
Article:
Sentchordi-Montané, L. orcid.org/0000-0002-4083-3352, Aza-Carmona, M., Benito-Sanz, S. et al. (17 more authors) (2018) Heterozygous aggrecan variants are associated with short stature and brachydactyly: Description of 16 probands and a review of the literature. Clinical Endocrinology, 88 (6). pp. 820-829.

https://doi.org/10.1111/cen.13581

CLINICAL ENDOCRINOLOGY

Heterozygous aggrecan mutations cause short stature with brachydactyly: Description of 16 probands and a review of the literature.

Journal:	*Clinical Endocrinology*
Manuscript ID	CEN-2017-001066.R1
Manuscript Type:	1 Original Article - UK, Europe
Date Submitted by the Author:	19-Jan-2018

Complete List of Authors:	SENTCHORDI-MONTANE, LUCIA; Hospital Infanta Leonor, Pediatrics; Hospital Universitario La Paz, Institute of Medical and Molecular Medicine, INGEMM; Hospital Universitario La Paz, Skeletal dysplasia Multidisciplinary Unit (UMDE) Aza-Carmona, Miriam; Hospital Universitario La Paz, Institute of Medical and Molecular Medicine (INGEMM); Hospital Universitario La Paz, Skeletal dysplasia Multidisciplinary Unit (UMDE); Instituto de Salud Carlos III, CIBERER Benito-Sanz, Sara; Hospital Universitario La Paz, Institute of Medical and Molecular Medicine (INGEMM); Instituto de Salud Carlos III, CIBERER Barreda-Bonis, Ana C.; Hospital Universitario La Paz, Pediatric Endocrinology; Hospital Universitario La Paz, Skeletal dysplasia Multidisciplinary Unit (UMDE) Sánchez-Garre, Consuelo; Hospital de Terrassa, Pediatric Endocrinology Prieto-Matos, Pablo; Hospital Universitario de Salamanca, Pediatric Endocrinology; Instituto de Investigación Biomédica Salamanca, Pediatrics Ruiz-Ocaña, Pablo; Hospital Universitario Puerta del Mar, Pediatric Endocrinology Lechuga-Sancho, Alfonso; Hospital Universitario Puerta del Mar, Pediatric Endocrinology Carcavilla-Urquí, Atilano; Hospital Virgen de la Salud, Pediatric Endocrinology Mulero-Collantes, Inés; Hospital Universitario Rio Hortega, Pediatric Endocrinology Martos-Moreno, Gabriel A.; Hospital Infantil Universitario Nino Jesus, Pediatric Endocrinology; Universidad Autónoma de Madrid, Pediatría; Instituto de Salud Carlos III, CIBER Fisiopatología de la Obesidad y Nutrición del Pozo, Angela; Hospital Universitario La Paz, Institute of Medical and Molecular Medicine (INGEMM); Instituto de Salud Carlos III, CIBERER Vallespín, Elena; Hospital Universitario La Paz, Institute of Medical and Molecular Medicine (INGEMM); Instituto de Salud Carlos III, CIBERER Offiah, Amaka; Sheffield Children's NHS Foundation Trust, Department of Oncology and Metabolism. Academic Unit of Child Health Parrón-Pajares, Manuel; Hospital Universitario La Paz, Pediatric Radiology; Hospital Universitario La Paz, Skeletal dysplasia Multidisciplinary Unit

	(UMDE) Dinis, Isabel; Centro Hospitalar e Universitario de Coimbra EPE, Pediatric Endocrinology Sousa, Sergio B.; Centro Hospitalar e Universitario de Coimbra EPE, Medical Genetics Unit Ros-Pérez, Purificación; Hospital Universitario Puerta del Hierro Majadahonda, Pediatric Endocrinology González-Casado, Isabel; Hospital Universitario La Paz, Pediatric Endocrinology; Hospital Universitario La Paz, Skeletal dysplasia Multidisciplinary Unit (UMDE) E. Heath, Karen; Hospital Universitario La Paz, Institute of Medical & Molecular Genetics; Hospital Universitario La Paz, Skeletal dysplasia Multidisciplinary Unit (UMDE); Instituto de Salud Carlos III, CIBERER
Key Words:	skeletal dysplasia, short stature, aggrecan, ACAN, brachydactyly

Heterozygous aggrecan mutations cause short stature with brachydactyly: Description of 16 probands and a review of the literature.

Short title: ACAN Clinical spectrum

Lucía Sentchordi-Montané[1,2,3], Miriam Aza-Carmona[2,3,4], Sara Benito-Sanz[2,4], Ana C. Barreda- Bonis[3,5], Consuelo Sánchez-Garre[6], Pablo Prieto-Matos[7], Pablo Ruiz-Ocaña[8], Alfonso Lechuga-Sancho[8], Atilano Carcavilla-Urquí[9], Inés Mulero-Collantes[10], Gabriel A. Martos-Moreno[11], Angela del Pozo[2,4], Elena Vallespín[2,4], Amaka Offiah[12], Manuel Parrón-Pajares[3,13], I. Dinis[14], Sergio B. Sousa[15], Purificación Ros-Pérez[16], Isabel González-Casado[3,5], Karen E. Heath[2,3,4]

1. Dept. of Pediatrics, Hospital Universitario Infanta Leonor, Madrid, Spain
2. Institute of Medical and Molecular Genetics (INGEMM), Hospital Universitario La Paz, Universidad Autonóma de Madrid, IdiPAZ, Madrid, Spain
3. Skeletal displasia Multidisciplinary Unit (UMDE), Hospital Universitario La Paz, Madrid, Spain
4. CIBERER, ISCIII, Madrid, Spain
5. Dept. of Pediatric Endocrinology, Hospital Universitario La Paz, Madrid, Spain
6. Dept. of Pediatric Endocrinology, Hospital de Terrassa, Terrassa (Barcelona), Spain
7. Dept. of Pediatrics, Hospital Universitario Salamanca, Instituto de Investigación Biomédica de Salamanca (IBSAL), Salamanca, Spain
8. Dept. of Pediatrics, Hospital Universitario Puerta del Mar, Cádiz, Spain
9. Dept. of Pediatrics, Hospital Virgen de la Salud, Toledo, Spain
10. Dept. of Pediatrics, Hospital Universitario Río Hortega, Valladolid, Spain

11. Dept. of Endocrinology, Hospital Infantil Universitario Niño Jesús, Universidad Autonóma de Madrid, Instituto de Investigación Sanitaria La Princesa; Dept. of Pediatrics, Universidad Autónoma de Madrid, and CIBEROBN, ISCIII, Madrid, Spain

12. Department of Oncology and Metabolism. Academic Unit of Child Health. Sheffield Children's NHS Foundation Trust, United Kingdom

13. Dept.of Pediatric Radiology, Hospital Universitario La Paz, Madrid, Spain

14. Dept. of Pediatric Endocrinology, Diabetes and Growth Unit, Hospital Pediátrico, Centro Hospitalar e Universitário de Coimbra, Portugal

15. Medical Genetics Unit, Hospital Pediátrico, Centro Hospitalar de Coimbra, Portugal

16. Dept. of Pediatrics, Hospital Universitario Puerta de Hierro Majadahonda, Madrid, Spain

Corresponding author: Karen Heath, Institute of Medical & Molecular Genetics (INGEMM); Paseo Castellana 261, 28046 Madrid, Spain. E-mail: karen.heath@salud.madrid.org; Fax: +34 91 207 1040.

Acknowledgements:

The authors would like to thank the families for their participation in this study. This work was supported in part by the following grants: SAF2012-30871, SAF2015-66831-R from the MINECO (to K.E.H) and Fundación SEEP (L.S-M and K.E.H). S.B-S was awarded a postdoctoral CIBERER fellowship.

Disclosure statement: The authors have nothing to disclose.

SUMMARY

Objective: Mutations in the aggrecan gene (*ACAN*) have been identified in two autosomal dominant skeletal dysplasias, Spondyloepiphyseal dysplasia, Kimberley type (SEDK) and osteochondritis dissecans, as well as in a severe recessive dysplasia, Spondyloepimetaphyseal dysplasia, aggrecan type. Next generation sequencing (NGS) has aided the identification of heterozygous *ACAN* mutations in individuals with short stature, minor skeletal defects and mild facial dysmorphisms, some of whom have advanced bone age (BA), poor pubertal spurt and early growth cessation as well as precocious osteoarthritis.

Design & methods: Clinical and genetic characterization of 16 probands with heterozygous *ACAN* variants, 14 with short stature and mild skeletal defects (group 1) and two with SEDK (group 2). Subsequently, we reviewed the literature to determine the frequency of the different clinical characteristics in *ACAN* positive individuals.

Results: A total of 16 *ACAN* variants were located throughout the gene, six pathogenic mutations and 10 variants of unknown significance (VUS). Interestingly, brachydactyly was observed in all probands. Probands from group 1, with a pathogenic mutation tended to be shorter and 60% had an advanced BA compared to 0% in those with a VUS. A higher incidence of coxa valga was observed in individuals with a VUS (37% v 0%). Nevertheless, other features were present at similar frequencies.

Conclusions: *ACAN* should be considered as a candidate gene in patients with short stature and minor skeletal defects, particularly those with brachydactyly, and in patients with spondyloepiphyseal dysplasia. It is also important to note that advanced BA and osteoarticular complications are not obligatory conditions for aggrecanopathies/aggrecan-associated dysplasias.

Key words: skeletal dysplasia, short stature, aggrecan, ACAN, brachydactyly.

INTRODUCTION

Longitudinal bone growth occurs at the growth plate as a result of chondrogenesis. It is regulated by a complex network of signals from endocrine and paracrine systems as well as interactions between cellular growth factors and extracellular matrix. Mutations in many of these pathways result in growth delay and/or skeletal defects.

Short stature is one of the most common reasons for referral to a pediatric endocrinologist. Next generation sequencing (NGS) has permitted the identification of genetic defects in subgroups of short stature individuals, including heterozygous mutations in the aggrecan gene (*ACAN*).

Aggrecan is a major structural component of the cartilage growth plate. Until recently, *ACAN* mutations had been observed in a few families with spondyloepiphyseal dysplasia, Kimberley type (SEDK, MIM 608361)[1], spondyloepimetaphyseal dysplasia, aggrecan type (SEMD, MIM 612813)[2], and osteochondritis dissecans (MIM 165800).[3] More recently, through the implementation of NGS, heterozygous *ACAN* mutations have been reported in individuals with a milder skeletal dysplasia, presenting with short stature and advanced bone age (BA).[4-10] This led to the creation of an International Aggrecan Consortium for the clinical and genetic evaluation of 103 ACAN heterozygotes from 20 families.[6] Height appeared to be less affected during childhood (median -2 SDS), and most had advanced BA compared to chronological age (CA).

Aggrecan consists of an N-terminal domain, two globular domains (G1 and G2), two inter-globular domains (CS and KS attachment regions), a selectin-like domain (G3) and a C-terminal domain.[11]Mutations are located throughout the protein and no genotype-phenotype correlations have been observed.[6,10] The pathogenic mechanisms for the accelerated bone maturation, cartilage degradation and the clinical heterogeneity remain elusive.

We present a retrospective study of the clinical and genetic findings of 16 probands with heterozygous *ACAN* variants, detected during routine genetic studies using a skeletal dysplasia NGS panel. We also review all cases reported in the literature to determine the frequency of the different clinical characteristics related to agrecanopathies.

PATIENTS AND METHODS

All participants provided informed consent for the performed studies and ethical approval was obtained from the Hospital La Paz ethical committee.

The 16 probands were referred for molecular study from Spanish and Portuguese endocrinology and genetic clinics. Ten formed part of a cohort of 100 children with short stature and mild skeletal defects in either the proband or one of their parents, in whom *SHOX* defects had been previously excluded using MLPA (P018G1, MRC Holland) and DNA sequencing. Endocrine disorders including GH-IGF1 related conditions were also excluded by biochemical analysis. The remaining six probands were referred for routine skeletal dysplasia genetic diagnosis (n>1000 patients). *SHOX* mutations were similarly excluded in four of these, not performed in probands 15-16. BA and skeletal surveys were performed. Blood samples were extracted from the proband and family members, when available.

All probands were analysed using a custom designed Skeletal dysplasia Next generation sequencing (NGS) panel, SKELETALSEQ.V3-6 (n=315-368 genes) and sequenced on a MiSeq/NextSeq sequencer (Illumina, San Diego, CA, USA). Bioinformatic analyses were performed as previously described.[11] Conservation, pathogenicity prediction analysis and population frequences of the identified *ACAN* variants was carried out using CADD V1.3 (http://cadd.gs.washington.edu/), GerpRS (http://mendel.stanford.edu/SidowLab/downloads/gerp/) and Alamut V2.10 (Interactive Biosoftware, France) and gnomAD database (http://gnomad.broadinstitute.org). Variants were subsequently validated by Sanger sequencing as was family testing. Kinship was confirmed using microsatellite marker analysis (Devyser Complete QF-PCR, Stockholm, Sweden).

After the identification of an *ACAN* variant, each referring clinician was asked to complete a specific aggrecanopathy clinical questionnaire including personal and familial records, anthropometric measures, facial dysmorphisms, age of puberty onset and pubertal spurt and other associated medical conditions, similar to that previously published.[6]

Advanced BA was defined as a BA greater than one year compared to the CA, whilst delayed BA was defined as BA less than one year relative to the CA. Brachydactyly was defined as short metacarpals and/or fingers. The clinical data, BA and skeletal surveys were then revised by three experts (LS-M, AO, MP). Subsequently, the 16 probands were divided into two clinical groups: those presenting with short stature and mild skeletal defects (group 1, probands 1-14) and two with SEDK (group 2, probands 15-16).

RESULTS

Molecular genetics

A total of 16 heterozygous *ACAN* variants were identified (Figure 1, Table 1). No other pathogenic mutation or variant of unknown significance (VUS) associated with short stature and skeletal defects, including brachydactyly, was detected in the probands. Six variants were classified as pathogenic mutations (4 nonsense, 1 frameshift and 1 splicing) whilst the remaining ten were classified as VUS (Table 1) using the American Society of College of Genetics and Genomics (ACMG) recommendations for classifying variants.[12]

Family testing was performed in all 16 probands. Mutations were inherited in all but one case (proband 9) which appears to have arisen as a *de novo* event or due to germinal mosaicism. The variants were identified in a total of 20 family members, 19 adults and one child, all with short stature and/or mild skeletal defects or SEDK (fathers of probands 15 and 16). Unfortunately, further cosegregation studies from multiple generations were not possible.

Clinical group 1 (patients 1-14) with short stature and mild skeletal defects

Thirteen of the 14 probands were children (age range 1.5-18 years, median 10.2 years). Probands 1 and 13 were previously included in the International Aggregation consortium study.[6] Clinical characteristics are shown in Table 2. Anthropometric measurements were assessed in the 13 children of group 1. The median height SDS was below average (-2.9), sitting height-to-height ratio was in the normal range (0.54) and BMI

was -0.31 SDS. Advanced BA was observed in three probands whilst equal or delayed BA with respect to CA was determined in ten. Three probands had reached their final height, two of whom had had a poor pubertal spurt. Examples of growth patterns are shown in Supp. Fig. 1. Radiological features and hand photos are shown in Supp. Fig. 2 and 3, respectively. Brachydactyly (short fingers and/or short metacarpals) was observed in all probands in group 1. Seven probands (53%) showed a similar phenotype with frontal bossing, depressed nasal bridge and/or mid-facial dysplasia (Table 2). Three of the patients (probands 2, 9, 12) have recently initiated growth hormone therapy, but no response data is currently available. To determine if the clinical characteristics were similar or different in individuals with a clearly pathogenic mutation compared to those with a VUS, we performed a comparison of the clinical and radiological characteristics of these probands from group 1 (Table 3).

Group 2 (probands 15-16) – SEDK

Heterozygous *ACAN* mutations were identified in the two probands with moderate skeletal anomalies including platyspondyly, whom were subsequently diagnosed as having SEDK (Table 1). Proband 15 was found to have a mutation in the canonical splice acceptor site of intron 1 (c.-7-2A>C) which is predicted to result in the removal of exon 2, where the initiation codon is located. We demonstrated, using a minigene assay, that this mutation indeed ablated the intron 1 splice acceptor site, thus, confirming the pathogenicity of this variant (Supp. Fig. 4). The second case, proband 16, has a missense variant (VUS) in *ACAN* (p.Thr533Ile). Clinical and radiological characteristics of both probands are shown in Table 4 and Supp. Fig.2, respectively.

DISCUSSION

A total of 16 heterozygous variants were detected throughout *ACAN*, 14 in individuals with short stature, mild skeletal defects and/or facial dysmorphisms (group 1) and two with SEDK (group 2). No other mutation/variant was identified in the skeletal dysplasia patient in the 16 probands which could explain their phenotype. As functional characterization is not

currently feasible for confirming the pathogenicity of the ACAN VUS variants, we compared the clinical features in probands from group 1 with a pathogenic mutation (nonsense, frameshift, splicing, n=5) and those with VUS (missense variants, n=8) (Table 3). Individuals with a pathogenic mutation were shorter (median -3.33 v -2.7 SDS) and 60% had an advanced BA compared to 0% in those with a VUS. Advanced BA:CA was only observed in 3/5 individuals with a pathogenic mutation, thus, two individuals had a BA equal or delayed with respect to the CA. To date, a total of 58 probands and 106 family members (total =164 heterozygous *ACAN* positive individuals with short stature and mild skeletal defects) have been reported in this study and in the literature (Table 5).[4-10]. Although advanced BA is a good indicator for the presence of mutations in *ACAN*, it cannot be the principal selection criteria. The other major difference was that a third of the individuals with a VUS had coxa valga whereas no individual with a pathogenic mutation presented with this clinical feature. Nevertheless, other features such as skeletal defects, facial dysmorphisms and precocious arthropathy or discopathy were present at similar frequencies in both variant classification groups. An interesting observation and in contrast to previous data, brachydactyly was observed in all probands.

After analysing the individuals according to the variant classification, we performed a study of the clinical features of the probands and affected family members from group 1 (n=32; 14 children and 18 adults). No sex or ethnic differences were observed. Only 23% of the children had advanced BA, once again significantly lower than that previously described (Table 5). The degree of short stature was also very variable. Another previously undescribed feature was the presence of mild hip abnormalities in five probands (38%). Parents of two of these probands suffer with osteoarthritis. Osteoarthritis and disc disease were uncommon in our cohort with only three parents having these medical complications (23%) (Table 5).

Clinical heterogeneity occurred in some families, as previously reported in a few cases.[6,10] Proband 3 (p.Ala248Thr- VUS) presents with normal stature although in the lower range (-1.8 SDS), BA equal to CA but has brachydactyly and minor skeletal defects. He has

not yet reached adult height and early growth cessation occurs in this growth disorder. His mother, with the same variant, presents with short stature (-3 SDS), precocious osteoarthritis and discopathy. Thus, the differences in height and clinical presentation are likely to be associated with age. In a similar way proband 12 (p.Glu2426Lys- VUS) presents with short stature (-2.5 SDS), BA equal to CA, mild dysmorphic features and skeletal defects. Her father has the same variant but has normal stature although within the lower limit (-1.79 SDS) and only brachydactyly. This clinical heterogeneity is similarly observed in other skeletal dysplasias such as those associated with heterozygous *SHOX* or *NPR2* mutations.[13]

Two heterozygous *ACAN* variants (1 pathogenic, 1 VUS) were identified in two individuals with SEDK. To date, only one SEDK case with an ACAN mutation in the CS1 domain, has been reported in the literature.[1] Prior to the implementation of NGS patients with this form of spondyloepiphyseal dysplasia were generally tested for mutations in *COL2A1* and if negative, remained molecularly undiagnosed. Thus, further cases may be identified in the future.

Interestingly, proband 6 presented with Madelung deformity and proband 15 and his father, considered to have SEDK, have curved radii and limited elbow extension. Madelung deformity is typically observed in individuals with Léri-Weill dyschondrosteosis (MIM 127300), isolated or due to post-traumatic conditions.[14] SHOX defects have been excluded in all probands. This observation is not that surprising since SHOX binds to SOX5 and SOX6 which along with SOX9 (SOX trio) activate an aggrecan enhancer, thus, participating in common regulatory pathways in chondrogenesis.[15]

The description of our cohort supports the undertaking of a detailed clinical examination and skeletal survey in short stature individuals with suspicion of a mild skeletal dysplasia. Our observation of an association with brachydactyly may help clinicians to request genetic analysis of *ACAN*.

For now, the diagnosis of this dysplasia is paramount for patients and their families. Individuals with *ACAN* mutations are at risk of short stature, early growth cessation and poor pubertal spurt, and other health related problems such as obesity and orthopaedic problems

should be prevented or their effects reduced. Careful monitoring of patients with *ACAN* mutations may help us to identify important genotype-phenotype correlations and to understand their long-term clinical outcomes. Additional familial studies, analysis of larger cohorts, generation of animal models and functional analysis will be also required in order to determine the incidence of ACAN mutations and the pathogenic mechanism(s)[16].

References

1. Gleghorn L, Ramesar R, Beighton P, Wallis G. A mutation in the variable repeat region of the aggrecan gene (AGC1) causes a form of spondyloepiphyseal dysplasia associated with severe, premature osteoarthritis. *Am J Hum Genet.* 2005; 77(3):484-490.
2. Tompson SW, Merriman B, Funari VA, et al. A recessive skeletal dysplasia, SEMD aggrecan type, results from a missense mutation affecting the C-type lectin domain of aggrecan. *Am J Hum Genet.* 2009; 84(1): 72-79.
3. Stattin EL, Wiklund F, Lindblom K, et al. A missense mutation in the aggrecan C-type lectin domain disrupts extracellular matrix interactions and causes dominant familial osteochondritis dissecans. *Am J Hum Genet.* 2010; 86(2):126-137.
4. Nilsson O, Guo MH, Dunbar N, et al. Short Stature, accelerated bone maturation, and early growth cessation due to heterozygous aggrecan mutations. *J Clin Endocrinol Metab.* 2014; 99(8): E1510-E1518.
5. Quintos JB, Guo MH, Dauber A. Idiopathic short stature due to novel heterozygous mutation of the aggrecan gene. *J Ped Endocrinol Metab.* 2015; 28(7-8):927-932.
6. Gkourogianni A, Andrew M, Tyzinski L et al. Clinical characterization of patients with autosomal dominant short stature due to aggrecan mutations.*J Clin Endocrinol Metab*. 2016; 102(2): 460-469.
7. Van der Steen M, Pfundt R, Maas SJWH, Bakker-van Waarde WM, Roelof J. Odink RJ, Hokken-Koelega ACS. ACAN gene mutations in short children born SGA and response to Growth Hormone treatment. *J Clin Endocrinol Metab*. 2017;102(5): 1458-1467.

8. Dateki S, Nakatomi A, Watanabe S et al.Identification of a novel heterozygous mutation of the aggrecan gene in a family with idiopathic short stature and multiple intervertebral disc herniation. *J Hum Genet.* 2017; 62(7):717-721.

9. Hu X, Gui B, Su J et al. Novel pathogenic ACAN variants in non-syndromic short stature patients. *Clin Chim Acta.* 2017;469*:* 126-129.

10. Hauer NN, Sticht H, Boppudi S et al. Genetic screening confirms heterozygous mutations in *ACAN* as a major cause of idiopathic short stature. *Sci Rep.* 2017;7(1): 12225.

11. Barraza-García J, Rivera-Pedroza CI, Hisado-Oliva A et al. Broadening the phenotypic spectrum of POP1-skeletal dysplasias: identification of POP1 mutations in a mild and severe skeletal dysplasia. *Clin Genet.* 2017; 92(1): 91-98.

12. Richards S, Aziz N, Bale S et al. ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015; 17(5):405-424.

13. Hisado-Oliva A, Garre-Vázquez AL, Santaolalla-Caballero F et al. Heterozygous *NPR2* mutations cause disproportionate short stature, similar to Léri-Weil dyschondrosteosis. *J Clin Endocrinol Metab.* 2015;100(8):E1133-1142.

14. Knutson EJ, Goldfarb CA, Madelung deformity. Hand (N.Y).2014; 9(3): 289-291.

15. Aza-Carmona M, Shears DJ, Yuste-Checa P et al. SHOX interacts with the chondrogenic transcription factors SOX5 and SOX6 to activate the aggrecan enhancer. *Hum Mol Genet.* 2011; 20(8): 1547-1559.

16. Gibson BG, Briggs MD. The aggrecanopathies: an evolving phenotypic spectrum of human genetic skeletal diseases. *Orphanet J Rare Dis.* 2016; 11(1):86.

Table 1: Details of the 16 heterozygous ACAN variants present in this cohort in individuals with short stature and mild skeletal defects (probands 1-14) and SEDK (probands 15, 16). Gene and protein domain localization indicated. Conservation, in silico pathogenicity predictions and number of affected family members shown for each mutation. ACMG classification also indicated.

Proband	Variant	Exon	Aggrecan domain	GerpRS	Amino acidconservation	CADD V1.3	SIFT	Polyphen	MutationTaster	gnomAD (MAF %)	Number of affected family members	ACMG classification
1	c.61G>T (p.Glu21*)	2	G1A	5.3	-	35	-	-		-	2	Pathogenic
2	c.371G>A (p.Arg124His)	3	G1A	5.36	High	24.9	Del	Possdam	Dis Caus	Fin:0.0046, Lat: 0.0029, NFE: 0.0017	1	VUS
3	c.742G>A (p.Ala248Thr)	5	G1B	5.36	High	24.9	Del	Probdam	Dis Caus	Eur: 0.0063 Lat: 0.0058 SA: 0.0032 Fin: 0.0011 EA: 0.0053	1	VUS
4	c.903G>C (p.Trp301Cys)	6	G1B´	5.56	High	24.7	Del	Probdam	Dis Caus	-	2	VUS
5	c.1608C>A (p.Tyr536*)	9	G2B	4.41	-	38	-	-	-	-	1	Pathogenic
6	c.1930G>A (p.Gly644Ser)	10	G2B´	5.11	High	25.2	Del	Probdam	Dis Caus	EA: 0.074 Afr: 0.0041 SA: 0.0032 NFE: 0.0008	1	VUS
7	c.1948G>A (p.Val650Met)	10	G2B´	5.11	High	25.4	Del	Prob dam	Dis Caus	NFE: 0.027 Lat: 0.026 SA: 0.0065 Afr: 0.0041	1	VUS
8	c.2218A>T (p.Thr740Ser)	12	KS	5.77	High	22.1	Del	Probdam	Dis Caus	NFE: 0.008	1	VUS
9	c.2369C>G	12	KS	5.77	-	22.1	-	-	-	-	0	Pathogenic

	(p.Ser790*)											
10	c.6142C>G (p.Pro2048Ala)	12	CS2	5.26	High	18. 5	Del	Possdam	Poly	NFE: 0.022 SA: 0.022 Lat: 0.0029	1	VUS
11	c.7269delG (p.Glu2424fs*5)	15	G3	-	-	-	-	-	-	-	2	Pathogenic
12	c.7276G>A (p.Glu2426Lys)	16	G3	5.69	High	35	Del	Probdam	Dis Caus	EA: 0.084 SA: 0.035 Afr: 0.021 NFE: 0.0031 Lat: 0.0029	1	VUS
13	c.7276G>T (p.Gly2426*)	16	G3	5.69	High	56	-	-	-	-	2	Pathogenic
14	c.7342G>A (p.Gly2448Arg)[π]	17	G3	5.31	High	23.5	Del	Probdam	Dis Caus	NFE: 0.026 Afr: 0.016 Lat: 0.014 SA: 0.0032	2	VUS
15	c-7-2A>C*	Intron 1	-	-	-	-	-	-	-	-	1	Pathogenic
16	c.1598C>T p.(Thr533Ile)	9	G2B	4.49	High	23.7	Del	Probdam	Dis Caus	-	1	VUS

The coordinates are according to *ACAN* transcript NM_013227.3. Aggrecan domains: G1 (A, B, B´) and G2 (B, B´) globular domains, chondroitin (CS) and keratin (KS) sulphate attachment regions, selectin-like domain (G3). Del: Deleterious, Tol: Tolerated, Prob dam: Probably Damaging, Poss dam: Possibly Damaging, Dis Caus: Disease causing, Poly: Polymorphism. CADD V1.3 values >14 were classified as deleterious. Patients 1 and 13 were included in the International Aggrecan Consortium (Gkourogianniet al, 2017). The gnomAD MAF: T: Total; NFE: Non-Finnish European; Lat: Latin; Afr: African; EA: East Asian; Fin: Finish; SA: South Asian. Highly conserved amino acid (AlamutV2.10).[π]Splicing tools in Alamut V2.10 predicted that it may affect splicing but a minigene assay did not confirm these

predictions.*Splicing tools predict the ablation of intron 1 canonical splice acceptor site, which was subsequently confirmed using a minigene assay.

Table 2: Clinical characteristics of probands from group 1 with short stature and mild skeletal defects. The total number presenting each clinical feature is based on paediatric cases (n=13), thus, excluding adult patient 14.

						Anthropometric data				Facial dysmorphisms										Skeletalfindings							
Proband	Geographic origin	Mutation cDNA (protein)	Age (years)	Gender (F/M)	SGA (Y/N)	Height (SDS)	Target height (SDS)	SH/H	BA v CA	Macrocephaly	Frontal bossing	Mid-facial hypoplasia	Depressed nasal bridge	Broadnose and philtrum	Thinlips	High archedpalate	Hypertelorism	Epicantus	Triangular face	Pubertyspurt (Menarche)	Brachydactyly	Hyperlordosis	Coxa valga	Otherskeletalfindings	Precocious arthropathy or discopathy in family member	Affectedfamilymembers (n)	Affected family members height (SDS)
1	Sp	c.61G>T (p.Glu21*)	4.5	F	N	-3.5	-3.0	0.56	+1.5	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	2	-3 -1.5
2	Sp	c.371G>A (p.Arg124His)	8.0	F	N	-3.7	-2.4	0.54	-2	-	-	-	+	+	+	-	-	-	-	-	+	+	+	-	-	1	-2.7
3	Ec	c.742G>A (p.Ala248Thr)	14.5	M	N	-1.8	-2.6	0.52	=	-	-	-	-	-	-	-	-	-	-	NA	+	-	+	Mildly flattened capital femoral epiphyses, slender femora	Osteoarthritis and discopathy in mother	1	-3
4	Sp	c.903G>C (p.Trp301Cys)	7.0	F	N	-3.5	-3.6	NA	=	-	+	+	+	-	-	-	-	-	-	-	+	-	+	Slender femora Osteochondral knee mild defects	Familiar osteochondritisdissecans in affected father and uncle	2	-4.4 -3.8
5	Sp	c.1608C>A (p.Tyr536*)	4.5	F	N	-3.5	-3.0	0.6	+1	-	-	-	-	-	-	-	-	-	-	-	+	-	-	Osteochondral knee mild defects	Osteoarthritis in father	1	-4.5
6	Ch	c.1930G>A (p.Gly644Ser)	16.0	F	N	-2.1	-2.0	0.57	=	-	-	-	-	-	-	-	-	-	-	NA	+	-	-	Madelung deformity, short femoral necks, mild epiphyseal knee defects	-	1	-3.7
7	Sp	c.1948G>A (p.Val650Met)	12.0	M	N	-2.6	-1.4	0.52	-3	-	-	-	+	-	+	-	-	+	-	-	+	-	-	-	-	1	-2.6
8	Sp	c.2218A>T (p.Thr740Ser)	3.0	M	N	-3.2	NA	0.57	-1	-	+	+	-	-	-	+	-	-	+	-	+	-	-	-	-	1	-3.7

9	Sp	c.2369C>G (p.Ser790*)	14.5	M	Y	-2.2	-0.6	0.52	+2	-	+	-	-	+	-	-	+	-	-	NA	+	+	-	-	-	-	-
10	Sp	c.6142C>G (p.Pro2048Ala)	12.5	F	Y	-2.2	-2.1	NA	+1	-	-	-	-	-	-	-	-	-	-	NA	+	-	-	-	-	1	-2.3
11	Sp	c.7269delG (p.Glu2424fs*5)	1.5	M	N	-3	-3.2	NA	+2	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	2	-5.8 -3.8
12	Sp	c.7276G>A (p.Glu2426Lys)	8.5	F	Y	-2.5	-1.8	NA	=	-	+	-	+	-	-	-	-	-	-	-	+	-	-	Cone-shaped epiphysis	-	1	-1.79
13	Sp	c.7276G>T (p.Gly2426*)	18.0	M	Y	-4.3	-3.4	0.54	=	+	-	-	-	-	-	-	-	-	-	Poor	+	-	-	Short femoral neck	-	2	-5.0 -3.7
14	Sp	c.7342G>A (p.Gly2448Arg)	46	F	NA	-3.7	-4.3	0.52	NA	-	+	+	-	-	-	-	-	-	-	Poor (11y)	+	-	-	-	-	2	-4.3 -3.5
		TOTAL (paediatric cohort, n=13)	**Median 10.2**	**7F 6M**	**4**	**-2.9**	**-2.5**	**0.54**	**3 Adv 10 Eq/Del**	**2**	**5**	**3**	**4**	**2**	**2**	**2**	**1**	**1**	**1**	**-**	**13**	**3**	**3**	**6**	**3 families**	**18**	**Median -3.77**

Geographic origin: Sp: Spain, Ec: Ecuador, Ch: China. Gender: M: Male; F: Female. SGA: N: No; Y: Yes. SH/H: Sitting height/height. BA vs CA: BA: Bone age; CA: Chronological age; +: BA>CA. =: BA=CA (equal), -: BA<CA. BA>1y CA: Advanced (Adv). BA +/- 1y CA: Equal, BA<1y: Delayed, Eq/Del: Equal or Delayed. NA: Not available. Facial dysmorphisms and skeletal findings: +: Present; -: Absent

Table 3. Comparison of clinical features observed in group 1 child probands (n=13) with pathogenic mutations (n=5) and VUS (n=8) as classified by ACMG.

Variant type (n=number of probands)	Inheritance/ De novo	SGA	Median height SDS	Target heightSDS	SH/H	BA v CA (Adv or Equal/delayed) (%)	Facial dysmorphisms (%)	Brachydactyly (%)	Hyperlordosis (%)	Coxa valga (%)	Otherskeletalfindings (%)	Precocious arthropathy or discopathy in family member(%)	Affected family members height SDS
Pathogenic mutation (nonsense/ frameshift) (n=5)	4 AD 1 de novo	2	-3.33	-2.64	0.55	3 Adv (60%) 2 Equal/Delayed (40%)	4 80%	5 100%	2 40%	0 0%	2 40%	1 20%	-5.8/-1.5
VUS (missense) (n=8)	8 AD	2	-2.7	-2.27	0.544	8 Equal/Delayed (100%)	5 62%	8 100%	1 12%	3 37%	4 50%	2 25%	-4.4/-1.79

AD: Autosomal dominant, SGA: Small for gestational age, SH/H: Sitting height/height, BA vs. CA: Bone age v Chronological age, Adv: Advanced BA (>1 year),Equal/Delayed: BA equal to CA or delayed (<1 year).

Table 4. Clinical and genetic features of probands 15 and 16, both with SEDK.

Proband	Geographic origin	Mutation	Gender (M/F)	Age (years)	SGA (Y/N)	Height SDS	Target height SDS	SH/H	BA v CA	Facial dysmorphisms	Morphologicfindings	Skeletalfindings	Affectedfamilymembers
15	Pt	c.-7-2A>C (intron 1)	M	7.5	Y	-4.20	-3.6	0.53	-2	No	Stocky appearance, short neck, limited elbow extension	Brachydactyly, curved radius, mild platyspondyly	Father´s height -3.66 SDS Stocky appearance, obese, brachydactyly with shortened metacarpals, curved radius, limited extension of elbows platyspondyly, coxarthrosis,
16	Sp	c.1598C>T (exon 8) p.(Thr533Ile) (G2 domain)	M	10	N	-0.76	-0.54	NA	=	No	Obesity, short trunk, waddling gait	Bilateral irregular femoral epiphyses, mild thoracic platyspondyly	Father´s height -0.38 SDS, limp.

Geographic origin: Pt: Portugal, Sp: Spain. BA vs CA: BA: Bone age; CA: Chronological age; +: BA>CA (Advanced), =: BA=CA (Equal), -: BA<CA (delay); NA: Not available.

Table 5. Overview table of main molecular and clinical characteristics of individuals with heterozygous *ACAN* variants/mutations and short stature (excluding SEDK and Familial osteochondritis dissecans) reported in this current study and previously in the literature.

Reference (ref number)	Number of patients (Children/adults) from X families	SGA	Range of height (SDS) Children Adults	Advanced BA in children	Frontal bossing	Flat nasal bridge	Mid facial hypoplasia	Brachydactyly	Short thumbs, and/or short first metacarpal	Broad great toes	Hyperlordosis	Hip anomalies	Mild osteochondral knee defects	Earlygrowthcessation (adults)	Early-onset arthritis /OD (families)	Intervertebral disc disease (families)
Nilsson et al, 2014 (ref 4)	14 (5/9)* 3 families	2/4	-4/-1.2 -3.8/-2.3	3/5	NR	NR	6/9	6/9	3/9	NR	NR	NR	NR	5/5	1/3	0/3
Quintos et al, 2015 (ref 5)	3 (1/2)** 1 family	0/1	-2.7 -4.7/-2.6	1/1	NR	NR	1/3	NR	NR	NR	NR	NR	NR	2/2	0/1	0/1
Manouk van der Steen et al, 2016 (ref 7)	10 (4/6) 3 families	3/3	-3.7/-2.4 -5.4/-3.7	3/4	NR	NR	9/10	NR	3/10	6/10	3/10	NR	NR	NR	3/3	0/3
Gkourogianni et al, 2017 (International Aggrecan Consortium) (ref 6)	102 ¥ (32/70) 20 families	NR	-4.2/-0.6 -5.9/-0.9	19/23 probands	2/32 probands	8/20	8/20	5/20	3/20	NR	NR	NR	NR	23/70	13/20	8/20
Dateki et al, 2017 (ref 8)	4 (2/2) 1 family	0/2	-2.7/-2.5 -3.1/-3	2/2	NR	NR	3/4	NR	NR	NR	1/4	NR	NR	NR	0/1	1/1
Hu et al, 2017 (ref 9)	9(3/6) 3 families	1/1	-4.3/-2.9 -5.4/-2.9	0/3	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	0/3	0/3

Hauer et al, 2017 (ref 10)	11(6/5) 6 families	1/6	-3.9/-2 -3.8/-1.8	2/5	3/6	NR	NR	3/6	2/6	2/6	NR	1/6	NR	NR	1/6	NR
Current study	32 (14/18)*** 14 families	4/13	-4.3/-1.86 -5.4/-1.79	3/13	5/13	4/13	3/13	12/13	11/13	NR	3/13	5/13	3/13	6/6	3/14	1/14
SUMMARY[§]	**164 (59/105) 45 families**	**11/40**	**-4.7/-0.6 -5.9/-0.9**	**28/45**	**9/49**	**4/13**	**22/39**	**19/28**	**19/38**	**8/16**	**7/27**	**5/13**	**3/13**	**27/74**	**20/45**	**10/39**

Ns: nonsense mutation, Fs: frameshift mutation, Spl: splice donor site variant, Mis: missense variant, NR: not reported.* Three families are included in the International Aggrecan Consortium (5 children, 9 adults)[6]; **The family reported in this study is included in the International Aggrecan Consortium (1 child, two adults)[6]; *** Two families (two children, two adults) are included in the International Aggrecan Consortium [6]. Results shown here are related to the paediatric cohort. ¥ Gkourogianni et al. 2017, reported a large cohort of probands and relatives (International Aggrecan Consortium). Clinical and molecular characteristics are related to the 20 families, not to individuals as with the other studies. In addition, data was compiled from both adults and children. We have attempted to separate this data when possible. [§] Combined data from this current study and previous studies. The numbers documented for type of mutation, early onset arthritis/OD and intervertebral disc disease data are given as the number of families whilst the other characteristics are totals for the number of individuals (probands or adults). Once again, most of the data from the International Aggrecan Consortium (¥ ref 6) cannot be summarized as the data is presented for families rather than individuals. Patients included in three earlier studies (*, **, ***) have not been duplicated in this data.

Figure Legends

Figure 1: Structure of ACAN and the locations of the variants identified in the 16 probands. The G1 region encoded by exons 3-6, the IGD region by exon 7, the G2 region by exons 8-10, the GAG (KS-CS1-CS2) attachment region encoded by exons 11-12 while the G3 region encoded by exons 13-19. Missense variants written in black whilst premature truncating (nonsense, frameshift, splicing) mutations written in red. Mutations corresponding to SEDK written in blue. Families with osteoarthritis and/or discopathy underlined.

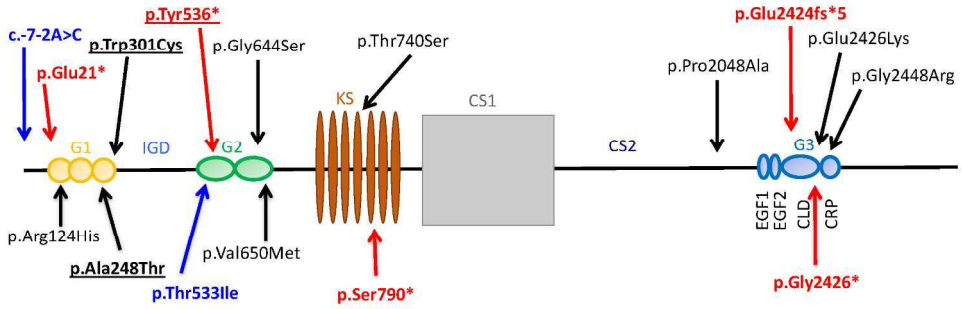
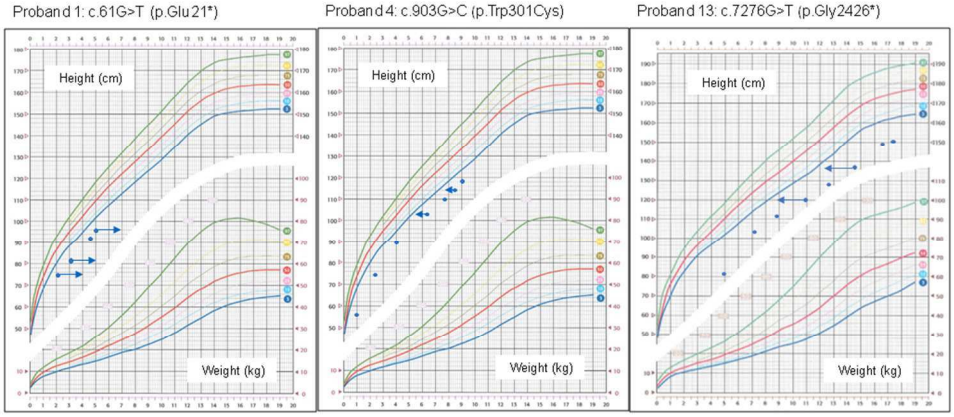


Figure 1: Structure of ACAN and the locations of the variants identified in the 16 probands. The G1 region is encoded by exons 3-6, the IGD region by exon 7, the G2 region by exons 8-10, the GAG (KS-CS1-CS2) attachment region encoded by exons 11-12 while the G3 region is encoded by exons 13-19. Missense mutations are written in black. Truncating (nonsense and frameshift) mutations are written in red. Mutations corresponding to Kimberley type dysplasia are written in blue. Families with osteoarthritis and/or discopathy are underlined.

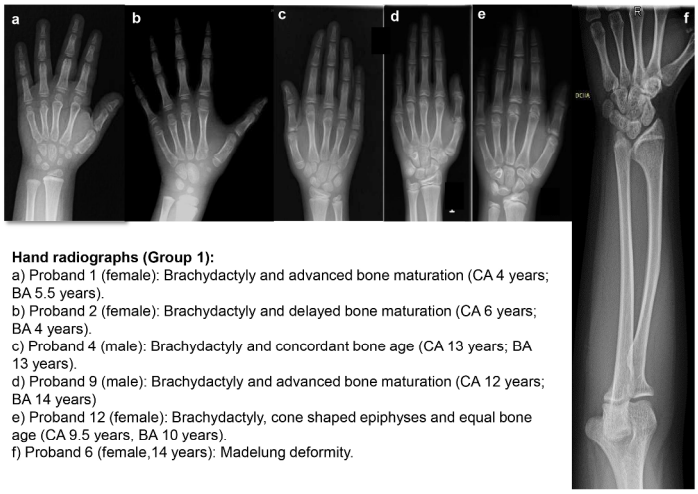
338x190mm (200 x 200 DPI)

Supp Fig 1: Growth charts of 3 from 13 probands from group 1. The closed circles indicate height at the chronological ages whilst the arrows indicate the bone age for those chronological ages. Proband 1 has advanced bone age, proband 4 has equal bone age and proband 13 has delayed bone age.



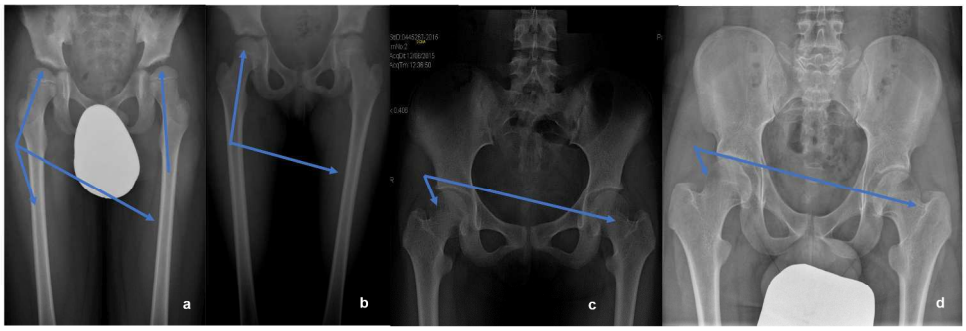
285x190mm (96 x 96 DPI)

Supp Fig 2: Main skeletal findings in probands from both group 1 and 2.



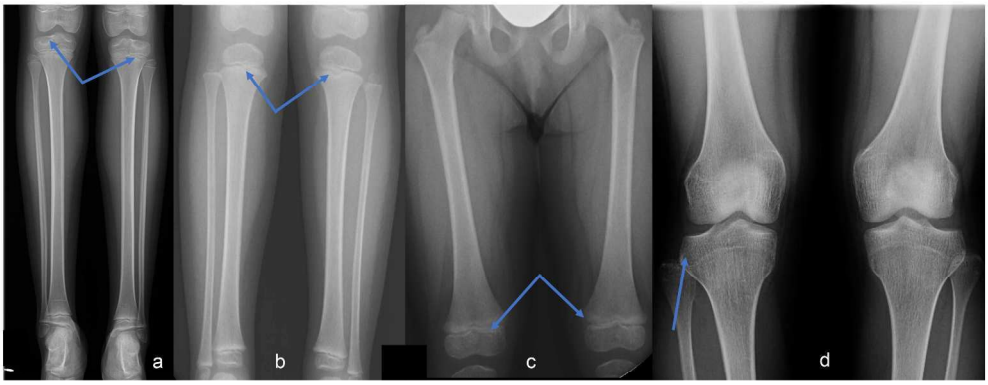
Hand radiographs (Group 1):
a) Proband 1 (female): Brachydactyly and advanced bone maturation (CA 4 years; BA 5.5 years).
b) Proband 2 (female): Brachydactyly and delayed bone maturation (CA 6 years; BA 4 years).
c) Proband 4 (male): Brachydactyly and concordant bone age (CA 13 years; BA 13 years).
d) Proband 9 (male): Brachydactyly and advanced bone maturation (CA 12 years; BA 14 years)
e) Proband 12 (female): Brachydactyly, cone shaped epiphyses and equal bone age (CA 9.5 years, BA 10 years).
f) Proband 6 (female,14 years): Madelung deformity.

285x190mm (300 x 300 DPI)



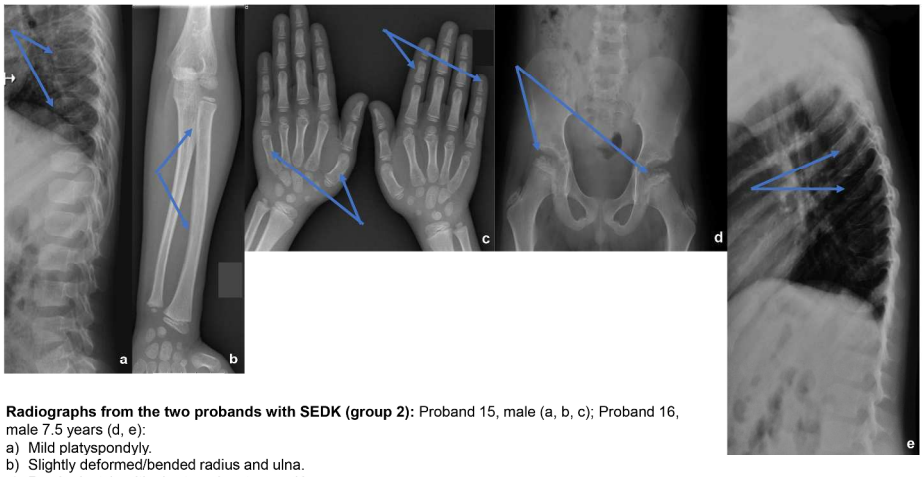
Hip radiographs:
a) Proband 3 (male, 14 years): Coxa valga, mildly flattened capital femoral epiphyses, slender femora.
b) Proband 4 (female, 7 years): Coxa valga, slender femora.
c) Proband 6 (female, 14 years): Short femoral necks.
d) Proband 13 (male, 18 years): Short femoral necks.

285x190mm (300 x 300 DPI)



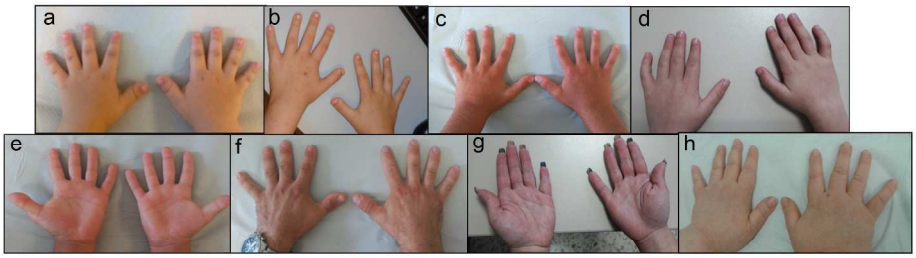
Knee radiographs:
a) Proband 4 (female 7 years): Osteochondral knee mild defects.
b) and c) Proband 5 (female, 4.5 years): Osteochondral knee mild defects.
d) Proband 6 (female, 14 years): Mild epiphyseal knee defects.

285x190mm (300 x 300 DPI)



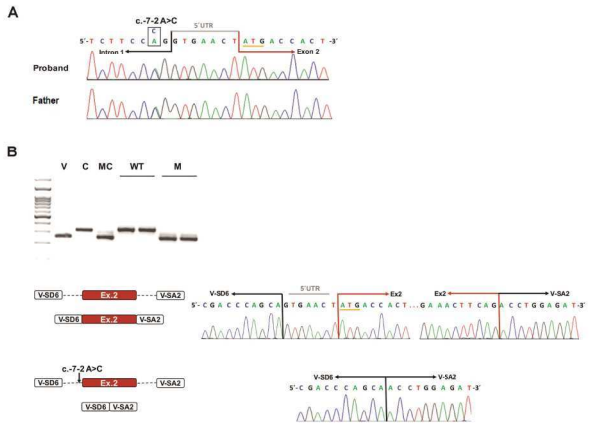
Radiographs from the two probands with SEDK (group 2): Proband 15, male (a, b, c); Proband 16, male 7.5 years (d, e):
a) Mild platyspondyly.
b) Slightly deformed/bended radius and ulna.
c) Brachydactyly with shortened metacarpal bones.
d) Irregularity in capital femoral epiphysis.
e) Moderate platyspondyly and kyphosis.

285x190mm (300 x 300 DPI)



Supp Fig 3: Hand photographs of various probands and parents from group 1, all showing brachydactyly.
Upper panel: Dorsal images of hands from paediatric cohort of patients with heterozygous *ACAN* mutations.
a) Patient 1, b) Patient 4, c) Patient 7, d) Patient 8.
Lower panel: Ventral and dorsal images of hands from parents with heterozygous *ACAN* mutations:
e and f): Proband´s 7 father, g) Proband´s 8 mother, h) Proband´s 13 mother.

285x190mm (300 x 300 DPI)



Supp. Figure 5. Genetic and functional analysis for the *ACAN* c.-7-2A>C variant. A) Sequence chromatogram of the c.-7-2 A>C variant detected in exon 2 of *ACAN* in the proband and his father. B) In vitro assay for the splicing mutation c.-7-2A>C using a minigene assay. On top panel, gel electrophoresis of splicing products generated from the amplification of cDNA isolated from transfected HEK293 cells (the same results were observed with COS7, data not shown) using two vector primers (PSD6 and PSA2) for empty pSPL3vector (band V), wild-type control (band C), mutant splicing control (band MC), ACAN wildtype c.-7-2A (band WT) and variant, c.-7-2C, in the non-coding portion of exon 2 (band M). On the bottom panel, schematic representation and chromatograms of the sequences obtained for the splicing PCR products for *ACAN* wild type (c.-7-2A) and variant (c.-7-2C). Firstly, sequencing of band D corresponds to the wild-type exon 2 of *ACAN*. Secondly, sequencing of band E corresponds to an aberrant transcript which demonstrated that the exon 2 splice acceptor site was ablated, thus, only the pSPL3vector sequence is observed (V-SD6/V-SA2).

285x190mm (300 x 300 DPI)