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GDF5 mutation case report and a systematic review of molecular and clinical spectrum: Expanding current knowledge on genotype-phenotype correlations

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ABSTRACT

Introduction: Brachydactyly is a bone development abnormality presenting with variable phenotypes and different transmission patterns. Mutations in *GDF5* (*Growth and Differentiation Factor 5*, MIM *601146) account for a significant amount of cases. Here, we report on a three-generation family, where the proband and the grandfather have an isolated brachydactyly with features of both type A1 (MIM #112500) and type C (MIM #113100), while the mother shows only subtle hand phenotype signs.

Materials and methods: Whole Exome Sequencing (WES) was performed on the two affected individuals. An in-depth analysis of *GDF5* genotype-phenotype correlations was performed through literature reviewing and retrieving information from several databases to elucidate *GDF5*-related molecular pathogenic mechanisms.

Results: WES analysis disclosed a pathogenic variant in *GDF5* (NM_000557.5:c.157dup; NP_000548.2:p.Leu53Profs*41; rs778834209), segregating with the phenotype. The frameshift variant was previously associated with Brachydactyly type C (MIM #113100), in heterozygosity, and with the severe Grebe type chondrodysplasia (MIM #200700), in homozygosity. In-depth analysis of literature and databases allowed to retrieve *GDF5* mutations and correlations to phenotypes. We disclosed the association of 49 *GDF5* pathogenic mutations with eight phenotypes, with both autosomal dominant and recessive transmission patterns. Clinical presentations ranged from severe defects of limb morphogenesis to mild redundant ossification. We suggest that such clinical gradient can be linked to a *continuum* of GDF5-activity variation, with loss of GDF5 activity underlying bone development defects, and gain of function causing disorders with excessive bone formation.

Conclusions: Our analysis of *GDF5* pathogenicity mechanisms furtherly supports that mutation and zygosity backgrounds resulting in the same level of GDF5 activity may lead to similar phenotypes. This information can aid in interpreting the potential pathogenic effect of new variants and in supporting an appropriate genetic counseling.

1. Introduction

Brachydactyly (BD), literally "the shortness of the fingers and toes", can occur either as an isolated malformation or as a part of a complex disorder, with a large number of associated syndromes [1]. It was described as the first human dominant trait in 1903 [2], and since then, different classifications have been proposed. The current classification, proposed by Temtamy and McKusick [3], expands the previous one (by Julia Bell, 1951) [4] and classifies brachydactyly into five types (BDA–BDE) and three subgroups (BDA1–BDA3), based on the phalangeal digital segments involvement and features affecting metacarpal bones [1]. Radiographs are essential to assess specific bone shortening

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and morphology, identifying the type of brachydactyly, as well as any associated malformation.

In 2001, *IHH* (*Indian Hedgehog,* MIM *600726) was the first gene whose mutations have been associated with BDA1 [5]. Since then, a network of genes directing the development of digits and joints has been uncovered, suggesting to consider brachydactylies as a "molecular disease family" [1].

In this context, many genes acting in the BMP (Bone Morphogenetic Proteins) pathway are mutated in different forms of brachydactyly, *i.e. BMPR1B* (*Bone Morphogenetic Protein Receptor 1B*, MIM *603248) in BDA1 and BDA2, *ROR2* (*Receptor Tyrosine Kinase-Like Orphan Receptor 2*, MIM *602337) in BDB1, and *HOXD13* (*Homeobox D13*, MIM *142989) in BDD and BDE [1,6]. *GDF5* (*Growth and Differentiation Factor 5*, MIM *601146) encodes for a soluble growth factor belonging to this pathway. Reported pathogenic mutations in *GDF5* cause BMP signaling dysregulation and different types of brachydactyly [1].

In recent years, Next Generation Sequencing (NGS) technologies have enabled the identification of several disease-causing genes [7], uncovering the molecular bases underlying several limb phenotypes, including brachydactylies. Their broad phenotypic spectrum makes the classification based solely on clinical features challenging.

Here, we report on a three-generation family whose members showed a phenotype with features of both BDA1 (MIM #112500) and BDC (MIM #113100). Whole Exome Sequencing (WES) allowed to detect a pathogenic variant in *GDF5* gene, segregating with the trait.

Moreover, as a growing number of *GDF5* pathogenic mutations has been associated with a broad clinical spectrum, we propose an in-depth analysis of *GDF5* mutations reported in literature and related disorders, highlighting the complex genotype-phenotype correlations.

2. Materials and methods

2.1. Study subjects

We studied a three-generation family with recurrence of an isolated form of brachydactyly. Detailed information on pedigree, anamnesis and clinical assessment were collected for all subjects (I:1, I:2, II:1, II:2, III:1, III:2, III:3; Fig. 1). Hands and feet radiographs were obtained for I:1, II:2 and III:2. Informed consents for DNA storage and genetic analyses were obtained for the propositus (III:2) and all the analyzed family members (I:1, II:1, II:2, III:1, III:3; Fig. 1); permission to publish photographs was given for all subjects reported in this work. All clinical and genetic studies were conducted according to the ethical principles defined in the Declaration of Helsinki. Genomic DNA was extracted from circulating leukocytes, saliva or buccal mucosa cells using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany), as per manufacturer's protocol.

2.2. Whole Exome Sequencing

WES of subjects I:1 and III:2 was performed on DNA extracted from

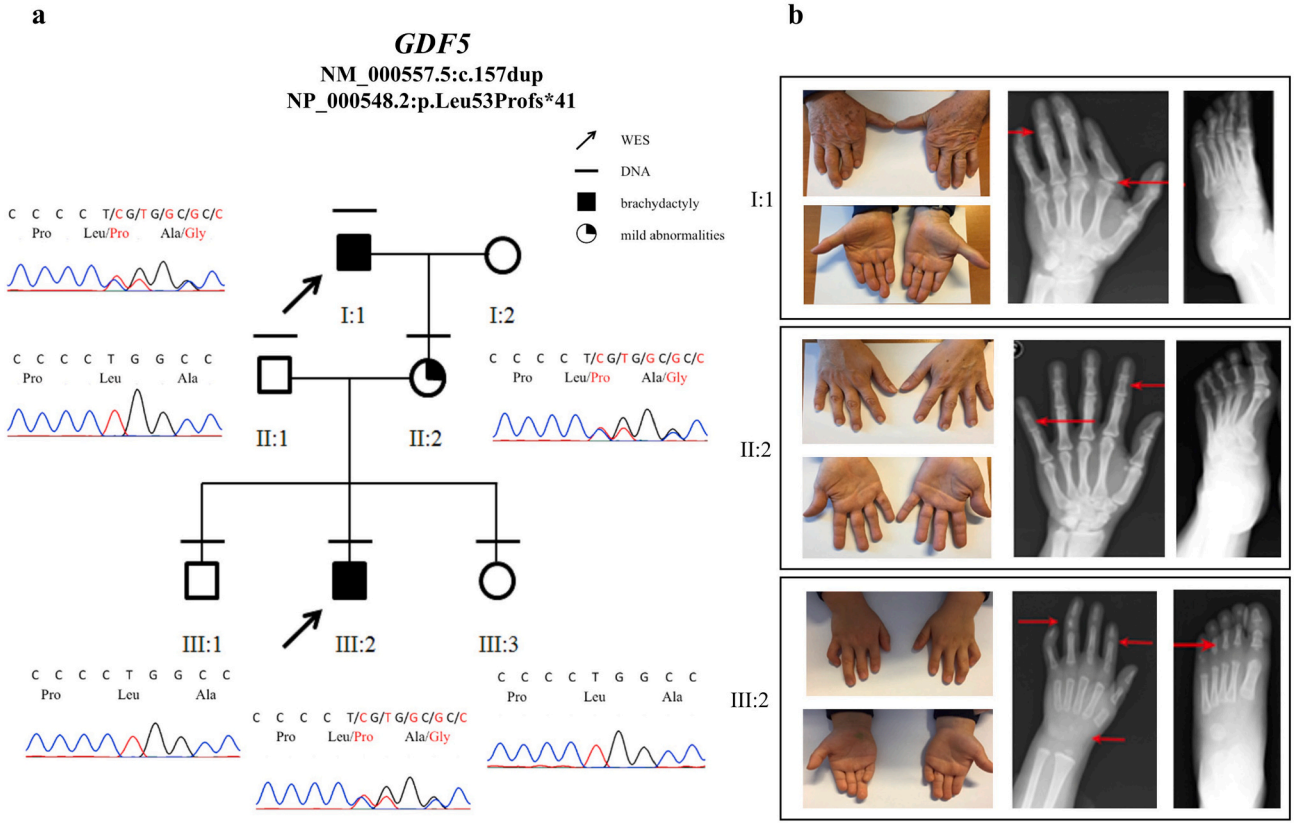

Fig. 1. Pedigree and hands pictures.
a) Pedigree of the family. Black lines indicate individuals for whom DNA was collected; arrows indicate individuals who underwent WES. Electropherograms showing genotypes of *GDF5* (c.157dup) variant are reported. b) Photos and radiographs of the hands of I:1, II:2, and III:2 are shown. Arrows point at key radiological features. The proband's (III:2) left hand radiograph shows absent (digits 2,5) or severely hypoplastic (digit 3) intermediate phalanges. The 4th finger has short dysplastic middle phalanx. 2nd finger displays ulnar deviation. Short first metacarpal is evident and there is a considerable delay in carpal bone ossification. The proband's left foot shows short distal phalanges, absent or severely hypoplastic intermediate phalanges, dysmorphic proximal phalanges. Right single palmar crease is present. The mother's (II:2) left hand shows subtly shortened middle phalanx of 2nd and 5th fingers. The mother's left foot shows hallux valgus and 5th finger clinodactyly. Left hand single palmar crease is present. The grandfather's (I:1) left hand shows absent intermediate phalanx of the 2nd finger, clinodactyly of the 5th finger, short first metacarpal, ulnar deviation of the 2nd finger. The grandfather's left foot shows short distal phalanges, absent or severely hypoplastic intermediate phalanges, 5th finger clinodactyly. Both hand and foot show osteoarthritic changes.

circulating leukocytes (Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Foggia, Italy). Targeted enrichment was performed using the SureSelect Clinical Research Exome kit (Agilent, Santa Clara, CA, USA). The libraries were sequenced using a NextSeq platform (Illumina, San Diego, CA, USA). Adaptors sequences were removed and the reads were aligned to the reference genome (UCSC GRCh37/hg19) using the Burrows-Wheeler Aligner (BWA v0.7.12) [8]. The duplicated reads were removed using Picard tool (http://broadinstitute.github.io/picard). The variant calling and recalibration steps were performed using the Genome Analysis Toolkit (GATK v3.7) [9]. Single Nucleotide Variants (SNVs) and small insertions/deletions (indels) were identified using the GATK HaplotypeCaller tool, according to the GATK's best practices [9]. Variants of subjects III:2 and I:1 were combined in a single file. Functional annotation of variants and genes was performed using ANNOVAR (July 2017 release) [10] and dbNSFP (v3.5a) [11]. High-quality variants (according to GATK hard filters) with an effect on the coding sequence and in splice site regions (+10 bp) were maintained. These variants were filtered retaining only those not annotated or with a minor allele frequency (MAF) $\leq$ 4% in gnomAD v2.0.2 [12] (the most frequent forms of brachydactyly have a frequency of 2%). The functional impact of variants was predicted by CADD v1.6 (Combined Annotation Dependent Depletion) [13], using 10 as a threshold value. Variant classification was based on criteria provided by the American College of Medical Genetics and Genomics (ACMG) [14].

2.3. Variants prioritization and validation

Shared genetic variants between the two affected subjects were prioritized based on the transmission pattern, hypothesizing an X-linked or an autosomal dominant transmission model with variable expressivity. To improve the prioritization step, we used Phenolyzer [15] including the following Human Phenotype Ontology (HPO) terms: "brachydactyly" (HP:0001156), "delayed ossification of carpal bones" (HP:0001216), "single transverse palmar crease" (HP:0000954), "long proximal phalanx of finger" (HP:0006127), "short metacarpal" (HP:0010049), "aplasia/hypoplasia of the middle phalanges of the hand" (HP:0009843), "abnormality of the distal phalanges of the toes" (HP:0010182), "abnormality of the middle phalanges of the toes" (HP:0010183) and "clinodactyly of the 5th finger" (HP:0004209).

The selected candidate variant was validated using Sanger sequencing approach. Genomic DNA was amplified by using GoTaq G2 Flexi DNA polymerase (Promega, Madison, WI, USA) and custom primers (forward: 5′-CTGGATACGAGAGCATTTCCAC-3′; reverse: 5′-CTCCCTTTGCCCTGGCATTG-3′; annealing temperature: 62 °C). The amplicon was checked through 2% agarose gel electrophoresis and purified using MSB Spin PCRapace (Stratec Molecular, Berlin, Germany). Sanger sequencing was performed using the ABI BigDye Terminator Sequencing Kit (v3.1) and run on a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence electropherograms were analyzed using ChromasPro (v1.7.5; Technelysium Pty Ltd., Brisbane, Australia).

2.4. Literature review methods

We searched the PubMed (www.ncbi.nlm.nih.gov/pubmed) database for "*GDF5* OR *CDMP1*" AND "mutation OR variant" and the data-mining tool MARRVEL v1.2 [16] for "*GDF5*", and analyzed results according to PRISMA guidelines [17]. *GDF5* variants were also annotated by consulting the following resources: OMIM (www.omim.org), HGMD (www.hgmd.cf.ac.uk/ac/index.php) [18], ClinVar (www.ncbi.nlm.nih.gov/clinvar) [19], DECIPHER (https://decipher.sanger.ac.uk) [20]. *GDF5* pathogenic variants were reported based on the GRCh37/hg19 version of the human genome. Variants were annotated with information on protein domain from Uniprot (P43026; https://www.uniprot.org), deleterious effect using CADD, variant identifiers from dbSNP, allele frequency as reported by gnomAD (v2.0.2), HGMD mutation class, MIM code of the associated phenotype, allele origin (inherited or *de novo*) and zygosity status, related phenotypes, reference paper and functional studies, when available in literature. For frameshift variants, the resulting downstream stop codon was predicted using the ExPASy Translate tool (www.expasy.org) [21]. Variant nomenclature was updated to the latest HGVS v20.5 (Human Genome Variation Society, www.hgvs.org) [22] nomenclature guidelines for nucleotide change on cDNA and amino acid substitution (reference transcript: NM_000557.5; reference protein: NP_000548.2).

3. Results

3.1. Clinical presentation

We report on an Italian family of three generations, referred to the Clinical Genetics Unit of Policlinico Umberto I Hospital (Rome, Italy). The proband (III:2, Fig. 1) was a 5-year-old boy with isolated brachydactyly, with both hands showing remarkable shortening of digits 2–5 and bilateral 5th-finger clinodactyly (Fig. 1). The 4th finger of the right hand appeared noticeably longer than the others. In addition, a single palmar crease was evident in the right hand, and 2nd, 3rd, 5th fingers lacked distal interphalangeal crease bilaterally. Radiographs showed brachydactyly with features of both BDA1 and BDC, with hypoplastic or absent intermediate phalanges, short first metacarpal and ulnar deviation of the 2nd finger (Fig. 1). The proband had two siblings (III:1, III:3) presenting normal ranges for height and normal limb morphology. The 43-year-old mother (II:2) had apparently normal fingers with no remarkable digit anomalies upon clinical inspection, but with single palmar crease on her left hand and X-ray findings of mild shortening of 2nd and 5th digit middle phalanges without any further anomaly (Fig. 1).

The 76-year-old grandfather (I:1) showed a similar clinical and radiological hand phenotype of the proband (Fig. 1), *i.e.* short first metacarpal, absent intermediate phalanx of the 2nd finger, and clinodactyly of the 5th finger. Both hands and feet showed normal palmar creases. Osteoarthritic changes were detectable. Both mother (II:2) and grandfather (I:1) underwent total body X-ray examination, confirming isolated brachydactyly. The proband (III:2) did not undergo further X-ray examination for radiation protection. Abdominal ultrasound showed no further anomalies of the three subjects.

3.2. WES data analysis

Exome sequencing experiments resulted in adequate coverage and depth. 61840 total variants shared between the two affected subjects were obtained and subsequently filtered. We firstly retained 13579 high-quality variants with an effect on the coding sequence and splice site regions. Variants were then filtered, considering only those not annotated or annotated with unknown/or $\leq$4% MAF in gnomAD (v2.0.2) database (*i.e.* 1644). Among those variants, we considered only the 1077 variants with a CADD score $\geq$ 10. According to different inheritance models (X-linked recessive and autosomal dominant), 11 shared variants on the X chromosome and 261 shared heterozygous autosomal variants were identified. The filtering and prioritization steps allowed to identify a single nucleotide insertion causing a frameshift effect in the *GDF5* gene (NM_000557.5:c.157dup; NP_000548.2:p.Leu53Profs*41). This variant had a CADD score of 23.2 and was annotated in gnomAD (v2.1.1) with a frequency of 0.0026%, and in dbSNP (v151, rs778834209) with a frequency of 0.012%. The frameshift mutation is annotated in ClinVar database (VCV000817904) and classified as pathogenic, and in HGMD (CI025313) as 'disease mutation' for BDC [23], with Grebe type chondrodysplasia annotated as 'additional phenotype' [24]. Based on the ACMG classification guidelines, the variant can be classified as pathogenic. The variant was reported in literature as 158insC and as c.157_158dupC [23,24].

Sanger sequencing confirmed the occurrence of the *GDF5* variant in heterozygosity in subjects I:1, II:2 and III:2, and excluded its presence in

II:1, III:1, III:3 (Fig. 1). No further variant possibly related to the phenotype and segregating with the trait was identified. Moreover, no putative variant/s that could represent a candidate genetic modifier, *e.g.* coding variant in a gene of the BMP and related pathways, have been detected.

3.3. Literature review

Literature search, to retrieve *GDF5* mutations and correlations to phenotypes, yielded n = 134 papers matching search criteria. Among them, n = 64 full-text articles describing *GDF5* mutations causing phenotypes with Mendelian inheritance patterns and providing clinical descriptions were assessed for eligibility and included in the review. The remaining papers were excluded because they did not report mutations or variants involved in Mendelian-inheritance phenotypes or did not feature relevant clinical and/or molecular information.

3.3.1. GDF5 pathogenic mutations and related phenotypes

Literature revision disclosed the association of *GDF5* mutations with eight phenotypes with Mendelian inheritance [Grebe type chondrodysplasia (MIM #200700), Hunter-Thompson type acromesomelic dysplasia (MIM #201250), Brachydactyly types A1 (MIM #112500), A2 (MIM #112600) and C (MIM #113100), Proximal Symphalangism type 1B (MIM #615298), Multiple Synostoses syndrome type 2 (MIM #610017), and Du Pan syndrome (MIM #228900)].

Overall, 49 pathogenic mutations were identified in *GDF5* gene, including small indels, causing frameshift or in-frame mutations, and single nucleotide variants causing missense or nonsense mutations (Fig. 2). The complete list of allelic variants, the corresponding phenotype, the zygosity status, the predicted deleterious effect (CADD v1.6 score) and protein domain localization are reported in Supplementary Table 1.

Phenotypes caused by *GDF5* mutations can be classified in three categories, summarized in Table 1.

The first category includes acromesomelic dysplasias, three very rare recessive severe disorders characterized by markedly defective limb development with proximal-distal gradient of increased involvement, *i.e.* Grebe type chondrodysplasia, Hunter-Thompson acromesomelic chondrodysplasia, and Du Pan syndrome. Grebe type chondrodysplasia (MIM #200700) and Hunter-Thompson acromesomelic chondrodysplasia (MIM #201250) are characterized by disproportionate short stature, marked shortening and deformities of the limbs (longer in the Hunter-Thompson type acromesomelic chondrodysplasia) without axial or craniofacial anomalies [24]. Lower limbs are more severely affected and hands and feet present with fingers and toes appearing as ball-shaped cutaneous bulges in Grebe type chondrodysplasia and showing marked BDC features in Hunter-Thompson type acromesomelic chondrodysplasia [25].

Du Pan syndrome or Fibular Hypoplasia and Complex Brachydactyly (MIM #228900) is characterized by a milder phenotype with autosomal

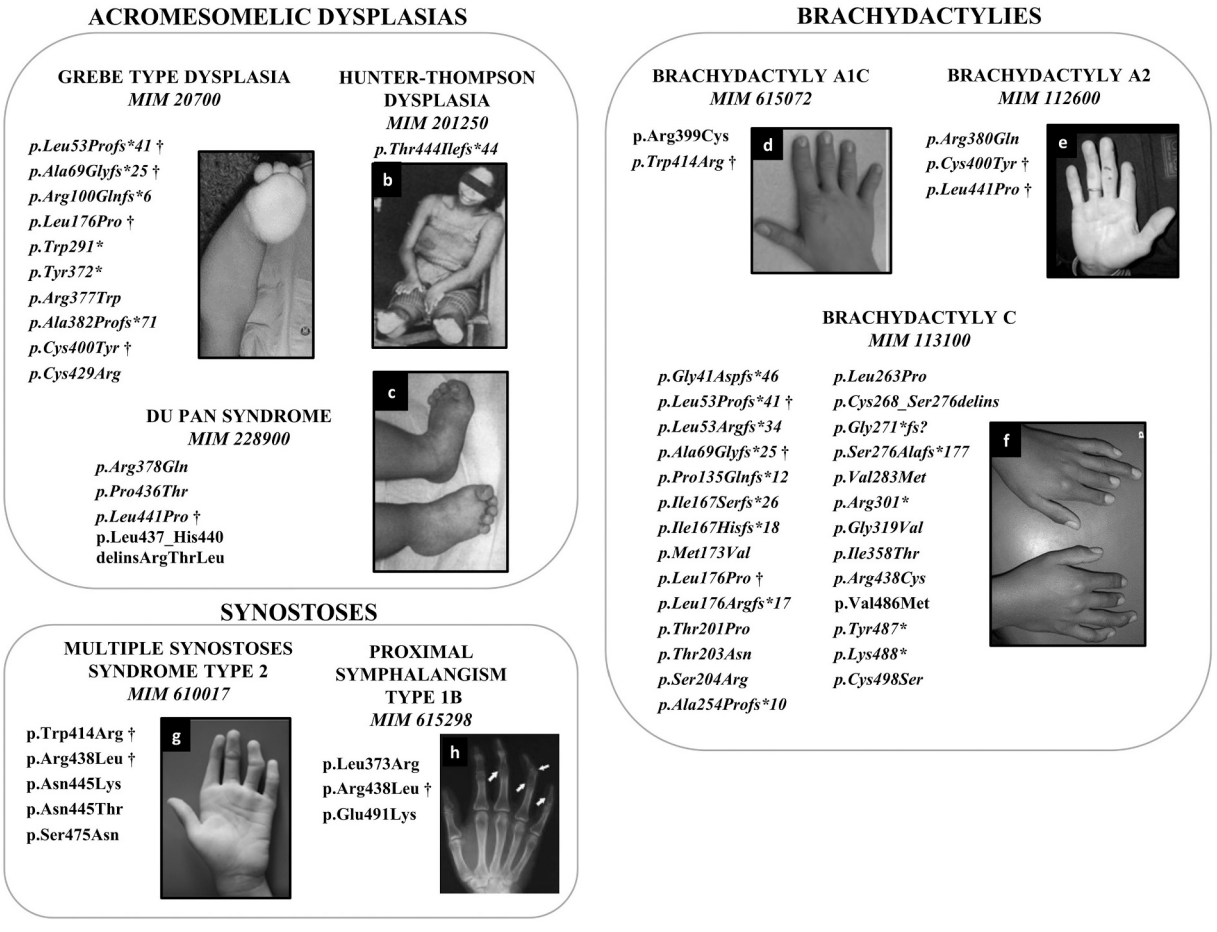

Fig. 2. *GDF5*-related phenotypes and causative variants.
The upper left quadrant represents acromesomelic dysplasias. The right quadrant displays brachydactylies. The lower left quadrant shows synostoses syndromes. For each phenotype reported variants and a representative picture retrieved from literature are provided. a) Grebe Type chondrodysplasia, upper limb [69]; b) Hunter-Thompson acromesomelic chondrodysplasia, whole body [56]; c) Du Pan syndrome, feet [28]; d) BDA1C, hand [29]; e) BDA2, hand [62]; f) BDC, hands [32]; g) Multiple Synostoses syndrome type 2, hand [42]; h) Proximal Symphalangism type 1B, hand X-ray [35]. Variants recurring in different conditions are marked with a † sign. Variants reported in biallelic state are displayed in italic.

Table 1
GDF5-related phenotypes with Mendelian inheritance: overview and key clinical features.

ACROMESOMELIC DYSPLASIAS	MIM	INHERITANCE	HANDS AND FEET	LIMB MAIN JOINTS	DEFINING FEATURES	ADDITIONAL FEATURES
Grebe type chondrodysplasia	200700	AR	Bulge-shaped digits Hypoplastic/absent carpals/tarsals	Valgus deformity (knee, elbow, ankle)	Extreme limb shortening Hands/feet presentation	Severely shortened and dysmorphic long bones
Hunter-Thompson chondrodysplasia	201250	AR	Severe Brachydactyly type C features	Knee, elbow, ankle dislocation	Joint dislocations Hands/feet presentation	Shortened and dysmorphic long bones
Du Pan syndrome	228900	AR, AD	Complex brachydactyly	Valgus deformity (knee, ankle)	Absent/hypoplastic fibula	Mildly shortened and dysmorphic long bones
BRACHYDACTYLIES	**MIM**	**INHERITANCE**	**DIGIT PATTERN**	**PHALANGES**	**METACARPALS**	**ADDITIONAL FEATURES**
Brachydactyly type A1C	615072	AD, SD	II,III,IV,V digit shortening	Hypoplastic/absent intermediate phalanges	Variable shortening	Short stature Clubfoot
Brachydactyly type C	113100	AD, AR	II,III,V digit shortening and clinodactyly IV finger less/not affected	Hypoplastic/absent intermediate phalanges Phalangeal hypersegmentation	Short I and II metacarpal(s)	Short stature Clubfoot Madelung deformity
Brachydactyly type A2	112600	AD	II digit shortening and clinodactyly	Hypoplastic/absent intermediate phalanx	Short II metacarpal	Inconstant V finger clinodactyly
SYNOSTOSES SYNDROMES	**MIM**	**INHERITANCE**	**HAND/FOOT JOINT FUSION**	**OTHER JOINTS**	**BRACHYDACTYLY**	**ADDITIONAL FEATURES**
Proximal Symphalangism type 1B	615298	AD	Proximal interphalangeal (finger V especially) Carpal/tarsal bones	Not involved	Occasionally, type A or C	None
Multiple Synostoses syndrome type 2	610017	AD	Interphalangeal joints Carpal/tarsal bones	Knee, elbow, ankle, wrist Cervical spine	Occasionally, type A or C	Dysmorphic features Broad, hemicylindrical nose

recessive inheritance, in most cases. Patients show mild disproportionate short stature, lower limbs being more affected than upper ones, knee and ankle valgus deformities and hands and feet anomalies. Fingers present complex features of various brachydactyly types, toes can present the ball-shape seen in Grebe type chondrodysplasia [26–28].

The second group of disorders consists of three different types of brachydactylies: BDA1C (MIM #615072), BDC (MIM #113100), and BDA2 (MIM #112600). BDA1C is a type A brachydactyly form characterized by shortening of the middle segment of fingers 2, 3, 4 and 5 in hands and feet, all digits bearing the same degree of affection. Dominant and semidominant transmission patterns have been described [29,30].

BDC shows a complex and variable phenotype, for which both autosomal dominant and recessive transmission have been reported. Patients present variable shortening and clinodactyly of the 2^{nd}, 3^{rd}, 5^{th} fingers and toes, short and deformed metacarpals/metatarsals (especially 1^{st} and 2^{nd}), with the distinct preservation of the 4^{th} finger in most cases [6]. Some BDC patients can also manifest clinical features of Angel-Shaped Phalango-Epiphyseal Dysplasia (ASPED), an autosomal dominant skeletal abnormality characterized by a typical angel-shaped phalanx, brachydactyly, specific radiological findings, abnormal dentition, hip dysplasia, and delayed bone age [31,32]. BDA2 is a mild form of brachydactyly with an autosomal dominant transmission characterized by shortening of the second finger and toe, and inconstant clinodactyly of the 5^{th} finger [1,33].

The third category is represented by syndromes with abnormal bone fusion, with autosomal dominant transmission. Proximal Symphalangism type 1B (MIM #615298) is characterized by reduced mobility of finger joints, worsening with age. Axial skeleton is not involved [34], hands and feet can be normal in morphology or show various BDA or BDC features [35]. Multiple Synostoses syndrome type 2 (MIM #610017) is a disorder causing deformities due to progressive bone fusion of spine (cervical segment), wrists and ankles. Patients have normal height but dysmorphic facies with broad hemicylindrical nose and fingers may present brachydactyly features [36,37].

GDF5-related phenotypes additionally include various mild skeletal anomalies. Subtle features reminiscent of BDA or BDC can be observed in *GDF5* mutations carriers, but detailed information is rarely available in clinical reports.

3.3.2. *GDF5 functional studies reported in literature*

We collected the results of several functional studies reported in literature to investigate the effect of 14 variants (Table 2) [23,29,30,33,36–42]. These studies demonstrated, through molecular, cellular and biochemical approaches, both *in vitro* and *in vivo*, that the pathogenic mechanisms caused by mutations altering the protein sequence in the prodomain were due to GDF5 secretion impairment, intracellular dimer retention (p.Leu176Pro [38]), and decreased chondrogenic potential (p.Thr201Pro and p.Leu263Pro [40]; p.Arg380Gln [33]). Missense pathogenic mutations, occurring in the active domain, affected protein activity (p.Arg399Cys [29]), BMPR family receptors' binding (p.Trp414Arg [30]; p.Leu441Pro [41]; p.Ser475Asn [37]) or caused insensitivity to the BMP inhibitor NOG (Arg438Leu [41]; p.Asn445Thr and p.Asn445Lys [37,42]; p.Ser475Asn [37]; p.Trp414Arg [30]).

4. Discussion

We studied an isolated form of brachydactyly in a three-generation Italian family with radiological features of both BDA1 and BDC. Through an exome sequencing approach, we identified a heterozygous frameshift variant in the *GDF5* gene (NM_000557.5:c.157dup; NP_000548.2:p.Leu53Profs*41; rs778834209) segregating with the trait. The variant is a C nucleotide duplication, occurring within a stretch of seven C nucleotides, that cause a frameshift (p.Leu53Profs*41) creating a premature stop 41 codons downstream. The predicted product is a 92 amino acids peptide, lacking ~80% of the full-length wild-type 501 amino acid protein, including most of the prodomain and the whole active domain (Fig. 3) [24]. This variant (ClinVar VCV000817904) has been associated with BDC [23] and Grebe type chondrodysplasia [24], depending on the genotype status. No functional evidence regarding transcript stability or truncated protein activity is available.

Table 2
Functional studies of *GDF5* variants.

Variant (NP_000548.2)	Domain	Zigosity	Phenotype	Function	Experimental data	Ref
p.Ala69Glyfs*25	Prodomain	*het*	**BDC**	*Loss*	Non detectable protein in transfected cells	[23]
		hom	**CGT**			
p.Leu176Pro	Prodomain	*het*	**BDC**	*Loss*	Intracellular GDF5 dimer retention	[38]
		hom	**CGT**			
p.Thr201Pro	Prodomain	*het*	**BDC**	*Loss*	Reduced intracellular and extracellular GDF5 levels Reduced SMAD signaling Reduced chondrogenic potential	[40]
p.Leu263Pro	Prodomain	*het*	**BDC**	*Loss*	Reduced intracellular and extracellular GDF5 levels Reduced SMAD signaling Reduced chondrogenic potential	[40]
p.Arg380Gln	Prodomain	*het*	**BDA2**	*Loss*	Impaired cleavage Reduced chondrogenic potential	[33]
p.Arg399Cys	Active domain	*het*	**BDA2**[a]	*Loss*	Reduced chondrogenic potential	[29]
		hom	**BDA1C**			
p.Cys400Tyr	Active domain	*het*	**BDA2**	*Loss*	Intracellular GDF5 dimer retention Reduced signal transduction	[39]
		hom	**CGT**			
p.Trp414Arg	Active domain	*het*	**BDA1C**	*Gain + loss*	Reduced BMPR1A affinity Reduced NOG inhibition sensitivity	[30]
			SYNS2			
p.Arg438Cys	Active domain	*het*	**BDC**	*Loss*	Impaired dimerization	[23][36]
p.Arg438Leu	Active domain	*het*	**SYM1B**	*Gain*	Higher BMPR1A affinity Increased chondrogenic potential	[41]
			SYNS2			
p.Leu441Pro	Active domain	*het*	**BDA2**	*Loss*	Reduced BMPR1A and BMPR1B affinity	[41]
		hom	**DPS**		Reduced SMAD signaling Reduced chondrogenic potential	
p.Asn445Lys	Active domain	*het*	**SYNS2**	*Gain*	Reduced NOG inhibition sensitivity Increased chondrogenic potential	[42][37]
p.Asn445Thr	Active domain	*het*	**SYNS2**	*Gain*	Reduced NOG inhibition sensitivity Increased chondrogenic potential	[42][37]
p.Ser475Asn	Active domain	*het*	**SYNS2**	*Gain*	Reduced BMPRII affinity Reduced NOG inhibition sensitivity Increased chondrogenic potential	[37]

This table shows *GDF5*-disease causative variants for which functional studies were performed and reported in literature. For each variant, zygosity, phenotypes, functional effect and experimental data are shown. BDC=Brachydactyly type C. CGT = Chondrodysplasia Grebe type. BDA2 = Brachydactyly type A2. BDA1C=-Brachydactyly type A1C. SYNS2 = Multiple Synostoses syndrome type 2. SYM1B=Proximal Symphalangism type 1B. DPS = Du Pan syndrome.

[a] The authors describe a heterozygous carrier phenotype matching BDA2, but the definite diagnosis is not specified.

The two-exon gene *GDF5 (Growth and Differentiation Factor 5*, MIM *601146) maps on the chromosome 20q11.22, and encodes two transcripts (NM_000557 and NM_001319138) that differ in the 5′-UTR, coding for proteins (NP_000548 and NP_001306067) of the same sequence and length. GDF5 protein, also known as Cartilage-Derived Morphogenetic Protein 1 (CDMP1), is a soluble growth factor belonging to the BMPs, the largest subgroup of the Transforming Growth Factor Beta (TGFβ) superfamily [43,44]. During embryonic development, *GDF5* is predominantly expressed in long bones, while in the adult is mainly expressed in salivary glands and at lower level in fat, lung, prostate, endometrium, brain, testis, thyroid, gall bladder and urinary bladder (GTEx v7; www.gtexportal.org). The protein has an N-terminal signal peptide domain (amino acids (aa) 1–27), a prodomain (aa 28–381) and a C-terminal active domain (aa 382–501) (Fig. 3) containing six highly conserved cysteine residues, the cystine knot motif, and a cysteine that connects two monomers *via* a disulfide bond [45]. The pre-pro-protein is proteolytically processed to form the active dimer [45].

During limb morphogenesis, GDF5 regulates the differentiation of chondrogenic tissue and controls the size of cartilage condensations and joint development [30]. It positively regulates differentiation of chondrogenic tissue by binding its high-affinity BMPR1B receptor and the lower-affinity BMPR1A receptor, leading to intracellular signal transduction by the phosphorylation of SMAD transcriptional factors [45]. This pathway is negatively regulated through its direct interaction with NOG, a GDF5 soluble inhibitor, which can mask the receptor binding sites of GDF5, impeding signaling transduction (Fig. 4) [30].

Mutations in *GDF5* have been associated with disorders characterized by defective or excessive cartilage and bone development involving especially the distal part of the limbs. Both autosomal recessive and dominant transmission models have been described for *GDF5*-related phenotypes, varying in severity and degree of skeletal involvement. Incomplete penetrance, *i.e.* the absence of a given phenotype in some individuals harboring the pathogenetic mutations [46], and variable expressivity, *i.e.* different degrees of involvement in individuals bearing the same mutation and manifesting the related phenotype [47], have been reported for the dominant forms. Furthermore, some cases of semidominance, *i.e.* inheritance pattern manifesting with a definite phenotype in homozygosity and a less marked presentation of the same phenotype in heterozygosity, have been described [29]. It has been proposed that a variable degree of GDF5 loss of function is the mechanism underlying bone development defects, while gain-of-function mutations cause disorders with excessive bone formation [30,48]. The role of *GDF5* in bone disorders is not limited to Mendelian inheritance conditions. For example, a functional single nucleotide polymorphism (NM_000557.5:c.-275T>C; rs143383) in the 5′ untranslated region of the gene is a known locus for osteoarthritis susceptibility [48].

The family we report in this study presents a previously undescribed phenotype with both BDA1 and BDC features. Variable expressivity has been observed, as the proband and his grandfather displayed the overt phenotype, while the proband's mother only showed subtle signs. Currently, no data are available in literature on the extent of the variable expressivity for these phenotypes in other families. Regarding the frameshift Leu53Profs*41 mutation, it has been associated with isolated BDC, in heterozygous state [23], and with the more severe phenotype of Grebe type chondrodysplasia, in homozygous state [24]. These observations suggest that a complex interplay among GDF5 protein levels and putative genetic modifiers concur to determine a variable phenotype. This represents a crucial issue to consider for appropriate genetic counseling, *e.g.* on prenatal diagnosis and on predicted effect on the

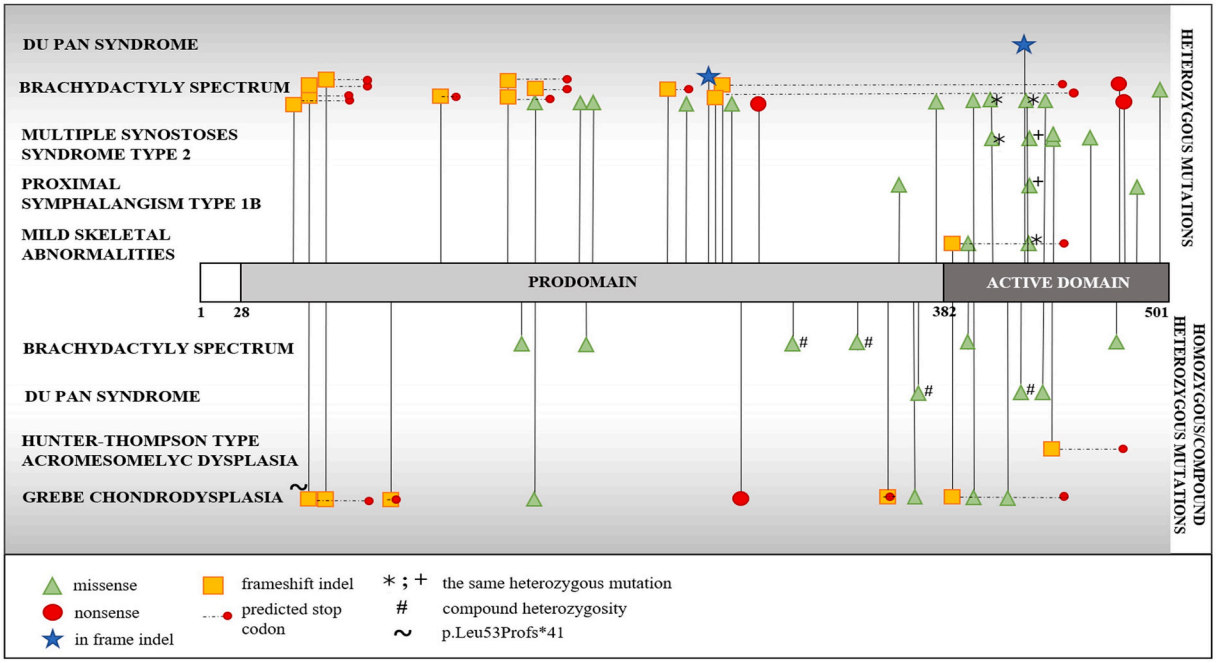

Fig. 3. Schematic genotype-phenotype correlations in the *GDF5* mutational spectrum.
The picture features all the reported *GDF5* mutations in literature, a list of all *GDF5*-related phenotypes and a schematic representation of the GDF5 protein. In the middle part of the figure is represented the GDF5 protein (protein domains: 1–27 signal peptide, 28–381 prodomain, 382–501 active domain). Mutations are represented by symbols indicating their functional class. They are displayed along the horizontal axis at the residue in which the change occurs, dotted lines indicate the predicted stop codon for frameshift variants. Mutations are reported along the vertical axis at the level of the associated phenotype. Grey gradient represents the different severity of the phenotypes. Mutations reported in heterozygosity or in biallelic state are displayed above and below the protein, respectively. The # symbol marks compound heterozygosity. The * and + symbols mark two specific mutations that are known to cause more than one phenotype in heterozygosity. The ~ symbol indicates the p.Leu53Profs*41 mutation reported in this work. The "Mild skeletal abnormalities" phenotype refers to subtle bone development anomalies reported in the reference paper without a formal diagnosis.

following generations, which is difficult to predict as those modifiers have not been characterized yet.

The above mentioned results and the relative issues raised, led us to review the molecular mechanism of *GDF5* mutations by performing an extensive literature analysis of all the *GDF5*-related genotypes, phenotypes and functional studies reported to date. *GDF5* genotype-phenotype correlations show that mutations in *GDF5* can be divided in many subclasses, depending on the mutation (missense, nonsense, frameshift, in-frame deletions-insertions mutations), affected protein domain (prodomain, active domain), and genotype (Supplementary Table 1). A single splicing variant has been reported as causative of BDC in heterozygosity, without further details [49]. A comprehensive representation of molecular and phenotype data about *GDF5* variants is shown in Figs. 2 and 3. The highest degree of GDF5 signaling impairment is caused by homozygous frameshift mutations causing a premature stop codon and the transcription of mRNAs that are likely to undergo Nonsense Mediated Decay (NMD). These mutations are reported as causing Grebe type chondrodysplasia in homozygosity [24,50,51] and BDC in heterozygous state [23,46]. This class includes the frameshift mutation identified in this work.

The Grebe type chondrodysplasia is also caused by homozygous nonsense or frameshift mutations localized downstream that presumably cause truncated proteins [24,52] and by homozygous missense mutations [38,39,53–55] that exert a significant effect on protein function [38]. A dominant-negative effect has been proposed to explain the brachydactyly phenotype for the heterozygous missense variants causative of Grebe type chondrodysplasia phenotype in homozygosity [39].

A remarkable impact on GDF5 function can also be caused by the p.Thr444Ilefs*44 homozygous frameshift mutation that introduces a late stop codon and a potentially truncated protein with altered folding [50,56], causing Hunter-Thompson type acromesomelic chondrodysplasia [56]. Recently, a pathogenetic mutation in *BMPR1B*, a GDF5 receptor, has been reported as causative for the same phenotype [57].

Missense mutations with significant impact on GDF5 function have also been described in compound heterozygosity (p.Arg378Gln and p.Pro436Thr) as causative for Du Pan syndrome [27]. The first one (p.Arg378Gln) affects the RRKRR protease recognition motif, like the p.Arg377Trp mutation causing Grebe type chondrodysplasia in homozygous state [53] and the p.Arg380Gln causing BDA2 in heterozygous state [33]. The heterozygous carriers of p.Arg378Gln showed BDA radiographic features. The functional effect of the second mutation (p.Pro436Thr) is unknown, and the heterozygous carrier did not show bone affection [27].

Interestingly, a heterozygous complex indel causing two missense mutations and an in-frame deletion has been reported associated with Du Pan syndrome phenotype. The authors hypothesized a dominant-negative effect to explain such a severe phenotype for a heterozygous mutation [28]. For Du Pan syndrome too, the phenotype can be caused by a mutation in *BMPR1B* [58].

Based on these observations, we propose a phenotype gradient, ranging from severely impaired bone development (as in Grebe type chondrodysplasia) to subtle changes in phalangeal morphology (as in BDA2) or even redundant ossification (as in Proximal Symphalangism 1B or Multiple Synostoses syndrome 2), corresponding to a parallel *continuum* of GDF5 activity variation, both excessive and defective (Fig. 5).

In this complex scenario, several *GDF5* loss of function mutations causing different types of brachydactyly have been reported. We propose that any allele variant in *GDF5*, resulting in the reduction of protein product or in a function comparable to the loss of an allele (such as

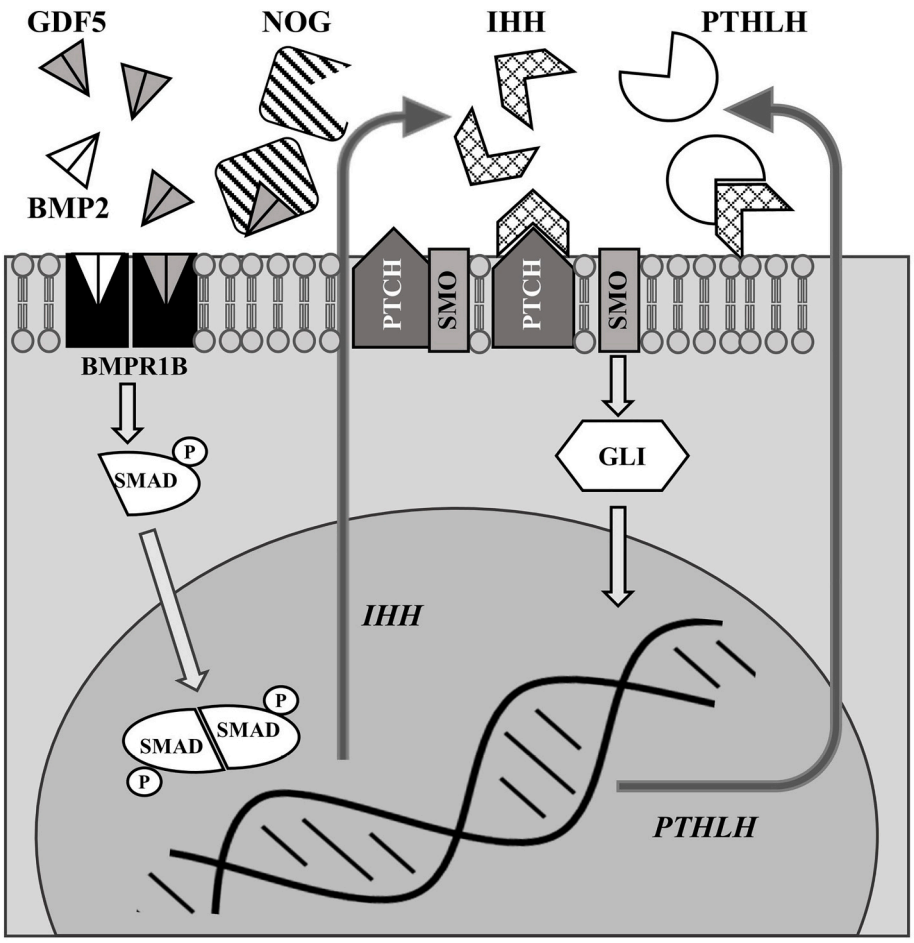

Fig. 4. GDF5 pathway.
The image depicts the GDF5 signaling pathway. GDF5 soluble dimers can bind to different BMPR family receptor dimers [45]. The main GDF5 receptor is BMPR1B [57]. BMP2 is another bone morphogenic protein binding and activating the BMPR1B receptor [45]. The binding of GDF5 and BMP2 to the receptors can be impaired by the soluble inhibitor NOG [42]. Once the receptor is activated, phosphorilated SMAD family proteins dimerize and translocate to the nucleus, where they act as transcriptional factors, inducing the transcription of *IHH* [45]. IHH, soluble morphogen of the hedgehog family, transduces its signal by binding to PTCH family receptors. This interaction frees the SMO transmembrane protein to transduce the signal to the nucleus by activating GLI family transcriptional factors, thus promoting bone development. This pathway also induces the production of the soluble hedgehog inhibitor PTHLH, downregulating its own activity with a negative feedback loop.

heterozygous gene deletions or heterozygous frameshift mutations) may cause a phenotype belonging to this spectrum. Indeed, chromosomal deletions involving the 20q11.22 *GDF5* genomic region are reported to cause brachydactyly [59]. The evaluation of these cases is complex, as limb anomalies can vary along with the extension of the deletion [60]. Frameshift mutations in the prodomain are known to cause BDC in heterozygosity [23,61] and Grebe type chondrodysplasia in homozygosity [24].

Brachydactylies can also be caused by heterozygous missense loss of function mutations affecting maturation or receptor binding [23,33,38,40,41,62,63] (Fig. 3). Interestingly, some missense mutations are associated with brachydactyly in homozygous state [48,64] and mild anomalies in heterozygous state [29], putatively resulting, when combined, in a signaling decrease comparable to that of a null allele. Mutations with a gain-of-function effect have also been reported, all of them in heterozygosity, as causative of Proximal Symphalangism type 1B or Multiple Synostoses syndrome type 2 [34–37,41,65,66]. These phenotypes are also caused by mutations in *NOG*, the main GDF5 inhibitor [67,68].

To further underline the complexity of the above reported molecular mechanisms, opposite phenotypes characterized by defective or excessive cartilage and bone development have been described as caused by single mutations exerting both loss and gain of function effects [30]. The reported examples support the hypothesis that conditions resulting in comparable loss or gain of GDF5 activity lead to similar phenotypes.

This correlation is pictured in the model proposed in Fig. 5. GDF5 protein activity variation can be the result of mutations that cause gain or loss of function, the more pronounced the effect the more severe is the phenotype, that is the excess or defect in GDF5 protein levels and/or activity parallel excess or defect in bone development. Furthermore, GDF5 is embedded in a signaling pathway regulating bone development which is composed of several players, acting for example as receptors (*e.g.* BMPR receptors) or inhibitors (*e.g.* NOG), therefore GDF5 mutations exert their role through the perturbation of a fine regulated signaling pathway. A gain of function mutation could, for example, cause insensitivity to the effect of the inhibitor, while a loss of function could act as causing the lost of binding to the BMP receptor. Moreover, this implies that the dysregulation of this pathway, introduced by mutations at different crucial points, could cause the same or similar phenotype caused by GDF5 perturbation.

A further level of complexity adds to this picture and is represented by putative modifier elements, *e.g.* genetic factors that could exert a modulatory effect on the expression level and/or protein activity of GDF5 and related members of BMP and associated pathways. Variants altering protein coding sequences with a mild effect on GDF5 and possible modifier genes' structure/function and variants occurring in *GDF5* regulatory regions could alter, when occurring in combination, the expression and the activity of those proteins and result in the wide clinical spectrum observed for these conditions. In conclusion, we identified a known pathogenic *GDF5* mutation, previously associated in heterozygosity with BDC [23], in a family presenting an isolated brachydactyly characterized by both BDA1 and BDC features and a remarkable variable expressivity. The same variant has been associated with the more severe phenotype of Grebe type chondrodysplasia in homozygosity [24], an important aspect to consider for appropriate genetic counseling.

The in-depth literature analysis strengthens the relationship between GDF5 activity variation and specifically related phenotypes, but it also highlights how faint are the borders defining individual clinical presentations. These should not be regarded as strict clinical entities, but

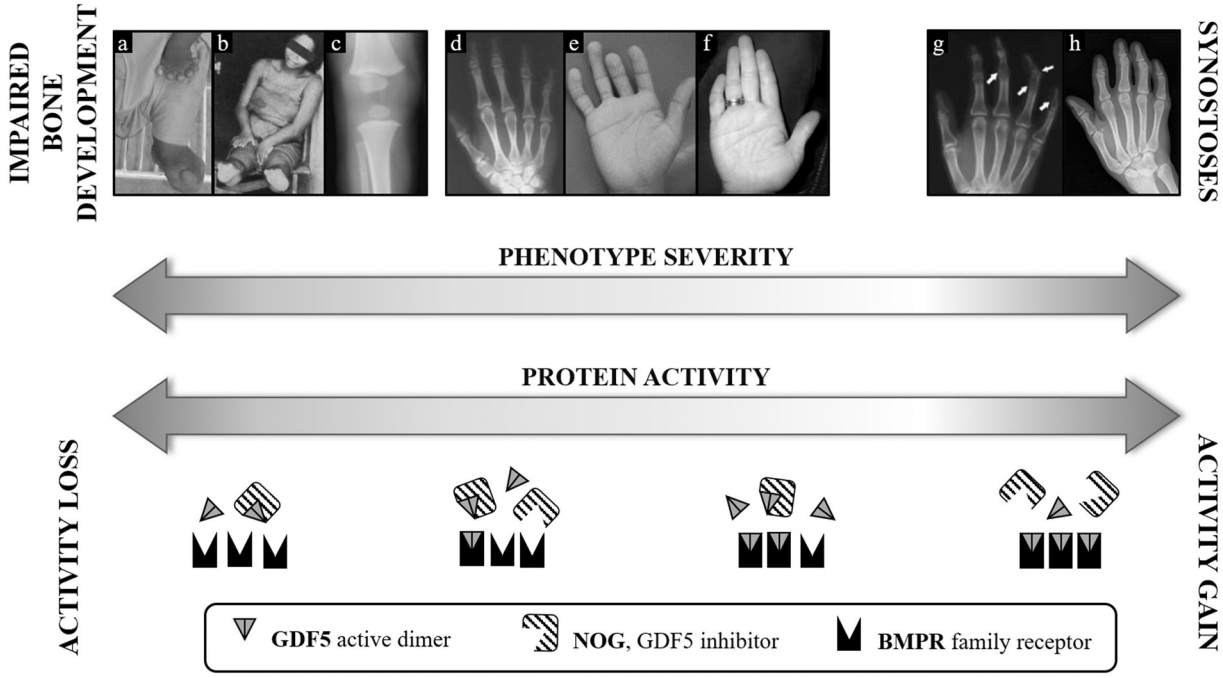

Fig. 5. GDF5 parallel molecular and clinical gradient.
The picture depicts the correlation between the variation in GDF5 signaling activity resulting from mutations in gene(s) coding for GDF5 or its interactors, and the main related phenotypes. The upper part shows the *GDF5*-related phenotypic spectrum, sorted by severity and bone involvement (excessive or defective). Pictures were retrieved from literature: a) Grebe type chondrodysplasia [69]; b) Hunter-Thompson acromesomelic chondrodysplasia [56]; c) Du Pan syndrome [27]; d) BDA1C [29]; e) BDC [32]; f) BDA2 [62]; g) Proximal Symphalangism type 1B [35]; h) Multiple Synostoses syndrome type 2 [42]. The lower part shows different levels of activity and expression of GDF5 and its main interactors (BMPR family receptors and the soluble inhibitor NOG) that parallel phenotype manifestations. GDF5 protein activity variation can be the result of mutations that cause gain or loss of function, the more pronounced the effect the more severe is the phenotype, in terms of excess or defect in bone development. GDF5 is embedded in a complex interplay regulating bone development, including BMPR receptors and NOG, an inhibitor of GDF5. The dysregulation of this pathway introduced by mutations at different crucial points could cause the same or similar phenotype caused by GDF5 perturbation.

need to be considered as part of a whole *continuum* of *GDF5*-related disorders, to which also mutations in genes encoding for GDF5 interactors (*NOG*, MIM *602991; *BMPR1B*, MIM *603248) contribute. Our results confirm the phenotype variability in *GDF5*-related disorders, pointing out the current complexity in the diagnosis of these diseases due to the interconnection between clinical phenotypes and molecular patterns.

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CRediT authorship contribution statement

Maria Luce Genovesi: Writing – original draft, Investigation, Visualization. **Daniele Guadagnolo:** Writing – original draft, Visualization. **Enrica Marchionni:** Writing – original draft, Visualization. **Agnese Giovannetti:** Data curation, Writing – review & editing. **Alice Traversa:** Investigation, Writing – review & editing. **Noemi Panzironi:** Investigation, Writing – review & editing. **Silvia Bernardo:** Investigation, Writing – review & editing. **Pietro Palumbo:** Investigation, Writing – review & editing. **Francesco Petrizzelli:** Data curation, Writing – review & editing. **Massimo Carella:** Resources, Writing – review & editing. **Tommaso Mazza:** Data curation, Writing – review & editing. **Antonio Pizzuti:** Resources, Supervision, Writing – review & editing. **Viviana Caputo:** Conceptualization, Visualization, Supervision, Writing – review & editing.

Declaration of competing interest

None.

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