OPEN ACCESS

Original research

Recurrent, founder and hypomorphic variants contribute to the genetic landscape of Joubert syndrome

Valentina Serpieri ,[1] Giulia Mortarini,[2] Hailey Loucks,[3] Tommaso Biagini,[4] Alessia Micalizzi,[5] Ilaria Palmieri,[1] Jennifer C Dempsey,[3] Fulvio D'Abrusco,[2] Concetta Mazzotta,[2] Roberta Battini,[6,7] Enrico Silvio Bertini ,[8] Eugen Boltshauser,[9] Renato Borgatti,[10,11] Knut Brockmann,[12] Stefano D'Arrigo ,[13] Nardo Nardocci,[13] Rita Fischetto,[14] Emanuele Agolini ,[5] Antonio Novelli,[5] Alfonso Romano,[15] Romina Romaniello ,[11] Franco Stanzial,[16] Sabrina Signorini,[11] Pietro Strisciuglio,[15] Simone Gana,[1] Tommaso Mazza ,[4] Dan Doherty,[3] Enza Maria Valente [1,2]

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jmg-2022-108725).

For numbered affiliations see end of article.

Correspondence to
Professor Enza Maria Valente, Department of Molecular Medicine, University of Pavia, Pavia 27100, Italy; enzamaria.valente@unipv.it

Received 24 May 2022
Accepted 8 January 2023

To cite: Serpieri V, Mortarini G, Loucks H, *et al*. *J Med Genet* Epub ahead of print: [please include Day Month Year]. doi:10.1136/jmedgenet-2022-108725

ABSTRACT

Background Joubert syndrome (JS) is a neurodevelopmental ciliopathy characterised by a distinctive mid-hindbrain malformation, the 'molar tooth sign'. Over 40 JS-associated genes are known, accounting for two-thirds of cases.

Methods While most variants are novel or extremely rare, we report on 11 recurring variants in seven genes, including three known 'founder variants' in the Ashkenazi Jewish, Hutterite and Finnish populations. We evaluated variant frequencies in ~550 European patients with JS and compared them with controls (>15 000 Italian plus gnomAD), and with an independent cohort of ~600 JS probands from the USA.

Results All variants were markedly enriched in the European JS cohort compared with controls. When comparing allele frequencies in the two JS cohorts, the Ashkenazim founder variant (*TMEM216* c.218G>T) was significantly enriched in American compared with European patients with JS, while *MKS1* c.1476T>G was about 10 times more frequent among European JS. Frequencies of other variants were comparable in the two cohorts. Genotyping of several markers identified four novel European founder haplotypes.
Two recurrent variants (*MKS1* c.1476T>G and *KIAA0586* c.428delG), have been detected in homozygosity in unaffected individuals, suggesting they could act as hypomorphic variants. However, while fibroblasts from a *MKS1* c.1476T>G healthy homozygote showed impaired ability to form primary cilia and mildly reduced ciliary length, ciliary parameters were normal in cells from a *KIAA0586* c.428delG healthy homozygote.

Conclusion This study contributes to understand the complex genetic landscape of JS, explain its variable prevalence in distinct geographical areas and characterise two recurrent hypomorphic variants.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Joubert syndrome (JS) is a mainly recessive neurodevelopmental ciliopathy caused by pathogenic variants in over 40 JS-associated genes.
⇒ While most variants are novel or extremely rare, some are recurring in several patients as well as healthy individuals, suggesting a founder effect.

WHAT THIS STUDY ADDS

⇒ We report on 11 recurring variants in seven JS genes, compare their frequencies in large JS and control cohorts and identify four novel founder haplotypes.
⇒ We functionally characterise in vitro the impact of two variants found in homozygosity in healthy individuals (*MKS1* c.1476T>G and *KIAA0586* c.428delG), demonstrating their hypomorphic effect.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study furthers knowledge on the complex genetic landscape of JS, explains its variable prevalence in distinct geographical areas and characterises two recurrent hypomorphic variants.

INTRODUCTION

Joubert syndrome (JS) is a rare, mostly recessively inherited neurodevelopmental ciliopathy whose diagnostic hallmark is a unique mid-hindbrain malformation (the 'molar tooth sign'). Typical features are cerebellar vermis hypo-dysplasia, elongated, thick and horizontalised superior cerebellar peduncles, and a deeper interpeduncular fossa.[1] JS phenotypical spectrum includes neonatal hypotonia, ataxia, abnormal ocular movements (mainly congenital ocular-motor apraxia and nystagmus), developmental delay and intellectual disability. This neurological presentation can occur either in isolation or combined with involvement of other organs, such as the retina, kidneys, liver and skeleton.[2 3] Its allelic condition, Meckel syndrome (MKS), is a lethal ciliopathy characterised by cystic dysplastic kidneys, encephalocele, congenital liver fibrosis and polydactyly.[4]

JS is genetically heterogeneous: over 40 causative genes are known, accounting for 65%–75% cases. While most pathogenetic variants are extremely rare or unique, some variants were found to recur

in certain populations due to founder effects, leading to a higher disease prevalence. Among Ashkenazi Jews, the prevalence of JS has been estimated between 1:34 000 and 1:40 000 (compared for instance to the Italian prevalence of ~1:200 000).[5 6] This enrichment has been ascribed to the founder variant c.218G>T in the *TMEM216* gene, whose carrier frequency reaches 0.5% among Ashkenazim.[6 7] Likewise, the founder variant c.52C>T in the *TMEM237* gene was identified in the Hutterite population with a carrier frequency up to 6%,[8 9] while the c.1408–34_1408-6del variant in the *MKS1* gene was commonly detected in Finnish patients with either JS or MKS.[10 11] Finally, the founder variant c.1575+1G>A in *TMEM67* was found on a shared haplotype in three MKS Mirpuri families from Pakistan.[10] Besides these, several other variants in JS-associated genes have been reported to recur in JS or MKS families, yet a founder effect has only been suggested for some of them.[2 12–24]

Here, we report on 11 recurrent variants in seven JS genes, attempt to identify founder haplotypes and perform a functional characterisation of two variants found in homozygosity in healthy individuals, to explore a potential hypomorphic effect.

PATIENTS AND METHODS

Identification of recurrent variants in JS genes

Recurrent variants in JS genes were searched in a large cohort of JS probands of European descent (mainly Italian) recruited in the Valente Lab. Genetic testing was performed in 551 probands, either based on Sanger sequencing of the most common JS genes, targeted sequencing of a custom panel of ~100 ciliopathy-related genes or whole-exome sequencing (WES). Overall, biallelic causative variants had been identified in 359 patients (65%). Ethical approval was in place, and a written informed consent had been signed by all families.

We focused on 11 recurrent variants detected in this cohort, including 10 previously reported and one novel variant in *KIAA0586*, found in three subjects from Sardinia, a known genetic isolate (table 1).

We compared allele frequencies in four independent non-JS control cohorts. These included: (1) Probands (mainly Italian) referred for non-JS diagnostic testing to the Mondino Foundation in Pavia (n=987) or to (2) The Bambino Gesù Paediatric Hospital in Rome (n=12 848); (3) Italian subjects (mainly healthy) from the Network of Italian Genomes (http://www.nig.cineca.it/) (n=1680); (4) Subjects from the genome aggregation database gnomAD V.2.1.1 (https://gnomad.broadinstitute.org) (n ~1 25 000). This cohort can be further divided according to ethnicity to extrapolate frequencies in selected geographical subcohorts.

Recurrent variants were also searched for in a replication cohort of ~600 patients with JS recruited in the Doherty lab. This cohort mainly includes patients from the USA, but families referred from other countries are also included. Causative biallelic variants in JS genes had been identified in 385 subjects (64%).

Comparison of allele frequencies was performed using χ^2 test with Fisher's correction, as appropriate.

Microsatellite analysis and haplotype reconstruction

We were able to genotype 138 individuals from 44 unrelated European JS families, as well as 13 non-JS subjects, carrying the variants of interest. We selected 8–10 highly polymorphic microsatellites for each locus, spanning ~4 Mb around each variant (online supplemental table 1). Markers were PCR-amplified using fluorescent primers, mixed with formamide and GeneScan 500 LIZ Size Standard (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and run on 3500 Genetic Analyzer (Thermo Fisher Scientific). Raw data were analysed using GeneMapper Software V.6 (Thermo Fisher Scientific). Haplotypes were reconstructed by phasing alleles according to familial segregation. The haplotype around *TMEM67* c.755T>C could not be analysed due to the lack of sufficient DNA samples.

Estimation of age of origin of founder variants

We were able to identify recognisable haplotypes for four of the seven variants. The DMLE+ (Disease Mapping Using Linkage Disequilibrium) V.2.3 software package was used to provide an estimated dating of these variants.[24] Input data to DMLE included an encoded description of the full haplotypes of variant carriers and non-carriers, the latter being used as controls from

Table 1 Recurrent variants and number of carriers in JS and non-JS cohorts

Variant		Patients with JS (European+US cohorts)		non-JS subjects (Italian controls+gnomAD)	
cDNA	Protein	het	hom	Het	hom*
TMEM216 c.218G>T†	p.(Arg73Leu)	0+4	2+11	1+44‡	–
TMEM237 c.52C>T§	p.(Arg18*)	2+0	–	1+16	–
MKS1 c.1408–34_1408-6del¶	–	3+2	–	7+308**	–
MKS1 c.1476T>G	p.(Cys492Trp)	8+1	–	11+14	1 parent of JS proband
KIAA0586 c.428delG	p.(Arg143Lysfs*4)	19+29	3+5	163+781	1 parent of JS proband†† + 2
KIAA0586 exons8-10 del‡‡	early termination	4+1	–	n.a.	n.a.
KIAA0586 c.863_864delAA	p.(Gln288Argfs*7)	3+3	–	0+8	–
KIAA0586 c.1006C>T	p.(Gln336)	2+0	–	3+2	–
RPGRIP1L c.1843A>C	p.(Thr615Pro)	2+4	2+0	1+17	–
CC2D2A c.4667A>T	p.(Asp1556Val)	10+16	–	17+51	–
TMEM67 c.755T>C	p.(Met252Thr)	3+2	–	3+23	–

*Parents of JS probands included.
†Ashkenazi Jewish founder variant.
‡Including 35 Ashkenazi Jewish.
§Hutterite founder variant.
¶Finnish/Northern European founder variant.
**Including 161 Finnish.
††Unaffected parent reported by Pauli *et al*.[17]
‡‡Fequency not available (n.a.) for non-JS cohorts.
JS, Joubert syndrome.

the general population. Genetic distances between markers were retrieved from the Marshfield Comprehensive Human Genetic Maps (https://www.biostat.wisc.edu/~kbroman/publications/mfdmaps/) or estimated based on physical distances given in the UCSC Genome Browser (https://genome-euro.ucsc.edu/GRCh37/hg19 assembly), considering 1 Mb~1 cM. The proportion of sampled variant-carrying chromosomes was calculated considering the minimum ratio between the number of current carrier subjects and the product P_{ti}*prev, where where P_(t_i) is the 2021 population of the ith country from where the cohorts considered in this study were originated (source: https://www.worldometers.info/population/europe/), and prev=4.7×100 000 population, which corresponds to the crude prevalence of JS as previously estimated.[5] A generation was assumed to be 25 years long.

Cell cultures

We obtained skin-derived primary fibroblasts from two *MKS1* c.1476T>G carriers (a healthy homozygous mother and her compound heterozygous affected son), three unrelated *KIAA0586* c.428delG carriers (one healthy and one affected homozygotes, and one affected compound heterozygote) and four healthy controls (online supplemental table 2). Cells were cultured in Dulbecco's Modified Eagle Medium high glucose (DMEM without L-glutamine) (Carlo Erba Reagents, Cornaredo, Italy) supplemented with inactivated 10% fetal bovine serum (FBS), 1X pen/strep (Euroclone, Pero, Italy), 1% L-glutamine 200 mM at 5% CO2 and 37°C. Fibroblasts were grown until confluency, changing the medium three times per week, and then detached with Trypsin-EDTA with Phenol Red (Euroclone) to be subsequently replated for primary cilium experiments, pelleted or cryopreserved.

Analysis of the primary cilium

To evaluate primary cilium formation and length, 2×10^5 fibroblasts were plated onto coverslips inside a 12-well plate in DMEM with 10% FBS. At 80% confluency, cells were starved for 24 hours using DMEM without FBS, and then fixed in cold methanol for 5' at 4°C. Cells on coverslips were treated in DPBS (Dulbecco's Phosphate Buffered Saline) with 10% bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA) and 1% goat serum (Carlo Erba Reagents), and incubated overnight with primary antibodies antiacetylated-α-tubulin mouse 1:500 (Sigma Aldrich) and anti-γ-tubulin rabbit 1:5000 (Thermo Fisher Scientific). The day after, cells were rinsed three times and incubated with secondary antibodies goat antirabbit IgG daylight 550 and 488 1:500 (Thermo Fisher Scientific) for 1 hour at room temperature. Finally, cells were incubated with DAPI (4′,6-diamidino-2-phenylindole) 1:6000 (Thermo Fisher Scientific) and coverslips were mounted on microscope slides with prolonged gold antifade reagent (Thermo Fisher Scientific). Images were acquired using a Zeiss Inverted microscope (Axioskope 2) and analysed using Fiji. The percentage of ciliated cells was calculated by dividing the number of cells showing a primary cilium for the total number of cells for each field. Each experiment was performed in triplicate, and we evaluated up to 15 fields per experiment. The ciliary length was measured from the centrosome (γ-tubulin) to the tip of the cilium (acetylated-α-tubulin). The experiment was performed in triplicate and up to 100 primary cilia per sample were counted in total. Statistical analysis was performed using a two-tailed Student's t-test.

RESULTS

Several recurrent variants are enriched in patients with JS compared with the general population

We first searched for recurrent variants in JS genes in our cohort of 551 European patients. We identified 11 recurrent variants in seven genes (*TMEM216*, *TMEM237*, *MKS1*, *KIAA0586*, *RPGRIP1L*, *CC2D2A* and *TMEM67*), including three already known founder variants: the Ashkenazim *TMEM216* c.218G>T variant;[6 7] the Hutterite *TMEM237* c.52C>T variant;[8] and the Northern European *MKS1* c.1408–34_1408-6del variant.[10 11] The predicted effect of these variants at the protein level and crude numbers of heterozygous and homozygous carriers in patients and controls are shown in table 1.

For the large deletion across *KIA0586* no control data were available. We compared allele frequencies of the remaining 10 variants in our European JS cohort versus >140 000 control subjects, including 15 000 Italian non-JS subjects and >125 000 subjects from gnomAD. Interestingly, although each variant was detected in some controls, they were all strongly enriched in patients with JS ($p<0.005$ to <0.00001) (table 2, figure 1 and online supplemental material). As expected, the majority of non-JS carriers of *TMEM216* c.218G>T and *MKS1* c.1408–34_1408-6del belonged to the Ashkenazim and Finnish gnomAD subcohorts, respectively, inflating global gnomAD control frequencies. Indeed, the *TMEM216* founder variant was about 100 times more frequent in the Ashkenazi gnomAD subcohort than in all other subcohorts (0.34% vs 0.003%), while the *MKS1* founder variant was about 10 times more common among Finnish than non-Finnish gnomAD cohorts (0.70% vs 0.06%). For this reason, to properly compare allele frequencies of these two variants with the mainly Italian JS cohort (which is not enriched in either Ashkenazim or Finnish cases), these two gnomAD subcohorts have been excluded from the control count for statistical purposes (table 2).

We next compared the variants' frequencies in the European JS cohort versus an independent cohort of about 600 patients with JS mainly recruited in the USA. The Ashkenazim *TMEM216* c.218G>T founder variant was significantly overrepresented in the US cohort compared with the European one, while *MKS1* c.1476T>G was much more common in the European than in the US JS cohort. Two variants (*TMEM237* c.52C>T and *KIAA0586* c.1006C>T) were detected only in two European and none of the US patients with JS, while the remaining seven variants had comparable allele frequencies in both cohorts (table 2).

A founder effect can be established only for some variants

We next asked whether, besides the three known founder variants, other recurrent variants could also represent founder mutations inherited from common ancestors. To explore this hypothesis, we genotyped several microsatellite markers spanning the genomic regions around seven variants in available carriers including patients and parents from the European JS cohort as well as some non-JS Italian controls. We were able to identify four recognisable haplotypes shared by individuals not known to be related; for these, we evaluated the geographical origin of carriers and attempted to date the origin of the founder variant (figure 2).

The first haplotype spanned ~2.29 Mb around the *MKS1* c.1476T>G variant and was shared by eight JS families, all originating from countries of the Mediterranean region except one family of Romanian origin. The mutation age was estimated around 12 generations ago (95% credible set: 10–14). Of note, three of these families (from Puglia in Southern Italy and

Table 2 Allelic frequencies (%) of recurrent variants in two JS and four non-JS cohorts

	JS		Non-JS				
	European JS (n~551)	US JS (n~600)	WES-Mondino (n=987)	WES-OPBG (n=12 848)	NIG* (n~1685)	gnomAD† (n~1 25 000)	P value‡
TMEM216 c.218G>T§	0.36	2.16	0.05	0	0	0.003	p<0.00001 p<0.0001
TMEM237 c.52C>T¶	0.18	0	0.05	0	0	0.006	p<0.0001 ns
MKS1 c.1408–34_1408-6del**	0.27	0.16	0.10	0.02	0	0.06	p<0.005 ns
MKS1 c.1476T>G	0.73	0.08	0	0.04	0.03	0.006	p<0.00001 p<0.05
KIAA0586 c.428delG	2.27	3.26	1.06	0.44	0.84	0.31	p<0.00001 ns
KIAA0586 exons8-10 del††	0.36	0.10	n.a.	n.a.	n.a.	n.a.	- ns
KIAA0586 c.863_864delAA	0.27	0.25	0	0	0	0.003	p<0.00001 ns
KIAA0586 c.1006C>T	0.18	0	0.05	0.008	0	0.0008	p<0.00001 ns
RPGRIP1L c.1843A>C	0.55	0.32	0	0.004	0	0.006	p<0.00001 ns
CC2D2A c.4667A>T	0.91	1.32	0.05	0.06	0.03	0.02	p<0.00001 ns
TMEM67 c.755T>C	0.27	0.16	0	0.008	0.06	0.008	p<0.00001 ns

*NIG: Network of Italian Genomes.
†gnomAD: genome aggregation database global frequencies (V.2.1.1). For the Ashkenazim and the Norther European/Finnish founder variants, the relative subpopulations have been excluded from gnomAD count to avoid an incorrect inflation of the control frequencies due to founder effect bias.
‡Upper line European JS versus all controls; lower line: European JS versus US JS. P values were calculated using χ^2 test (with Fisher's correction for TMEM237 c.52C>T and KIAA0586 c.1006C>T).
§Ashkenazi Jewish founder variant.
¶Hutterite founder variant.
**Finnish/Norther European founder variant.
††Being a large deletion, AF (alternative frequency) from non-JS cohorts are not available (n.a., not applicable).
JS, Joubert syndrome.

Albany) shared a much larger haplotype >3.84 Mb, suggesting closer relatedness. A second haplotype spanned ~2.02 Mb across the *KIAA0586* c.1006C>T variant was detected in two JS families and one non-JS unrelated carrier, all from Sardinia. Mutation age was estimated around two generations ago (95% credible set: 1–3). A third haplotype spanned ~1.40 Mb across

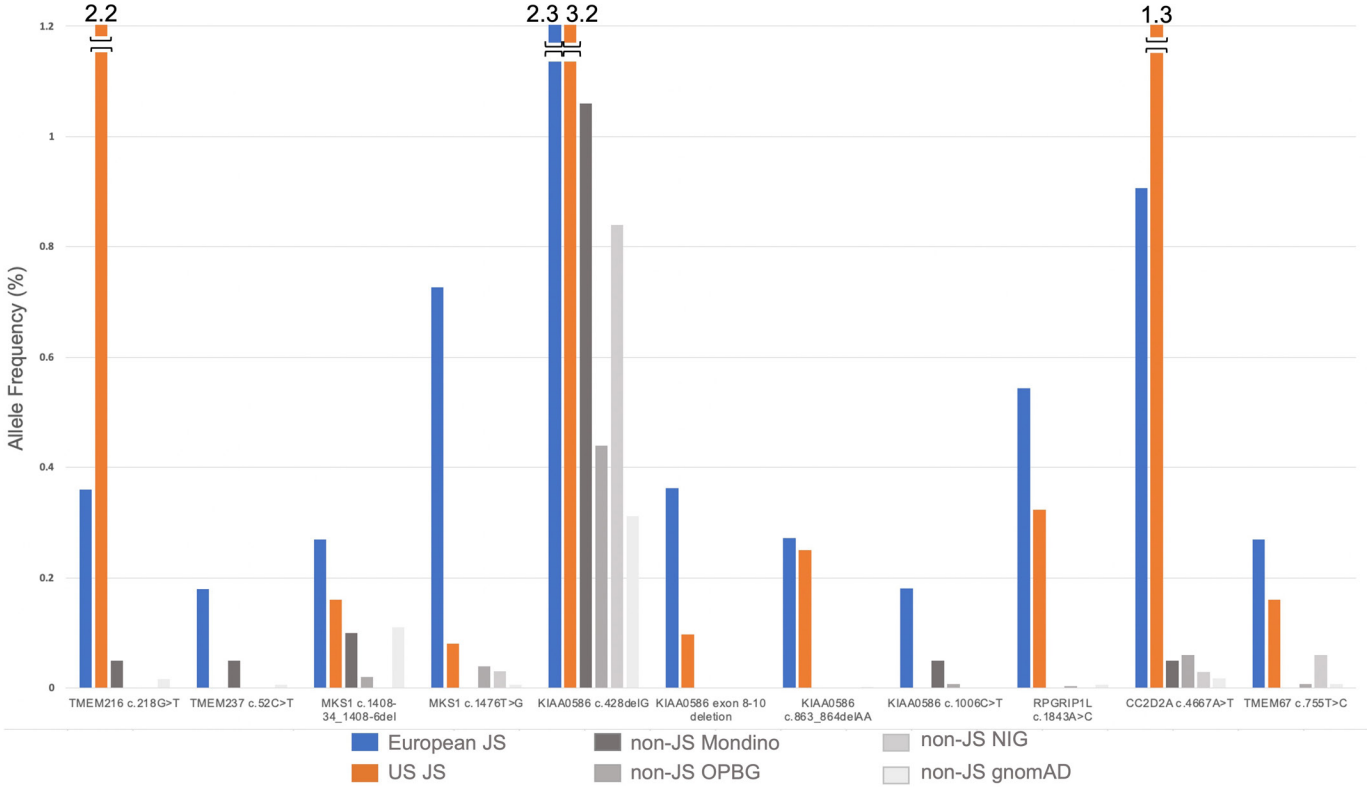

Figure 1 Allele frequencies of 11 recurrent JS variants. Allelic frequencies of recurrent variants in the European JS cohort (blue), the US JS cohort (orange) and four distinct non-JS control cohorts (grey shades). JS, Joubert syndrome; NIG, Network of Italian Genomes.

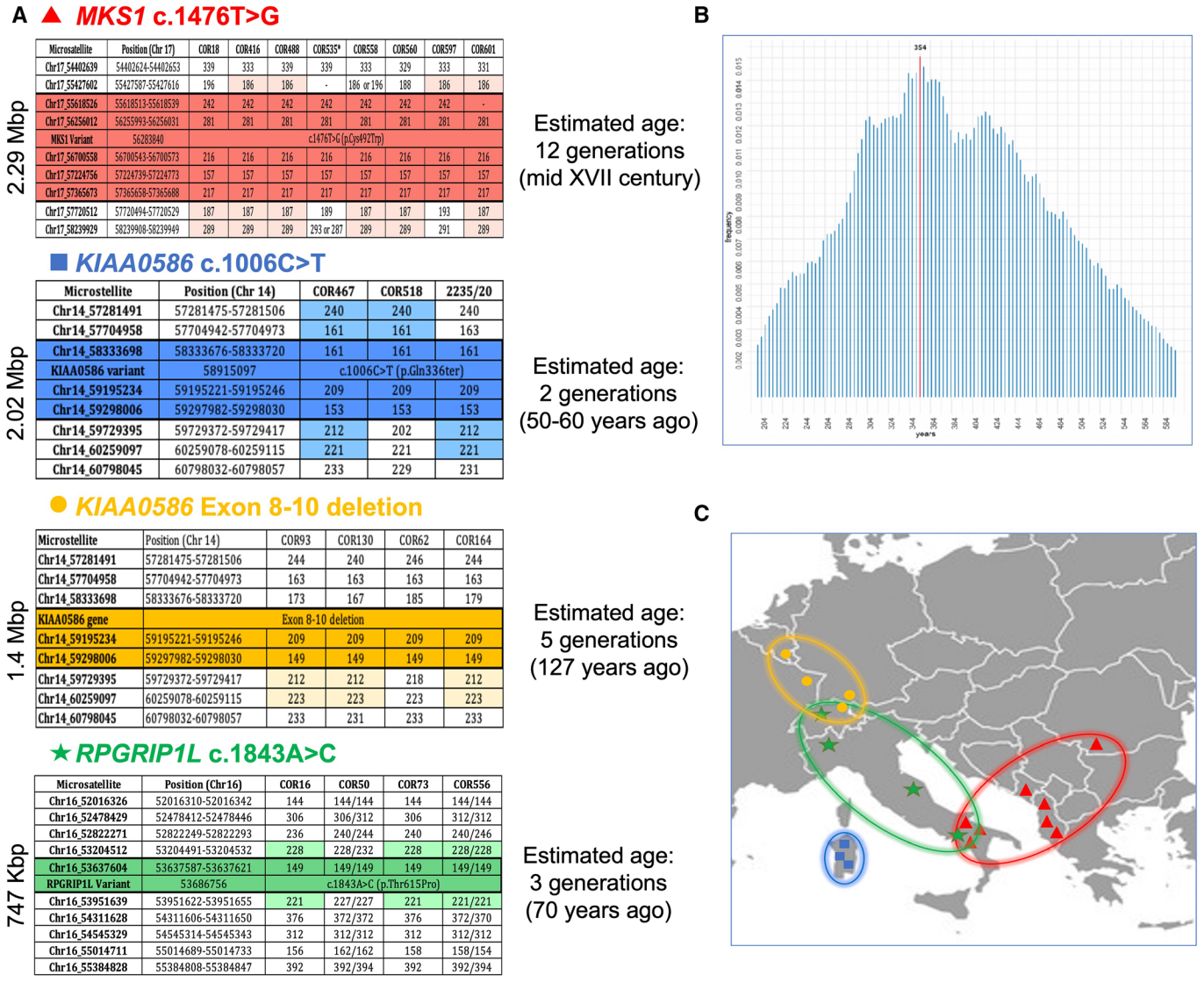

Figure 2 Schematics of the four novel founder haplotypes identified in this study. (A) Shared haplotypes across families, genomic size and estimated age of founder variants; (B) Example of dating plot (for founder variant *MKS1* c.1476T>G); (C) Geographical origin of families carrying the four variants.

KIAA0586 exon 8–10 deletion was detected in four JS families originating from central Europe (Southern Germany, Switzerland, Belgium and Northern France). This variant was estimated to have arisen about five generations ago, with a broader 95% credible set (2–14 generations). A fifth carrier was identified in the US JS cohort, but the geographical origin is not available. Finally, a small 747 Kb haplotype was identified across *RPGRIP1L* c.1843A>C in four JS families of Italian or Swiss origin. While this haplotype was too small to attempt a dating, the three Italian families shared a larger 1.49 Mb haplotype, which we were able to approximately date three generations ago (95% credible set: 2–5).

Conversely, we failed to identify recognisable shared haplotypes among the European JS families sharing the recurrent variants *CC2D2A* c.4667A>T, *KIAA0586* c.428delG and *KIAA0586* c.863_864delAA.

Functional characterisation of founder variants *MKS1* c.1476T>G and *KIAA0586* c.428delG

Two founder variants, *MKS1* c.1476T>G and *KIAA0586* c.428delG, were particularly interesting as both have been identified in homozygosity in one or more healthy individuals (controls and/or unaffected parents of JS probands).

Of note, the *MKS1* c.1476T>G variant has never been detected in the homozygous state in affected individuals, while we identified one instance of homozygosity in the unaffected parent of one JS male child who was compound heterozygous for *MKS1* c.1476T>G and a canonical splice site variant (c.1024+1G>A). Conversely, *KIAA0586* c.428delG has been reported in the homozygous state in several patients with JS as well as in two healthy controls from gnomAD and in one unaffected parent of a patient with JS.

To assess the impact of these two recurrent variants, we evaluated ciliogenesis and cilia morphology in skin fibroblasts from affected and unaffected carriers and compared them with four healthy controls. For each variant, we obtained fibroblasts from one homozygous healthy subject and one patient with JS carrying the variant in compound heterozygosity with a deleterious variant (*MKS1* c.1024+1C>A and *KIAA0586* c.863_864delAA, respectively); we also obtained fibroblasts from one *KIAA0586* c.428delG homozygous patient with JS, in whom no additional pathogenic variants could be detected by WES (either in

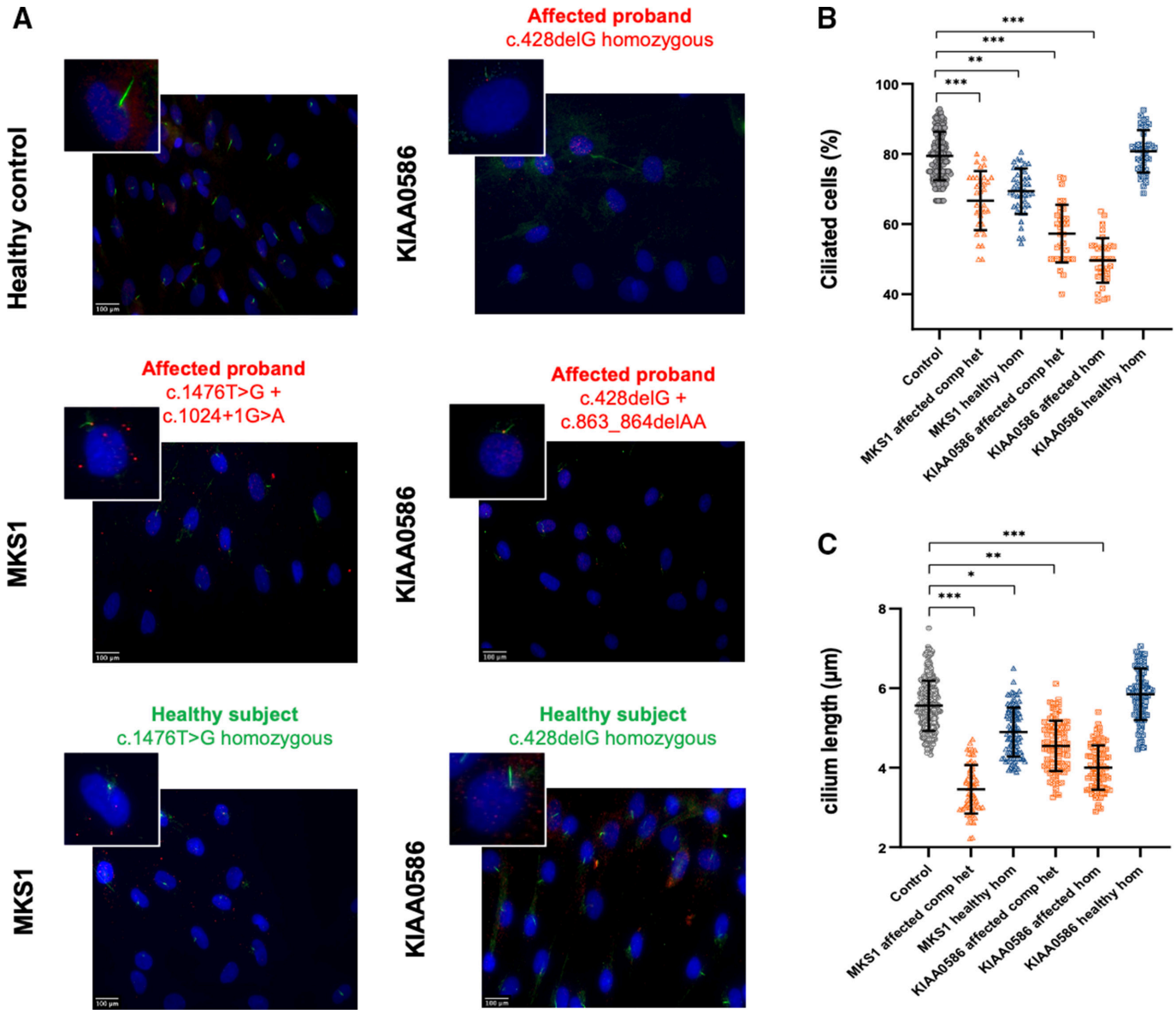

Figure 3 Functional assessment of *MKS1* c.1476T>G and *KIAA0586* c.428delG variants. Count of ciliated cells and ciliary length in four healthy controls, patients and healthy subjects carrying either variant in the homozygous or in the compound heterozygous state, as stated. (A) Representative images of immunofluorescence experiments showing primary cilia marked with γ-tubulin and acetylated-α-tubulin antibody in serum starved fibroblasts; (B–C) Box plots showing the percentage of ciliated fibroblasts (B) and ciliary length (C) in tested fibroblasts. Black circles: healthy controls; Orange: JS affected subjects; Blue: healthy subjects; Triangles: carriers of *MKS1* c.1476T>G variant; Squares: carriers of *KIAA0586* c.428delG variant. *p<0.01; **p<0.001; ***p<0.0001. JS, Joubert syndrome

KIAA0586 or other ciliary genes). We measured two standard parameters, respectively the percentage of cells able to form cilia on starvation, and the average ciliary length.

All three patients with JS, including the *MKS1* compound heterozygous (figure 3B, C, orange triangles) and the *KIAA0586* compound heterozygous and homozygous carriers (figure 3B, C, orange squares) showed a statistically significant reduction of both the percentage of ciliated cells and ciliary length compared with controls. Of note, *KIAA0586* patients had a more marker reduction of ciliated cells compared with the *MKS1* patient, which reached statistical significance for the *KIAA0586* homozygous patient.

However, intriguingly, the two homozygous healthy subjects displayed very different cellular phenotypes: fibroblasts from the *MKS1* homozygous healthy mother (figure 3B, C, blue triangles) showed a similarly significant reduction of ciliation but a much milder (although still significant) reduction of ciliary length as her compound heterozygous affected son; conversely, cells from the *KIAA0586* homozygous healthy subject (figure 3B, C, blue squares) were indistinguishable from healthy controls, both with regard to ciliation and ciliary length.

DISCUSSION

JS is characterised by wide genetic and allelic heterogeneity, with the majority of pathogenic variants being private to single families. Yet, some recurrent variants have been reported, and four of them have been shown to be founder variants in the Ashkenazi Jewish, Hutterite, Finnish and Mirpuri Pakistani populations, respectively.[6–8 10 11]

To better characterise the JS genetic landscape, we focused on 10 recurrent variants detected in our European JS cohort. Frequencies of these variants ranged from 0.18% to 0.91%, with the exception of the highly prevalent *KIAA0586* c.428delG. Interestingly, all these variants (except *KIAA0586* exon 8–10 deletion, which could not be assessed in controls) were present in gnomAD, although their frequencies were significantly lower than those detected in European patients with JS. This is a relevant observation, since recurrent variants could be erroneously downgraded using American College of Medical Genetics and Genomics (ACMG) criteria due to their relative high frequency (and also possible occurrence of homozygotes), in population databases such as gnomAD, unless it is demonstrated that they occur significantly more commonly in patients than in controls.

When comparing variant frequencies in the two JS cohorts, the Ashkenazim founder variant was the only one significantly overrepresented in US patients with JS, with a nearly 10 times higher frequency than European ones (2.16% vs 0.36%). This observation likely finds its historical roots in the large waves of immigration of Jewish diaspora communities from Europe between the late nineteenth century and the end of World War II, which made USA home for one of the largest Jewish populations in the world. Conversely, we could not observe a significantly different distribution of the Finnish founder variant between European and American JS cohorts. This suggests a more widespread worldwide distribution of this mutation, possibly explained by its very ancient origin, which was dated back approximately to 162 generations (~4050 years) ago.[10] The Hutterite founder variant was absent in the US cohort and very rare in the European cohort, likely reflecting the limited admixture of this inbred community with their neighbouring populations both in central Europe, where they originated, and in the USA and Canada, where Hutterites migrated towards the end of the nineteen century. Only one variant (*MKS1* c.1476T>G) was significantly enriched in European versus American patients, while the remaining seven had comparable allele frequencies in the two cohorts. The most common one is *KIAA0586* c.428delG, with an impressive allele frequency of 2.27%–3.26% in patients with JS. We were surprised to see that the allele frequency of this variant in the Ashkenazi Jewish gnomAD subgroup (0.83%) was even higher than that of the *TMEM216* founder variant (0.34%), which is considered the most common genetic cause of JS among Ashkenazim.[6] Yet, the proportion of Ashkenazi Jewish patients with JS carrying *KIAA0586* c.428delG has not been reported to date.

To search for novel founder haplotypes, we were able to genotype microsatellite markers in affected and healthy carriers of seven recurrent variants.

MKS1 c.1476T>G, the only variant enriched in European versus US JS, was detected in eight patients from the European and only in one patient from the US JS cohort, all coming from countries of the Mediterranean area (Southern Italy, Albany, Montenegro and Greece) with the exception of a single family from Romania. In line with this, the allele frequency of this variant among Italian non-JS controls was about five times higher than among gnomAD carriers, all non-Finnish Europeans (0.04% vs 0.006%), and we detected a large 2.29Mb haplotype, indicating *MKS1* c.1476T>G as a recent founder variant, whose origin could be approximately dated 12 generations ago. It is tempting to speculate that all these patients, originating from countries abutting the Mediterranean Sea, have inherited the same mutation from a common ancestor living in the late seventeenth century, a time in which the Mediterranean Sea was swept by endless market trades across its coasts.

The two affected and the single healthy carriers of the *KIAA0586* c.1006C>T variant were all of Sardinian origin, and shared a large 2Mb haplotype indicating this as a recent founder variant, which likely originated in Sardinia only few generations ago. Indeed, no variant carriers were identified among the US patients with JS and only two carriers were reported in Italian non-JS controls and two in gnomAD, of non-Finnish European descent. A more complex scenario regards the *RPGRIP1L* c.1843A>C variant: we could detect a recent 1.5Mb founder haplotype, which was shared by three Italian JS families carrying such a variant, while a fourth patient from Switzerland only shared a smaller portion of this haplotype, suggesting an older origin. The same variant was detected in four US patients with JS (two white non-Hispanic, respectively, from US and Australia, one white Hispanic from Argentina and one African-American), and had been previously reported in several patients with JS originating from the USA, Switzerland and France.[20 21] While we failed to detect this variant in Italian non-JS controls, it was reported in 17 gnomAD individuals, all non-Finnish Europeans except one of Latin-admixed American origin. The available data do not allow us to determine whether all carriers inherited the same founder variant from a single ancestor or whether the variant has arisen independently in different populations, and further studies would be needed to clarify this aspect.

Finally, although we were not able to assess the frequency of *KIAA0586* exon 8–10 deletion in non-JS controls, it is interesting to note that such deletion was reported in five patients with JS from central Northern Europe and in four patients recruited in the USA.[18 19] Despite the identification of a shared 1.4 Mb region, the exact break points of the deletion have not been well described and may vary among patients, suggesting independent mutational events.

Interestingly, we failed to identify recognisable shared haplotypes for the other three recurrent variants (*KIAA0586* c.428delG and c.863_864delAA, CC2D2A c.4667A>T). This suggests either that these might represent very ancestral variants (with founder haplotypes too small to be detected), or alternatively, they could have arisen several times as independent events, representing mutational hotspots. This issue is not easy to resolve. For instance, the six JS heterozygous carriers of *KIAA0586* c.863_864delAA have distinct origins (Southern and Northern Italy, Switzerland, Germany, USA and Brazil), and similarly the eight heterozygous carriers of this variant in gnomAD are from different subgroups (African/African American, Latino/admixed American, non-Finnish European, South Asian and other). Haplotype analysis in carriers of different geographical origins would be required to better clarify this issue.

One factor possibly contributing to the enrichment of certain founder variants is their hypomorphic nature. This term refers to variants whose pathogenic impact on the protein function is relatively mild and, as such, they result in a disease phenotype only when in compound heterozygosity with a more deleterious variant, or in presence of additional genetic modifiers (eg, a third pathogenic variant on a distinct gene). Indeed, real hypomorphic variants are occasionally detected in the homozygous state in healthy individuals, challenging the assessment of their pathogenicity in a diagnostic setting.

This is already known for the *KIAA0586* c.428delG variant, reported in homozygosity in a few patients with JS as well as in three unaffected individuals.[13 14 17] Here, we attempt to functionally characterise this variant as well as another potentially hypomorphic recurrent variant (*MKS1* c.1476T>G), which we found in homozygosity in the unaffected mother of a child with JS. The affected son carried the variant in trans with a canonical

splice site variant, previously reported in compound heterozygosity with the Finnish founder variant in a fetus affected by MKS.[25] Of note, the *MKS1* c.1476T>G variant was formerly described in a patient with mild Bardet-Biedl syndrome (in compound heterozygosity with a 1-amino acid deletion), and was suggested to act as a hypomorphic variant through a rescue experiment on mks1 morphant zebrafish embryos.[12]

To characterise these two variants, we evaluated ciliogenesis and average ciliary length, known to be impaired in *MKS1* or *KIAA0586* defective cells.[13 16 19 26 27] We compared these measures in fibroblasts from controls, healthy subjects homozygous for either variant, and patients with JS. Surprisingly, we obtained strikingly different results for the two variants. For the *MKS1* variant, ciliogenesis was similarly impaired in the healthy homozygous mother and in her compound heterozygous affected son; however, in the latter, the combination with the more severe splice site variant resulted in a more marked reduction of ciliary length, while this was less affected in the mother, although still significantly compared with controls. This observation suggests that *MKS1* c.1476T>G is a hypomorphic pathogenic variant, able to impair ciliary functioning but not enough to reach a critical threshold of disease, which can manifest only when the variant is combined with a more deleterious one.

Conversely, while both affected subjects carrying *KIAA0586* c.428delG showed marked deficits both in ciliogenesis and ciliary length (regardless of the homozygous or compound heterozygous state), these parameters were fully normal in the healthy homozygote's fibroblasts, challenging the pathogenicity of this variant at least in the homozygous state. A first possible explanation is the existence of a second 'cryptic' *KIAA0586* variant missed by the genetic testing in affected homozygotes, and indeed we had previously identified two patients with JS who were homozygous for the c.428delG variant but also carried a distinct *KIAA0586* heterozygous pathogenic variant.[13 17] An alternative explanation is that c.428delG is a low-penetrance variant, whose impact needs to be enhanced by as yet unidentified modifier factors. In this line, Bachmann-Gagescu and collaborators reported a 4-year-old patient with JS homozygous for *KIAA0586* c.428delG who was also heterozygous for a deleterious variant in the *CPLANE1* gene,[14] a combination possibly recalling the model of triallelic/digenic inheritance already proposed for another ciliopathy, Bardet-Biedl syndrome.[28]

These experiments suggest that the clinical variability seen in patients with JS may be influenced, at least in part, by the relative pathogenic impact of each variant on the protein function, resulting in a grading of the cellular phenotype in terms of ciliogenesis, ciliary length and related ciliary functioning. In the absence of reliable bioinformatic tools able to accurately predict the functional outcome of variants (especially those classified as 'variants of unknown significance'), the development of simple and quantifiable functional assays will represent an essential advancement to better understand the complexity of ciliopathy phenotypes and provide the patients and families with more reliable prognostic indications and counselling.

Author affiliations
[1]Neurogenetics Research Centre, IRCCS Mondino Foundation, Pavia, Italy
[2]Department of Molecular Medicine, University of Pavia, Pavia, Italy
[3]Department of Pediatrics, University of Washington, Seattle, Washington, USA
[4]Bioinformatics Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, S. Giovanni Rotondo, Foggia, Italy
[5]Laboratory of Medical Genetics, IRCCS Bambino Gesù Children's Hospital, Rome, Italy
[6]Department of Neuroscience, IRCCS Stella Maris Foundation, Pisa, Italy
[7]Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
[8]Unit of Neuromuscular and Neurodegenerative Disorders, IRCCS Ospedale Pediatrico Bambino Gesù, Rome, Italy
[9]Departement of Pediatric Neurology, University Children's Hospital Zürich, Zurich, Switzerland
[10]Department of Brain and Behavioral Sciences, University of Pavia, Pavia, Italy
[11]Child Neurology and Psychiatry Unit, IRCCS Mondino Foundation, Pavia, Italy
[12]Interdisciplinary Pediatric Centre for Children with Developmental Disabilities and Severe Chronic Disorders, University Medical Centre, Georg August University, Göttingen, Germany
[13]Department of Pediatric Neuroscience, Fondazione IRCCS Istituto Neurologico "C Besta", Milan, Italy
[14]Clinical Genetics Unit, Department of Pediatric Medicine, Giovanni XXIII Children's Hospital, Bari, Italy
[15]Section of Pediatrics, Department of Medical Translational Sciences, University of Naples Federico II, Naples, Italy
[16]Genetic Counseling Service, Department of Pediatrics, Regional Hospital of Bozen, Bozen, Italy

Twitter Tommaso Mazza @irongraft

Acknowledgements The authors thank all patients and families for their continuous support and participation to JS research projects.

Contributors VS, DD and EMV planned the study; VS, GM, HL, TB, AM, IP, JCD, FDA, CM and EA collected the data and performed molecular or bioinformatic analyses; VS, TB and TM performed statistical analyses; VS, TM and EMV wrote the first draft of the manuscript, tables and figures; RoBa, EnBe, EuBo, ReBo, KB, SDA, NN, RF, AN, AR, RR, FS, SS, PS, SG and DD clinically diagnosed and followed up the patients. All the authors revised the manuscript for important intellectual content and approved the final version. EMV is responsible for the overall content, as guarantor.

Funding This research was funded by Telethon Foundation (grant number GGP20070); Italian Ministry of Health (Ricerca Corrente to IRCCS Mondino Foundation, Fondazione IRCCS Casa Sollievo della Sofferenza and IRCCS Stella Maris Foundation; Ricerca Finalizzata grant number RF-2019-12369368). JS research in the Doherty Lab is supported by grants from NIH (R01HD100730, R01NS064077), as well as support from the NIH-funded UW Intellectual and Developmental Disabilities Research Center (U54HD083091 PI Michael Guralnick, Genetics Core and subproject 6849 to DD, and P50HD103524 PI Michael Guralnick, Genetics Core).

Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s)

Ethics approval This study involves human participants and was approved by the ethics committee of the University of Pavia (Protocol n. 20180077857/12-09-2018). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

ORCID iDs
Valentina Serpieri http://orcid.org/0000-0002-3553-0066
Enrico Silvio Bertini http://orcid.org/0000-0001-9276-4590
Stefano D'Arrigo http://orcid.org/0000-0001-5188-9418
Emanuele Agolini http://orcid.org/0000-0001-6543-6225
Romina Romaniello http://orcid.org/0000-0002-8709-6732

Tommaso Mazza http://orcid.org/0000-0003-0434-8533
Enza Maria Valente http://orcid.org/0000-0002-0600-6820

REFERENCES

1 Maria BL, Hoang KB, Tusa RJ, *et al*. "Joubert syndrome" revisited: key ocular motor signs with magnetic resonance imaging correlation. J Child Neurol 1997;12:423–30.
2 Bachmann-Gagescu R, Dempsey JC, Phelps IG, *et al*. Joubert syndrome: a model for untangling recessive disorders with extreme genetic heterogeneity. J Med Genet 2015;52:514–22.
3 Romani M, Micalizzi A, Valente EM. Joubert syndrome: congenital cerebellar ataxia with the molar tooth. Lancet Neurol 2013;12:S1474-4422(13)70136-4:894–905.:.
4 Salonen R. The meckel syndrome: clinicopathological findings in 67 patients. Am J Med Genet 1984;18:671–89.
5 Nuovo S, Bacigalupo I, Ginevrino M, *et al*. Age and sex prevalence estimate of joubert syndrome in italy. Neurology 2020;94:e797–801.
6 Edvardson S, Shaag A, Zenvirt S, *et al*. Joubert syndrome 2 (JBTS2) in ashkenazi jews is associated with a TMEM216 mutation. Am J Hum Genet 2010;86:93–7.
7 Valente EM, Logan CV, Mougou-Zerelli S, *et al*. Mutations in TMEM216 perturb ciliogenesis and cause joubert, meckel and related syndromes. Nat Genet 2010;42:619–25.
8 Huang L, Szymanska K, Jensen VL, *et al*. TMEM237 is mutated in individuals with a joubert syndrome related disorder and expands the role of the TMEM family at the ciliary transition zone. Am J Hum Genet 2011;89:713–30.
9 Boycott KM, Parboosingh JS, Scott JN, *et al*. Meckel syndrome in the hutterite population is actually a joubert-related cerebello-oculo-renal syndrome. Am J Med Genet A 2007;143A:1715–25.
10 Khaddour R, Smith U, Baala L, *et al*. Spectrum of MKS1 and MKS3 mutations in meckel syndrome: a genotype-phenotype correlation. mutation in brief #960. Online. Hum Mutat 2007;28:523–4.
11 Kyttälä M, Tallila J, Salonen R, *et al*. MKS1, encoding a component of the flagellar apparatus basal body proteome, is mutated in meckel syndrome. Nat Genet 2006;38:155–7.
12 Leitch CC, Zaghloul NA, Davis EE, *et al*. Hypomorphic mutations in syndromic encephalocele genes are associated with bardet-biedl syndrome. Nat Genet 2008;40:443–8.
13 Roosing S, Hofree M, Kim S, *et al*. Functional genome-wide sirna screen identifies KIAA0586 as mutated in joubert syndrome. Elife 2015;4:e06602.
14 Bachmann-Gagescu R, Phelps IG, Dempsey JC, *et al*. KIAA0586 is mutated in joubert syndrome. Hum Mutat 2015;36:831–5.
15 Stephen LA, Tawamie H, Davis GM, *et al*. TALPID3 controls centrosome and cell polarity and the human ortholog KIAA0586 is mutated in joubert syndrome (JBTS23). Elife 2015;4:e08077.
16 Alby C, Piquand K, Huber C, *et al*. Mutations in KIAA0586 cause lethal ciliopathies ranging from a hydrolethalus phenotype to short-rib polydactyly syndrome. Am J Hum Genet 2015;97:311–8.
17 Pauli S, Altmüller J, Schröder S, *et al*. Homozygosity for the c.428delg variant in *kiaa0586* in a healthy individual: implications for molecular testing in patients with joubert syndrome. J Med Genet 2019;56:261–4.
18 Sumathipala D, Strømme P, Gilissen C, *et al*. Sudden death in epilepsy and ectopic neurohypophysis in joubert syndrome 23 diagnosed using snvs/indels and structural variants pipelines on WGS data: a case report. BMC Med Genet 2020;21:96.
19 Malicdan MCV, Vilboux T, Stephen J, *et al*. Mutations in human homologue of chicken talpid3 gene (KIAA0586) cause a hybrid ciliopathy with overlapping features of jeune and joubert syndromes. J Med Genet 2015;52:830–9.
20 Delous M, Baala L, Salomon R, *et al*. The ciliary gene RPGRIP1L is mutated in cerebello-oculo-renal syndrome (joubert syndrome type B) and meckel syndrome. Nat Genet 2007;39:875–81.
21 Wolf MTF, Saunier S, O'Toole JF, *et al*. Mutational analysis of the RPGRIP1L gene in patients with joubert syndrome and nephronophthisis. Kidney Int 2007;72:1520–6.
22 Szymanska K, Berry I, Logan CV, *et al*. Founder mutations and genotype-phenotype correlations in meckel-gruber syndrome and associated ciliopathies. Cilia 2012;1:18.
23 Bachmann-Gagescu R, Ishak GE, Dempsey JC, *et al*. Genotype-phenotype correlation in CC2D2A-related joubert syndrome reveals an association with ventriculomegaly and seizures. J Med Genet 2012;49:126–37.
24 Reeve JP, Rannala B. DMLE+: bayesian linkage disequilibrium gene mapping. Bioinformatics 2002;18:894–5.
25 Consugar MB, Kubly VJ, Lager DJ, *et al*. Molecular diagnostics of meckel-gruber syndrome highlights phenotypic differences between MKS1 and MKS3. Hum Genet 2007;121:591–9.
26 Weatherbee SD, Niswander LA, Anderson KV. A mouse model for Meckel syndrome reveals MKS1 is required for ciliogenesis and hedgehog signaling. Hum Mol Genet 2009;18:4565–75.
27 Dawe HR, Smith UM, Cullinane AR, *et al*. The meckel-gruber syndrome proteins MKS1 and meckelin interact and are required for primary cilium formation. Hum Mol Genet 2007;16:173–86.
28 Katsanis N, Ansley SJ, Badano JL, *et al*. Triallelic inheritance in bardet-biedl syndrome, a mendelian recessive disorder. Science 2001;293:2256–9.