Genetic evidence that mutations in the *COL1A1*, *COL1A2*, *COL3A1*, or *COL5A2* collagen genes are not responsible for mitral valve prolapse

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SUMMARY   DNA markers were used to assess the segregation of genes encoding the collagen types that predominate in the mitral valve (types I, III, and V) in two family pedigrees that are phenotypically different but showed dominantly inherited mitral valve prolapse. The inheritance of these markers was compared with the segregation of the phenotype for mitral valve prolapse in both families. In one family it was shown that the *COL1A1*, *COL1A2*, *COL3A1*, and *COL5A2* genes segregated independently of the phenotype; in the other family the results for *COL1A1*, *COL1A2*, and *COL5A2* were similar but analysis at the *COL3A1* locus was not possible.

These data indicate that in these families mitral valve prolapse does not arise from a defect in one of these collagen genes.

Mitral valve prolapse is remarkably common. Its prevalence has been studied in women and men separately[1][2] and in general surveys[3-5] and estimates range from 5% to 15%. Mitral valve prolapse is inherited as an autosomal dominant trait and the gene shows incomplete penetrance with age and sex dependent expression.[6] The major pathological changes include an increase of the cusp area with elongation or rupture of the chordae tendineae, resulting in prolapse of the valve into the left atrium during systole. The clinical presentation is variable—in many cases mitral valve prolapse is little more than an auscultatory abnormality, but in some patients complications arise.[7]

Quantitative cross sectional echocardiographic techniques have showed two different patterns of mitral valve abnormality in patients with mitral valve prolapse, and evidence suggested that these were associated with distinct phenotypic features and risks of complications.[8][9] These subtypes may reflect genetic heterogeneity within the mitral valve prolapse syndrome, and certain complications, such as chordal rupture, may be associated with specific mutations.

The pathogenesis of mitral valve prolapse is unknown. It is, however, a common manifestation of various systemic connective tissue disorders,[3] for example some of the Ehlers-Danlos syndromes and Marfan syndrome, in which there is evidence of a collagen defect.[10]

Histologically the lesion is characterised by fragmentation of the collagenous bundles within the valve fibrosa.[7] Several studies have implicated collagen defects as the primary events causing this abnormality, and mutations in collagen genes have been shown in some disorders that are associated with mitral valve prolapse.[10] Collagen types I, III, and V predominate in the mitral valve and, with the exception of type III collagen which is the product of a single gene (*COL3A1*) located on chromosome 2, these proteins are encoded by more than one gene.[11] Type I is the product of two genes, *COL1A1* and *COL1A2*, located on chromosomes 17 and 7 respectively; type V is thought to be encoded by three

genes, of which only one (*COL5A2* on chromosome 2) has been cloned.[11] Heterogeneity in mitral valve prolapse might reflect a variety of mutations affecting different genes which could determine the severity of the lesion, as is seen in osteogenesis imperfecta.[10 12]

The use of DNA markers to study the inheritance of specific genes is now well established and has been successfully used to study various inherited diseases.[13] These restriction fragment length polymorphisms are inherited as codominant traits and can be used as markers in segregation analysis. We studied collagen gene restriction fragment length polymorphisms in two families in which mitral valve prolapse was segregating as an autosomal dominant trait.

Patients and methods

SUBJECTS
Because prolapse is not uncommon all the members of each family were given a thorough physical examination and underwent M mode and cross sectional echocardiography to check for the possibility that mitral valve prolapse might also be present in a spouse. Standard criteria were used for the interpretation of the echocardiograms.[14 15] Specifically, mitral valve prolapse was considered to be present when M mode tracings targeted on cross sectional echocardiography showed late systolic displacement of the continuous mitral leaflet interfaces by at least 2 mm behind the line between the points of valve closing and opening, or when systolic billowing of one or both leaflets across the mitral annular plane in systole was demonstrated in the parasternal long axis view.[14 15] The apical four chamber view was not used because of recent evidence that apparent leaflet billowing in this view may be normal. Use of this view leads to false positive diagnosis of mitral valve prolapse because of the non-planar ("saddle") shape of the mitral annulus.[16] The echocardiograms for both families were read by the same investigator (RBD) to exclude any variation in the definition of mitral valve prolapse.

RESTRICTION ENZYME ANALYSIS OF DNA
Genomic DNA was extracted from leucocytes contained in 10–20 ml of anticoagulated blood, as described previously.[17] Samples of DNA were digested to completion by various restriction endonucleases, the fragments were separated electrophoretically on 1% agarose gels, and blotted on to nylon or nitrocellulose support membranes according to established protocols.[18]

COLLAGEN GENE RESTRICTION FRAGMENT LENGTH POLYMORPHISMS
Polymorphisms associated with the genes encoding the pro-α1 and pro-α2 chains of type I (*COL1A1* and *COL1A2*), pro-α1 chain of type III (*COL3A1*), and pro-α2 chain of type V (*COL5A2*) collagen were used in these studies. Variation at the *COL1A1* locus was detected in *Msp*I and *Rsa*I digests using the genomic probes p1A1H17526 and p2FC6 respectively.[19] For *COL1A2*, an *Eco*RI polymorphism was detected with a 6·75 kb genomic clone NJ3[20] and an *Rsa*I marker was visible with the cDNA Hf32.[21] For the *COL5A2* gene, an *Msp*I polymorphism[22] was used and detected with a 3·5 kb genomic clone, DMC2. Two polymorphic markers were used for the *COL3A1* gene, both located in the 3′ untranslated region: one was detected by *Ava*II and the cDNA probe pIII-33[23] and the other by *Eco*RI with a 2·1 kb fragment of genomic clone IdF17.[24]

To estimate the likelihood that a marker was linked to the mutant locus we calculated the ratio of the probability for that association at a given genetic distance (the recombination fraction, θ) to the probability of association at a distance where the two are not linked ($\theta = 0.5$)—that is where the association could have arisen by chance alone. This ratio is usually expressed as a logarithm, the lod (log of odds) score for linkage. A lod score of 3 or greater indicates odds of 1000:1, or better, in favour of linkage and is taken as formal proof of linkage. Conversely lods of −2 or more are taken to indicate exclusion.

LINKAGE ANALYSIS
These calculations were performed by the computer program LINKAGE,[25] and we took into account the incomplete penetrance and variable sex dependent expression of the phenotype. A gene frequency based on a 5% population prevalence was used, together with estimates of 50% penetrance for males and 90% for females, based on the data of Devereux *et al*.[6 15] No data were available to show what proportion of young unaffected individuals develop mitral valve prolapse later on in life and, therefore, no allowance could be made for age dependant expression.

Results

CLINICAL SUMMARIES
Quantitative echocardiographic methods were not used in these studies and, therefore, no conclusive distinction between different subtypes of mitral valve prolapse could be made.

Family H—The figure shows the pedigree of this three generation family. Of the five siblings in the second generation, two (II.3 and II.7) had clear mitral valve prolapse shown by echocardiography and a third (II.5) showed signs of slight prolapse. No information was available on the clinical state of the
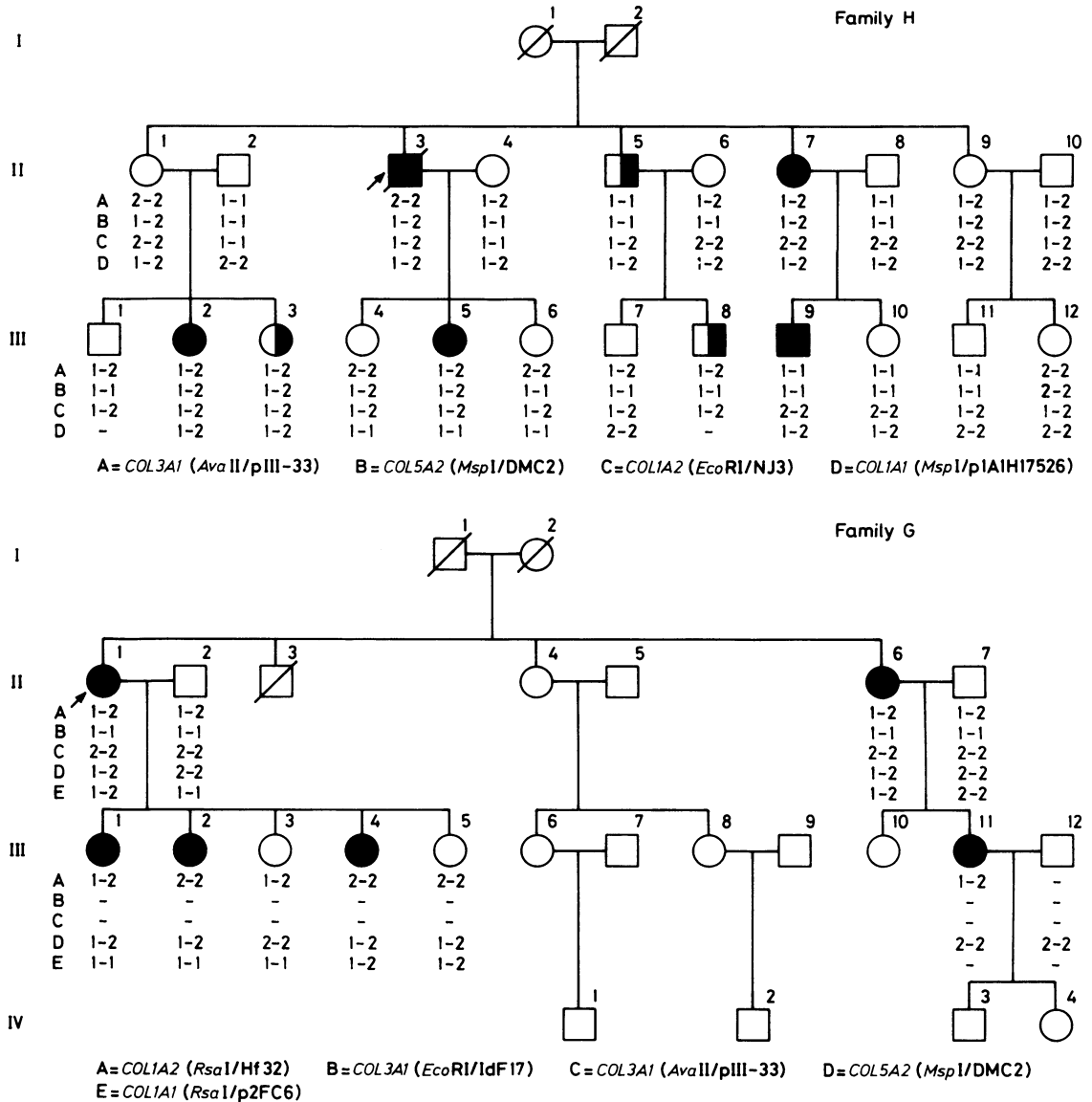
Family H

A= COL3A1 (Ava II/pIII-33)   B = COL5A2 (MspI/DMC2)   C=COL1A2 (EcoRI/NJ3)   D=COL1A1 (MspI/p1AIH17526)

Family G

A= COL1A2 (Rsa I/Hf 32)   B = COL3A1 (EcoRI/IdF 17)   C = COL3A1 (Ava II/pIII-33)   D= COL5A2 (MspI/DMC2)
E = COL1A1 (Rsa I/p2FC6)

Figure *Pedigrees of two families showing the segregation of the mitral valve prolapse phenotype. Circles denote females, squares are males, and black symbols identify affected individuals. Half shaded symbols indicate mild prolapse. The genotypes for each of the collagen genes are listed under each individual and are identified by the appropriate key.*

two individuals in the first generation. The proband had undergone surgery to replace his mitral valve after rupture of chordae to both the anterior and posterior cusps. He has since died. In addition, there were other manifestations in this pedigree that are characteristic of connective disorders (table 1). In the second generation, three of the five siblings had increased armspan-height differences (II.3,
+13·5 cm; II.5, +6·3 cm; II·7, +9·0 cm), all five had early onset varicose veins severe enough to require operation, and three (II.3, II.5, and II.7) had hyperextensible skin. Of the 12 individuals in the third generation, three had mitral valve prolapse shown by echocardiography (III.2, III.5, and III.9) and in two others (III.3 and III.8) the echocardiograms suggested a degree of prolapse. One of those

Table 1 *Description of phenotypes seen in the two families*

	Armspan* − height (cm)	JHS	Echo	Auscultation	Skeletal†	Vascular‡	Skin§
Family H:							
II. 1	3·0	0	—	—	—	+	—
II. 3	13·5	0	Valve replaced		—	+	+
II. 5	6·3	0	±	—	—	+	+
II. 7	9·0	1/9	+	+	—	+	+
II. 9	3·5	1/9	—	—	—	+	—
III. 1	ND	0	—	—	—	—	—
III. 2	6·0	0	+	+	+	—	—
III. 3	2·0	6/9	±	—	—	—	+
III. 4	3·2	0	—	—	—	—	—
III. 5	5·0	0	+	—	—	—	—
III. 6	2·5	0	—	—	—	—	—
III. 7	3·0	3/9	—	—	—	—	—
III. 8	3·0	0	±	—	—	—	—
III. 9	1·0	0	+	—	—	—	—
III.10	−2·5	0	—	—	—	—	—
III.11	7·5	0	—	—	+	—	—
III.12	3·0	2/9	—	+	—	—	—
Family G:							
II. 1	7·8	N/A	—	—	—	N/A	—
II. 2	0·0	N/A	+	+	—	N/A	—
II. 4	N/A	N/A	—	—	—	N/A	N/A
II. 6	−5·0	N/A	+	+	—	N/A	—
II. 7	5·0	N/A	±	—	—	N/A	—
III. 1	−4·0	N/A	+	—	+	N/A	—
III. 2	1·0	N/A	+	—	—	N/A	—
III. 3	1·0	N/A	+	+	—	N/A	—
III. 4	4·0	N/A	+	+	—	N/A	—
III. 5	1·0	N/A	±	—	—	N/A	—
III.10	N/A	N/A	—	—	—	N/A	N/A
III.11	−0·5	N/A	+	+	+	N/A	N/A
III.12	N/A	N/A	—	—	N/A	N/A	N/A
IV. 3	6·5	N/A	—	—	—	N/A	—
IV. 4	4·0	N/A	—	—	—	N/A	—

*A difference of > 7·5 cm was regarded as clinically significant. JHS, joint hypermobility score calculated as described by Beighton and Horan.[26] ± denotes a suggestion of prolapse that does not fully satisfy diagnostic criteria. † + = kyphosis, scoliosis, or pectus deformities. ‡Early onset varicose veins. §Evidence of hyperextensible skin. N/A, not available.

mildly affected (III.3) also had excessively hyper-mobile joints and hyperextensible skin. Skeletal defects were present in two individuals: III.2 had an increased armspan-height difference (+ 6·0 cm), kyphosis, and pectus excavatum, while III.11 also had abnormally long arms (+ 7·5 cm), kyphosis, and scoliosis. The vascular manifestations seen in the parents were not obvious in their offspring.

Family G—There was no information on the first generation of this family (fig). Of the four siblings in the second generation, two of the three who were available for examination were affected (II.1 and II.6), and one of them (II.1) has a history of cerebrovascular disease. No information was available on the clinical state or genotype of the dead brother. The nuclear family of an unaffected sibling (II.4) was not studied. All the offspring in the third generation were female; four of them were clearly affected. Two siblings had mild mitral valve prolapse: III.3 showed a suggestion of prolapse on one echocardiogram and met diagnostic criteria in a second study, but was normal on auscultation. She had mild left ventricular dysfunction and electrocar-

diographic changes consistent with a previous episode of myocarditis, which could explain the positive echocardiogram. Her sister III.5 was com-pletely normal on first examination, but four years later an echocardiogram showed slight evidence of prolapse and there was a discernible systolic murmur with a possible click. In the absence of firm evidence showing mitral valve prolapse, this individual was regarded as normal for the purposes of these analyses. In the fourth generation, the two siblings studied were normal. Two individuals with mitral valve prolapse in this family had mild scoliosis; no one with prolapse had an armspan-height difference of more than 7·5 cm and none had hyperextensible skin (table 1).

GENETIC ANALYSIS
The genotypes for each of the collagen loci are listed below each individual in the two pedigrees presented in the figure, and table 2 shows the calculations of the lod scores for each locus at various recombination distances.

In family H, exclusion of the *COL1A1* gene is

Table 2 *Lod scores for the collagen loci calculated at various recombination fractions*

Family	Locus	Recombination fraction (θ)									
		0	0·05	0·10	0·15	0·20	0·25	0·30	0·35	0·40	0·45
G	COL1A1	−0·74	0·03	0·12	0·17	0·17	0·14	0·10	0·07	0·03	0·01
H	COL1A1	−1·44	−0·81	−0·53	−0·35	−0·23	−0·14	−0·09	−0·08	−0·02	−0·005
G	COL5A2	−1·09	−0·99	−0·79	−0·57	−0·40	−0·26	−0·16	−0·09	−0·04	−0·01
H	COL5A2	−4·24	−1·91	−1·12	−0·68	−0·41	−0·23	−0·12	−0·05	−0·02	−0·001
G	COL1A2	−0·62	−0·41	−0·28	−0·19	−0·13	−0·08	−0·05	−0·03	−0·01	−0·002
H	COL1A2	−3·24	−1·23	−0·74	−0·48	−0·31	−0·20	−0·12	−0·06	−0·03	−0·01
G	COL3A1	Not informative									
H	COL3A1	−1·90	−1·22	−0·80	−0·54	−0·36	−0·23	−0·14	−0·08	−0·03	−0·01

explained as follows. The proband (II.3) is hetero-zygous 1–2 and has an affected child who is homozy-gous for the 1 allele, ruling out an association with the 2 allele. The 1 allele is also excluded because the homozygous affected individual III.5 has unaffected siblings with the same genotype, and their affected cousin III.2 has inherited the 1 allele from her unaffected mother (lod score −1·44 at θ = 0). The COL1A2 locus was also excluded. The proband has a genotype of 1–2 for the COL1A2 locus and he has passed the 2 allele on to all three of his daughters, only one of whom is affected. This effectively excludes the involvement of the 1 allele. Three of his siblings (II.1, II.7, and II.9) were homozygous 2–2, but only one was affected, suggesting that this allele is also not involved (lod −3·24 at θ = 0). This could be due to incomplete penetrance of the gene, but the exclusion of the 2 allele is based on the fact that the mildly affected heterozygote III.8 inherited the 2 allele from his unaffected mother and not from his father. Similarly, in the COL5A2 analysis, the heterozygous proband passed the 2 allele on to two of his daughters, of whom only one was affected. This could be due to incomplete penetrance, but the presence of the homozygous 1–1 affected cousin (III.9), excludes linkage. The inheritance of the 1 allele by the affected daughter (III.5) from her mother (II.4) and not her affected father (III.3) excludes linkage of the trait to this locus (lod −4·24 at θ = 0). Therefore, tight linkage has been excluded at the COL1A1, COL1A2, and COL5A2 loci in this family. In the COL3A1 analysis, two affected individuals were homozygous for the 1 (III.9) or 2 (II.3) allele. A lod score of −1·9 at θ = 0 for this analysis strongly suggests exclusion at this locus. Although this does not quite achieve the score that is accepted as formal proof of exclusion (lod −2·0), the trait is clearly not cosegregating with the COL3A1 locus.

No analysis at the COL3A1 locus was possible in family G, as the individuals in generation II were all homozygous for both restriction fragment length polymorphisms and consequently were not infor-mative. Linkage to the COL5A2 locus was excluded on the basis that the three affected heterozygous sisters (III.1, III.2, and III.4) inherited the 1 allele from their affected mother, but the presence of the homozygous 2–2 affected cousin (III.11) rules out the involvement of this allele. The 2 allele is excluded because the three affected daughters of the proband (III.1, III.2, and III.4) inherited the 1 allele from their mother and not the 2 allele (lod score −1·09 at θ = 0). We also showed discordance with the COL1A1 locus. The homozygous affected individuals III.1 and III.2 inherited the 1 allele from their affected mother, ruling out linkage to the 2 allele; but the affected individual III.4 inherited the 2 allele from her mother and not the 1, excluding linkage with this allele as well (lod −0·74 at θ = 0). For COL1A2, the mitral valve prolapse locus could not be linked to the 1 allele because there are affected individuals homozygous for the 2 allele (III.2 and III.4). Exclusion of the 2 allele is based on the unaffected individual III.5 who is homozygous 2–2, as are her affected sisters III.2 and III.4 (lod −0·62 at θ = 0). It has been shown that the mitral valve prolapse gene is not completely penetrant and its expression varies with age and sex,[6] so this exclusion cannot be certain. Although these data do not provide any evidence to support linkage of the phenotype with any of these loci, the lod scores in family G do not reach the value (−2·0) that is required for formal statistical proof of exclusion.

Discussion

Mitral valve prolapse is a common feature of various inherited connective tissue disorders in which collagen defects have been described.[3] This associa-tion taken together with histological evidence of an abnormal collagenous matrix[5 7] supports the view that abnormal collagen could be involved in the aetiology of mitral valve prolapse. Data from various studies on isolated valves that show a reduction or absence of type III collagen in the tissue,[27] abnor-mally high rates of collagen synthesis,[28] and increased

collagen production[29] are also consistent with this hypothesis. Additionally, in the family of a proband with clinically diagnosed Ehlers-Danlos type IV syndrome, mitral valve prolapse was clearly associated with abnormal synthesis of type III collagen.[30] Thus there is evidence favouring the involvement of a collagen defect, perhaps of type III collagen specifically, in the pathogenesis of mitral valve prolapse.

A rapid method for testing the hypothesis that mitral valve prolapse arises from a heritable collagen gene defect is available through the use of collagen gene polymorphisms and segregation analysis in affected families. Because the markers are so closely associated with the genes in this particular case they are ideal for use in segregation analysis because the probability of a crossover occurring between marker and gene in any given meiosis is extremely small. Thus any examples of discordance between the candidate gene and the abnormal phenotype effectively exclude that gene as the mutant locus.

In sequential studies of 45 and 48 patients with mitral valve prolapse who had 179 and 171 first degree relatives respectively it was shown that mitral valve prolapse was transmitted in an autosomal dominant fashion, that its expression was dependant on age and sex, and that the gene showed incomplete penetrance.[6 15] These points complicate the analysis of genetic linkage. For example, exclusions based on unaffected individuals in a particular kindred can never be certain, even if age based probability curves are available. This problem arose in the exclusion of one of the *COL1A2* alleles in family G and also in the exclusion of one of the *COL1A1* alleles in family H. The woman (III.5) in family G was assessed as normal when she was first examined. But four years later there was slight echocardiographic evidence of prolapse, a late systolic murmur, and possibly a midsystolic click. Her electrocardiogram was normal and she had no symptoms. Because she is younger (27) than her two homozygous affected sisters (40 and 30) prolapse could develop later. But, as her exact clinical state was uncertain and there was no clear indication of mitral valve prolapse, she was regarded as normal. In the case of family H, the *COL1A1* 1 allele was excluded because two homozygous siblings (III.4 and III.6) were unaffected and one of them was three years older than her affected sister. It may be that mitral valve prolapse will develop later in these individuals, so exclusion cannot be certain. However, the presence of an affected heterozygous cousin III.2 who has inherited the 1 allele from her unaffected mother adds weight to the argument because, with an estimated penetrance of 90% for females,[6 15] at the age of 55 the mother should have shown signs of prolapse if it were going to develop. These con-

clusions are supported by the negative lod scores calculated for these loci (table 2), although in neither case do the genes achieve the $-2 \cdot 0$ score that is taken as formal proof of exclusion. Because this is a segregation analysis of candidate genes rather than random markers, a single recombinant or negative lod score at zero recombination distance is sufficient to exclude the candidate locus.

Phenotypic heterogeneity has been established within the mitral valve prolapse syndrome, and two separate patterns of prolapse could be defined by quantitative cross sectional echocardiography.[8 9] One of these patterns of prolapse was associated with a distinct habitus that included low body weight and bony abnormalities of the thorax, which could reflect an underlying mild defect in systemic connective tissue. Although mitral valve prolapse was segregating as an autosomal dominant trait in both families in this study, the two were clearly phenotypically different. Family H showed various abnormal systemic features whereas G did not, indicating the possibility that each family reflected a separate subtype. These two families were assessed at different centres and, although this did not affect the diagnosis of mitral valve prolapse by standardised echocardiographic methods and a single experienced investigator reviewing the studies, it was not possible to apply the quantitative echocardiographic techniques used by Pini *et al*[8 9] to family H to verify whether or not these two families did reflect heterogeneity of mitral valve prolapse.

The manifestations seen in family H were consistent with features observed both in Ehlers-Danlos syndrome (varicose veins, hyperextensible skin, and joint hypermobility) and in the Marfan syndrome (abnormally long arms and pectus deformity). Biochemical analysis of the resected valve from the proband showed abnormal type III/III + I collagen ratios, but it was probable that this was due to the development of secondary fibrosis on the valve, rather than an inherent deficiency in the expression of one of these collagen types.[31] The segregation analysis confirms the fact that the trait is not linked to the *COL1A1*, *COL1A2*, *COL3A1*, or *COL5A2* loci. That is to say mitral valve prolapse in this family was not caused by a structural defect in any of these collagen genes. In contrast, family G seemed not to have any signs of a generalised connective tissue deficiency, but did have systolic billowing of the mitral leaflets in a qualitative evaluation of cross sectional echocardiograms. This pattern of prolapse coupled with the absence of any systemic signs (table 1) suggests that this family may represent "type I" mitral valve prolapse as defined by Pini *et al*.[8 9] No biochemical data were available for this family, but again there was no evidence to support linkage of

mitral valve prolapse to three of the four collagen loci studied. It is possible that the trait could be caused by a defect in the *COL3A1* gene, because the family was not informative for either of the polymorphisms at this locus, and it will be restudied as and when new *COL3A1* gene polymorphisms are found.

It has been suggested that a collagen abnormality is responsible for the Marfan syndrome.[32] Studies on Marfan syndrome pedigrees, however, also failed to establish an association for this disorder with the major fibrillar collagen genes,[33 34] and mitral valve prolapse is frequently seen in patients with this disorder. It was suggested that other genes coding for extracellular matrix components are possible candidates for the Marfan phenotype,[33] and this could be equally likely for mitral valve prolapse. An alternative hypothesis is that mitral valve prolapse is a polygenic disorder, where the phenotype arises from the interaction of more than one gene, and the individual genes responsible cannot be identified because alone they do not cause major changes in the phenotype.[35] It is also feasible that an error in the enzymes responsible for the complex post-translational processing of collagen could be responsible for the formation of a valve with a weakened extracellular matrix, leading eventually to mitral valve prolapse. But this seems to be unlikely because enzyme deficiencies are generally recessive traits, although a few are dominantly inherited.

Therefore, despite the biochemical and clinical evidence implicating collagen, it seems that mitral valve prolapse in these families does not arise from a defect in the structural collagen genes that we studied. The remaining type V collagen genes for which probes are not yet available remain potential candidates, as do the genes encoding other components of the connective tissue matrix such as elastin and proteoglycans.

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