a basis of comparison, we recalculated the Kaplan-Meier survival curves and confidence intervals for EDS type IV index patients and their relatives using the original data presented by Pepin et al.<sup>1</sup> None of the subjects included in this study were in the previous study.

## RESULTS

### Mutations and mutation outcome

We identified four nonsense mutations, nine frameshift mutations that led to downstream premature termination codons, and six splice-site mutations that created frameshifts and downstream premature termination codons (Table 1). Cultured cells from eight of these individuals (Families 4, 6, 8, 10, 11, 12, 15,

and 18) produced about half the normal amount of type III procollagen and no abnormal molecules, as predicted by the type of mutation. In cells from individuals in four of the families (1, 2, 3, and 9), we could not distinguish the amount of type III procollagen produced from the control, and no abnormality was detected in the mobilities of type III collagen chains.

In all families for which cultured fibroblasts were available, the mutation was shown to result in nonsense-mediated mRNA decay (Table 1). For the remainder, the effect was inferred from the predicted outcome of the other mutations.

### Survival

The index patients were identified at a younger age than were their affected relatives (Table 2). A total of seven subjects died: two index patients (2/19; 10%) and five relatives (5/35; 14%). In the natural history study of individuals with EDS type IV by Pepin et al.,<sup>1</sup> a similar proportion of index patients died (26/220; 11.8%), but a much larger proportion of relatives (105/199; 53%) were not alive at the time of ascertainment. Pepin et al.<sup>1</sup> attributed the higher death rate of relatives to the method of ascertainment (using the family history records of younger index patients for diagnosis and inclusion). Using the same method of ascertainment, we found, unexpectedly, a lower death rate in relatives in this group than in those with other types of mutations (Fig. 1).

The ages of death were 31, 36, 38, 41, 53, 58, and 68 years in the patients who died in the null cohort. Despite the small sample size, there is a difference in survival between the groups (Figure, Supplemental Digital Content 1, <http://links.lww.com/GIM/A176>). A log-rank test of the Kaplan-Meier analysis confirmed a significant difference in survival ( $P < 0.005$ ,  $\chi^2$  value was 21.2, with a degree of freedom of 1).

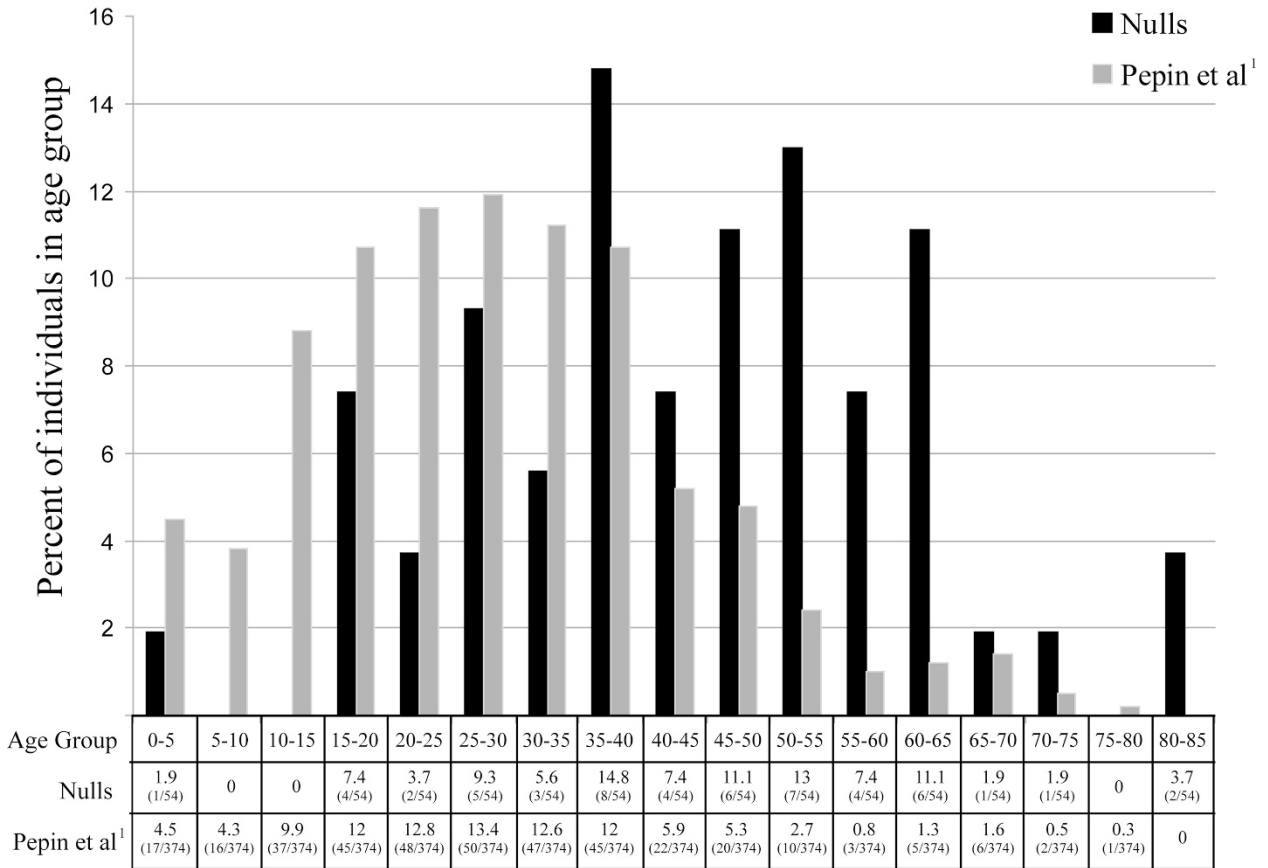
All deaths in this study resulted from arterial dissection or rupture, compared with 79% of deaths from arterial complications, 10% from organ rupture, 7% from gastrointestinal rupture, and 4% other causes in the study by Pepin et al.<sup>1</sup>

### Medical complications

At the time of ascertainment, 18 of 19 index patients (95%) and 10 of 35 relatives (28%) had had a single vascular complication (arterial dissection, aneurysm, or rupture). One index patient (5%) and 25 of 35 relatives (71%) had not had any type of vascular, bowel, or gastrointestinal complication at the time of ascertainment.

### Arterial complications

Of the 18 index patients with arterial complications, 12 had had dissections (three carotid artery dissections, three abdominal aortic dissections, two thoracic aortic dissections, two renal artery dissections, one coronary artery dissection, and one unknown). Four of the index patients had an arterial aneurysm as their first complication (common hepatic artery aneurysm, abdominal aortic aneurysm, splenic artery aneurysm, and aneurysms at multiple locations), whereas the remaining two index patients had multiple arterial ruptures (celiac, superior mesenteric, hepatic, splenic, renal, iliac, and femoral) as their primary complication. Of the 10 relatives with arterial complications, three had dissections (thoracoabdominal aortic, extracranial vertebral, and internal carotid), three had arterial ruptures (two aortic ruptures and one femoral artery rupture after catheterization), one had a hepatic artery aneurysm, and the specific details were not available for the remaining three. Of the 25 relatives without a complication at the time of ascertainment, one individual had a normal abdominal ultrasound, but no others were

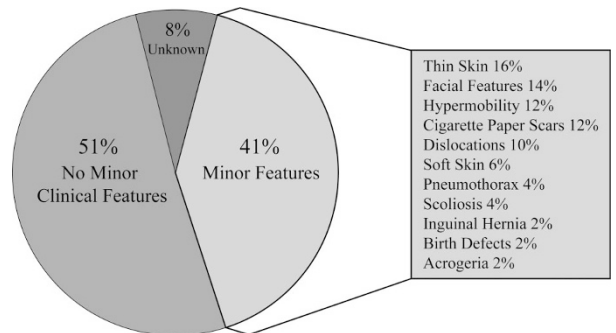


**Fig. 1.** Distribution by age of individuals with COL3A1 null mutations and other types of mutations in COL3A1. The distribution is arranged in 5-year intervals. Each box contains the number of individuals at their last known age (numerator), the total number in the group (denominator), and the percentage of the total.

studied by magnetic resonance imaging or computed tomography for evidence of arterial abnormalities.

**Minor features of EDS type IV**

One or more of the minor diagnostic criteria for the vascular type of EDS (as defined by Beighton et al.<sup>9</sup>) were reported as present in 41% of null subjects, with thin skin (16%), characteristic facial features (14%), joint laxity (12%), or unusual scarring (12%) being the most common (Fig. 2). In none of the patients with null mutations were friable tissue and poor wound healing reported, characteristics common in other individuals with EDS type IV. More than half of the null cohort (51%) was reported to have no minor clinical features of EDS type IV.



**Fig. 2.** Minor diagnostic criteria of EDS type IV in 54 individuals with COL3A1 null mutations.

**DISCUSSION**

Our study indicates that most individuals with COL3A1 null mutations have a different clinical course than individuals with missense or splice-site mutations that result in the production of abnormal type III procollagen molecules. Compared with individuals with mutations that produce abnormal molecules, those with COL3A1 null mutations had an extended life span, the average age of first complication was delayed by almost 15 years, and primary complications were limited to vascular events (dissections, aneurysms, or ruptures).

Families with null mutations were ascertained at a later age than those with mutations that altered protein structure (44.4 years vs. 28.7 years). Although 18 of 19 of these families were ascertained after a vascular complication in a single individual, only 28% of relatives who had the same mutation reported a vascular complication. Thus, most relatives with COL3A1 null mutations did not have the complications associated with EDS type IV, and only a minority was thought to have even the minor clinical features of the condition.

One limitation of this study is that we relied on available medical history to determine whether individuals with the mutation had complications of EDS type IV. Because those without complications had no reason to suspect they were at risk before mutation characterization, they would not have had detailed imaging for aneurysms or undetected dissections in major vessels. Thus, we could not identify the true incidence of vascular complications in this group, only in those who were symptomatic. Additionally, we were not able to study individuals in these families who had no complications consistent with EDS type IV and no testing. This exclusion biases ascertainment toward those with complications (in some instances assumed to result because they had mutations). Finally, because the ascertainment of null families seems to be far below what we would expect, this study may overestimate the risk of arterial complications of heterozygosity for null mutations.

We remain uncertain why analysis of the proteins produced by the cultured cells is incompletely sensitive to the presence of null mutations; it may reflect the relative expression of the intact *COL3A1* allele. The diminished sensitivity of protein-based studies in detection of *COL3A1* mutations that result in premature termination codons suggests direct DNA analysis as the preferred mode of diagnostic testing. However, in rare instances, analysis of the outcome of some mutations, particularly those that affect splice sites, may be required to prove that the ultimate effect is to reduce mRNA stability. These studies can only be done in cultured cells.

Null mutations in genes that encode the dominant protein of a tissue, such as the collagen genes, often have a milder phenotype. In families with OI that results from mutations in the *COL1A1* gene, almost 50% of affected individuals who we studied have haploinsufficiency mutations and have OI type I, the mildest OI phenotype (unpublished data). However, *COL3A1* null mutations represent only 4% of mutations we have identified in that gene. Because null mutations in *COL3A1* are likely to be as common or nearly as common as those in *COL1A1*, the penetrance of this class of mutation is lower than that of missense and splicing mutations. This suggests that there may be a second variant (perhaps the relative expression of the intact *COL3A1* allele) that predisposes individuals with the primary mutation to develop arterial events. We are uncertain where the bulk of individuals with *COL3A1* null mutations are to be found, but if the analogy with OI holds, then perhaps they are represented among individuals with late-onset arterial aneurysms. It is appropriate to launch a research study to determine the extent to which such mutations account for late-onset aneurysms and dissections.

In current practice, it is the presence of a family history of vascular or organ complication and the presence of at least some of the minor clinical features of EDS type IV that drives the decision to test for mutations in *COL3A1*. Our study suggests that lowering the threshold for such testing to include individuals with aneurysms or dissections at a later age and individuals with aneurysms or dissections and a positive family history of aneurysms or dissections may identify some of the individuals with this class of mutations. Finally, because ascertainment on the basis of clinical findings alone has a low yield, genetic testing for *COL3A1* mutations should be offered to all first-degree relatives of an individual identified with a *COL3A1* null mutation.

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