Thesis

PHENOTYPIC SPECTRUM OF COLLAGEN 1-RELATED CONNECTIVE TISSUE DISORDERS

submitted by

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Graz, 28.11.2022

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 28.11.2022 *Julia Gesselbauer m.p.*

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1. Table of Content

Abbreviations 5
List of Figures 7
List of Tables 7
Zusammenfassung 8
Abstract 10
1. Introduction 12
2. Material und Methods 13
3. Collagen I associated diseases – review of the literature 15
3.1. Collagen 15
3.1.1. The collagen family 15
3.1.2 Type I Collagen 15
3.2 Diseases associated with *COL1A* 23
3.2.1. Osteogenesis imperfecta 23
3.2.2. Ehlers-Danlos syndromes 29
3.2.3 Caffey Disease 34
3.2.4. COL1-related overlap disorder 35
4. Clinical Report 49
4.1. Patient presentation 49
4.2. Genetic analysis 51
5. Discussion 52
5.1. COL1-related disorders – phenotype-genotype relations 52
5.2. Phenotype-genotype associations in C1ROD 55
5.3. Comparison of the proband to other C1ROD phenotypes 58
5.4. Approaches for future testing and classification 59
6. Bibliography 62

Abbreviations

3Hyp	3-Hydroxyprolin
aEDS	Ehlers-Danlos-Syndrome, arthrochalasia type
AGEs	Advanced glycation end products
Array-CGH	Array Comparative genomic hybridization
BCS	Brittle cornea syndrome
BMD	Bone mineral density
BMP1	Bone morphogenetic protein 1
C1ROD	COL1-related overlap disorder
CD	Caffey disease
cEDS	Classical Ehlers-Danlos-Syndrome
clEDS	Classical-like Ehlers-Danlos-Syndrome
clEDS2	Classical-like Ehlers-Danlos-Syndrome 2
CRTAP	Cartilage-associated protein
CT	Computed tomography
C-terminal	Carboxy-terminal
cvEDS	Cardio-valvular Ehlers-Danlos-Syndrome
CyPB	Cyclophilin B
dEDS	Dermatosparaxis
DI	Dentinogenesis imperfecta
DNA	Deoxyribonucleic acid
DXA	Dual-energy x-ray absorptiometry
ECM	Extracellular matrix
ECG	Electrocardiogram
EDS	Ehlers-Danlos Syndromes
ER	Endoplasmic reticulum
FACIT	Fibril-associated collagens with interrupted triple helices
gDNA	Genomic DNA
GG-Hyl	Glucosylgalactosylhydroxylysine
G-Hyl	Galactosylhydroxylysine
GJH	General joint hypermobility
HCTD	Hereditary connective tissue disorder
hEDS	Hypermobile Ehlers-Danlos-Syndrome
HSP47	Heat shock protein 47
kEDS	Kyphoscoliotic Ehlers-Danlos-Syndrome
LH	Lysyl hydroxylases
LOX	Lysyl oxidase
mcEDS	Musculocontractural Ehlers-Danlos-Syndrome
mEDS	Myopathic Ehlers-Danlos-Syndrome
MRI	Magnetic resonance imaging
NGS	Next-Generation-Sequencing
N-terminal	Amino-terminal
OI	Osteogenesis imperfecta
P3H1	P3-hydroxylase 1
P4H	Prolyl 4-hydroxylase
pEDS	Periodontal Ehlers-Danlos-Syndrome
RNA	Ribonucleic acid
SD	Standard deviation

sEDS	Spondyloplastic Ehlers-Danlos-Syndrome
TEM	Transmission electron microscopy
vEDS	Vascular Ehlers-Danlos-Syndrome
WES	Whole-Exome sequencing

List of Figures

Figure 1 – Structure of collagen[5] 18
Figure 2 – Collagen ultrastructural view[21] 19
Figure 3 – Overview of the described C1ROD-associated genes and their distribution among *COL1A1* and *COL1A2*. 54

List of Tables

Table 1 – Overview of the patient‘s characteristics and missing clinical features in comparison to other patients with C1ROD 50

Zusammenfassung

Ziel dieser Diplomarbeit ist das Verfassen eines Fallberichts über eine Betroffene mit einer Mutation im *COL1A1*-Gen. Dieses Gen kodiert zusammen mit *COL1A2* für das extrazelluläre Matrixprotein Kollagen I. Mutationen in *COL1A1* und *COL1A2* verursachen eine Reihe unterschiedlicher Bindegewebserkrankungen, allen voran die Osteogenesis imperfecta (OI) und das Ehlers-Danlos-Syndrom (EDS). Aufgrund des breiten Spektrums an möglichen resultierenden Phänotypen und oft nicht eindeutig zuordenbaren Symptomen ist eine Diagnosestellung anhand klinischer Merkmale oftmals erschwert. In den letzten Jahren wurde durch einzelne Publikationen eine neue Entität, die „combined Osteogenesis imperfecta and Ehlers-Danlos syndrome 1 and 2" (COL1-related overlap disorder, C1ROD) beschrieben, was die Komplexität der Charakterisierung und Beschreibung dieser Erkrankungsbilder verdeutlicht.

Im Rahmen dieser Arbeit wird eine systematische Literaturrecherche in PubMed mit dem Fokus COL1-bedingter Bindegewebserkrankungen durchgeführt. Der Fallbericht wird in das Spektrum der vorhandenen klinischen Beschreibungen eingeordnet, bzw. ihnen gegenübergestellt. Nach Abschluss der Arbeit wird der Fallbericht weiter adaptiert um ihn als Publikation einzureichen.

Methoden

Die systematische Literaturrecherche wurde in PubMed durchgeführt. Die klinische Darstellung der 33-jährigen Betroffenen mit Symptomen sowohl einer OI als auch eines EDS basiert auf der medizinischen Vorgeschichte, Röntgenbildern, der Beschreibung der phänotypischen Auffälligkeiten und den Ergebnissen der molekulargenetischen Untersuchungen. Die genetischen Daten umfassen die Ergebnisse einer Whole-Exom Sequenzierung, welche nach dem Einverständnis der Patientin durchgeführt wurde.

Ergebnisse

Durch die klinische Beurteilung einer Betroffenen mit Kleinwuchs, Hypermobilität von Gelenken, Subluxationen und ausgeprägten fazialen Auffälligkeiten, konnte keine eindeutige Diagnose gestellt werden. Mittels Exom-Analyse wurde eine zuvor in der Literatur noch nicht beschriebene Variante (NM_000088.3: c.2885G>T) im

COL1A1-Gen identifiziert. Diese wurde in den elterlichen Blutproben nicht gefunden, weshalb die Variante als de-novo klassifiziert wurde.

Die retrograde Phänotypisierung nach Erhalt der genetischen Resultate ist zum Teil mit Symptomen einer OI, als auch mit EDS-spezifischen Auffälligkeiten vereinbar, und die Diagnose einer C1ROD konnte molekulargenetisch gestellt werden.

Conclusio

In der Vergangenheit existierten mehrere Versuche COL1-assoziierter Auffälligkeiten als abgrenzte Krankheitsentitäten zu definieren. Fallberichte wie dieser zeigen die mögliche Bandbreite an klinischen Auffälligkeiten in Zusammenhang mit *COL1A1*-Varianten und bestätigen die Sinnhaftigkeit der neu definierten Entität C1ROD. Der Fallbericht zeigt auch, dass in manchen Fällen eine Diagnose molekulargenetisch gestellt werden muss, da der Phänotyp nicht den klassischen Beschreibungen in der Literatur etablierter Krankheitsentitäten entspricht.

Abstract

Objective

The main objective of this thesis is to write a clinical report about a patient carrying a mutation in *COL1A1,* one of the two genes encoding collagen I, an extracellular matrix protein. Mutations in *COL1A1* and *COL1A2* have been known to cause different hereditary connective tissue disorders with a broad spectrum of phenotypes, most importantly Osteogenesis imperfecta (OI) and Ehlers-Danlos syndrome (EDS). However, not all patients may be easily categorized due to inconclusive presentation of symptoms. In recent years a new entity, i.e., combined Osteogenesis imperfecta and Ehlers-Danlos syndrome 1 and 2 (COL1-related overlap disorder, C1ROD), has been evolved, which further illustrates the complexity in diagnosing these diseases.

In this work, a systematic review of the literature, regarding COL1-related hereditary connective tissue disorders is conducted in PubMed. The clinical symptoms of our patient will be compared with the existing clinical descriptions and preexisting categories of COL1-related diseases. The case report of our patient will be further adapted and submitted for publication.

Methods

The review of the literature was performed in PubMed. The clinical presentation is based on medical history, radiographs, clinical descriptions, and genetic results of a 33-year-old patient with symptoms of both of OI and EDS. The genetic results include data from Whole-Exome sequencing which were performed after informed consent was obtained.

Results

A clear clinical diagnosis based on the patient's phenotype, i.e., short stature, joint hypermobility, several subluxations, and distinct facial features, could not be made. Whole-Exome sequencing was performed, a yet unknown variant in *COL1A1* (NM_000088.3: c.2885G>T) was detected. The variant was not found in the parents' blood samples and was therefore assumed to be de-novo. As most of the symptoms of the patient are consistent with both OI and EDS a diagnosis of C1ROD was made retrospectively.

Conclusion

There have been many attempts to define COL1-related diseases as independent entities, however, this has proven to be difficult. Case reports, as the one presented in this thesis, show the broad spectrum of phenotypes associated with variants in *COL1A* and underline the purpose of the introduction of the new defined entity C1ROD. In addition, it emphasizes the importance of genetic testing as some phenotypes are not consistent with the classic clinical descriptions of established entities available in the literature.

1. Introduction

Osteogenesis imperfecta (OI) is a rare hereditary connective tissue disorder with the main clinical manifestations bone fragility and susceptibility to fractures. Other features as hearing loss, blue sclerae, small body stature, dentinogenesis imperfecta or bone deformities may be present. Currently there are 22 subdivisional groups based on clinical severity and underlying genetic reason (OI type XXII: MIM #619795). The clinical outcome varies and may reach from asymptomatic or very mild to severe and lethal phenotypes. Mutations in the genes *COL1A1* and *COL1A2*, encoding for the α1(I) and α2(I) chains of collagen I, make up for approximately 85-90% of OI. However, due to evolving possibilities of biochemical testing new associated genes have been found.

The Ehlers-Danlos syndromes (EDS) comprise a group of genotypically and phenotypically heterogenous connective tissue disorders with main clinical features of hyperextensible skin, generalized joint hypermobility, abnormal scarring and/or easy bruising. Secondary features differ between different subtypes and include fragility of soft tissue, in some cases with severe blood vessel ruptures, or involvement of the inner organs and musculoskeletal system. Genetic causes for EDS are more diverse compared to OI, and only some of the known subtypes are caused by mutations in *COL1*-genes.

Clinical reports over the last decades indicate an overlap between these two hereditary connective tissue disorders. These so-called combined Osteogenesis imperfecta and Ehlers-Danlos syndromes (OIEDS) 1 (MIM # 619120) and 2 (MIM #619115) are already classified as a new entity in OMIM and are caused by mutations in either *COL1A1* or *COL1A2*. Affected patients may present mainly with OI phenotypes (susceptibility to bone fractures, blue sclerae, and other), others show primarily EDS-like symptoms (joint hyperextensibility, skin hyperextensibility, abnormal scarring, vascular fragility, easy bruising). Hence, in most patients no definite classification as either OI or EDS can be made. Current knowledge is based on clinical reports, but some patients do not correspond to literature and present with mild or even atypical symptoms. Although several studies indicate an association between distinct phenotypes to specific locations within the responsible genes, no exact genotype-phenotype correlation exists.

Herein, the clinical case of a 33-year-old patient referred to genetic counseling due to a suspected hereditary connective tissue disorder is presented. She showed distinct signs of OI and EDS combined, such as joint hypermobility, several joint subluxations, blue sclerae and distinct facial features. However, these allowed no diagnosis of either EDS or OI, as there was no history of bone fracture, skin hyperextensibility, fragile blood vessels or other manifestations expected to occur in solitary one of these diseases.

The aim of this thesis is to review available literature for COL1-related disorders with main focus on the overlapping phenotype of OI and EDS, and to compare our patient to this small cohort. This may contribute to further understanding and defining the newly introduced entity of combined Osteogenesis imperfecta and Ehlers-Danlos syndromes.

2. Material und Methods

The clinical report is based on patient data collected when the patient was admitted to the Department of Human Genetics of the Medical University of Graz due to a suspected hereditary connective tissue disorder. The report includes medical history, X-rays and the clinical description done by a physician geneticist at the department and biomolecular results. Informed consent was obtained for the following study and publication.

A systematic **literature review** was conducted on October 2nd, 2021 with the following search term: ("Collagen Type I/genetics*"[Mesh] OR (COL1A* AND (Diagnosis[Filter] OR Genetic Counseling[filter] OR Clinical Description[Filter]))) AND "Mutation"[Mesh] AND "Humans"[Mesh]. At that time this yielded 555 results. This was repeated in April 2022 with 578 results. Further literature review was performed in PubMed and Ovid with the terms “Osteogenesis imperfecta”, “Ehlers-Danlos-Syndromes”, “collagen I”, “collagen family”, “Caffey disease”, “atypical osteogenesis imperfecta”, “Osteogenesis imperfecta Ehlers-Danlos overlap”, “COL1-related disease”, “COL1A1” and “COL1A2”.

The clinical report will be further adapted for planned publication (not included in this thesis).

Genetic analysis

Exome sequencing was performed in a diagnostic setting. DNA was extracted from leucocytes using the QIASymphony DSP DNA Midi Kit on a QIASymphony SP instrument (QIAGEN, Hilden, Germany). Nextera DNA Flex Library Prep Kit was used for the library preparation. Sequencing was performed on a NextSeq 550 (Illumina, San Diego, California, USA). Sequence alignment of raw FASTQ files to the human reference sequence (GRCh37/hg19 assembly) and variant calling were performed with the DRAGEN Germline Pipeline V.3.2.8 on Illumina BaseSpace (https://basespace.illumina.com/). Variant annotation, filtering and prioritization were performed using VarSeq V.2.2 (Golden Helix, Bozeman, Montana, USA, www.goldenhelix.com). The following human phenotype ontology (HPO) terms were used for this purpose: HP:0008873 Disproportionate short-limb short stature, HP:0010485 Hyperextensibility at elbow, HP:0045086 Knee joint hypermobility, HP:0045087 Hip joint hypermobility, HP:0032153 Joint subluxation, HP:0000475 Broad neck, HP:0100558 Hemiatrophy of upper limb, and HP:0100840 Aplasia/Hypoplasia of the eyebrow.

Sanger sequencing was performed in the parents of the patient for segregation analysis of the *COL1A1* variant.

3. Collagen I associated diseases – review of the literature

3.1. Collagen

3.1.1. The collagen family

Collagen I is one of 28 currently accepted members of the collagen family.[1] They represent the most abundant proteins in the human body, making up about 30% of total proteins.[2] Their characteristic feature is the presence of at least one triple helical domain.[1] These helices comprise of three α- or polypeptide chains. Depending on the involved polypeptides either homotrimeric helices - consisting of three identical polypeptides - or heterotrimeric helices - consisting of different polypeptides - are formed. Non-collagenous domains may also be present.[3] At least one domain of the α-chains consists of repeated sequences of three aminoacids, starting with glycine. This glycine in every third position of the Gly-Xaa-Yaa sequence guarantees the winding of the helix.[3] On position Xaa and Yaa any other aminoacid can be found. The Xaa position is often occupied by Prolin whereas Yaa is occupied by 4-Hydroxy-Prolin. Collagens are further divided into 9 subfamilies based on their supramolecular assembly and ability to form supramolecular networks. Collagen I, II, III, V, XI, XXIV and XXVII are “fibril-forming” collagens, characterized by their ability to form fibrils due to one major triple helical domain.[1,3]

3.1.2 Type I Collagen

Characteristics

Collagen I is the most abundant protein in the connective tissue and the major component of extracellular matrix found in many tissues like bone, cornea, tendon, skin and ligaments. The precursor form, procollagen I, is encoded by two different genes, namely *COL1A1* and *COL1A2*.[4]

Collagen I has a heterotrimeric structure comprising two α1-chains and one α2-chain, with a total length of approximately 300nm and thickness of about 1,5nm.[2] These three chains form a central right-handed triple helix embedded between two global ends, the amino- (N-)terminal and the carboxyl- (C-) terminal propeptides.[5]

The precursor form undergoes a series of intracellular and extracellular posttranslational modifications, including the cleavage of the two propeptides at the end. These modifications are crucial for the biomechanical properties and functionality of the later mature collagen I, which is incorporated into the extracellular matrix (ECM).[2]

COL1A1 and *COL1A2*

COL1A1 (MIM 114000), encoding for the α1(I)-chain, is located on chromosome 17 and consist of 51 exons. *COL1A2* (MIM 120160), encoding the α2(I)-chain, is located on chromosome 7 and comprises 52 exons.[4] The triple helical domain of α1(I) as well as α2(I) consist of 1014 amino acids. The corresponding exons always display a multiple of 9 base pairs, ensuring the structurally necessary sequence of three repeating amino acids Gly-Xaa-Yaa.[4]

Mutations in *COL1A1* or *COL1A2* are known to cause a range of hereditary connective tissue disorders, e.g., the autosomal dominant form of Osteogenesis imperfecta (OI), as well as the arthrochalasia (aEDS) and cardio-valvular (cvEDS) types of Ehlers-Danlos syndromes (EDS) and Caffey disease (CD).

Collagen I synthesis and processing

The proα chains of collagen I are synthesized in the endoplasmic reticulum (ER). The formation of the triple helix takes place following several posttranslational modifications including hydroxlylation of lysin, and prolin and O-linked glycosylation.[2] Once the triple helix is folded the residues are no longer accessible for the responsible enzymes.[6] A number of different enzymes located in the ER are responsible for these complex modifications. They include Prolyl 4-hydroxylase (P4H), the prolyl 3-hydroxylase complex, and three lysyl hydroxylases (LH).[7]

P4H hydroxylates the majority of prolin residues in the Yaa position of the triple helix.[7] The resulting 4-Hydroxyprolines are responsible for the thermal stability of the helix.[8]

Hydroxylation of lysin, which is crucial for the later process of crosslinking is catalyzed by LH. In contrast to hydroxyproline, the percentage of hydroxylated lysin is tissue-specific and even differs within the same tissue, depending on different physiological or pathological states.[2]

Hydroxylation to 3-Hydroxyprolin (3Hyp) requires a whole complex of proteins, that is 3-hydroxylase 1 (P3H1), cyclophilin B (CyPB), and the cartilage-associated protein (CRTAP).[6] CyPB belongs to the family of Peptidyl-prolyl cis-trans isomerases, a group of catalysts with cis/trans prolyl isomerase activity.[9] The isomerization of cis-peptid bonds to trans configuration is required for further folding of the helix.[10] In collagen I only one prolin residue per chain is hydroxylated by this complex, namely prolin 986 in the α1(I) chains and prolin 707 in the α2(I) chain.[11]

Glycosylation of hydroxylysine residues is catalyzed by the glycosyltransferases procollagen galactosyltransferasere 1 and collagen glucosyltransferases 1.[5] There are two steps of glycosylation. In the first step galactose is added and produces galactosylhydroxylysine (G-Hyl). In the second step some of these products are then further glycosylated by adding glucose, which leads to glucosylgalactosylhydroxylysine (GG-Hyl).[2] Lysyl hydroxylase 3 (LH3), in addition to its lysyl hydroxylase activity mentioned above, also has galactosyltransferase[12] and glucosyltransferase[13] activity. However, the main function of LH3 in osteoblasts seems to be GGT activity, rather than GT or LH activity.[14]

Some proteins function as chaperones and ensure correct folding of collagen, such as heat shock protein 47 (HSP47). This collagen-specific stress protein, encoded by *SERPINH1*, provides stability for the later established triple helix by preventing aggregation.[15,16]

Still in the ER, triple helix formation begins at a specific domain in the C-propeptide and continues towards the N-terminal propeptide, forming the procollagen I molecule.[5,17] This precursor consists of an Amino-(N-)terminal propeptide, followed by a telopeptide, a central triple helix, another telopeptide and the Carboxy-(C-)terminal-propeptide.[5]

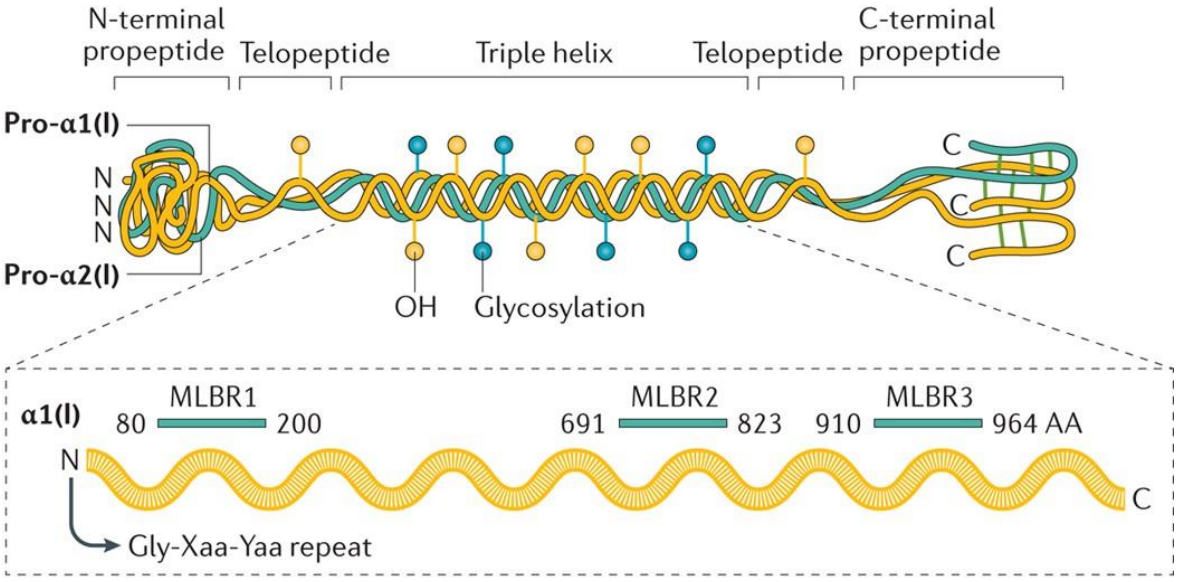

FIGURE 1 – Structure of collagen[5]

The soluble procollagen is then transported to the Golgi-network, where it is packed into secretory vesicles and later exported into the ECM.[2]

After secretion both propeptides are cleaved off by specific metalloproteinases. ADAMTS2 (a disintegrin and metalloproteinase with thrombospondin motifs) is responsible for cleaving off the N-terminal-[18], and BMP1 (bone morphogenetic protein 1) the C-terminal-propeptide.[1] The cleavage sites for the proteinases are encoded by exon 6 of *COL1A1* and *COL1A2*.[19] On either side of the protein a short, non-helical telopeptide remains which is involved in the further crosslinking and assembling processes.[8,20]

Enzymatic cleavage of the propeptides induces fibrillogenesis.[5] During this process lysine and hydroxylysine residues in specific positions are further modified by LOX, a copper-dependent lysiyl oxidase. This results in aldehydic forms of lysine and hydroxylysine. In collagen I there are only two residues in the nonhelical C-telopeptide and three in the N-telopeptide region of the α1(I) chain where this oxidative deamination is possible.[2]

Mature collagen molecules spontaneously assemble into parallelly aligned fibrils, as procollagen monomers form a longitudinal line with a characteristic gap of about 40nm between two consecutive strands resulting in a consecutive axial periodicity of 67nm in bone tissue.[21] The consecutive alternating packing density leads to the distinct banding pattern seen on the ultrastructural level.[2]

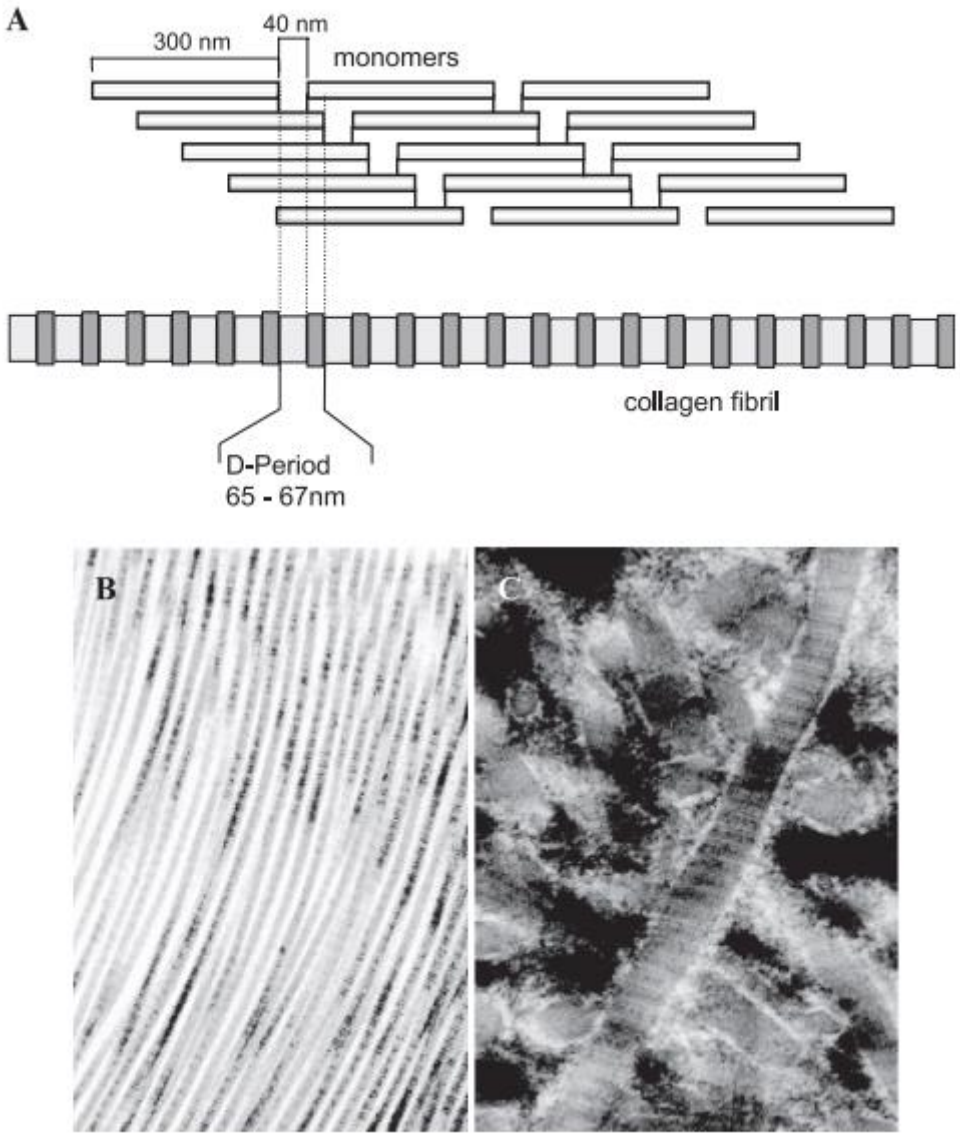

FIGURE 2 – Collagen ultrastructural view[21]

Finally, crosslinks between the previously developed aldehydes of lysins and hydroxylysines are established, which leads to the formation of covalent and subsequently complex multivalent intra- and intermolecular crosslinks. This step is crucial for the final biomechanical stability.[2]

Collagen in bone tissue

Bone is a metabolically active connective tissue.[22] Microscopically, two types of bones can be distinguished: Woven bone is a product of primary bone formation whereas collagen fibrils are displayed in an unorganized manner. It is later replaced by the stronger lamellar bone.[23] In lamellar bone collagen fibrils are tightly packed

with alternating orientation resulting in a more resilient tissue.[23] In the skeleton of an adult human two main types of lamellar bone are distinguished. The first type is the dense and more solid cortical or compact bone, making up for around 80% of total bone weight. The second type is the cancellous bone, also called trabecular bone forming a honeycomb-like meshwork.[23,24]

Anatomically, lamellar bone consists of a dense layer of cortical bone, surrounding trabecular bone with the bone marrow inside.[22] The main functional units are cylinder shaped osteons. In the center of each osteon lies a longitudinal canal, the so-called Haversian canal, which contains the vessels that are important for the blood supply of the bone cells. Approximately four to twenty concentric layers of mineralized collagen fibers form an osteon, which is further surrounded by the so-called cement line. Volkman's canals run vertically to the Haversian and contain blood vessels, thereby connecting the inner bone marrow with the periosteum.[22] The outside bone is coated by periosteum, a thin layer of fibrous connective tissue with many blood vessels and numerous innervations.[24]

Bone tissue constitutes of approximately 10% bone cells and 90% bone matrix. There are different types of bone cells: Osteoprogenitor cells are undifferentiated progenitor cells that can differentiate into Osteoblasts.[24] Osteoblasts are responsible for producing organic bone matrix by secreting a fluid called osteoid.[22] Osteocytes originate from osteoblasts and are immured in mineralized bone matrix located in lacunae. Osteoclasts are large multi-nucleated cells responsible for phagocytosis and therefore allowing remodeling of the bone matrix.[24]

Bone matrix comprises of approximately 50-70% mineral or inorganic matrix, 20-40% organic matrix, 5-10% water and <3% lipids.

The main occurring mineral is hydroxyapatite, with smaller amounts of carbonate, magnesium and acid phosphate.[23] The gaps in the collagen fibril structure enable calcium apatite crystals to attach.[25] Thereby they merge and further grow alongside the collagen fibrils.[26]

Collagen I makes up for approximately 80% of the total content of proteins and about 95% of all collagen molecules in the bone.[27] The mineralized collagen fibers form planar sheets called lamellae that have an average size of 3-7µm. They also build up the walls of Haversian canals.[28] Other types of collagens appearing in smaller

amounts are collagen III, V and FACIT collagens.[23] Collagen III and V seem to be responsible for the regulation of fibril diameter.[29] An in vitro study on chick embryos showed a decreasing fibril diameter with increasing percentage of type V collagen[30], and there is evidence that a significant part of collagen V in the bone is crosslinked to type I collagen.[27] The acronym FACIT, short for “fibril-associated collagens with interrupted triple helices”, describes a group of non-fibrillar collagens that are interconnected with the surface of collagen fibrils.[1]

Non-collagenous proteins comprise exogenously derived serum proteins like albumin and α2-HS-glycoprotein and endogenously synthesized proteins e.g., proteoglycans, glycosylated proteins like alkaline phosphatase and more.[23]

The strength of bone is determined not only by bone mass, but also by the quality of tissue, which is depending on general architecture, properties of the materials, composition and bone turnover.[25] The major role of the collagen network appears to be the provision of toughness to the bone structure by its ability to absorb energy, whereas the inorganic mineral components are responsible for stiffness and rigidity.[23,25,31]

As collagen represents a major part of organic matrix, different parts of collagen biosynthesis may influence final bone strength. Homotrimeric collagen, comprising of three identical α1-chains, is usually not found in healthy adult tissue.[29] Mann et al. showed that the Sp1 binding site polymorphism in *COL1A1,* leading to the secretion of abnormal amounts of α1-chains, results in increased risk of osteoporotic fractures and overall reduced bone strength.[32] Mutations in the *PLOD* genes (OMIM #153454, #601865, #603066), encoding for LH1-LH3, lead to different connective tissue disorders. Signs of reduced bone strength are thereby seen in Bruck syndrome[33] or a unique phenotype described by Salo et al.[34] Disturbances in glycosylation might have an impact on bone strength as well, since this process has been shown to influence fibrillogenesis and crosslinking.[14] The orientation of collagen fibrils is also considered to influence bone strength.[25,29]
Most types of osteogenesis imperfecta are explained by impaired collagen biosynthesis, leading to a qualitatively dysfunctional or reduced amount of collagen, as described in the corresponding section.[5]
Aging impacts collagen network by typical age-related posttranslational

modifications such as isomerization, development of advanced glycation end products (AGEs) and crosslinks.[25,35]

3.2 Diseases associated with *COL1A*

3.2.1. Osteogenesis imperfecta

Osteogenesis imperfecta (OI), also known as “brittle bone disease” is a phenotypically heterogenous group of rare hereditary connective tissue disorder with main clinical manifestations of bone fragility and susceptibility to fractures. Other features such as hearing loss, blue sclerae, low height, dentinogenesis imperfecta or bone deformities may be present.[5,36] The incidence is estimated to be approximately 1:10.000-20:000.[19,36]

Classification

OI was originally classified in 1979 by Sillence et al.[37], based on clinical and radiographic features as well as assumed patterns of inheritance due to unavailable possibilities of genetic testing at that time. For their study, 180 patients were recruited and revised. This led to the original classification of OI into four types:

- Type I: dominantly inherited OI with blue sclerae
- Type II: lethal perinatal OI with radiographically crumpled femora and beaded ribs
- Type III: progressively deforming OI with normal sclerae
- Type IV: dominantly inherited OI with normal sclerae

With the help of pedigree analysis, these four types of OI were assigned to distinctive patterns of inheritance. For types I and IV a predominantly dominant type of inheritance was assumed, whereas in pedigrees with OI type II and III also recessive inheritance patterns were found.[37] Introduction of genetic testing enabled assignment to mutations in *COL1A1* or *COL1A2* genes.[38] In clinical practice the Sillence classification is still broadly used as phenotypical description due to its convenience. However, the detection of new causative mutations in genes other than *COL1A1* and *COL1A2* have led to a new understanding of the pathomechanisms and the need for a new, adapted genetic classification.[19]

In the commonly used classification system, the long-known types I-IV are still used and ascribed to autosomal dominant mutations in *COL1A1* and *COL1A2*. They make up for about 85-90% of current known types of OI.[36] Other, recently

discovered types are integrated into this classification system based on associated gene and biomolecular mechanism.[5,36]

Types of OI

Type I

OI type I is characterized by a mild phenotype with blue sclera and normal stature. It often stays unrecognized until early childhood and gets diagnosed when children start to walk, and multiple fractures occur due to mild trauma. The susceptibility to fractures normally decreases with age, typically after puberty, but may increase again in the 5th decade of life. Fractures generally heal without remaining deformities. Adults are often shorter than predicted by parental height, but attain physiological stature in most cases. Joint hypermobility may be present, as well as scoliosis. Hearing loss is more frequent, and applies to about half of affected individuals.[39] Although blue sclerae are a classifying feature, they may be absent in some individuals.[36]

Type II

Infants with type II OI, also called the “perinatal lethal form”, usually die within weeks after birth or in some cases intrauterine. Survival past one year of age is extremely rare. OI type II is characterized by severe bone deformities with multiple fractures or short, bowed bones and a typical abduction of the legs in a so-called “frog-leg-position”. Weight and length are low for gestational age. The skull seems large compared to the rest of the body and is poorly ossified. The anterior fontanelle is abnormally large as well. At birth the infants often show distinct facial features with a flat and triangular face and dark blue-gray sclerae. Death usually occurs due to pulmonary infections or respiratory failure.[36,39]

Type III

The progressive deforming type is the most severe form of OI compatible with life after infancy. Affected individuals suffer from multiple fractures and deformities, whereby deformities may even be present in absence of fractures. Hundreds of fractures may be acquired in lifetime and most affected patients are dependent on walking aids or wheelchairs. This type is typically diagnosed shortly after birth, as fractures occur even by simply handling the child. Affected adults have distinct small stature and usually achieve normal life expectancy. Secondary features like DI,

hearing loss, macrocephaly, severe scoliosis and thorax deformities are common.[36,39] Death is often the result of severe chest wall deformities leading to cardio-respiratory failure.[38]

Type IV

This type shows a broad spectrum of clinical severity, but is defined as moderate severity. Patients may suffer from multiple fractures in their lifetime or present only very mild symptoms, complicating the process of diagnostics. Characteristics are mild to severe forms of DI, hearing loss and normal or greyish sclerae.[39] The severity has shown to be variable even within the same family.[38]

Type V

The dominant inherited type V is phenotypically very similar to type IV with occurrence of frequent fractures and bone deformities.[36] However, there are distinct clinical features characteristic for this type, most strikingly the calcification of the interosseus membranes of the forearms. Further differences are distinct bone histology and in some patients the formation of hypertrophic callus.[40,41]

Recessive types of OI

Together with type V, the recessive types make up for approximately 15-20% of OI. Often, they are assigned to the clinically used types I-IV by severity with additional presence of distinct clinical features.[36]

Pathophysiology and mechanisms

Modified by the classification of Sillence et al.[37] 22 different types of OI and 25 causative genes are described in OMIM[42] (OI type XXII: MIM #619795) however, the number is constantly growing. They can be further grouped into 5 functional clusters based on biomolecular mechanisms, as described below.

Defects in collagen I biosynthesis and structure

Mutations in *COL1A1* and *COL1A2* lead to autosomal dominant inherited structural defects in collagen, whereas two different types can be distinguished. Quantitative defects are characterized by a reduced amount of otherwise normally functioning collagen I.[43] These are usually the result of loss-of-function mutations by a premature stop codon.[36,44,45]

Qualitative defects are characterized by the production of altered, or rather

structurally damaged collagen I fibrils, who are integrated into the ECM.[43] Most commonly the glycine within the strict Gly-Xaa-Yaa sequence responsible for the triple helix formation is replaced by another amino acid, which further leads to delayed helix folding.[19] Other pathomechanisms include splice site alterations, changes in the triple helical structure such as duplications, insertions and deletions, or alterations in the Carboxy-terminal propeptide.[19,43]

Quantitative defects lead to the milder type I OI, whereas abnormal fibrils caused by qualitative defects often result in more severe types, i.e. OI type II-IV.[39]

Defects in posttranslational modification and processing

The recessive types VII, VIII and IX are caused by mutations in genes encoding the P3H complex, which are important for later occurring extracellular folding of collagen.[19] That is *CRTAP* for type VII[46], *P3H1* for type VIII[47] and *CyPB*, also called peptidyl-prolyl isomerase B or short PPIB, for type IX[48]. Mutations in *TMEM38B* lead to OMIM type XIV[19,49] and OMIM type XIII is caused by mutations in *BMP1*.[49]

Defects in collagen folding and crosslinking

Two other autosomal recessive types of OI are attributed to mutations in the genes *SERPHIN1*, encoding for HSP47, and *FKBP10*, encoding for the 65kDA FK506-binding protein, leading to OMIM OI types X and XI.[19,49]

Impaired bone mineralization

The autosomal dominant type V is caused by mutations in Interferon-induced transmembrane protein 5 (*IFITM5*), encoding for a membrane protein specific to osteoblasts. The exact underlying mechanism of its role in mineralization in vivo is not yet resolved.[40]

Mutations in *SERPINF1*, encoding the osteoblast and osteoclast regulating protein PEDF (pigment epithelium-derived factor), are causative for type VI OI.[50]

Defects in differentiation and function of osteoblasts

OMIM types XII, caused by *SP7*[51], and types XV to XVIII are due to mutations in genes relevant for the differentiation of osteoblasts. Responsible genes for OMIM types XV to XVIII are *WNT1*, *CREB3L1*, *SPARC* and *TENTS*.[51] *MBTPS2* leads to OMIM type XIX, a X-linked type of OI.[52]

Unclassified causative genes

Recently discovered mutations in *MESD*[53], *KDELR2*[42] and *CCDC134*[54] are defined as OMIM types XX, XXI and XXII.

Clinical manifestation and secondary features

OI offers a broad spectrum of symptoms, ranging from barely identifiable conditions like early onset osteoporosis to severe perinatal forms. Main clinical characteristics are defined by alterations in bone integrity and consecutive increased susceptibility to fractures or bone deformation.[36]

One very common secondary feature is short stature or stature shorter than predicted. Severity varies between different subtypes.[36] Hearing loss, if present, often starts after puberty and is progressive with age. However, early onset hearing loss can be observed in about 7% of affected children. It often starts as conductive hearing loss due to fractures of middle ear bones which later gets aggravated by additional sensorineural hearing loss.[39] Scleral blueish hue is a classification criterion formerly used to distinguish between OI types I-IV.[37] As it may be absent in some types and can differ interindividually, it is now considered more a secondary than a defining feature.[36,39] Dentinogenesis imperfecta (DI) describes an abnormal tooth development with reduced mineralization and therefore discolored and especially fragile teeth.[5] Distinct facial features such as a triangular-shaped face, frontal bossing, platybasia with following basilar impression, prognathia or macrocephalia are also associated with OI.[39] Other possible clinical signs include easy bruising, respiratory insufficiency, cardiovascular manifestations, gross motor development delays or bleeding diathesis, as well as secondary skeletal deformities such as scoliosis.[5]

Clinical management

At present there is no cure for OI. The main therapeutic goal is to preserve quality of life via medical, physical, and surgical treatment. The administration of bisphosphonates is the standard procedure in patients with moderate to severe OI, although in childhood this happens as “off-label-use”, as there is no approval for children.[55]

Genotype-phenotype relation of *COL1A1* and *COL1A2* related OI

A definitive genotype-phenotype correlation does not exist. Common principles have been proposed, based on observations and evaluation of cohorts with dominant types of OI.[43,56-58] However, there are multiple exceptions to these rules. In general, the phenotype depends on the type of mutation, resulting amino acid, location and whether α1(I) or α2(I) is affected.[43]

Quantitative mutations resulting in reduced amount of one α chain usually cause a milder clinical type.[39,56] Splice site mutations, which make up for approximately 20% of mutations in *COL1A1*, generally show a milder phenotype as well. However, lethal outcomes have been described.[43]

The substituting amino acid has great impact on clinical outcome. Most commonly the glycine, appearing in the first position of the repeating Gly-Xaa-Yaa sequence, is replaced by serin, cysteine or arginine. Generally, branched (valine) or charged (aspartic acid, glutamic acid or arginine) amino acids lead to a more severe or even lethal phenotype than neutral polar (serin and cysteine) or apolar (alanine) ones.[56] Substitutions with serin and cysteine within α1(I) chain show a mixed clinical outcome with lethal and non-lethal types.[43] Charged or branched aminoacids are more likely to lead to lethal phenotype in α1(I), whereas in α2(I) this happens to a lesser extent.[43] In the C-terminal 200 residues of α1(I) almost all valine or charged residues are lethal, whereas in α2(I) substitutions with valine are lethal only in 17% of cases.[43] Substitutions by alanine usually result in mild phenotypes in both chains.[43,58] As they seem to be underrepresented in clinical data, it was presumed that they may elude genetic testing due to undetectable mild clinical presentation.[43]

Mutations in α1(I), compared to α2(I), seem to exhibit a more severe clinical phenotype.[43,56,57] Glycine substitutions with branched or charged sidechains in the α1(I) chain primarily lead to lethal outcomes. If the α2(II) chain is affected, the outcome is often non-lethal.[19] Serin substitutions in α1(I) chain lead to a clinical more severe type compared to same substitutions in α2(I) chain.[56]

It was observed that the clinical severity increases from the N-terminal to C-terminal end, possibly due to the coherent assembling direction of the triple helix.[56] Substitutions in the α1(I) chain, located in the N-terminal fifth of the chain, are mostly

non-lethal, which similarly applies to the α2(I) chain.[43] Interestingly, substitutions in the first N-terminal 90 helical residues often lead to a combined OI-Ehlers-Danlos syndrome phenotype.[59]

Mutations altering the cleavage sites for C-propeptide or N-propeptide lead to distinct types of OI. If C-propeptide cannot be cleaved off the resulting product is pC-collagen, a structurally defect collagen with extant C-propeptide. This usually results in a mild phenotype.[5] Mutations which cause the removal of the N-terminal cleavage site and therefore collagen fibrils with N-propeptide still attached (i.e. pN-collagen) result in a distinct type of Ehlers-Danlos syndrome.[60]

Lethal phenotypes seem to be mainly caused by glycine substitutions or splice site alterations, as other types of mutations like frameshift or nonsense mutations have been associated with non-lethal phenotypes. Moreover, mutations in 1(I) chains are more often lethal compared with 2(I) chains.[56] 8 lethal clusters for the α2(I) have been proposed in distinct sections which are involved in the interactions between matrix proteoglycans and collagen fibrils[43]. Some of these have been disproven. Rauch et al.[58] identified one patient and Maioli et al. 8 persons with non-lethal phenotypes in some of these clusters.[56] On the contrary no such clusters have been proposed for α1(I) chains. Rather regions of particular importance have been defined, where alterations in the sequence are supposed to cause only lethal outcomes.[43] Part of this assumption was disproved by Maioli et al. as well.[56]

3.2.2. Ehlers-Danlos syndromes

The Ehlers-Danlos syndromes (EDS) comprise a group of genetically heterogenous heritable connective tissue disorders that share distinct clinical features. Main clinical characteristics are joint hypermobility, hyperextensible skin and tissue fragility. Secondary features depend on the respective subtype and some of them are important criteria used for classification. These include cardiac-valvular involvement, arterial rupture, skin abnormalities, skeletal deformities such as scoliosis, or weakness of tissues with predisposition for ruptures or herniation.[61] Overall, prevalence is expected to be approximately 1:5.000. However, only few case reports exist whereas for rarer types, which describe affected families or single patients.[62]

Classification

The 2017 international classification identified alterations in 19 causative genes that lead to an overall number of 13 distinct EDS subtypes.[61] Studies performed after 2017 reported another subset of EDS, which is classified as classical-like EDS 2 (clEDS2, OMIM #618000) in OMIM. The number of known causative genes is thereby increased to 20.[63]

The classification mentioned above is broadly used in clinical practice. It is based on clinically defined major and minor criteria associated with the corresponding subtype, as well as underlying genetic and pathogenic mechanism. Major criteria are characterized by the presence in most affected individuals and/or their distinct appearance in the respective subset, which allows differentiation from other subtypes or even other connective tissue disorders. Hence, they have high diagnostic specificity. Minor criteria are less specific but may support final diagnosis. Thus, the diagnostic pathway is primarily based on clinical symptoms. However, final diagnosis should always be confirmed by genetic testing with a causative alteration in the corresponding genes. The importance of genetic testing is emphasized, particularly considering genetic and phenotypic heterogeneity of EDS, clinical overlaps between different subtypes, as well as similarities to other hereditary connective tissue disorders.[61] However, there is one subtype of EDS which cannot be assigned to a causative gene, namely the hypermobile EDS (hEDS). hEDS displays the most common type and is expected to make up for approximately 80-90% of all EDS cases and affecting up to 1-3% of overall population. It is assumed to have an autosomal dominant pattern of inheritance, but diagnosis can be hindered due to broad spectrum of symptom severity, high prevalence of GHJ in other connective tissue disorders and the lack of genetic verifiability.[64]

Pathophysiology and mechanisms

Similar to OI, subtypes of EDS can be divided by underlying mechanism and are distributed regarding their correspondent step in biosynthesis of ECM and tissue. The majority of EDS subtypes is caused by alterations in either collagen synthesis itself, or adjacent processes.[61]

Clinical manifestations and secondary features

Skin hyperextensibility is a distinct feature in EDS and should be assessed by pinching and lifting the skin in defined locations, i.e., dorsal side of feet and hands, and at the volar and middle part of the non-dominant forearm. Skin is considered hyperextensible if at least 3 locations are affected. The cut-offs are 1,5cm for the above-described positions at feet, hands, and non-dominant forearm or 3cm for knee, neck, or elbows.[61]

Other skin-related conditions are easy bruising, atrophic scarring with a broad spectrum of severity, whereas in some patients even hemosideric scarring may occur, and abnormality of skin texture, manifesting from soft and doughy, to translucent and velvety skin with increased visibility of veins.[61]

Joint hypermobility can affect the whole body, i.e. generalized joint hypermobility GJH, or particular joints, i.e. localized hypermobility. GJH is evaluated via the Beighton score, a clinical scoring system with 9 attainable points. According to the classification, a Beighton score of ≥5 points corresponds with the presence of GJH, whereas patients with a score of <5 may still be positive for GJH depending on their family history and a five-point questioning respectively.[61] However, the Beighton score has limitations and its usage as diagnostic tool is controversially discussed, as some major joints are not included and the upper limbs are overrepresented compared to lower limbs.[65]

Types of EDS

Classical EDS (cEDS)

In over 90% cEDS is caused by mutations in either *COL5A1* or *COL5A2* genes, leading to a distinct phenotype with major criteria of skin hyperextensibility, atrophic scarring and GJH. Inheritance is autosomal dominant. Minor criteria include other skin conditions such as easy bruising, soft and doughy skin, mulloscoid pseudotumors or subcutaneous spheroids. Other features such as epicanthal folds, hernia, complications of joint hypermobility or a family history of persons with fulfilled criteria may be present.[61]

In rare cases, a specific mutation in *COL1A1*, causing a substitution of an arginine residue by cysteine (p.Arg312Cys) leads to a similar phenotype and is classified within the same clinical entity of cEDS.[66-68] However, this amino acid substitution

causes another specific phenotype as well, which is characterized by arterial rupture at young age and missing or mild signs of other EDS-typical features.[66,69] The p.Arg312Cys mutation in *COL1A1* is therefore known as a causative variant both in cEDS, as well as in vEDS (vascular EDS).[61]

Cardiac-valvular EDS (cvEDS)

cvEDS is characterized by severe and progressive cardiac-valvular problems, caused by biallelic *COL1A2* mutations which result in total absence of proα2(I)-chains. Major criteria include the defining feature of cardiac-valvular involvement, as well as several skin-related conditions such as skin hyperextensibility, atrophic scarring, easy bruising, thin skin, or joint hypermobility.[61,66]

Vascular EDS (vEDS)

The autosomal dominant inherited vEDS is attributed to heterozygous mutations in *COL3A1*. Major criteria include a family history of vEDS with detected causative *COL3A1* alteration, arterial rupture at young age, spontaneous sigmoid colon perforation, uterine rupture in pregnancy in absence of other risk factors, or presence of a Carotid-cavernosus sinus fistula without preceding trauma. Minimal criteria, which should be considered as vEDS if they appear combined, comprise a group of unspecific features, e.g., congenital hip dislocation, thin and translucent skin, easy bruising, spontaneous pneumothorax, hypermobility of small joints or tendon and muscle ruptures.[61] Rare cases of vEDS are ascribed to specific alterations in *COL1A1*, which lead to a substitution of arginine to cysteine.[66,69]

Arthrochalasia EDS (aEDS)

aEDS, formerly EDS type VIIA or VIIB, is characterized by congenital hip dislocation in combination with either skin hyperextensibility or GJH together with at least two minor criteria. Minor criteria include muscle hypotonia, kyphoscoliosis, mild osteopenia, tissue fragility and easy bruising. It is caused by a partial or entire loss of exon 6 in either *COL1A1* or *COL1A2* most commonly due to splice site alterations.[61,66] Exon 6 encodes a region containing the N-terminal cleavage site on both genes.[60]

Non COL1A-related EDS subtypes

The classical-like EDS (clEDS) is an autosomal recessive inherited subtype with a phenotype similar to cEDS. It is caused by mutations in *TNXB*, leading to complete absence of the corresponding gene product Tenascin B.[61,66] Recently, mutations in the *AEBP1* gene have been ascribed to a similar phenotype which is regarded as classical-like EDS 2 (clEDS2).[63]

Diagnosis of Hypermobile EDS (hEDS) can only be made clinically, since there are currently no causative genetic variants described. Due to great heterogeneity of clinical symptoms and overlapping phenotypes with other hereditary connective tissue disorders, making a diagnosis can be challenging. Therefore, the importance of fulfilled criteria defined for hEDS is emphazied.[61]

Dermatosparaxis (dEDS) is caused by autosomal recessively inherited mutations in *ADAMTS2*, encoding the enzyme responsible for cleaving off the N-terminal propeptide of procollagen I. Main symptoms are extreme skin fragility with skin tears, distinct craniofacial features and either one other major criterion or at least three minor criteria. Together with other conditions typical for the clinical spectrum of EDS, minor criteria also include teeth abnormalities and osteopenia.[61,66]

Kyphoscoliotic EDS (kEDS) is characterized by congenital muscular hypotonia, congenital or early onset kyphoscoliosis and severe GJH with recurring dislocations or subluxations. Causative genes are *PLOD1* or *FKBP14*, both of which lead to gene-specific minor criteria.[61,66]

Other rare types of EDS are spondyloplastic EDS (sEDS), brittle cornea syndrome (BCS), musculocontractural EDS (mcEDS), myopathic EDS (mEDS) and periodontal EDS (pEDS).[61]

Clinical management

There is no cure for neither subtype of EDS at present. Clinical management aims to reduce symptoms, prevent complications, and guarantee best possible quality of life depending on the respective phenotype.[62] Each patient suspected with EDS should undergo genetic testing to confirm diagnosis, except for hEDS for which to date no causative mutation is known. This is especially important for specific subtypes of EDS with possible life-threatening conditions, such as vEDS.[61,62]

General treatment strategies include multidisciplinary pain management and physiotherapy. Due to skin fragility, surgical interventions are often avoided, and conservative approaches favored.[62]

Genotype-phenotype relation of *COL1A1* and *COL1A2* related EDS

As COL1A-related EDS-forms are often only described by single case reports, general phenotype-genotype relations do not exist.

Alanine-to-cysteine substitutions in specific locations of *COL1A1* are associated with distinct phenotypes. The mutation p.(Arg312Cys) leads to cEDS with or without vascular fragility[66-68], whereas p.(Arg574Cys) and p.(Arg1093Cys) result in vEDS[66,69]. However, other replacements of arginine by cystein within COL1A are described, which in contrast lead to different connective tissue disorders such as the OI/EDS overlap syndrome[70,71] or Caffey disease.[72] Entire absence of the proα2(I) chains leads to cvEDS.[61] Skipping of exon 6 on either *COL1A1* or *COL1A2* results in the clinic type of arthrochalasia, whereas mutations in *COL1A1* are responsible for more severe clinical phenotypes in comparison to *COL1A2*. This is ascribed to the general composition of a collagen molecule, which consists of two α1(I) and only one α2(I) chain.[60,66]

3.2.3 Caffey Disease

Caffey disease (CD), or infantile cortical hyperostosis (OMIM 114000), is a hereditary collagenopathy characterized by episodes of subperiosteal new bone formation during the first five months of life, which typically resolves spontaneously within the first two years of life. Affected bones include diaphysis of long bones, mandibles, and clavicles. In addition, painful swelling and fever may occur during these episodes.[73] Prevalence is unknown and it is supposed to be underdiagnosed due to spontaneous resolution during early childhood.[74]

The autosomal dominant inherited CD has been connected to a missense mutation in exon 41 in *COL1A1*, encoding one part of the triple-helical domain. In observed individuals and pedigrees this p.(Arg836Cys) substitution has shown incomplete penetrance. Interestingly, some individuals additionally present with clinical signs similar to Ehlers-Danlos-syndromes, i.e., hyperextensible skin and joint hyperlaxity. In one family an increased incidence of fractures was apparent, whereas other signs of collagenopathies were not observed.[72] Analysis of a 3-generation family

described persistent bone deformities as well as short stature in 5 affected members with molecular diagnosis of CD, further expanding the clinical spectrum of CD.[75] Another distinct subtype of CD is expected to have autosomal recessive inheritance and shows a more severe and often prenatal lethal phenotype.[72] To date, there is no evidence of a causative gene.[74]

3.2.4. COL1-related overlap disorder

The term COL1-related overlap disorder (C1ROD), as proposed by Morlino et al.,[76] describes an evolving term of an autosomal dominant inherited connective tissue disorder with causative mutation in either *COL1A1* or *COL1A2*, which leads to clinical symptoms of EDS with or without OI. To date there exist only few case reports, which describe both phenotype and genotype of affected individuals. In OMIM, these phenotypes are categorized as a new entity, namely “combined osteogenesis imperfecta and Ehlers-Danlos syndrome 1 and 2” (OIEDS Syndrome 1, OMIM #619115; OIEDS Syndrome 2, OMIM #619120). However, there are considerations whether it should be in fact classified as an additional EDS subtype, as most patients present with clinical symptoms mainly resembling features of this collagenopathy.[76]

Classification

Recently, classification criteria for C1ROD have been proposed by Morlino et al. Similar to the classification of EDS[61], major and minor criteria can be distinguished. In addition, eight exclusion criteria have been described. The objective of these criteria is to screen patients for the presence of C1ROD in order to initiate genetic testing, rather assigning a diagnosis. Genetic testing is recommended if at least three major criteria, or two major and one minor criteria, or one major and at least five minor criteria are fulfilled. The four major criteria include blue sclerae, GJH according to age, significantly soft and doughy and/or hyperextensible skin, and flatfeet with valgus deformity of the hindfoot. Minor criteria comprise the following seven: dolichostenomelia, hearing loss, short stature (<2 SD), two or more atrophic (non-papyraceous) scars, two or more fractures in the prepubertal age, two or more joint dislocations and two or more injuries and/or ruptures of ligaments, tendons and/or muscles. If at least one exclusion criterion is present, suspected diagnosis should be reconsidered. This does not necessarily exclude the presence of

alterations in the *COL1* genes, however, in those cases other diseases such as OI, cEDS, cvEDS or aEDS should be considered. Exclusion criteria are congenital fractures, DI, molluscoid pseudotumors, papyraceous scars, a prepubertal fracture rate >1.00, progressive or severe heart valve disease, platyspondyly and long bone deformities.[76]

Clinical reports suspected of C1ROD

Many patients described in clinical reports have been referred to genetic testing due to EDS-like symptoms.[71,76-78] As the term C1ROD is still evolving and, although classification criteria have been proposed, there is no definite diagnostic tool for this entity yet. Clinical reports resembling assumptive phenotype found through literature review will be described in the following. Excluded are phenotypes resulting from mutations causing partial or entire loss of exon 6, which therefore are classified as aEDS[60,61], as well as other phenotypes with unambiguous assignment to either EDS or OI subtypes.

Sippola et al. reported a five-year-old proband with bilateral hip dislocation, severe laxity of all joints, scoliosis, yellow tinged teeth and blue sclerae. Radiographic images showed wormian bones in the skull, platyspondyly and generalized osteoporosis. His mother was of noticeable short stature and had blue sclerae as well. However, she had no history of joint laxity or fractures. Within the same family other similar phenotypes were described, including blue sclerae, bilateral hip dislocation, and multiple fractures, whereas the manifestations of symptoms varied interindividually regarding severity. Analysis of cultured skin fibroblasts from the proband showed a suspected mutation affecting the α2(I) chain, located near the N-terminal end of the triple helical domain. It lies within α2-CB4, a cyanogen bromide peptide, comprising amino acids 7 to 327. This alteration led to a deletion of about 30 amino acids. Further testing showed impaired N-propeptide cleavage and decreased thermal stability of the resulting collagen chain, resulting in shortened α2(I) chains in fibroblast culture.[79] Following studies yielded a 19-bp-large deletion between the last codon of exon 10 to the first codon of exon 12 in COL1A2, causing an in-frame RNA splicing. As exon 6 is not affected, this variant cannot be classified as aEDS. Hence, it was defined as atypical OI by the authors.[80]

Two affected individuals from a family with suspected autosomal dominant inherited connective tissue disorder were referred to genetic testing due to atypical signs of OI and symptoms primarily typical of EDS, respectively. The patients had blue sclerae, short stature, skin hyperextensibility, easy bruising and late onset of fractures in early adulthood, either spontaneously or due to mild trauma. There were also signs of premature osteoporosis in radiological examination. Further investigation showed an intronic deletion c.432+3_432+13del in *COL1A2,* which leads to skipping of the entire exon 9.[81]

In 1997, Feshenko et al. described a patient who presented with bilateral hip dysplasia, muscular hypotonia and umbilical hernia after birth. In infancy, one dislocation of a shoulder occurred, and marked general joint laxity was noticed. Further features included blue sclerae, joint laxity, and frontal bossing. There were no signs of DI, hearing loss or bone fragility. His mother was notably of short stature with blue sclerae, abnormal scarring and GJH, and his older sister showed blue sclerae and GJH as well. Genetic testing revealed a heterozygous splice donor site mutation, which leads to skipping of exon 9. Analysis showed that this results in shorter in-frame α2(I) chains.[82]

A partial *COL1A2* gene duplication, leading to the addition of 477 amino acids, was detected by Raff et al. in an 8-year-old boy with congenital bilateral hip dislocation and club feet. In further development he showed general joint hypermobility, mild skin hyperextensibility, prominent forehead with an anterior fontanel of 3x3cm, umbilical hernia, blue-greyish sclerae, general opalescence of teeth and a dislocation of one shoulder. In childhood he sustained one fracture, however, bone density appeared normal. His parents and three siblings showed no signs of connective tissue disorders. Genetic testing revealed a heterozygous duplication of 159 triplets in the center of the triple helical domain of α2(I). The affected exon sequence includes 20 exons, ranging from exon 12 to exon 32, and does not lead to a frameshift. Analysis showed a maintained Gly-Yaa-Xaa pattern and alignment of the helix, except for 90 amino acids at the N-terminal end of the chain and one elongated chain by additional 387 amino acids. However, the resulting prolonged chain does not change the secretion, leading to small but otherwise unaffected fibrils. This report is interesting, as on the one hand the clinical phenotype is mild in comparison to the phenotype expected from biochemical analysis, and on the other

hand it represents a case with congenital bilateral hip dislocation not categorized as aEDS due to absent affection of exon 6.[83]

Nicholls et al. reported a nine-year-old girl with marked GJH, pes planus, pale blue sclerae and mildly increased bone fragility. At her premature birth ligamentous laxity and muscle hypotonia were noticed. She had a history of recurrent patellar luxation and multiple fractures of skull, clavicle, three fingers and one toe due to mild trauma. Genetic testing yielded a homozygous splice site mutation in *COL1A2* which resulted in total absence of α2(I) chains. Her mother was a heterozygous carrier and had mild joint laxity. Her older sister was also found to carry one mutant allele, however, she displayed no clinical signs of either EDS or OI.[84] Complete absence of α2(I) chains is known to cause cvEDS.[61] However, cardiovascular conditions where not reported in this family. This may be attributed to the very young age of the reported proband, for which reason cvEDS should not be excluded on basis of this study.

Cabral et al. described seven children with main clinical symptoms of skeletal fragility, classified either as OI type III or IV with additional features of EDS such as severe joint hypermobility and early onset scoliosis. All patients had short stature and blue sclerae and five of them showed a Beighton score of 5/9 or higher. Causative mutations were found in the first 90 N-terminal amino acids of the triple helix in α1(I) without affecting exon 6. One lead to the skipping of exon 7, the others were glycine substitutions in exons 7-11. The affected region was assumed to be a highly stable domain, providing an 85 amino acid long anchor important for N-terminal helix nucleation. The adjacent 5 amino acids are characterized by a lack of residues crucial for stability of the triple helix, e.i. Hyp and Pro. They are therefore proposed to display a flexible microunfolding region. Analysis showed that mutations in this specific 90 amino acid long region interfered with N-propeptide processing. Furthermore, the nearer to exon 6, the more impact mutations seemed to have on the final collagen product. If exon 7 was affected, in ¾ of the collagen chains pN-propeptide was integrated into the ECM, whereas in exons further towards the center of the helix this percentage dropped. Mutation in exon 11 only resulted in a delay of the cleavage process. The authors concluded that the distinct phenotype found in these patients was a result of this incomplete or delayed N-propeptide cleavage and incorporation of pN-collagen into the ECM.[59]

A ten-year-old girl was reported by Symoens et al. with perinatal fracture of clavicle and pneumothorax, Beighton score of 8/9 at the age of 7 years, mild blue sclerae, mild skin hyperextensibility, genu recurvatum and mild joint hypermobility mainly at hands and wrists. There were no signs of atrophic scarring or teeth abnormalities. Genetic testing detected a p.(Met1264Val) mutation, leading to alternative splicing and generation of two altered transcripts due to a substitution of methionine by valin. The alteration is located in the C-terminal end in exon 49 of pro-α1(I), a region in the C-propeptide which has been known to be highly conserved. Interestingly, regarding the first transcript, no evidence of structural abnormalities of collagen (I) chains was found in further analysis. This led to the assumption that either no mutant chains were synthesized, or if produced, they would not interfere with the formation of fibrils. The second transcript due to activation of a cryptic splice site was supposed to result in a functional haploinsufficiency because of immediate degradation after synthesis. The authors considered the clinical phenotype to be the result of either the reduced amount of collagen α1(I) or the consequence of both transcripts combined.[85]

Cabral et al. reported 4 affected family members in a small pedigree with prepubertal long bone fractures, light blue sclerae, large joint hyperextensibility and moderately decreased DXA score. The causative mutation in *COL1A1* leads to a substitution of arginine by cysteine in the Y-position of the helix. Individuals showed no other signs associated with C1ROD.[70]

A 4-year-old boy has been referred to genetic testing due to suspected EDS. Clinical examination showed GJH with Beighton score of 9/9, bilateral flat feet, soft and translucent skin and many bruises. He had a history of one fracture in early childhood. His sclerae were blue at the age of 4 years, however, at later consultation with 7 years they appeared white. No signs of DI or skin hyperextensibility were noticed. Following genetic testing revealed a heterozygous mutation in *COL1A1*, resulting in an arginine to cysteine substitution at position 858 within the triple helical domain. His mother and sister were carrier of this variant as well and both presented a Beighton score of 7/9, similar skin texture, rather short stature for their age (both at the 10th percentile), and a history of fractures in childhood. However, their sclerae were white.[71]

7 patients with predominantly clinical signs of EDS and only mild symptoms of OI were summarized by Malfait et al. They all presented with generalized joint hypermobility, skin hyperextensibility and/or translucency, short stature, blue sclerae and osteopenia and/or a history of infrequent fractures. In addition, some patients showed signs of vascular fragility. Genetic testing yielded variable mutations leading to exon skipping or glycine substitutions in *COL1A1* or *COL1A2*. However, one common feature was a location close to the N-terminal procollagen cleavage site, without affecting exon 6. Thus, the authors proposed that the clinical overlap between OI and EDS may be the result of impaired N-propeptide cleavage and therefore integration of pN-collagen into ECM due to adjacent mutations.[78] However, this assumption could later be partly disproven by Morlino et al.[76]

One of two patients described in a study by Reuter et al., a 3-year-old boy, was suspected of a connective tissue disorder due to combined OI and EDS symptoms. At birth he had large fontanelles, blue sclerae, bilateral clubfeet, a unilateral inguinal hernia, bilateral hydrocele testis and mild muscular hypotonia. At 7 months X-ray indicated wormian bones and streaky lucencies in the skull, however radiographs of spine and extremities conducted two months later showed no signs of impaired skeletal mineralization. Assessment at three years of age showed relative macrocephaly, frontotemporal alopecia, opalescent teeth and three posttraumatic broken incisors, as well as soft skin and marked joint hypermobility. Beighton score was 6/9. Serum parameters were normal and there was no history of fractures. However, the authors stated that the proband was still very young and as it is not unusual for type I OI to develop first fractures once children start to walk and fall, final diagnosis may change at older age. His mother had a unilateral hip dislocation, however there was no other family history of joint hypermobility or other signs of a HCTD. Genetic testing revealed a de-novo mutation c.1316G > A (p.Gly439Asp) in exon 23 of *COL1A2*.[86]

In a clinical report by Vandersteen et al., one 26-year-old woman was examined due to suspected OI and/or EDS. She, her mother and her sister had blue sclerae, signs of hypermobility, arthralgia, joint dislocations and subluxations, and suffered from excessive menstrual bleeding. The proband reported delayed wound healing and atrophic scarring, however she had no history of fractures. Her mother was reported to have tendency for excessive hematomas and sustained three fractures during

her lifetime. Her sister reportedly suffered more fractures; however, the exact number was not known. Cardiac involvement was only reported for their mother, who underwent cardiac surgery due to mitral regurgitation. Genetic testing yielded a c.643G > A mutation, which is located in exon 9 of *COL1A1*. Interestingly, this exact same c.643G > A alteration in *COL1A1* was found in another patient, a 7-year-old girl, also reported in this study. In clinical examination this affected girl showed blue sclerae and mild signs of EDS, i.e., a Beighton score of 7/9 and anamnestic joint instability. However, she had no history of fractures and bone mineral density appeared normal. She was referred to genetic testing after the sudden death of her father at the age of 51 years after complications during cardiac surgery. He suffered from a myxomatous mitral valve degeneration, which was later suspected to be due to a connective tissue disorder in histological examination, rather than an infection. He was reported to have suffered from joint hypermobility, bilateral hallux valgus and hallux rigidus, posterior uveitis of unknown cause, and one prepubertal fracture. He had blue sclerae as well.[87]

Shi et al. described a pedigree comprising thirteen affected patients. They all presented with distinct blue sclerae, and some exhibited fractures and joint dislocations, especially at young age. However, no sign of scoliosis, DI, skin extensibility, atrophic scarring or other skin conditions were described. There was also record of three abortions, one of which occurred spontaneously in the 10th week of pregnancy. The other two fetuses were medical indicated abortions due to limb deformities seen in antenatal 4D color Doppler ultrasound. A causative mutation was found *COL1A1* and lead to a substitution of alanine to valine at position 1174.[88]

Two individuals with overlapping OI/EDS phenotype were reported in a large genotype-phenotype correlation population study in the Swedish population by Lindahl et. al. Two pathogen mutations, c.563G>A (p.Gly188Asp) in exon 8 (7) of *COL1A1,* and c.326G>A (p.Gly109Asp) in exon 8 of *COL1A2* were revealed. However, no additional information regarding the phenotype of these two patients was provided.[57] Both mutations were previously described by Malfait et al. in patients with C1ROD[78].

From a large cohort of patients with suspected HCTD and EDS respectively, two families who belong to the same tribe showed features resembling both EDS and

OI. Most noticeable were the similarities between the two reported patients, comprising one 6-month-old boy and a 7-month-old girl. Both showed severe skin and joint laxity, respiratory distress at birth, distinct craniofacial features, such as high forehead, blue sclerae, mid-face hypoplasia, saggy cheeks and microstomia, and severe hypotonia with absent reflexes. A wrinkled skin over the abdomen, hands and feet were noticed. Additionally, the boy showed brachydactyly. The girl was also reported to have a cleft palate and skeletal examination showed s-shaped kyphoscoliosis, generalized osteopenia, and subluxations of the hip joint with coxa vara. Both of them carried a homozygous missense mutation c.2050G>A (p.Glu684Lys) in *COL1A1*. Interestingly, both of their parents were reportedly clinically unaffected, whereas an autosomal recessive inheritance pattern was assumed by the authors.[89]

In three individuals with a clinically diagnosed OI/EDS overlap a *COL1A1* mutation was identified. A seven-year-old girl with pelvic fracture, blue sclerae, hyperextensible skin, joint hypermobility and a respective Beighton score of 6/9 was reported with a pathogenic mutation c.643G>A (p.Gly215See) in exon 9. p.Gly215Arg. A 28-year-old woman was clinically evaluated because of a clinical diagnosis of Sillence type I OI and suspected aEDS respectively, due to repeated fractures, deafness, blue sclerae, kyphosis, early postural hip problems, and a Beighton score of 5/9. Next-Generation-Sequencing (NGS) revealed a deletion c.1265delG (p.Gly422AlafsX119) in *COL1A1*, which leads to a frameshift. The third patient, a 48-year-old male, was assessed due to fractures, slight presenile conductive hearing loss, blue sclerae, easy bruising, hypermobility, and a significant family history of vasculopathy. Beighton score was 4/9. Genetic testing yielded a pathogenic mutation c.662G>C (p.Gly221Ala) in exon 9. Another patient with predominantly signs of EDS, i.e., hypermobility with hyperextensible skin and marked pelvic floor weakness additionally had a history of fractured tibia and fibula. Genetic testing revealed a likely pathogenic variant c.2980C>T (p.Arg994Cys).[90]

A family with one maternal and one paternal inherited mutation each in *COL1A1*, led to a compound heterozygosity with severe phenotype in the three children. The mother had a history of low bone mass measurement (BMD) with lumbar Z-score of -3.8 at 9 years, assessed by dual-energy x-ray absorptiometry (DXA) and three

prepubertal fractures. She had light blue sclerae and a triangular shaped face. The father showed no signs of a HCTD. All children were born at term and had a triangular shaped face with light blue sclerae. The first child suffered from recurrent fractures during early childhood and marked joint hypermobility. DXA showed a Z-score of -2,6. The second child had a history of three long bone fractures due to minimal trauma and showed mild joint hypermobility during early childhood. The third sibling had four prepubertal fractures of long bones and showed no signs of joint hypermobility. All children, as well as their mother, underwent bisphosphonate therapy. Molecular genetics revealed two different mutations in *COL1A1*. The first, a frameshift mutation (c.2522delC; p.Pro841-Leufs*266), was inherited by their mother, and the second mutation (c.3196C>T; p.Arg1066Cys) by their unaffected father.[91] The c.3196C>T alteration was already described by Cabral et al. to lead to an OI/EDS overlapping phenotype with reportedly significant large joint hypermobility, moderately decreased DXA score and long bone fractures.[70] However, joint involvement in these patients seems to be more severe than in those three affected children described by Ackermann et.al.

Symoens et al. reported a young female adult with suspected EDS due to mild combined symptoms of EDS and OI. In clinical examination she presented a Beighton score of 3/9, however, she had a history of recurrent joint dislocations, mainly of the right shoulder, fingers, and toes. She had fragile and vulnerable skin, delayed wound healing with “cigarette-paper”-like scars, easy bruising, pes planus, a history of five traumatic fractures, and one tendon rupture. There were no signs of skin hyperextensibility, abnormalities in radiographs of thorax, finger, foot, elbow, and shoulder, and echocardiography seemed normal. Analysis showed a small in-frame deletion in exon 44 of *COL1A1*, which was only present in approximately 9% of genomic DNA (gDNA) in the patient’s fibroblasts. However, it was not detected in gDNA taken from leucocytes in her blood. The same mutation in heterozygous appearance was previously described in lethal forms of OI. The authors therefore concluded that the mild symptoms present in this patient result from the mosaic state of the mutation.[92]

Report of a 4-year-old boy with a clinical phenotype of EDS/OI with brachydactyly described a de novo missense mutation in *COL1A2*. He was born in the 35th gestational week after premature rupture of membranes with a birth weight of 1850g.

Clinical and radiographic examination performed at birth and shortly postnatal showed body deformity, tachypnea, bowed legs, congenital fracture of the right femoral shaft, bell shaped chest, blue sclerae, prominent forehead, plagiocephaly and low set ears. One further fracture occurred in childhood. A distinct skin hyperextensibility, GJH, DI, brachydactyly, flat feet, and genu recurvatum were also noticed. Biochemical analysis revealed a missense mutation c.3296G > A (p.Gly1099Glu) in exon 49, which is located in a highly conserved region at the C-terminal end of the triple helix.[93]

One Chinese man was referred to genetic testing due to severe signs of OI, categorized as OI type III, combined with more severe than expected symptoms of EDS. The patient had short stature, a history of 60 fractures by the age of 30 years, grayish blue sclerae, tooth loss, severe kyphoscoliosis, asymmetric thorax and respiratory distress, bilateral radial head dislocations, marked GJH, mild skin hyperextensibility, easy bruising, dislocations of finger joints, and distinct facial features, i.e. protuberant eyes and jaw, and an oval-shaped face. No signs of hearing or visual impairment were detected. At clinical examination Beighton score was 6/9, and a radiograph showed bone deformities of the lower limbs. Genetic testing yielded a heterozygous p.Gly224Asp mutation, which lies in exon 9 of *COL1A1*. The authors ascribed the severe phenotype to the charged amino acid aspartate which replaces glycine within the triple helical domain and presumably leads to a severe disruption of helix formation.[94]

Duong et al. reported two patients from a five-generation family with overall 8 affected members with diagnosis of cEDS. Genetic testing revealed a p.Arg312Cys mutation in exon 14 of *COL1A1*, a mutation which is listed both under cEDS and vEDS in the 2017 Classification of EDS.[61] The first patient showed clinical signs consistent with diagnosis of cEDS. However, the second patient, a 59-year old woman, additionally showed mild signs of OI. She had a history of GJH with recurrent ankle dislocations in childhood, atrophic scarring, vulnerable skin with over 200 sutures during her lifetime, prolonged wound healing after perineal tear at vaginal delivery of her second child, and fractures of elbow, left and right ankle before adulthood and one complex comminuted fracture of her right forearm due to mild trauma at the age of 45 years. (Reportedly due to a fall from standing height). Clinical examination showed a Beighton score of 0/9, loose joints, mildly

hyperextensible skin, pes planus, severe bilateral hallux valgus, varicose veins, hemosiderose deposits over her shins, and normal body stature.[67]

A clinical report published by Lin et al. described a family with two mutations in *COL1A1* and *COL1A5* respectively, which led to a clinical phenotype of C1ROD. The proband, an 18-year-old female, suffered from three fractures after the age of 12 and one traumatic knee ligament rupture. Additionally, she had blue sclerae, joint hypermobility, atrophic scarring, prominent ears, and easy bruising. Her mother showed similar characteristics, including recurrent fractures of long bones, joint hypermobility and joint laxity, easy bruising after mild trauma, prominent ears, atrophic scarring, and blue sclerae. The proband's brother presented with multiple fractures of long bones and blue sclerae at the age of 14 years, however there were no signs related to EDS. Her father was healthy. Genetic analysis yielded two heterozygous mutations, namely a deletion (c.2010delT, p.Gly671Alafs*95) in *COL1A1* and a missense mutation (c.5335A>G) in *COL5A1*, which is located in the C-propeptide. The *COL5A1* mutation was classified as uncertain significance and likely benign in ClinVar database, whereas the *COL1A1* variant was previously described in type I and IV OI. The proband and her mother carried both mutations, however her brother inherited only the *COL1A1* variant. Their father carried none of them. The authors concluded that the diverse phenotypes shown by the proband and her mother, in comparison with the proband's brother, may be the result of the *COL5A1* only present in these two individuals with both OI and EDS phenotype, as *COL5A1* is generally known to cause cEDS. They concluded that this phenotype may also result from combined mutations, where one is responsible for the OI phenotype, and the other results in EDS-like symptoms.[95]

In the work of Morlino et al., in which the term C1ROD was proposed to be a new EDS subtype with distinct clinical criteria, altogether twenty-one individuals from thirteen different pedigrees were studied and categorized as C1ROD. Common characteristic of the patients was a primarily suspected EDS with causative mutation in *COL1A1* or *COL1A2*, without diagnosis of an COL1-related EDS subtype such as aEDS, cEDS or cvEDS, or non-deforming OI with blue sclerae. Interestingly, two out of the five mutations found in *COL1A1*, and one of eight mutations affecting *COL1A2* were not located within the N-terminal end of the triple helix. Most mutations were substitution of Glycine, and two led to the skipping of exon 9. Clinical findings with

high prevalence (>75%) included soft and/or doughy skin, flatfoot and positive Beighton score. Additionally, all but one patient showed blue sclerae with variable intensity. Other associated features with lesser appearance were easy bruising, piezogenic papules, joint pain, mitral valve prolapse, joint dislocations, neonatal hypotonia, muscle ruptures, hyperextensible skin, short stature, soft tissue lesions, myopia, hearing loss, atrophic scarring, a history of bone fractures and dolichostenomelia. The authors concluded that most variants associated with C1ROD are indeed located near the N-terminal cleavage part, however, exceptions exist.[76]

A 30-year-old woman with C1ROD was reported by Gnoli et al. with diagnosis of EDS at the age of 23 years. In clinical examination she showed GJH with a Beighton score of 9/9, recurrent joint dislocations, especially in shoulders, wrists, and knees, one enlarged hypertrophic scar after laparoscopy, right carotid artery kinking, mild valve insufficiency, and anamnestic easily bruising and prolonged bleeding. There were no signs of blue sclerae, DI or visual impairments, and body height was normal. However, she had a mild hearing loss at high frequencies and early onset osteopenia, detected at the age of 28 years (DXA Z-score of -2.1, measured at femoral neck and lumbar). She reported to have flat feet in childhood, however there were no medical records available. Genetic testing revealed two heterozygous mutations: The first one, p.Leu45Val, was located in exon 2 of *COL1A1* and was classified as likely benign. The second variant, p.Gly901Ser, was found in exon 42 of *COL1A2* and was further classified as pathogenic, and it has already been associated with moderate-severe and lethal cases of OI. Both variants have also been found in the patient's mother, who showed small body stature (10° percentile) and mild osteopenia in DXA scan. She was otherwise healthy, especially no signs of GJH, blue sclerae or fractures were reported.[77]

Foy et al. reported three individuals from one family with diagnosis of C1ROD. The proband was first examined at the age of 38 years and diagnosed with hEDS, after fulfilling the 2017 classification criteria. By that time, she had suffered from recurrent joint dislocations and instability with Beighton score of 6/9, falls, chronic fatigue, joint pain, easy bruising, flatfeet, skin hyperextensibility (at least 3cm, location not mentioned), large scars, velvety skin, piezogenic papules and gingivorrhagia. There were no signs of atrophic scarring. Osteodensitometry showed mild osteopenia and

cardiovascular examination revealed mild mitral insufficiency. Her daughter and cousin both met the classification criteria for hEDS as well and showed clinical symptoms of joint hypermobility (both with a Beighton score of 7/9), recurrent small joint instabilities and sprains, chronic joint pain, falls, chronic fatigue, genu and/or elbow recurvatum, easy bruising, blue sclerae, gingivorrhagia, piezogenic papules, and gastrointestinal disorders. However, skin involvement differed from the proband. Her daughter showed dystrophic scarring and easy bruising, whereas her cousin had mild skin hyperextensibility, and thin and translucent skin. Her cousin had a small dilatation of the thoracic aorta and her daughter had a microfistula between ascending aorta and right pulmonary artery. Osteodensitometry showed mild osteopenia in her cousin as well. The proband was referred to genetic testing due to her significant skin involvement, mainly for the exclusion of cEDS. A heterozygous mutation, c935G>T in exon 14 of *COL1A1* was detected, which lead to the substitution of arginine by leucine in position 312 of the triple helix. This position has previously been described, however in those cases the resulting amino acid was cysteine, and not leucin. The variant was detected in the other two affected individuals, whereas healthy family members did not carry it.[96]

Mouse model for combined OI and EDS

A mouse model for combined OI and EDS was reported by Chen et al. These Col1a1^{Jrt}/+ mice harbored a dominantly inherited T to C transition in a splice donor site of *COL1A1*, leading to the skipping of exon 9 and an 18-bp large deletion within the N-terminal region of the triple helical domain of α1(I) chain. According to the authors, this mutation is located in a region comparable to the associated region of C1ROD in humans. Phenotypically, these mice have been described to be of smaller size with reduced bone mass measurement (BMD), reduced trabecular number and brittle bones that easily fractured. Additionally, EDS features such as fragile skin and tendons, kyphosis, and early development of osteoarthritis have been reported. Analysis of bone, tendon and fibroblast cultures under TEM yielded collagen fibrils with smaller diameters compared to the tissues in +/+ mice, which is stated to be consistent with previous findings in human collagen. Furthermore, evidence for a greater amount of unprocessed collagen chains have been found, indicating impaired N-terminal cleavage. Due to clinical and genotypical comparability, the authors concluded that these Col1a1^{Jrt}/+ mice will help further

understanding phenotype-genotype relations and searching for therapeutic options in OI-EDS.[97]

4. Clinical Report

4.1. Patient presentation

The 33-year-old female patient was born to non-consanguineous parents with Austrian descent. Because of prolonged labor, a caesarean section was performed in the 40th week of pregnancy. Birth weight was 2.000g (<1 percentile), body length 43cm (<1 percentile) and occipito-frontal circumference 32cm (<1 percentile). Distinct facial dysmorphias, including small triangular face, deep-seated eyes, lower position of ears, prominent chin, beaked nose, widened suturae, prominent superficial veins and sparse hair were noticed postpartum. Further motor and cognitive development was without abnormalities. At 16 years of age she introduced at the pediatric department due to short stature and potential growth retardation. Her body size at that time was 141cm (10cm <3rd percentile), whereas estimated size based on her parents' size was 166cm (father 175cm, mother 170cm). A pterygium colli, frontal bossing, longer distal parts of extremities compared to proximal, cubita valga, pelvic obliquity and general extensibility of joints were noticed. However, at that time it was decided that no further diagnostics should be performed due to missing therapeutical consequences. Conducted karyogram showed a regular female chromosome set of 46,XX. Measured bone age by Greulich and Pyle showed a 99% completed growth at that time. She was referred to genetic testing after the diagnosis of breast cancer diagnosed at the age of 33 years. Due to distinct body features further genetic consultation was offered.

The female proband presented at the age of 33 years, with a body size of 142cm, body weight of 49kg, head circumference of 52,5cm and arm-spread of 137cm. She reported no abnormalities in motor, cognitive or pubertal development. Menarche was at 14 years of age. During physical examination several features were observed. Distinct hyperextensibility of both elbows and knees of approximately 10°, genua valga on both sides, as well as subluxations of thumb, finger joints, wrist, ankle and shoulder on the right side, as well as subluxations of the hips on either side, were witnessed. Furthermore, she showed hemihypoplasia of the right hand, with a difference in length of 1cm, a radialdeviation of the right index finger and ulnardeviation of the right middle finger. There was no sign of scoliosis or other spinal affections. Skin showed no hyperextensibility or atrophic scarring, and she

reported no wound healing issues or tendency to easy bruising. Distinct facial features included blue sclerae, sparse eyebrows and frontal bossing. She had no history of fractures, flat feet or ruptures of tendons, muscles, or ligaments. Furthermore, there were no signs of hearing loss or eye affections. Dentinogenesis imperfecta was not present, however, she had tooth agenesis with one maxillar tooth missing, one maxillar tooth with short root and one retained tooth in the mandible. Because of elevated resting heart rate, she previously underwent echocardiography and ECG, which showed no abnormalities. Medical imaging conducted during recent years included X-ray of the spine, cranial MRI and CT scan and sonography of the right shoulder. Abnormal configuration of skulls, as well as small rotational alterations of the spine were noticed. There were no signs of platyspondyly. DXA scanning results were not available.

In Table 1 the most characteristic features of the patient, as well as some “missing features” which are described in other patients with C1ROD, are summarized. These missing features were found in other patients with C1ROD in several publications, e.g., Morlino et al.[76], and are therefore an indication for the presence of C1ROD. However, they are not universally present in all patients.

TABLE 1 – Overview of the patient‘s characteristics and missing clinical features in comparison to other patients with C1ROD

Summary of significant clinical features
Short stature (142cm) Hyperextensibility of elbows and knees Several subluxations (hips, right hand, right shoulder, right ankle) Blue sclerae
Missing clinical features
No skin involvement (skin hyperextensibility, abnormal scarring) No history of fractures or flat feet

Additional distinct clinical features not common in C1ROD
Several teeth-affections (one missing tooth, one tooth with short root, one retained tooth)
Hemihypoplasia of the right hand, deviation of fingers
Asymmetry of symptoms (right body half more affected than the left half)
Sparse eyebrows, frontal bossing

4.2. Genetic analysis

After informed consent was obtained, array analysis and whole exome sequencing (WES) were performed. Genomic DNA from leucocytes was isolated via QiaSymphony and WES was performed on a NextSeq 550 (Illumina, San Diego, California, USA). Furthermore, variant annotation, filtering, and prioritization using the human phenotype ontology (HPO) terms HP:0008873 Disproportionate short-limb short stature, HP:0010485 Hyperextensibility at elbow, HP:0045086 Knee joint hypermobility, HP:0045087 Hip joint hypermobility, HP:0032153 Joint subluxation, HP:0000475 Broad neck, HP:0100558 Hemiatrophy of upper limb, and HP:0100840 Aplasia/Hypoplasia of the eyebrow was performed using VarSeq V.2.2 (Golden Helix, Bozeman, Montana, USA, www.goldenhelix.com.)

WES showed a heterozygous mutation in *COL1A1* (NM_000088.3: c.2885G>T), which was classified as pathogenic using the ACMG criteria. The located mutation leads to a substitution of glycine by valin (p.Gly962Val) and is therefore suspected to interrupt helix confirmation. This variant has previously not been described in the literature or in international databases, and is located in a so-called hotspot region, where mutations are known to be causative to OI III and no benign variants are described. In the parents of the patient, Sanger sequencing was performed. Both parents lack this variant, therefore it was classified as a de-novo pathogenic mutation.

5. Discussion

5.1. COL1-related disorders – phenotype-genotype relations

There have been many attempts to establish phenotype-genotype relations in COL1-related disorders. Especially for OI types I-IV, large population-based studies were conducted to collect clinical and genetic data[43,56-58]. However, determining distinct phenotype-genotype relations for single variants has proven to be difficult in the past decades, as these HCTD do not only show interfamilial, but even intrafamilial variability.[43,56,98] Generally, the resulting phenotype depends on the type of mutation, location within the chain, substituting amino acid, whether αI(I) or α2(I) is affected and other currently unknown modifiers.[43,89]

In the last decades some phenotype-genotype relations of *COL1A1* and *COL1A2* could be established, however some of them have been proven to be less clear than first expected. Mutations which affect exon 6 in either gene lead to aEDS, which has main clinical characteristic of congenital, bilateral hip dislocation together with severe GJH and/or skin hyperextensibility.[61] Quantitative defects, mainly due to a functional null allele in *COL1A1*, lead to a smaller amount of otherwise structurally normal procollagenα1(I) and cause the clinical type I OI.[43-45] Nowadays the Sillence types I-IV are primarily used to describe a distinct mild phenotype rather than the biomolecular pathomechanisms. Hence, several mutations in *COL1A2* have been described to lead to OI type I as well.[43] In contrast, total absence of procollagen-α2(I) chains due to biallelic mutations in *COL1A2* causes distinct severe cardiovascular involvement, which is further classified as cvEDS.[61] However, one report of a nine-year-old girl with clinical diagnosis of C1ROD due to symptoms such as marked GJH, pes planus, pale blue sclerae and mildly increased bone fragility, showed the biomolecular result of an homozygous mutation in *COL1A2* which leads to the total absence of α2(I) chains.[84] Regarding the genetic pathomechanisms a cvEDS might be assumed.[61,66] As the patient is very young and cardiac-valvular complications may not have been developed at that time, clinical follow up data of the patient and her family might contribute to further knowledge of this distinct phenotype.

The N-terminal region was supposed to be of particular importance for the resulting OI/EDS overlapping phenotype. Cabral et al. described seven individuals with

OI/EDS phenotype and mutations between exons 7-11 in *COL1A1*. Analysis of skin fibroblast showed an impaired N-terminal processing which leads to incorporation of pN into the ECM, although the cleavage site itself remained intact. Furthermore, they noticed an overall increase in severity of impairment towards the cleavage site. The collected data depicted this region of the N-terminal 85 amino acids together with the adjacent 15 residues towards the triple helix of high importance due to their ascribed function as anchor for the N-terminal helix.[59] In a follow-up study by Makareeva et al. further evidence for the existence of this highly stable N-anchor region was provided by biochemical and biophysical studies of skin fibroblasts. The authors attributed the EDS-like symptoms in the clinical overlap of OI and EDS to the unfolding of this anchor and therefore impaired N-terminal cleavage.[99] Malfait et al. further supported this assumption by reporting seven patients with a diagnosis of C1ROD caused by mutations located in the N-terminal part of the triple helix and a phenotype with dominantly EDS-like symptoms and only mild signs of OI. Analysis of dermal fibroblasts presented results consistent with previous studies.[78] However, more recent studies indicate that an overlapping phenotype is not only associated with mutations in this N-terminal part of the helix[76]. As a result of the conducted literature review different variants associated with C1ROD were found, showing a distribution across the triple helical domain as well. In Fig.3 these variants are depicted.

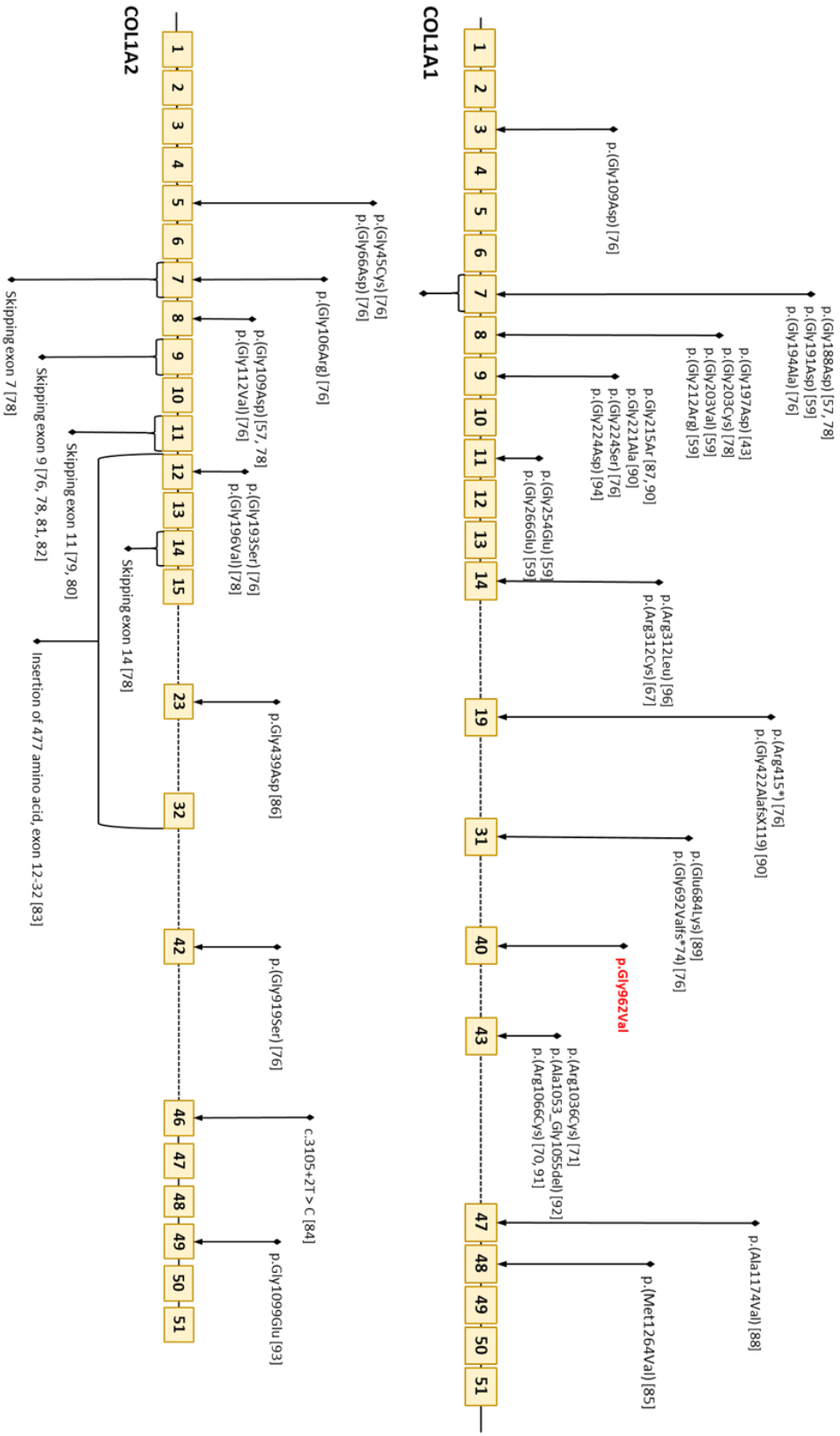

FIGURE 3 – Overview of the described C1ROD-associated genes and their distribution among *COL1A1* and *COL1A2*. The patient which is presented in this work is depicted in red.

Another assumption, which has been observed since the beginnings of genetic testing in HCTDs, is that the difference in severity is dependent on whether α1(I) or α2(I) is affected. Overall, mutations in the α1(I) chains are assumed to lead to a clinically more severe phenotype, because of overall composition of collagen I, which comprises two α1(I) chains and one α2(I) chain. Therefore, mutant chains are supposed to appear in 75% of heterotrimers if *COL1A1* is affected and 50% if *COLA2* is affected.[5,43,60] This relation of clinical severity and even lethality was described in several population studies which compared phenotypes with respective mutations.[43,57,58]

The localization of the mutation within the repetitive pattern of Gly-Xaa-Yaa seems to have an impact on phenotypical outcome as well, as analysis of skin fibroblasts indicate. In the study of Cabral et al., p.Arg1066Cys mutations in the Yaa position effect helix destabilization to a lesser extent compared to glycine substitutions in the same region of α1(I).[70] Moreover, non-glycine substitutions in Xaa or Yaa position seem to have a different clinical outcome than glycine substitutions and many are reported to lead to collagenopathies other than OI.[68-72,85,88,89,91] Lethal phenotypes are more likely to be seen in glycine substitution in α1(I) than in α2(I) chains[43,56] and respective mutations are unequally distributed along the two chains. Marini et. al proposed the presence of lethal clusters in α2(I), however, in following studies nonlethal outcomes and even patients with mild clinical phenotype have been described in these regions.[56,58]

5.2. Phenotype-genotype associations in C1ROD

The phenotypic variability seen in OI can be observed for mutations which have been reported to cause C1ROD in families with more than one affected family members as well. In some cases one part of the family is more severely affected and the other part rather mildly.[79,88] Interestingly, some of the C1ROD-related mutations have been previously described in other HTCDs, i.e., OI or EDS. One patient with the mutation p.Gly194Ala in *COL1A1* was diagnosed with C1ROD due to symptoms such as joint dislocation, muscle rupture, soft and doughy skin, flat feet, blue sclerae, and hyperextensible skin.[76] Alterations affecting the exact same codon have been previously described and classified as OI, i.e. OI type I (p.Gly194Arg[100], p.Gly194Ala[101]) and OI type III (p.Gly194Asp[102]), respectively. Another example may be the missense mutation in p.Arg312 in *COL1A1*, which is known to cause

either cEDS or vEDS[61]. Recently it has been described with a clinical phenotype of C1ROD as well.[96] It is assumed by some authors that there may be identified more similar cases with a wide phenotypic spectrum, which will further support the assumption that COL1-related diseases in fact represent a broad spectrum of possible phenotypes.[76,77]

In previous studies it was supposed that mutations in the N-terminal part of the triple helix lead to different phenotypic outcomes, depending on whether the α1(I) or the α2(I) chain is affected. Mutations in *COL1A1* were thereby assumed to lead to a more OI-like phenotype, whereas alterations affecting *COL1A2* were reported with a more severe EDS-like phenotype.[59,99] However, this turned out to be less strict than expected, as there are records of patients with primarily EDS-like phenotypes and mutations in the N-terminal part of *COL1A1*.[78]

One hallmark of C1ROD is general joint hypermobility. This feature is also present in EDS subtypes and even in OI, which further demonstrates the difficulties in classifying and distinguishing HCTDs.[76] In a large phenotype-genotype correlation study by Maioli et al., additional clinical signs primarily known for EDS such as hyperextensible skin, joint hypermobility, abnormal scarring, and easy bruising were assessed. Overall, 1,4% of the patients with a diagnosis of OI showed these features. Genetic testing revealed one deletion in *COL1A1* and three mutations within the N-terminal part of the triple helix of *COL1A2*.[56] Another association analysis in a population reported the prevalence of joint hypermobility in OI patients with glycine substitutions in *COL1A2* or *COL1A2* to be as high as 83,3%. Moreover, joint hypermobility was seen in all evaluated patients with either frameshift, nonsense or splicing variations in respective genes. However, no definition or procedure of assessment for joint hypermobility was provided.[103] GJH is a distinctive feature for hEDS as well, where no biochemical detectable evidence exists at the moment.[61] Therefor it may be possible that individuals with mutation in *COL1A* genes have been overlooked due to diagnosis of hEDS and further renunciative genetic testing.[96] However, hEDS represents the most common subtype of EDS[61] and genetic testing may therefore not be reasonable in all cases. One study conducted by Weerakkody et al. concluded, that genetic testing of patients with suspected hEDS without complications and no family history of

vascular involvement is unlikely to be of diagnostic value, as pathogenic variants can rarely be found in these patients and VUSs, who do not contribute to final diagnosis, are more common. They emphasized the importance of NGS panel testing if a history of vascular complication, marfanoid features, or a significant family history are present. This applies for patients with overlapping symptoms between OI and EDS as well, in whom further testing of the collagen encoding genes is encouraged.[90] To evaluate the presence of GJH in their clinical criteria for C1ROD, Morlino et al.[76] used the recommendations proposed by Juul-Kristensen et al. in their review. According to their recommendations, the cut-off-point for joint hypermobility for adults is set at 5/9 points, whereas in children it is 6/9.[104] Blue sclerae are part of the OI symptom complex and often described in patients with C1ROD as well[5,76]. Some studies indicate that mutations in the N-terminal part of the triple helical domain of α1(I) are associated with a high prevalence of blue sclerae.[56-58] As many causative mutations for C1ROD are located in this specific region, this might be one reason for the high prevalence of this trait in C1ROD.[76] Moreover, only few patients with alterations in the N-terminal domain show clinical signs of DI compared to other locations.[56,58] In C1ROD DI is not a common feature, and Morlino et al. defined it as exclusion criterion.[76] Bone fragility is one feature in OI and may lead to susceptibility to fractures.[36] However traumatic fractures in childhood are not uncommon. One longitudinal study in New Zealand estimated that approximately half of the children acquired at least one fracture until adulthood, whereas many were reported to have sustained more than one fracture during childhood.[105] In another Swedish study about 1/3 of children were reported to sustain at least one fracture until the age of 17 years.[106] Although these numbers vary between different populations, it can be concluded that fractures during childhood are not rare. Coincidental occurrence together with joint hypermobility without underlying bone fragility may therefore be possible. Then again, susceptibility to fractures may be seen in types of EDS as expression of bone fragility and impaired collagen incorporation as well. Byers et al. reported a cohort with aEDS in which some of them showed an increased incidence of fractures. He therefore included bone fractures in the clincal features of aEDS.[60] Moreover, mild osteopenia is described as minor criterion for aEDS in the 2017 International Classification,[61] although, there might be no evidence for a higher preposition to

frequent fractures in infants with EDS.[107] As this study only included children, it does not represent the overall population of patients with EDS.
An important, however rare, feature of some *COL1*-related diseases is vascular fragility which may occur in other entities beside vEDS. Malfait et al. reported patients with diagnosis of C1ROD, whereas in some of them various vascular involvements were noticed. These ranged from aortic dilation in a child to severe intracerebral arterial ruptures. Both patients with intracerebral hemorrhage had a glycine substitution in the triple helical domain in *COL1A1* and *COL1A2*.[78] Another, otherwise healthy patient with spontaneous cervical artery dissection was reported with a p.Gly191Ala substitution in *COL1A1*, who displayed blue sclerae in clinical examination. These were the only clinically evident features consistent with a HCTD.[108]

5.3. Comparison of the proband to other C1ROD phenotypes

The clinical phenotype of the proband comprises both signs of OI, i.e., short stature, blue sclerae and distinct facial dysmorphias, and EDS, i.e., GJH and several subluxations including the hips. Noticeably, her right body half was more affected and included a hemihypoplasia of the right hand with a difference in length of 1cm. According to the classification criteria proposed by Morlino et al.[76], the proband showed two major signs (blue sclerae, GJH) and two minor signs (two or more joint dislocations, short stature) of C1ROD and genetic testing is therefore encouraged.

The detected mutation c.2885G>T (p.Gly962Val) found in our proband is located in exon 41 of *COL1A1* and leads to a substitution of glycine by valine. As this does not affect the N-terminal region, this clinical report shows another example for C1ROD with a causative variant outside the important N-anchor domain associated with an OI/EDS overlapping phenotype.[59,78,99] Regarding alterations in the *COL1* genes, most commonly the glycine within the strict Gly-Xaa-Yaa sequence of the central triple helix is replaced by another amino acid.[19] These substitutions make up for approximately 79% of mutations in α1(I), and 75% in α2(I).[43] Regarding the type of amino acids, valine is an amino acid with a branched nonpolar side chain and is therefore associated with a higher incidence of lethal outcomes for OI. Marini et al. demonstrate that an overall 73% of substitutions by valine in the α1(I) chains are lethal. This percentage is significantly lower if the α2(I) chain is affected, in which

only 17% of valine substitutions lead to a lethal outcome.[43] In the probands found through literature review three other mutations with a substitution by valine in *COL1A1* were described.[59] In one of these individuals with predominantly signs of OI[59] an alteration in the N-terminal domain of *COL1A1* was detected. This region is associated with only rare cases of lethal outcomes.[43] The two other mutations in *COL1A1* affected the C-terminal part of the triple helix, i.e., exon 47 and 48, which in both cases lead to overall mild signs of OI and EDS.[85,88] At position c.2885 so far no other mutation has been described in international databases, however one mutation, which affects the same amino acid, has been reported by Marini et al. Unlike our proband, this patient had a substitution of glycine by aspartic acid (p.Gly962Asp) and was diagnosed with severe type III OI.[43]

One interesting aspect of this case is the asymmetry of the phenotype. One possible explanation for the unilateral occurrence of subluxations would be mosaicism of the detected variant. Even if there was no indication for mosaicism in the blood, mosaicism cannot be excluded for de novo variants, without testing further tissues. Hemihypoplasia can also be found in other syndromes, e.g. Silver Russel Syndrome (MIM #180860). This is a general problem of symptoms described in single cases, that additional genetic disorders cannot be excluded as a cause

5.4. Approaches for future testing and classification

Overall, literature and clinical reports for C1ROD are scarce and phenotype-genotype relations are therefore hard to establish. As the severity of clinical features differs considerably, there may be missed associations due to possible prenatal lethal outcomes on the one hand, and very mild symptoms such as early onset osteoporosis or mild hypermobile joints on the other hand.
It may be noted that many probands described in clinical reports are children who may develop more additional features during their lifetime. Especially signs of premature osteoporosis may be overseen due to refrained assessment of bone density, i.e. osteodensitometry, in children or later in the development evolving symptoms such as fractures. Therefore, follow-up studies of patients with C1ROD may contribute to the further understanding of the whole phenotypical spectrum. Further understanding of specific pathomechanisms and relations between the genotype and the resulting protein may be achieved by biochemical and biophysical

examination, especially as phenotypical predictions on mere knowledge of genotype and vice versa has proven to be difficult.

Classification is important for patients to help them understand their condition, to make a risk assessment and plan medical surveillance. Moreover, it helps with establishing new genotype-phenotype relations for COL1-related diseases. It may also help to guide the process of diagnosis, especially in patients with mild phenotypes. As the final diagnosis of COL1-related disorders is often based on genetic results[61], it is of interest for both patients and clinicians that suspected HCTDs are not overseen due to mild or atypical phenotypes, especially with regard to those rare cases with severe and even lethal complications such as arterial ruptures.[78,108]

The integration of the new entity of C1ROD as novel subtype into the current EDS classification was proposed by Morlino et al., as many patients present with predominantly EDS symptoms and only mild or even absent signs of OI. They concluded that this approach helps to guide therapeutic strategies and genetic counselling, as in this cohort the development of complications during lifetime is associated more closely with EDS subtypes than OI.[76] A screening for ocular, intraoral, ear and especially bone involvement is recommended. However, they pointed out that there is currently too few evidence to establish a specific follow-up scheme for vascular fragility.[76] Another advantage that comes with a distinct classification are defined criteria which allow future clinical reports to systemically examine and further describe patients. It may be noticed that some clinical reports describe only main clinical features and absent signs are not mentioned. Moreover, not all clinical symptoms are reproducible or well documented. This mainly applies to features characteristic for EDS, i.e., skin hyperextensibility and general joint hypermobility. Recommended diagnostic surveys exist for these criteria[61,104], however they are not universally used and documented. Predefined clinical criteria may therefore contribute to a better comparability between cases and better representation of the broad clinical spectrum depicted by COL1-related diseases.

In the future, more biochemical, genetic, as well as phenotypical data is needed to understand complex genotype-phenotype relations in genes encoding collagen I. Although we did not provide biochemical data, the reported clinical phenotype of our proband with C1ROD and the causative mutation p.Gly962Val may contribute to the

understanding and definition of the newly evolved entity of C1ROD as well as the overall spectrum of COL1-related diseases.

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