Supporting Information Tables and Figures

Variant filtering, digenic variants, and other challenges in clinical sequencing: a lesson from fibrillinopathies

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Supporting Information Table S1 Overview and descriptions of *in silico* tools and databases used in this study.

Tools	Description	Range / Classification	Reference
Ada / Random forest (dbscSNV)*†	Predicts effect of single nucleotide variants (SNVs) on splice sites, combining scores such as the Position Weight Matrix model and MaxEntScan, improving them by applying adaptive boosting and random forest learning methods, respectively.	[0 – 1] (applied cut-off in this study: >0.6) High values indicate higher probability of mis-splicing.	1
CADD v1.3 PHRED score	Combined Annotation Dependent Depletion (CADD) is a tool for scoring the deleteriousness of SNVs as well as insertion/deletion variants in the human genome based on the combination of 63 prediction algorithms.	[0 – 99] High values indicate higher probability of deleteriousness.	2
FATHMM*‡	The Functional Analysis Through Hidden Markov Models (FATHMM) predicts the functional effects of missense mutations by combining sequence conservation within hidden Markov models, representing the alignment of homologous sequences and conserved protein domains, with "pathogenicity weights", representing the overall tolerance of the protein/domain to mutations.	[-18.09 – 11.0] ≤-1.5: "Damaging", >-1.5: "Tolerated"	3
FATHMM MKL*‡	The successor of FATHMM, FATHMM-MKL uses the same model but also a machine-learning approach.	[0 – 1] >0.5: "Damaging", ≤0.5: "Neutral"	4
MutationAssessor*‡	Predicts the functional impact of amino acid substitutions in proteins, such as mutations discovered in cancer or missense polymorphisms. The functional impact is assessed based on evolutionary conservation of the affected amino acid in protein homologs.	[-5.545 – 5.975] >3.5: "High" (deleterious), 1.9 - 3.5: "Medium" (likely deleterious), 0.8 - 1.9: "Low" (likely benign), <0.8: "Neutral" (benign)	5
MutationTaster*‡	Evaluates disease-causing potential of sequence alterations trained with known polymorphisms and known disease-causing sequence variants.	[0 – 1] ≥0.31709: "Damaging", <0.31709: "Tolerated"	6
PhastCons100*	PhastCons is a program for identifying evolutionarily conserved elements in a multiple alignment, given a phylogenetic tree. It is based on a statistical model of sequence evolution called a phylogenetic hidden Markov model.	[0 – 1] Higher values indicate higher phylogenetic conservation.	7
PhyloP100*	Computes conservation or acceleration p-values based on an alignment and a model of neutral evolution.	[-10 – 10] Higher values indicate higher phylogenetic conservation.	8
PolyPhen2 HVAR*‡	Predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.	[0 – 1] ≥0.5: "Deleterious", <0.5: "Neutral"	9
SIFT*‡	The Sorting Intolerant From Tolerant (SIFT) algorithm predicts whether an amino acid substitution affects protein function. SIFT prediction is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences.	[0 – 1] <0.05: "Damaging", ≥0.05: "Tolerated"	10
SiPhy29*	Computes values according to the conservation pattern of genomic bases.	[0 – 25] Higher values indicate higher phylogenetic conservation.	11
Databases			
ClinVar 2019.5	ClinVar is a freely accessible, public archive for the clinical interpretation of human sequence variants.	Benign, likely benign, uncertain significance, pathogenic, drug response, association, risk factor, protective, affects, conflicting data from submitters, other, not provided.	12
HGMD professional 2019.1	The commercial Human Gene Mutation Database professional (HGMD Pro) constitutes a comprehensive core collection of data on germline sequence variants in nuclear genes underlying or associated with human inherited disease. The less up-to-date public version of HGMD is freely available for academic institutions/non-profit organizations.	Disease-causing (DM), likely disease-causing (DM?), disease-associated polymorphism (DP), *in vitro* or *in vivo* functional polymorphism (FP), disease-associated polymorphism with additional functional evidence (DFP), frameshift or truncating variant (FTV), retired record (R)	13
OMIM 05.2018	The Online Mendelian Inheritance in Man (OMIM) is a freely available, comprehensive, authorative compendium of human genes and genetic phenotypes. The full-text, referenced overviews in OMIM contain information on all known mendelian disorders and over 15,000 genes. OMIM focuses on the relationship between phenotype and genotype.	Autosomal, X-linked, digenic dominant, Multifactorial, susceptibility to, isolated cases.	Omim.org

* Available from dbNSFP v2.9 & 3.0[14]. † Used for filtering of splicing mutations (category II). ‡ Used for the assessment of missense variants for the *sensu lato* selection of sequence variants (category VI).

Supporting Information Table S2 Comparison of carrier frequencies of selected sequence variants among ExAC, gnomAD, and reported in the literature.

		ExAC		gnomAD		Literature		Reference
Gene	**Sequence variant**	**Carrier frequency**	**95% CI**	**Carrier frequency**	**95% CI**	**Carrier frequency**	**95% CI**	
BRCA1 (NM_007294.3) / *BRCA2* (NM_000059.3)	c.68_69del p.(Glu23Valfs*17) c.5266dupC p.(Gln1756Profs*74) / c.5946delT p.(Ser1982Argfs*22)	1/756 (80/60,514)*	0.001 - 0.0016	1/805 (172/138,502)*	0.001 - 0.0014	~1/400 - 1/800	n/a	15
CFTR (NM_000492.3)	c.350G>A p.(Arg117His)	1/325 (185/60,180)*	0.0027 - 0.0036	1/349 (396/138,335)*	0.0026 - 0.0032	1/422 (864/364,890)	0.0022 - 0.0026	16
	c.1521_1523del p.(Phe508del)	1/74 (823/60,648)*	0.0127 - 0.0146	1/71 (1945/138,491)*	0.0134 - 0.0146	1/65 (5608/364,890)	0.015 - 0.0158	
	c.3209G>A p.(Arg1070Gln)	1/646 (93/60,049)*	0.0012 - 0.0019	1/836 (147/122,859)*	0.001 - 0.0014	n/a	n/a	
GJB2 (NM_004004.5)	c.35delG p.(Gly12Valfs*2)	1/57 (583/33,343)†	0.0161 - 0.019	1/52 (1,199/62,276)†	0.0182 - 0.0204	1/46 (517/23,602)	0.0201 - 0.0239	17
	c.109G>A p.(Val37Ile)	1/23 (552/12,556)‡	0.0405 - 0.0478	1/18 (1,384/24,809)‡	0.053 - 0.0587	1/17 (446/7,710)	0.0527 - 0.0633	
HBB (NM_000518.4)	c.19G>A p.(Glu7Lys) c.20A>T p.(Glu7Val)	1/8 (648/5,202)§	0.1158 - 0.134	1/9 (1396/12,015)§	0.1106 - 0.1221	~10%	n/a	18

ExAC/gnomAD subpopulations were applied according to Song et al. (2016)[15]:
* All ExAC/gnomAD subpopulations.
† European (Non-Finnish) ExAC/gnomAD subpopulation.
‡ South Asian and East Asian ExAC/gnomAD subpopulation.
§ African ExAC/gnomAD subpopulation.
95% CI, 95% confidence interval; ExAC, Exome Aggregation Consortium; gnomAD, Genome Aggregation Database; n/a, information not available.

Supporting Information Table S3 Prevalence estimates and gnomAD-wide pathogenic variants per gene.
See separate multi-sheet Excel table.
Note that for prevalence calculations, the number of observed sequence variants in categories I and II in each gene were divided by the average number of individuals (observed number of alleles divided by two).

Supporting Information Table S4 Manual evaluation of genes with a gnomAD-based prevalence >1:2,000.

Gene (Highest Expressed Protein Coding Isoforms)*	Associated Disease(s)†	Gene Expression in Humans (Analyzed Lines)‡	gnomAD-based Prevalence in 10,000 Individuals	Excluded Variants	Is Loss of Function an established Disease Mechanism (References)
ATXN7 (NM_001177387/ENST00000538065, **NM_000333**/ENST00000295900)	Spinocerebellar ataxia 7	Inferred: biallelic (GM12878, K562, H1ESC, HSMM, HUVEC, HMEC, HCC1954, PBMC); Measured: biallelic (GM12878, B Cell)	10.59	None	Unclear; spinocerebellar ataxia 7 is caused by a heterozygous expanded trinucleotide (CAG) repeat, i.e. loss of function is not (yet) established as a disease mechanism (19)
NOTCH1 (**NM_017617**/ENST00000277541)	Adams-Oliver syndrome 5; Aortic valve disease 1	Inferred: biallelic (GM12878, K562, H1ESC, HSMM, HUVEC, HMEC, HCC1954, PBMC); Measured: biallelic (GM12878)	6.94	None	Yes (e.g. 20-23)
FLNC (NM_001127487/ENST00000346177, **NM_001458**/ENST00000325888)	Cardiomyopathy familial hypertrophic 26; Cardiomyopathy familial restrictive 5; Myopathy distal 4; Myopathy myofibrillar 5	Inferred: biallelic (K562, HSMM, HUVEC), monoallelic (HMEC); Measured: n/a	5.88 (6.01 before exclusion)	2 (frameshift)§	Yes (e.g. 24-27; ncbi.nlm.nih.gov/clinvar/?term=flnc[gene])
RYR2 (ENST00000542537, **NM_001035**/ENST00000366574,)	Arrhythmogenic right ventricular dysplasia 2; Ventricular tachycardia catecholaminergic polymorphic 1	Inferred: biallelic (H1ESC, HSMM, HUVEC); Measured: n/a	5.48	None	Yes (28-30)
MYH14 (NM_001077186/ENST00000425460, NM_024729/ENST00000376970, **NM_001145809**/ENST00000601313)	Peripheral neuropathy, myopathy, hoarseness, and hearing loss; Deafness autosomal dominant 4A	Inferred: biallelic (H1ESC), monoallelic (HCC1954); Measured: n/a	5.27	None	Yes (31,32; ncbi.nlm.nih.gov/clinvar/?term=myh14[gene])
CACNA1G (**NM_18896**/ENST00000359106, NM_NM_198387/ENST00000352832)	Spinocerebellar ataxia 42; Spinocerebellar ataxia 42, early-onset, severe, with neurodevelopmental deficits	n/a	5.09 (5.40 before exclusion)	1 (frameshift)§ 2 (splicing)§	Yes (33)
RERE (**NM_012102**/ENST00000337907, ENST00000377464)	Neurodevelopmental disorder with or without anomalies of the brain, eye, or heart	Inferred: biallelic (GM12878, K562, H1ESC, HSMM, HUVEC, HMEC, HCC1954, PBMC); Measured: biallelic (B Cell)	5.04	None	Yes (34; ncbi.nlm.nih.gov/clinvar/?term=rere[gene])

* According to GTEx (gtexportal.org); Bold, Isoform with the most entries in ClinVar.
† According to Online Mendelian Inheritance in Man (omim.org).
‡ According to the Database of Autosomal Monoallelic Expression (mae.hms.harvard.edu).
§ The variants were excluded because they are exclusively present in weakly expressed exons (median read count per base <10% of the highest expressed exon in tissues likely relevant to the disease) according to GTEx (gtexportal.org).
B Cell, summary data from multiple b cell clones; GM12878, lymphoblast cell line; HCC1954, breast cancer cell line; HMEC, mammary epithelium cell line; HSMM, skeletal muscle myocytes cell line; HUVEC, vascular epithelium cell line; H1ESC, embryonic stem cell line; K562, Acute myelocytic leukemia cell line; n/a, information not available; PBMC, peripheral blood monocytes.

Supporting Information Table S5 Examples of gnomAD sequence variants, which may be misinterpreted.

		gnomAD Genomes			gnomAD Exomes			OMIM	
Gene (Isoform)	**Sequence Variant**	**Alt. Alleles**	**No. Alleles**	**Hom.**	**Alt. Alleles**	**No. Alleles**	**Hom.**	**Disorder OMIM ID***	**Inheritance**
Sequence variants with the less frequent allele as reference allele in the genome build GRCh37/hg19									
KMT2B (NM_014727)†	c.3059dupG p.(Arg1021Profs)	30,912	30,912	15,456	172,850	172,852	86,424	617284	AD
SON (NM_138927)‡	c.7236dupA p.(Ala2413Serfs)	29,718	29,718	14,859	184,134	184,218	82,045	617140	AD
SON (NM_138927)‡	c.7248delA p.(Arg2416Serfs)	29,712	29,712	14,856	189,077	189,172	94,510	617140	AD
Sequence variants miscalled as two independent variants instead of a small insertion/deletion									
KAT6A (NM_006766)	c.1138G>T p.(Glu380*)	5	30,984	0	68	246,004	0	616268	AD
	c.1139A>C p.(Glu380Ala)	5	30,984	0	68	246,076	0		
SON (NM_032195)	c.5301_5302del p.(Ala1768Glnfs)	13	30,980	0	11	245,748	0	617140	AD
	c.5304_5316del p.(Ser1769Valfs)	13	30,976	0	10	245,734	0		
SON (NM_032195)	c.5910_5911del p.(Ser1971Profs)	5	27,886	0	n/a	n/a	n/a	617140	AD
	c.5913_5931del p.(Ser1971Argfs)	5	28,048	0	n/a	n/a	n/a		
TBX1 (NM_080647)	c.1122_1123insA p.(Gly375Argfs)	3	29,924	0	n/a	n/a	n/a	188400	AD
	c.1125_1126insCCGGCGGC p.(Ala376Profs)	3	29,910	0	n/a	n/a	n/a		
TSC2 (NM_000548)	c.3846_3847del p.(Ser1282Argfs)	21	30,948	0	14	245,388	0	607341	AD
	c.3850_3856del p.(Gln1284Serfs)	21	30,944	0	15	245,336	0		
Sequence variants exclusively located in weakly expressed exons§									
CACNA1G (NM_018896)	c.2911-1G>A	n/a	n/a	n/a	1	156,312	0	616795	AD
CACNA1G (NM_018896)	c.4759+1G>A	n/a	n/a	n/a	1	243,456	0	616795	AD
CACNA1G (NM_018896)	c.5868dupA p.(Gly1957Argfs)	n/a	n/a	n/a	1	235,394	0	616795	AD
CACNA1G (NM_018896)	c.5925+1G>A	3	30,942	0	49	231,928	0	616795	AD
CACNA1G (NM_018896)	c.6004del p.(Asp2002Metfs)	n/a	n/a	n/a	34	246,228	0	616795	AD
ELOVL5 (NM_001242828)	c.286C>T p.(Arg96*)	4	30,990	0	8	123,488	0	615957	AD
FLNC (NM_001458)	c.5212dupC p.(Leu1738Profs)	n/a	n/a	n/a	1	204,110	0	617047	AD
FLNC (NM_001458)	c.5277delC p.(Gly1760Alafs)	n/a	n/a	n/a	2	141,116	0	617047	AD

* Selected disorder.
† For this genomic position, the reference allele has been updated in the genome build GRCh38/hg38.
‡This pair of indels has been described.[35]
§ Exons with median read count per base <10% of the highest expressed exon in tissues likely relevant to the disease according to GTEx (gtexportal.org).
AD, autosomal dominant; Alt. Alleles, number of gnomAD non-reference alleles; gnomAD, Genome Aggregation Database; Hom., number of homozygotes in gnomAD; No. Alleles, total number of observed alleles; n/a, information not available; OMIM, Online Mendelian Inheritance in Man.

Supporting Information Table S6 All likely pathogenic *FBN1* and *FBN2* sequence variants in gnomAD including annotations.
See separate multi-sheet Excel table.

Supporting Information Table S7 Overview of overlapping and distinguishing features of Marfan syndrome (MFS) and congenital contractural arachnodactyly (CCA) (adapted from Godfrey 2012; Dietz 2017).[36-37]

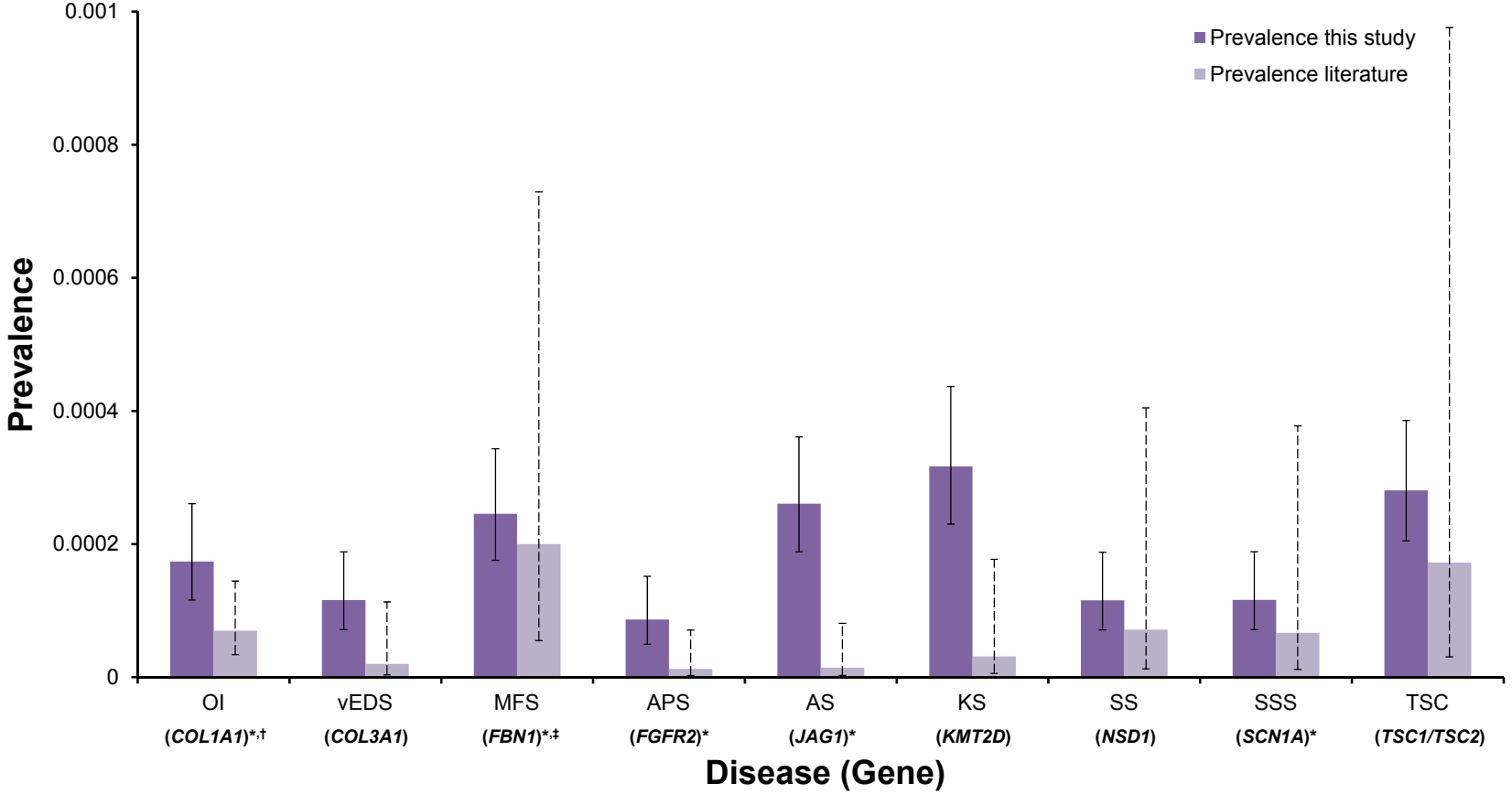
Features	MFS	CCA
Genetics		
Gene	*FBN1*	*FBN2*
Skeletal		
Marfanoid habitus	+	+
Arachnodactyly	+	+
Kyphoscoliosis	+	+
Pectus excavatum/carinatum	+	+
Joint laxity	+	–
Joint contractures	–	+
Pes planus	+	(+)
Crumpled ears	–	+
Ocular		
Ectopia lentis	+	–
High myopia	+	–
Cardiovascular		
Aneurysms	+	(+)
Dissections	+	–
Mitral valve involvement	+*	+†

* Patients with MFS tend to have mitral valve prolapse, which can lead to mitral valve regurgitation.
† Patients with CCA tend to have mitral valve regurgitation.
+, present; (+), rarely present; –, missing.

Supporting Information Table S8 *FBN1*/*FBN2* dual variants in two families.

Variant Info			Phylogenetic Conservation	Missense Predictions	CADD	ClinVar 2019.5	HGMD Pro 2019.1	gnomAD	ACMG	GTEx††
Chr:Position (hg19)	**Gene (pLi)**	**HGVS c. HGVS p.**	**PhastCons/PhyloP/SiPhy**	**N of 6 Predicted Damaging***	**PHRED Score**	**Disorder (Clinical Significance)**	**Phenotype (Class)**	**AC/AN/HOM**	**Classification using InterVar v.201904/hg19**	**Aorta / Fibroblasts (TPM)**
Family 1										
15:48703314	*FBN1* (pLi: 1) Exon 65	c.8489A>G p.(Gln2830Arg)	1/7.8/14.9	3/6†	22	n/a	n/a	0/~267000/0	Uncertain Significance (PM2, [PP1], PP3)	62.5 / 288
5:127670562	*FBN2* (pLi: 1) Intron 30	c.3974-26T>G p.(Asn1327_Val1368del)	n/a	n/a	13.2	Congenital Contractural Arachnodactyly (Pathogenic)	Contractural Arachnodactyly§ (DM)	0/~250000/0	Likely Pathogenic (PS3, PM2, [PP1], PP3, PP5)	0 / 135.7
Family 2										
15:48726812	*FBN1* (pLi: 1) Exon 53	c.6595G>A p.(Gly2199Ser)	1/7.8/20.3	5/6‡	35	n/a	Aortic Dissection Stanford Type A¶ (DM?)	3/246006/0	Uncertain Significance (PM1, [PP1], PP3)	62.5 / 287.5
5:127673806	*FBN2* (pLi: 1) Exon 27	c.3481G>A p.(Glu1161Lys)	1/7.6/18.6	6/6	35	Congenital Contractural Arachnodactyly (Uncertain)	Contractural Arachnodactyly** (DM)	0/~280000/0	Likely Pathogenic (PM1, PM2, [PP1], PP3, PP5)	0 / 135.7

* Number of classifications as "deleterious", “disease-causing” or "damaging" by the six used *in silico* missense prediction tools FATHMM, FATHMM-MKL, MutationTaster, MutationAssessor, PolyPhen2, SIFT (*cf*. Supporting Information Table S1).
† Predicted as non-deleterious by MutationAssessor, PolyPhen2, and SIFT.
‡ Predicted as non-deleterious by MutationAssessor.
§ Described by Maslen et al. (1997).[38]
¶ Described by Tan et al. (2017).[39]
** Described by Callewaert et al. (2009).[40]
†† Gene expression according to GTEx (gtexportal.org).
AC, Allele count in gnomAD; ACMG, American College of Medical Genetics; AN, Observed allele number in gnomAD; CADD, Combined Annotation Dependent Depletion; Chr, Chromosome; DM, Disease-causing mutation; DM?, Likely disease-causing mutation; GTEx, Genotype-Tissue Expression Project; gnomAD, Genome Aggregation Database; HGMD Pro, Human Gene Mutation Database Professional; HGVS, Human Genome Variation Society; HOM, Number of homozygotes in gnomAD; n/a, Information not available; TPM, Transcripts per million.



Supporting Information Figure S1 Comparison of gnomAD-based predisposition/prevalence estimates and prevalence rates reported in the literature (according to GeneReviews)[41] for selected autosomal-dominant disorders.

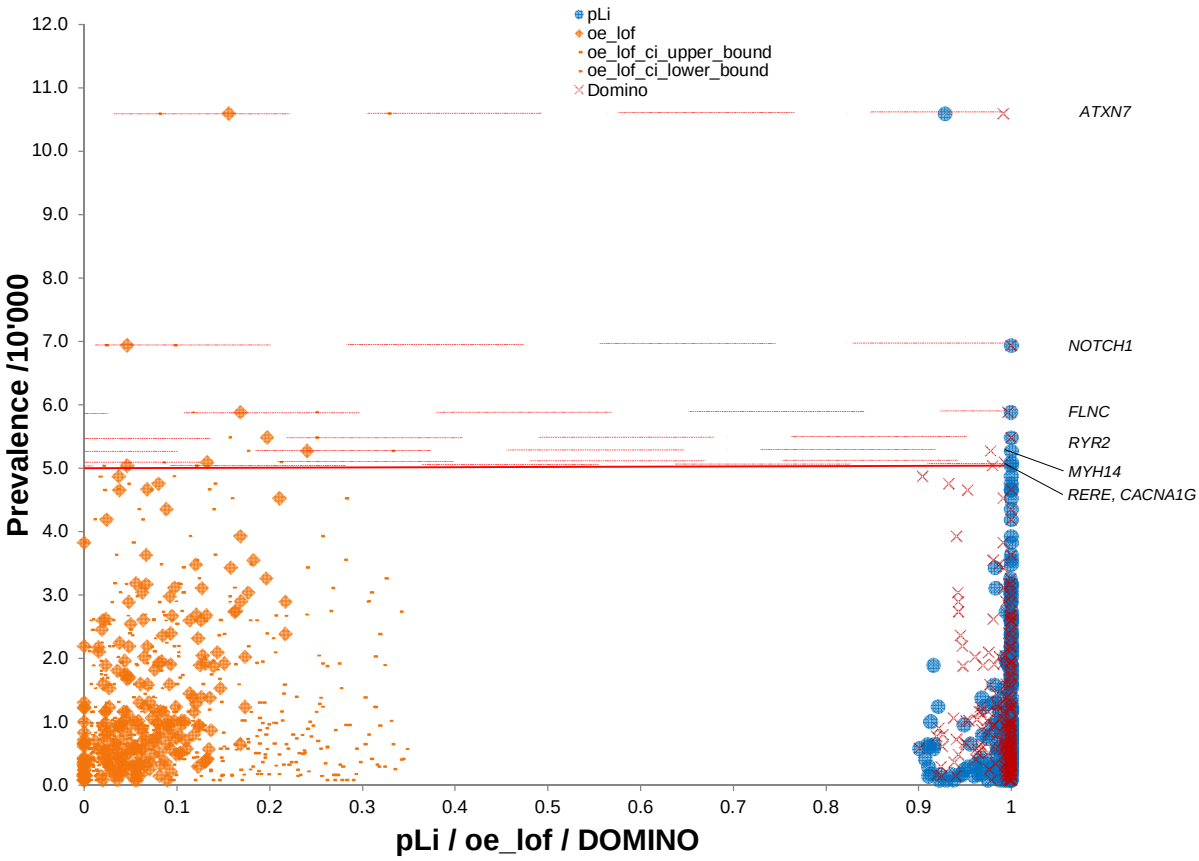
* Gene is the cause of more than one disease; however, the depicted prevalence from the literature is based only on the indicated disease, thereby likely underestimating the prevalence of all disorders caused by the corresponding gene.

† OI is caused by mutations in *COL1A1* and *COL1A2*, however, gnomAD variants in *COL1A2* did not pass our filters for categories I-II and are therefore not considered in the gnomAD-based estimate of OI.

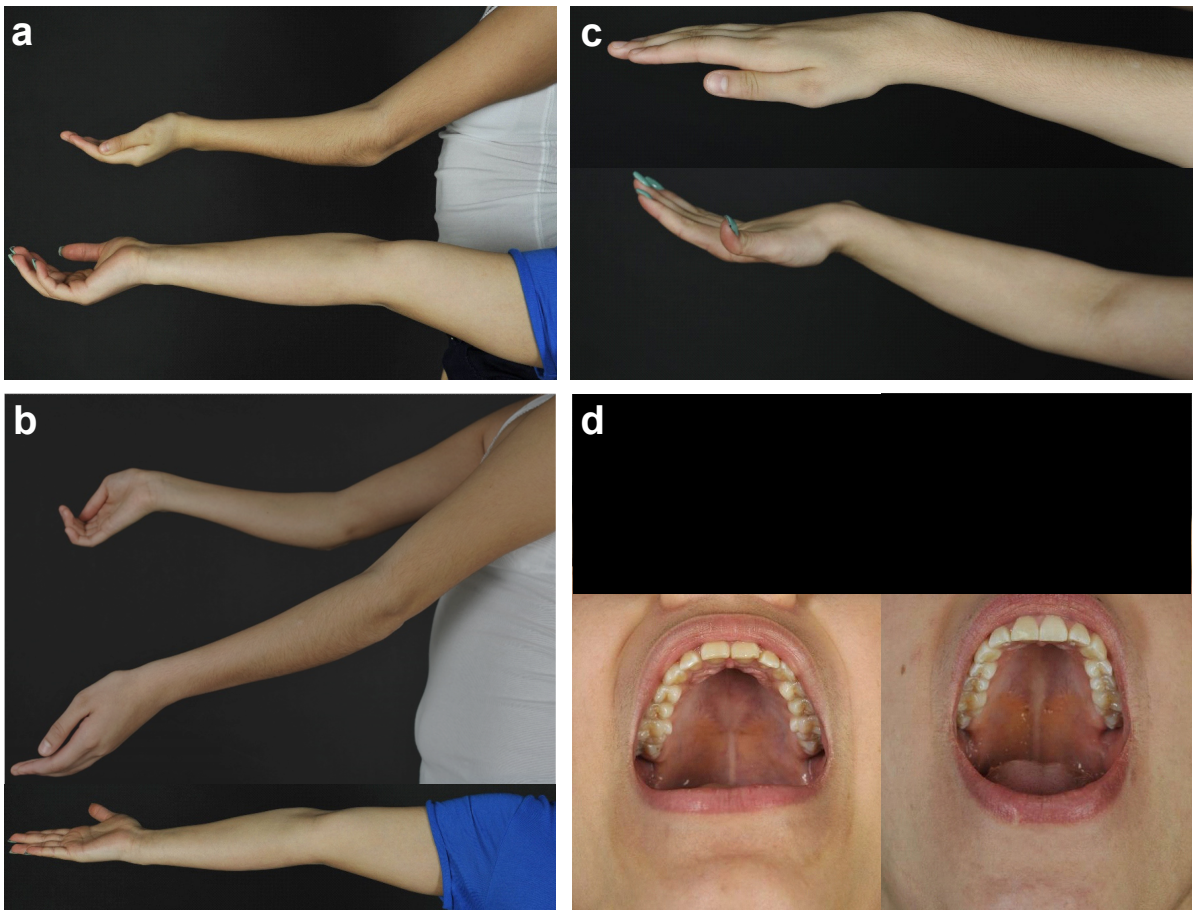
‡ For *FBN1*, the shown gnomAD-based prevalence estimate is based on categories I-II (Supporting Information Table S3).

Full error bars indicate 95% confidence intervals of relative allele frequencies in gnomAD, while dashed error bars indicate estimated 95% confidence intervals of reported relative frequencies (*cf.* actual sample sizes are unknown).

APS, Apert syndrome (and *FGFR*-related craniosynostosis syndromes); AS, Alagille syndrome; KS, Kabuki syndrome; MFS, Marfan syndrome; OI, Osteogenesis imperfecta; SS, Sotos syndrome; SSS, *SCN1A* seizure disorders; TSC, Tuberus sclerosis complex; vEDS, vascular Ehlers-Danlos syndrome.



Supporting Information Figure S2 Comparison of pLi, oe_lof, and DOMINO values with the prevalence of (likely) pathogenic variants for 253 genes considered in our gnomAD-wide analysis (*cf.* Supporting Information Table S3). The genes indicated above the red line (5:10,000) were analyzed manually to assess whether or not they are intolerant to loss of function (*cf*. Supporting Information Table S4). ci, 90% confidence interval; lof, loss of function; oe, observed/expected metric.



Supporting Information Figure S3 Selected clinical features in Family 1.
Elbow (a, b) and wrist/finger (c) mobility of Ab1 with *FBN1* mutation (bottom) and Ab2 with *FBN1*/*FBN2* dual mutations (top), (d) narrow-arched palate of Ab1 (right) and high-arched palate of Ab2 (left).

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