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Pathogenic mechanisms in genetically defined Ehlers-Danlos syndrome

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Abstract:	The Ehlers–Danlos Syndromes (EDS) are a group of rare heritable connective tissue disorders, with skin hyperextensibility, joint hypermobility, and generalized connective tissue fragility as common hallmarks. Currently, 13 EDS types are recognized, caused by defects in 20 genes, which consequently alter biosynthesis, organization and/or supramolecular assembly of collagen fibrils in the extracellular matrix. Molecular analyses on patient samples, mostly dermal fibroblast cultures, combined with studies on animal models highlighted that part of EDS pathogenesis can be attributed to impaired cellular dynamics. Although our understanding of the full extent of (extra)cellular consequences is still limited, this narrative review aims to provide a comprehensive overview of the current knowledge on the extracellular, pericellular and intracellular alterations implicated in EDS pathogenesis.

HIGHLIGHTS

EDS are a group of multisystemic conditions that share soft and hyperextensible skin, abnormal wound healing, easy bruising, joint hypermobility, and chronic pain, with variable involvement of cardiovascular, musculoskeletal and ocular systems.

Alterations in collagen fibril morphology and organization are a unifying feature in all EDS types, despite a diverse range of underlying disease genes and molecular defects.

A change in integrin receptors is found on the surface of dermal fibroblasts in most EDS types.

Emerging insights from transcriptome profiling studies highlight a role for disturbed intracellular signaling pathways in some EDS types such as the unfolded protein response and impaired TGFß signaling.

The complete range of molecular mechanisms contributing to the pleotropic phenotype and the connection between different EDS types are incompletely understood.

Pathogenic mechanisms in genetically defined Ehlers-Danlos syndromes

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ABSTRACT

The Ehlers–Danlos Syndromes (EDS) are a group of rare heritable connective tissue disorders, with skin hyperextensibility, joint hypermobility, and generalized connective tissue fragility as common hallmarks. Currently, 13 EDS types are recognized, caused by defects in 20 genes, which consequently alter biosynthesis, organization and/or supramolecular assembly of collagen fibrils in the extracellular matrix. Molecular analyses on patient samples, mostly dermal fibroblast cultures, combined with studies on animal models highlighted that part of EDS pathogenesis can be attributed to impaired cellular dynamics. Although our understanding of the full extent of (extra)cellular consequences is still limited, this narrative review aims to provide a comprehensive overview of the current knowledge on the extracellular, pericellular and intracellular alterations implicated in EDS pathogenesis.

EHLERS-DANLOS SYNDROMES: CLINICALLY AND GENETICALLY DIVERSE DISORDERS

The Ehlers–Danlos syndromes (EDS) encompass a collection of pleiotropic and multisystemic heritable connective tissue disorders in which skin hyperextensibility and fragility, abnormal wound healing, generalized joint hypermobility, and generalized connective tissue frailty predominate the clinical picture, although with variable severity. [1] The clinical manifestations may vary between and among EDS types, and can include potentially life-threatening fragility of the arterial tree and of hollow organs, involvement of the ocular system, musculoskeletal complications, and the development of chronic, widespread pain, which collectively contribute to marked disability, compromise the quality of life, and/or can even lead to early mortality (see Clinician's corner). [2] Pronounced inter- and intrafamilial variability in phenotypic presentation of affected individuals is observed. [3–5] At present, 13 distinct EDS types are recognized, of which 12 have been molecularly elucidated and are caused by defects in 20 different genes (summarized in Table 1 and Figure 1). [1,2] For the hypermobile EDS type (hEDS), no causative gene has been identified, hence diagnosis relies on stringent clinical criteria and exclusion of other (genetic) conditions that cause joint hypermobility. [1,6]

At present, a multitude of genetic causes have been discovered that elicit the pleiotropic phenotypes of the EDS types. Over the years valuable insights have been gained by the combination of ultrastructural, biochemical, and molecular studies on patient-derived samples, mainly skin biopsies or primary dermal fibroblast cultures derived thereof, as well as on specific tissues obtained from representative animal models of EDS. [7] Although these studies revealed that multiple factors are at play in the pathophysiology of EDS, our understanding of the pathways that link the genetic alterations to the associated clinical picture remains overall very limited. The aim of this narrative review is to provide a comprehensive overview of the current understanding of the pathological mechanisms involved in the pathogenesis of genetically defined EDS types. We highlight disturbances in collagen fibrillogenesis, alterations in **extracellular matrix (ECM) (see Glossary)** architecture and remodeling, altered adhesion properties and changes in cellular dynamics with involvement of different signaling cascades

as important players. Current knowledge on EDS pathogenesis showcases the complex interplay between extracellular, pericellular and intracellular events, but further investigations are highly necessary to grasp the full extent and interconnection of the (extra)cellular consequences of the genetic defects underlying EDS.

CONNECTIVE TISSUES: CROSSTALK BETWEEN EXTRACELLULAR MATRIX AND RESIDENT CELLS

Connective tissues are composed of cells that are embedded in significant quantities of surrounding ECM. The ECM forms a complex three-dimensional network composed of a wide variety of macromolecules and matrix-associated factors, collectively coined the matrisome. [8,9] Each of these components is synthesized and maintained by tissue-specific cells such as fibroblasts, osteoblasts and chondroblasts. [8] Although the ECM constituents are fundamentally identical, they assemble into tissue-specific (supra)structures with a unique composition and topology, providing different biochemical and mechanical cues adapted to meet the functional requirements of a particular tissue or organ. [8]

In addition to its structural role, the ECM plays a regulatory role and possesses the ability to modulate cell behavior. [10] The ECM represents a highly organized, dynamic and interactive network that is involved in many developmental and physiological processes, which rely on bidirectional crosstalk between the ECM and cells. [11] Central to this 'communication' are direct interactions between cells and ECM molecules mediated by cell surface receptors, such as **integrins** and syndecans, which alter cytoskeleton dynamics and activate signaling pathways that affect gene expression and diverse cellular responses, including cell adhesion, migration, proliferation, differentiation, and survival. [8,9,12] The importance of proper 'connections' between ECM molecules and resident cells is illustrated by a phenomenon called **anoikis**, a form of programmed cell death initiated by detachment of cells from their surrounding ECM. [13] Additionally, the ECM undergoes continuous remodeling (i.e., assembly and degradation), which is regulated by tightly controlled proteolytic cascades (e.g., mediated

by among others matrix metalloproteinases (MMPs)) that convert structural proteins to signaling molecules and functional fragments, release growth factors and cytokines that are sequestered to the ECM or directly degrade matrix constituents. [14] As such, the dynamic interplay between ECM components and the pericellular and intracellular compartment modulates cellular behavior and instructs ECM assembly and architecture. These processes are crucial to maintain tissue homeostasis. [15]

INSIGHTS INTO THE PATHOMECHANISMS UNDERLYING GENETICALLY DEFINED EHLERS-DANLOS SYNDROMES

Central to the pathogenesis of all EDS types, regardless of their genetic basis, is that collagen biosynthesis, fibrillogenesis and/or supramolecular assembly are impeded at some level (Box 1 and Figure 2). Nevertheless, the pathophysiological consequences underlying the different EDS types are still incompletely understood. Table 2 summarizes the currently known pathomechanisms for each genetically solved EDS type. Of note, findings in human samples are often based on small sample sizes, an inherent limitation of rare disorders, and mechanistic heterogeneity is suggested between as well as within EDS types.

Altered collagen fibril morphology and organization

Early evidence suggesting that the EDS phenotypes are caused by altered physical properties of the ECM came from ultrastructural analyses on skin biopsies of EDS patients which showed an altered collagen “wickerwork”. [16] The subsequent identification of biochemical alterations in collagen quality and quantity, followed by the elucidation of molecular defects and the study of their consequences in human cells and tissues, and in mouse (and zebrafish) models, have been closely intertwined with our knowledge and appreciation of the complexity and the regulation of collagen fibril formation and supramolecular organization of these fibrils in the ECM (Box 1). [17]

Collagen fibril morphology and organization are usually studied by transmission electron microscopy (TEM) on skin biopsies of EDS patients since skin represents a major affected organ and is easily accessible, or in other relevant tissues derived from representative animal models of EDS (e.g., ligaments, aorta). A variety of alterations in collagen fibril shape and diameter and/or fibril organization have been identified in EDS, contrasting the entirely round and tightly packed collagen fibrils observed in healthy tissues. [7,17] These ultrastructural alterations, although commonly observed in EDS, are not specific enough to distinguish between different EDS types. [1,18,19] Moreover, they are also found in genetically unresolved hEDS and other heritable connective tissue disorders, including osteogenesis imperfecta and Ullrich congenital muscular dystrophy. [3] As such, their presence is not sufficient to firmly establish a diagnosis of EDS.

Defects in type I collagen – Alterations affecting the pro-α1(I)- or the pro-α2(I)-chains have been identified in several EDS types and all compromise proper collagen fibril biosynthesis, either directly or indirectly through altered posttranslational modifications.

Defective lysyl hydroxylase 1 (LH1) activity results in uniform underhydroxylation of specific lysine residues and underglycosylation of hydroxylysyl residues in the triple helix of type I procollagen in kyphoscoliotic EDS (kEDS-*PLOD1)* patients. As a result of these altered posttranslational modifications, collagen molecules fail to efficiently form intermolecular crosslinks. TEM analysis of skin from kEDS-*PLOD1* patients and in skin and aorta of *Plod1*$^{-/-}$ mice revealed collagen fibrils with variable and overall increased diameters and irregular fibril spacing. [20–22]

Defective proteolytic removal of the amino (N)-propeptide of type I procollagen and consequential incorporation of pN-collagen molecules (containing the retained N-propeptide) into collagen fibrils is part of the pathogenic mechanism in several EDS types. Heterozygous variants that introduce the loss of the N-propeptide cleavage site in either the pro-α1(I)- or the pro-α2(I)-chains, as found in arthrochalasia EDS (aEDS), lead to collagen fibrils with smaller diameters and irregular contours in the dermis of affected individuals. These ultrastructural

alterations appear to be more pronounced in individuals with alterations in the pro-α1(I)-collagen chain, encoded by *COL1A1*, which is, to some degree, in line with the more severe phenotype associated with *COL1A1* pathogenic variants. [23–25] This is attributed to the stoichiometry of type I procollagen, which consists of two pro-α1(I)-chains and one pro-α2(I)-chain. Dermatosparaxis EDS (dEDS) is characterized by deficient activity of the catalytic N-propeptide protease, a disintegrin and metalloproteinase with thrombospondin motifs 2 (ADAMTS2), which results in the retention of the N-terminal propeptide of both pro-α1(I)- and pro-α2(I)-chains (and possibly altered proteolytic processing of other collagens and ECM molecules). [26,27] This severely compromises the proper stacking of collagen molecules by sterically interfering with cross-linking. Consequently, collagen fibrils with a striking hieroglyphic pattern are typically seen in the dermis of dEDS patients, [28] although some degree of variable severity is observed. [19] Finally, heterozygous variants in the first 85 N-terminal amino acids of the triple helix of either the pro-α1(I)- or pro-α2(I)-chains have been identified in patients with the so-called COL1-related overlapping disorder (C1ROD). This N-terminal region functions as an anchor for the stabilization and adequate folding of the triple helix. Pathogenic variants in this region introduce a conformational change in the procollagen N-proteinase cleavage site which results in delayed processing of the N-propeptide. Skin biopsies of C1ROD patients show overall smaller, sometimes irregular collagen fibril diameters and lower fibril density. [29–32]

Defects in type V collagen and type III collagen – Both type V and type III collagen are fibrillar collagens that assemble with type I collagen and play a regulatory role during collagen fibrillogenesis.

Defects in the genes coding for the pro-α1-chains or pro-α2-chain of type V collagen result in classic EDS (cEDS). The $[\alpha1(V)]_2\alpha2(V)$ heterotrimer forms heterotypic type I/V collagen fibrils with the entire triple helical domain of type V collagen embedded within the fibril. The partially processed N-propeptide of type V collagen protrudes between the type I collagen molecules to the fibril surface where it controls fibrillogenesis by sterically hindering

the addition of collagen monomers and interacting with other matrisome components. [29,37] TEM analysis of cEDS individuals consistently shows the existence of atypical large (cauli)flower-shaped dermal collagen fibrils on cross-section with variable inter-individual frequencies. Similar alterations in collagen fibril shape and reduced collagen fibril density were observed in dermis and tendons of the *Col5a1* haploinsufficient (*Col5a1*$^{+/-}$) mouse model. [33,34] Elegant studies on *Col5a1* knockout and haploinsufficient mouse models showed that these fibrils result from the unregulated assembly of type I collagen and uncontrolled lateral growth of the collagen fibrils due to the reduced availability of type V collagen molecules. [18,33–35] Mice haploinsufficient for *Col5a2* (*Col5a2*$^{+/-}$) did not show obvious differences in dermal collagen fibril morphology on cross sections, but irregular and some thicker fibril contours were detected on longitudinal views. [36] Together, these findings highlight the importance of type V collagen in controlling type I collagen utilization during the initiation of fibril formation.

The homotrimeric type III collagen is predominantly found in tissues with elastic properties (e.g., skin, blood vessels, gastrointestinal tract, uterus, etc.) and can also form heterotypic fibrils with type I collagen. Defects in type III collagen cause vascular EDS (vEDS). Ultrastructural studies in vEDS patients show a thinner dermis with aberrant diameters and distribution of collagen fibrils. [37] Depending on the location of the variant, the ultrastructural alterations may vary, with smaller collagen fibril diameters observed for variants located towards the C-terminal part of the triple helix [38] and more variable fibril diameters seen with variants near the N-terminal end of the triple helix [39]. TEM analysis on postmortem tissues from a vEDS individual showed a markedly reduced collagen content and decreased collagen fibril diameters in the media of all arteries and in the adventitia and intima of some, while they were increased in the vena cava. [37] Several murine models with either structural *Col3a1* variants [40–43] or *Col3a1* haploinsufficiency [44] also showed a reduced collagen fibril content in skin and/or aorta with widely variable fibril diameters. These findings, together with the observation that collagen fibrils with a higher collagen III:I ratio tend to be thinner, suggest that type III collagen limits fibril diameter. [45,46]

Defects in bridging molecules – Type I collagen fibrils interact with several ECM molecules which regulate fibril properties. For example, tenascin-X (TNX) and type XII collagen form flexible bridges between collagen fibrils and other ECM molecules. [47,48] Loss of TNX leads to classical-like EDS (clEDS) while disruption of type XII collagen results in a phenotype that overlaps with Bethlem myopathy and Ullrich congenital muscular dystrophy, currently coined as myopathic EDS (mEDS).

TEM analysis of the dermis of clEDS patients and skin, Achilles tendon and tail of *Tnxb*$^{-/-}$ mice revealed a markedly reduced collagen fibril density (albeit with a normal contour and size of collagen fibrils), and hence a decreased collagen content. [49] Ultrastructural analysis of an individual with mEDS also showed a reduced dermal collagen fibril density and a shift to larger fibril diameters. [50] Similar alterations in collagen fibril diameters and density were observed in the skin of *Col12a1*$^{-/-}$ mice but fibrils also displayed irregular contours. [51] Furthermore, tendon from *Col12a1*$^{-/-}$ mice showed decreased collagen fibril packing and collagen fibrils were diffusely dispersed throughout the endomysium of *Col12a1*$^{-/-}$ muscle. [52,53]

These findings contribute to the proposed model of TNX and type XII collagen as regulators of collagen fibril spacing by bridging collagen fibrils, thereby mediating the organization and mechanical properties of collagen fibrils. [47,48]

Defects in glycosaminoglycan biosynthesis – Elegant TEM-based visualization of glycosaminoglycan (GAG) chains in skin biopsies from mcEDS-*CHST14* patients and *Chst14*$^{-/-}$ mice revealed the presence of linear, rigid, rod-shaped, chondroitin sulfate (CS) GAG chains projecting from the outer surface of the collagen fibrils, as opposed to curved dermatan sulfate (DS) GAG chains that are in close contact with collagen fibrils in healthy control samples. [54,55] Decorin, a major small leucine-rich proteoglycan (SLRP) in skin, plays a key regulating role during collagen fibril formation, possibly via electrostatic interactions between its flexible DS chain and adjacent collagen fibrils. Therefore, substitution of these flexible DS chains by

rigid CS chains most likely contributes to the spatial disorganization of collagen fibrils in mcEDS-*CHST14* patients and *Chst14*$^{-/-}$ mice. [54,55] Disruption of the regulatory role of SLRPs during collagen fibrillogenesis likely also contributes to spEDS-*B4GALT7* and spEDS-*B3GALT6* where defects in the biosynthesis of the common tetrasaccharide linker region affect proper GAG assembly (of DS, CS and heparan sulfate (HS) GAGs). Although no ultrastructural analyses are available for spEDS-*B4GALT7*, TEM analysis in spEDS-*B3GALT6* patients and adult *b3galt6*$^{-/-}$ zebrafish indeed showed loosely packed dermal collagen fibrils with variable fibril diameters. [56,57]

Other EDS-associated defects – The collagen ultrastructural phenotype in the skin of brittle cornea syndrome (BCS) patients with pathogenic variants in *ZNF469*, encoding zinc-finger protein 469, ranges from normal to minor variation in the diameter of the fibrils, with sometimes slightly thinner fibrils. [58] In the cornea of *Zfp469*$^{-/-}$ mice, a model of BCS, collagen fibrils with smaller diameters and increased fibril density were observed. [59] Similarly, fewer and disorganized collagen fibrils were observed in the cornea of *znf469*$^{-/-}$ zebrafish. [60]

TEM analysis on skin from patients with periodontal EDS (pEDS) harboring missense variants in *C1R*, encoding complement component C1r, showed variable collagen fibril diameters with some abnormally shaped fibrils and increased interfibrillar spacing. [61,62]

Ultrastructural analysis of two patients with biallelic *AEBP1* variants, encoding adipocyte enhancer-binding protein 1, revealed dermal collagen fibrils with variable diameters and irregular contours, supportive of a role for AEBP1 during collagen fibril formation. [63]

The extracellular matrix in EDS: about collagens and more

In line with the ECM as an interconnected network, the observed alterations in collagen fibril architecture are expected to impact the structure and/or function of other ECM molecules. Hence, the extracellular consequences of the genetic defects stretch far beyond the initial defective molecule.

Altered assembly or availability of other ECM molecules – In some EDS types, TEM analyses revealed alterations in elastic fiber composition, such as fragmentation of elastin in skin from clEDS [64], kEDS-*FKBP14* [65] and spEDS-*B3GALT6* [57], and altered elastin appearance with ruffled edges in mEDS skin [50]. *In vitro* deposition of several ECM molecules, including types I, III, V and VI collagen, glycoproteins such as fibronectin and tenascins, and SLRPs (e.g., decorin, lumican, etc.), is often disturbed in different types of EDS (see Table 2). [17] Recent studies in skin and Achilles tendon of *Col5a1*$^{+/-}$ mice, a model of cEDS, demonstrated increased expression and protein levels of *Lum*, encoding the SLRP lumican, which likely reflects a compensation mechanism in response to disrupted fibril formation upon reduced type V collagen availability. [66] This further underscores that general disruption of ECM assembly due to a single molecular defect can elicit compensatory processes, which probably also contributes to the pathogenesis.

A role for ECM degradation and remodeling – In some EDS types there is evidence for increased ECM degradation and remodeling. Increased MMP9 expression was seen in the aorta of *Col3a1*$^{+/-}$ mice, a model of vEDS. [67,68] Long-term treatment of these mice with the nonspecific MMP inhibitor doxycycline normalized MMP9 activity, attenuated the decrease in aortic collagen content and prevented spontaneous and stress-induced aortic lesions. [67–69] Activation of MMP2 and MMP9 has also been detected in *Tnxb*$^{-/-}$ mice in the context of cancer metastasis research but could have implications for ECM remodeling in clEDS patients as well. [70,71] Transcriptome analysis on spEDS-*B3GALT6* fibroblasts also showed altered expression of genes encoding molecules involved in ECM remodeling. [72] A recent study on fibroblasts from pEDS patients showed that activated C1s can cleave and hence degrade type I procollagen, thereby demonstrating a direct effect of the altered functionality of a complement factor on the ECM. [73] Altered ECM degradation and remodeling could result in the release of functional fragments and/or growth factors that can communicate with resident cells, and further add to the distorted ECM homeostasis associated with EDS. However, this requires further investigation. [73]

Taken together, disrupted integrity and alterations in the physical properties of the ECM in many different tissues and organs cause connective tissue weakness which contributes to the phenotypic features (e.g., tissue fragility) observed in EDS patients. Additionally, inadequately sequestered matrisome components and/or released ECM fragments could potentially contribute to other EDS-related symptoms, such as pain (Box2).

A role for integrins in the pathophysiology of EDS

In normal conditions, the ECM can directly bind to different cell surface (co-)receptors thereby mediating cell anchorage and regulating various pathways involved in intracellular signaling and mechanotransduction. [74] The defective ECM surrounding the cells in EDS could cause disrupted cell-matrix communication and elicit abnormal signaling, which in turn could affect cellular behavior. Interestingly, the organization and/or recruitment of integrin heterodimers on the cell surface of fibroblasts derived from EDS patients was shown to be shifted *in vitro,* with the recruitment of the alternative fibronectin receptor, αvβ3 integrin, in several EDS types (Table 2). In these EDS types, the absence of proper collagen organization in the ECM is thought to result in the failure of collagen to engage with its α2β1 integrin receptor. [75] This, in turn, is assumed to lead to downregulation of this collagen receptor and of the canonical fibronectin receptor, α5β1 integrin, and upregulation/recruitment of the alternative fibronectin receptor, αvβ3 integrin. In cEDS and vEDS fibroblasts, crosstalk between αvβ3 integrin and the EGF receptor, mediated by the paxillin-p60Src pathway, is believed to function as a rescue mechanism to promote cell adhesion and thereby preventing anoikis. [76,77]

Altered integrin recruitment was shown to impact the migration capacity of dermal fibroblasts from cEDS patients. [78] A reduced migration capacity, decreased attachment to ECM components, and decreased proliferation rate was also observed for dermal fibroblasts of *Col5a1*$^{+/-}$ mice. These alterations could contribute to the slow wound healing and impaired cicatrisation typically observed in cEDS. [79]

Hence, altered signaling resulting from the observed 'integrin switch' or mediated by other (associated) pathways likely influences other cellular processes (e.g., wound healing) that contribute to cEDS pathogenesis. To date, no information is available on disrupted signaling from non-integrin surface receptors, e.g. syndecans, discoidin domain receptors (DDRs) or growth factor receptors.

Altered cellular dynamics and signaling pathways involved in EDS

While most of the early research in EDS focused on alterations in the ECM, our understanding of the pathomechanisms underlying EDS has benefited from transcriptome profiling of dermal fibroblast cultures from several EDS types (see Table 2).

Unsurprisingly, a finding common to all investigated EDS types is the presence of differential expression of genes coding for ECM molecules, is in line with the observed disruptions in ECM deposition. This also reflects compensatory mechanisms in response to the altered availability of certain ECM molecules. Additionally, transcriptome data suggested a role for altered expression of genes involved in cell cycle regulation in cEDS, in vascular integrity in kEDS-*PLOD1* and kEDS-*FKBP14* and in GAG biosynthesis in spEDS-*B3GALT6*. [22,72,80]

Increasing evidence suggests the involvement of particular signaling pathways in EDS, which are highlighted below.

Unfolded protein response – The endoplasmic reticulum (ER) enforces strict quality control on the correct folding of nascent proteins. Unfolded or incorrectly folded proteins can accumulate intracellularly and elicit ER stress [81], a cellular response that aims to restore ER homeostasis, known as the **unfolded protein response (UPR)**. Evidence for intracellular accumulation of abnormal collagen and/or other ECM molecules, leading to ER stress and other downstream consequences, such as disturbed autophagic pathways and/or increased apoptosis, have been observed in several EDS types, (e.g., cEDS, vEDS, kEDS-*FKBP14*, pEDS-*C1R* and mEDS).

ER dilation has been observed in skin fibroblasts of vEDS patients [39,82], as well as in fibroblasts within the aortic adventitia of *Col3a1* knock-in mice. [41] These findings, although not consistently observed in human vEDS patients, are suggestive for intracellular accumulation of type III collagen, at least for some specific defects. Gene expression analysis on vEDS dermal fibroblasts provided some evidence for altered ER homeostasis and activation of apoptosis. [83–86] In contrast, dermal fibroblasts from a transgenic *Col3a1* mouse model did not show any signs of ER stress. [40] A systematic in-depth analysis of the UPR has not been performed for vEDS.

Transcriptome profiling on dermal fibroblasts from cEDS patients supports a role for disruption of ER homeostasis and autophagy with decreased expression of molecular chaperones that facilitate protein folding, enzymes regulating post-Golgi collagen processing, and proteins acting as cargo receptors required for ER proteostasis and implicated in the autophagy process. [80]

TEM analysis on skin of a kEDS-*FKBP14* patient showed dermal fibroblasts with enlarged ER cisternae, suggestive for collagen accumulation in the ER. [65] This finding was reinforced by demonstration of intracellular retention of types III and VI collagen, but not type I collagen, in dermal fibroblasts of two unrelated kEDS-*FKBP14* patients. [87]

A dilated ER was also seen in skin and cultured dermal fibroblasts from pEDS patients, mimicking the findings in overexpression studies of mutant C1r. [62]

Intracellular retention of type XII collagen in fibroblast cultures of some (but not all) exon skips was described in mEDS. [50,88]

Based on these observations, targeting ER stress might reflect a therapeutic strategy at least in some EDS types. This will however largely depend on the specific genetic defect and mutant protein and will require further investigation.

TGFβ signaling – Excessive transforming growth factor-beta (TGFβ) signaling has previously been documented in other heritable connective tissue disorders such as Marfan syndrome and other hereditary aortic aneurysm syndromes, and osteogenesis imperfecta. [89,90] Several

reports also noted alterations in this signaling pathway in the context of EDS. Increased serum levels of TGFβ were observed in vEDS patients, but no evidence of increased canonical or non-canonical TGFβ mediated signaling was detected in patient dermal fibroblasts. [91] Deregulation of TGFβ signaling was also suggested in a cEDS mouse model where increased levels of TGFβ and its downstream signaling targets were observed in serum and skin of *Col5a1*$^{+/-}$ mice, respectively. [66] TNX was shown to interact with the small latent TGFβ complex and to regulate the bioavailability of TGFβ *in vitro*. [92] Interestingly, deregulation of the TGFβ pathway with upregulated pSmad1/5/8, and increased fibroblast-secreted TGFβ3 and plasma TGFβ2 was demonstrated in dermal fibroblasts and skin biopsies of patients with **congenital adrenal hyperplasia X-linked (CAH-X)** syndrome, suffering from a combination of TNX deficiency and CAH. These observations were attributed to TNX deficiency since patients with only CAH did not display altered TGFβ signaling. [93] Furthermore, studies in skin of *Col12a1*$^{-/-}$ mice, mimicking mEDS, demonstrated increased amounts of soluble TGFβ, and was shown to promote increased myofibroblast differentiation. [51] Altered expression of genes involved in the BMP/TGFβ signaling pathway were also detected in both spEDS-*B3GALT6* [72] and spEDS-*SLC39A13* [94]. This was associated with the intracellular localization of Smad proteins in connective tissue-forming cells in spEDS-*SLC39A13*. [94] Furthermore, TGFβ signaling was shown to mediate the transition of fibroblasts to myofibroblasts in lung of *Aebp1* knockout (*Aebp1*$^{-/-}$) mice. [95,96] Whether this pathway is also involved in other tissues and its contribution to the *AEBP1*-associated phenotype remains elusive.

In conclusion, evidence of a role for increased TGFβ signaling has independently been detected in several EDS types. Since TGFβ is involved in ECM protein synthesis and remodeling, its deregulation likely contributes to the observed alterations in ECM molecules. [97] Additionally, deregulation of the TGFβ pathway can also play a role in the wound healing issues observed in EDS. [51,66,98]

PLC/IP3/PKC/ERK signaling – Recent transcriptome analysis on thoracic aorta in two *Col3a1* knock-in mouse models of vEDS (i.e., the phenotypically mild *Col3a1*$^{G209S/+}$ mice and the severe *Col3a1*$^{G938D/+}$ mice) suggested a key role for increased signaling of the PLC/IP_3/PKC/ERK pathway. [41] The functional relevance of this pathway in the disease was illustrated by increased survival of the knock-in mice upon inhibition of IP3 (hydralazine), PKCβ (ruboxistaurin), or MEK/ERK (cobimetinib). [41] These findings initiated the Prevention of Rupture with Enzastaurin in Vascular Ehlers-Danlos Syndrome (PREVEnt) clinical trial, launched in 2022 by Aytu Biopharma (https://clinicaltrials.gov/study/NCT05463679). This study aimed to test Enzastaurin, a PKCβ inhibitor, in preventing cardiac or arterial events in vEDS patients, but this trial been indefinitely suspended by the company. Furthermore, the increased risk for life-threatening vascular events associated with puberty and pregnancy seen in vEDS patients [4,99] was shown to be rescued by inhibition of oxytocin signaling in mild *Col3a1*$^{G209S/+}$ female mice and androgen signaling in severe *Col3a1*$^{G938D/+}$ mice. [41]

Although research into these signaling pathways remains limited, these findings support the idea that altered ECM-to-cell signaling is part of the pathogenesis of EDS. This merits further research as these pathways (and possibly others) could help identify potential druggable targets.

CONCLUDING REMARKS

Taken together, a complex interplay between altered ECM and cellular dynamics exists in EDS. Careful interpretation of (some of) the results is warranted, because they are often based on (very) small sample sizes, a limitation inherent to the study of these rare disorders. Additionally, some findings were only observed in *in vitro* assays and *in vivo* data are lacking or were only detected in animal models and not (yet) confirmed or studied in patient samples. Adding to the complexity is the suggested mechanistic heterogeneity between and most probably within EDS types. It is possible that for a given gene and its encoding protein,

mechanistic consequences are specifically linked to the type and location of the identified sequence variant, which further adds to the complexity and can introduce discrepancies between studies. Furthermore, easy access to dermal samples and resident fibroblasts via a skin biopsy makes it the most studied tissue in human EDS. Although these samples are and will be relevant to examine the underlying consequences in EDS, they are not necessarily representative for other cell types. Therefore, the effects observed in skin and dermal fibroblasts cannot merely be extrapolated to other tissues. To fully grasp the extent of alterations that occur in the extracellular, pericellular and intracellular environment as well as their pathophysiological connection within and across EDS types, more in-depth studies are necessary. Hence, an integrated investigation of tissues from both EDS patients and suitable animal models across all EDS types using a combination of biochemical and 'omics' techniques, including (single-cell) transcriptomics and proteomics studies, are necessary to tackle knowledge gaps (Outstanding Questions). Moreover, a comprehensive understanding of deregulated pathways is anticipated to uncover potential therapeutic targets and pave the way for more effective treatment strategies. A recent study provided a promising (personalized) treatment strategy for autosomal dominantly inherited EDS types for which haploinsufficiency results in a milder phenotype than structural defects, like in vEDS. [100] Allele-specific RNA interference in fibroblasts from a vEDS patient was shown to specifically silence (>90%) the mutant allele, which was accompanied by a reduced UPR, with restoration of collagen fibril formation *in vitro*. [85] Furthermore, the possibility of enzyme replacement therapies for some EDS types caused by enzyme deficiencies, such as dEDS, requires investigation. Future therapeutic endeavors will largely benefit from the creation of international patient registries, collecting both clinical and molecular data, to overcome the challenges working with these rare, phenotypically variable and genetically heterogeneous conditions.

AUTHOR CONTRIBUTIONS

Delfien Syx: Conceptualization; Visualization; Writing – original draft; Writing – review & editing. **Fransiska Malfait**: Conceptualization; Writing – review & editing

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DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. Clinical characteristics associated with the different EDS types. (A) A variable clinical presentation can be observed between EDS types and within EDS types and virtually every organ or tissue can be affected. **(B)** The major and minor clinical characteristics in human EDS patients as defined by the *International Classification of the Ehlers-Danlos Syndromes*, published in 2017, are depicted according to organ system and their presence is indicated with a dark grey and light grey background, respectively. * and ** not included in the classification but criteria based on [31] and [2,63,101,102], respectively. For a detailed summary of the clinical features, the reader is referred to previously published work. [1] Figure partially created with Biorender.com.

Figure 2. **The biosynthetic pathway of fibrillar collagens and proteoglycans.** Molecules implicated in EDS pathogenesis are depicted in bold and the corresponding EDS type is indicated with a black background. **(1)** Transcription and translation of pro-α-chains. **(2)** Enzymatic co- and post-translational modifications of nascent pro-α-chains in the endoplasmic reticulum (ER), including proline and lysine hydroxylation by prolyl hydroxylases (PH) and lysyl hydroxylases (LH), respectively, and addition of sugar residues by galactosyltransferases (GLT). **(3)** Association of three pro-α-chains at their C-terminal propeptides and propagation towards the N-terminus initiates triple helix formation, assisted by molecular chaperones. **(4)** Transport of laterally aggregated trimeric procollagen molecules towards the extracellular environment. **(5)** Mature collagen molecule formation by proteolytic removal of N- and C-propeptides (e.g., ADAMTS-2 and BMP-1/mTLD for type I procollagen). **(6)** Assembly of collagen fibrils requires the concerted action of several assisting proteins, including *organizers* (e.g., fibronectin and integrins), *nucleators* (e.g., type V collagen) and *regulators* (e.g., SLRPs, fibril-associated collagens with interrupted triple helices (FACIT) and glycoproteins). [103] **(7)** Gap and overlap regions account for the striated aspect of collagen fibrils seen on ultrastructure as alternating dark and light bands on electron microscopy.

Proteoglycan biosynthesis starts with the synthesis of a core protein which is enzymatically modified in the Golgi apparatus. A common tetrasaccharide linker (one xylose (Xyl), two galactose (Gal) and one glucuronic acid (GlcA)) is formed onto which glycosaminoglycan (GAG) chains are synthesized and modified by epimerization and sulfation. Figure adapted from [104], used under the Creative Commons License (CC BY 4.0).

GLOSSARY

Anoikis: a form of programmed apoptotic cell death caused by disruption of cell–matrix interactions. Anoikis is regulated through the action of integrins and represents a critical mechanism to prevent dysplastic cell growth or attachment to an inappropriate extracellular matrix. It is a physiological process essential for tissue homeostasis and development. In the context of EDS, the abnormal ECM hampers proper adhesion of fibroblasts to their surrounding ECM, but alterations in the recruitment of alternative integrin receptors on the cell surface of fibroblasts are believed to counteract this process.

Congenital adrenal hyperplasia X-linked (CAH-X) syndrome: This contiguous gene syndrome combines signs of congenital adrenal hyperplasia (CAH) and Tenascin X (TNX) deficiency linked to classical-like EDS (clEDS). CAH is caused by 21-hydroxylase deficiency due to pathogenetic variants in the *CYP21A2* gene. The gene encoding TNX, *TNXB*, partially overlaps with *CYP21A2*. In rare cases, patients with severe, salt-wasting CAH have deletions in *CYP21A2* (often a 30-kb deletion) that extend into *TNXB*, creating chimeras of *TNXB* and its pseudogene *TNXA* resulting in a CAH-X syndrome. CAH-X patients are more likely to have joint hypermobility, chronic joint pain, multiple joint dislocations and a structural cardiac valve abnormality than age-matched and sex-matched patients with *CAH* without involvement of *TNXB*.

Extracellular matrix (ECM): an intricate network composed of molecules that provide strength and stability to tissues and organs but also functions as a biorepository for diverse growth factors.

Integrins: heterodimeric cell surface receptors consisting of an α and β subunit that mediate cell-cell and cell-ECM interactions.

Unfolded protein response (UPR): a cascade of adaptive cellular responses that act in a coordinated attempt to reduce the intracellular accumulation of misfolded of unfolded proteins by upregulating ER-resident chaperones to increase protein folding capacity and protein degradation pathways.

TEXT BOXES

BOX 1. Collagen biosynthesis, fibrillogenesis and ECM assembly

Collagens are the most abundant proteins in the ECM and to date 28 types are known which are categorized into several subgroups. [11] The biosynthesis of fibrillar procollagens (such as types I, III and V procollagen) is a complex process requiring many modifying enzymes, chaperones and regulatory (ECM) proteins (Figure 2). Type I collagen is the most abundant protein in the human body and its biosynthesis is widely studied. In brief, three post-translationally modified pro-α-chains associate at their carboxy- (C-) terminal propeptides and assemble intracellularly into a trimeric procollagen molecule propagating to the amino- (N-) terminus in a zipper-like manner. These trimeric proteins contain either three identical (homotrimer) or three genetically distinct (heterotrimer) pro-α-chains, which are composed of repeating Gly-Xaa-Yaa triplets with a glycine (Gly) residue and two other amino acids forming a long uninterrupted triple helical domain flanked by globular N- and C-terminal propeptides. These propeptides are proteolytically removed resulting in mature collagen molecules that subsequently engage to form highly ordered cross-striated fibrils and fibers in the extracellular environment. [11] Collagen fibrils typically contain different collagen types, e.g., type I and type V collagen form heterotypic type I/V collagen fibrils. [105] The assembly of collagen fibrils into highly ordered striated fibrils occurs in a tissue-specific way and requires the assistance of several proteins. Fibril assembly is mediated at the plasma membrane by fibronectin and integrins which serve as *organizers*. At the cell surface, some collagens function as *nucleators* (e.g., type V collagen) and initiate immature fibril assembly. Deposition of intermediate fibrils into the ECM and stabilization by interactions with *regulators* (e.g., small leucine-rich proteoglycan (SLRP) decorin, glycoprotein tenascin X and fibril-associated collagens with interrupted triple helices (FACIT) such as type XII collagen), which control the rate of assembly, size and structure of the collagen fibrils (together with other molecules). [103] As fibrillogenesis proceeds, fibrils grow linearly and laterally through fusion of inter-mediate collagen fibrils,

followed by the formation of intra- and intermolecular crosslinks to stabilize these structures. [106]

BOX 2. Chronic pain, a major problem in EDS

One of the main reasons why EDS patients seek medical attention is the presence of chronic, widespread pain, which severely impairs daily functioning, quality of life and psychosocial functioning. [44–46,48,49] Initial questionnaire studies indicated that 90% of patients across EDS types report pain. [107,108] More recent questionnaire studies reported (variable) chronic pain in the majority of cEDS [109] and 67% of vEDS patients. [110] Our group provided the first evidence of lower mechanical pain thresholds, higher vibration detection thresholds, reduced thermal sensitivity, and impaired endogenous central pain modulation in cEDS. [109] EDS patients often use a broad spectrum of analgesics, ranging from non-steroidal anti-inflammatory drugs (NSAIDs) to opioids, which result in moderate pain relief at best and hold serious health risks. [111–113] A multidisciplinary approach with inclusion of physical therapy and cognitive behavioral therapy to adapt coping strategies is recommended. [111]

Interestingly, studies showed a central role for ECM organization in painful conditions [114] and painful peripheral tissue injuries have been associated with ECM remodeling and changes in ECM modifying enzymes, including MMPs. [115,116] Therefore, it is tempting to hypothesize that the abnormal ECM architecture and organization seen in EDS contributes to the observed pain phenotype. Defective ECM can contribute in two ways: (a) by compromising the normal development and/or support of the nervous system, leading to increased vulnerability of the peripheral nerves; and (b) by inducing molecular and/or cellular alterations in the nociceptors and the peripheral nervous system. A variety of sequestered ECM proteins or their degradation products are able to act as damage-associated molecular patterns (DAMPs) [117], which are endogenous danger signals and are recognized by pattern recognition receptors such as Toll-like receptors (TLRs), which can induce painful pathways upon activation. [118–121]

In depth research into the origins and mechanisms of EDS-related pain is scarce. However, studies in mouse models, including the *Col5a1*$^{+/-}$ model of cEDS and the *Tnxb*$^{-/-}$ model of clEDS, showed the presence of pain-related behavior with altered sensory processing showing increased sensitivity to mechanical stimuli in both models and altered nociceptive innervation in the skin of *Col5a1*$^{+/-}$ mice. [122,123] Recently, mechanical allodynia in *Tnxb*$^{-/-}$ mice was attributed to hypersensitivity of Aδ- and Aβ-fibers, and it is induced by constitutive activation of TLR5. [124] In addition, *Tnxb*$^{-/-}$ mice showed more P-ERK and NADPH-diaphorase positive neurons in the dorsal horn of the spinal cord, which is indicative of central sensitization. [123] Nevertheless, the mechanisms linking defective ECM in EDS and chronic pain remain incompletely understood.

CLINICIAN'S CORNER

An incidence of about 1 in 5,000 individuals for all forms of EDS was proposed, with similar incidence rates in males and females and no apparent ethnic predisposition. Classical and vascular EDS are the most common genetically defined EDS types, with incidences of 1:20,000 and 1:50,000-1:200,000, respectively. For the other molecularly solved EDS types, incidence estimates are lacking, but below 100 people were reported worldwide. Since patients with milder forms frequently do not seek medical attention, incidence rates are likely an underestimation.

For each EDS type, major and minor clinical criteria were defined to help guide diagnosis, but in view of important clinical overlap, only a genetic test can confirm the definitive diagnosis (except for hEDS for which the genetic basis remains elusive) (Figure 1). A precise molecular diagnosis is crucial for accurate genetic counselling, and guiding prevention and management strategies. Multigene next generation sequencing (NGS) panels are the preferred diagnostic approach in case of complex phenotypes, as they are more time- and cost-effective than gene-by-gene Sanger sequencing and can identify large and small genomic deletions in the covered

610 regions. In some individuals presenting an EDS phenotype no molecular defects are found in
611 any of the known EDS-related genes (e.g., hypermobile EDS (hEDS)), therefore it is
612 anticipated that additional disease genes will be identified.
613
614 To date, no curative treatment options are available for any type of EDS. Hence management
615 strategies predominantly focus on prevention and symptom-based treatment, which depends
616 on the EDS type and the clinical features. Given the phenotypic complexity of EDS a
617 multidisciplinary approach is recommended, including a primary care physician, geneticist,
618 physical therapist, and health care professionals for medical and surgical interventions,
619 combined with education and social support which can also be provided through patient
620 communities. Being a rare disease encompassing several disease entities (e.g., EDS types),
621 makes the design of clinical trials assessing the effect of potential therapeutics challenging.
622
623 For hEDS, the diagnosis relies solely on clinical criteria and exclusion of other (genetic)
624 conditions that cause joint hypermobility. hEDS forms a phenotypic continuum with the
625 Hypermobility Spectrum Disorders (HSD), a group of conditions characterized by symptomatic
626 joint hypermobility and a variety of extra-articular comorbidities including among others fatigue,
627 chronic pain, headaches, dysautonomic features and functional gastrointestinal disorders.
628 hEDS and HSD are diagnosed more commonly in females, but it remains unknown whether
629 this is truly an increased incidence, or it reflects more severe manifestations.
630

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944 **Table 1 | Overview of the different EDS types and their characteristics as defined by the *2017 International EDS Classification*. [1]**

945

EDS type	Protein	Gene	IP [(a)]	Pathological consequences	Variant type [(a)]
Defects in collagen structure and processing					
Classical (cEDS)	Pro-α1-chain of type V collagen Pro-α2-chain of type V collagen	*COL5A1* *COL5A2*	AD	Decreased availability of functional type V collagen in the ECM which compromises the assembly of heterotypic type I/V collagen fibrils	NS, FS, SS, MS, Gdel, Gdup FS, SS, MS, Indel, Gdel
Vascular (vEDS)	Pro-α1-chain of type III collagen	*COL3A1*	AD	Qualitative or quantitative alterations in extracellular availability of type III collagen which compromises the assembly of heterotypic type I/III collagen fibrils	NS, FS, MS, SS, Indel, Gdel
Arthrochalasia (aEDS)	Pro-α1-chain of type I collagen Pro-α2-chain of type I collagen	*COL1A1* *COL1A2*	AD	Partial or complete loss of exon 6 of the *COL1A1* or *COL1A2* gene, which results in defective cleavage of the N-propeptide of either the pro-α1(I) or pro-α2(I) chain	SS*, Gdel* SS*, Gdel*
Cardiac-valvular (cvEDS)	Pro-α2-chain of type I collagen	*COL1A2*	AR	Loss-of-function variants in *COL1A2* inducing nonsense-mediated mRNA decay which results in complete loss of pro-α2(I)-chains forcing the formation of $[\alpha1(I)]_3$ homotrimers	NS, FS, SS
Dermatosparaxis (dEDS)	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 2 (ADAMTS-2)	*ADAMTS2*	AR	Retention of the N-propeptide of both pro-α1(I) and pro-α2(I) chain of type I procollagen and defective cleavage of other substrates (e.g., types II, III and V collagen, reelin, etc.)	NS, FS, Gdel, TIC
Defects in collagen folding and cross-linking					

Kyphoscoliotic (kEDS)	Lysyl hydroxylase 1 (LH1)	*PLOD1*	AR	First described congenital error of human collagen metabolism leading to underhydroxylation of lysine residues in the triple helical region thereby compromising subsequent glycosylation and crosslinking reactions	NS, FS, MS, SS, Indel, Gdel, Gdup
Kyphoscoliotic (kEDS)	FK506 Binding Protein, 22 kDa (FKBP22)	*FKBP14*	AR	Lack of fully functional FKBP22 protein, which results in decreased molecular chaperone activity and improper quality control of procollagen folding	FS, SS, MS, indel, TIC
Defects in ECM bridging molecules					
Classical-like (clEDS)	Tenascin X (TNX)	*TNXB*	AR	Loss-of-function variants resulting in a lack of function of the glycoprotein tenascin X compromising collagen fibril organization	NS, FS, MS, Gdel
Myopathic (mEDS)	Pro-α1-chain of type XII collagen	*COL12A1*	AD/AR	Qualitative or quantitative alterations in type XII collagen which compromises collagen fibril organization	SS, MS, Gdel
Defects in glycosaminoglycan biosynthesis					
Musculocontractural (mcEDS)	Dermatan 4-*O*-sulfotransferase 1 (D4ST1)	*CHST14*	AR	Defective sulfation of N-Acetylgalactosamine (GalNAc) allowing back-epimerisation of IdoA to GlcA in DS/CS chains leading to lack of dermatan sulfate (DS) and excessive amount of chondroitin sulfate (CS) glycosaminoglycan (GAG) chains on proteoglycans	NS, FS, MS
Musculocontractural (mcEDS)	Dermatan sulfate epimerase-1 (DS-epi1)	*DSE*	AR	Defective conversion of GlcA to IdoA in CS chains leading to severely decreased levels of dermatan sulfate (DS) and excessive amount of chondroitin sulfate (CS) glycosaminoglycan (GAG) chains on proteoglycans	NS, FS, MS
Spondylodysplastic (spEDS)	Galactosyltransferase I (β4GalT7)	*B4GALT7*	AR	Decreased β4GalT7 activity, which results in absence of the first galactose residue in the proteoglycan linker region and	NS, FS, MS, SS

				severely impairs overall GAG biosynthesis (of DS, CS and heparan sulfate (HS))	
Spondylodysplastic (spEDS)	Galactosyltransferase II (β3GalT6)	*B3GALT6*	AR	Decreased β3GalT6 activity, which results in absence of the second galactose residue in the proteoglycan linker region and severely impairs overall GAG biosynthesis (of DS, CS and heparan sulfate (HS)) with formation of a proteoglycan linker region consisting of three instead of four sugar residues.	FS, MS, Indel, TIC
Defects in intracellular processes					
Spondylodysplastic (spEDS)	Zrt/Irt-Like Protein 13 (ZIP13)	*SLC39A13*	AR	Underhydroxylation of lysine and proline residues in collagen and aberrant collagen crosslinking	FS, MS, Indel
Brittle cornea syndrome (BCS)	Zinc Finger Protein 469 (ZNF469)	*ZNF469*	AR	Loss of ZNF469 function, which results in defective regulation of collagen fibril synthesis and organization, but the underlying mechanism remains unknown	NS, FS, MS, Gdel
Brittle cornea syndrome (BCS)	PR/SET Domain 5 (PRDM5)	*PRDM5*	AR	Supposedly defective transcriptional regulation of multiple collagen and ECM genes	NS, FS, SS, MS, Gdel
Defects in the complement pathway					
Periodontal (pEDS)	Complement component C1r (C1r)	*C1R*	AD	Gain-of-function variants presumably leading to altered interactions with ECM constituents	MS, indel
Periodontal (pEDS)	Complement component C1s (C1s)	*C1S*	AD	Gain-of-function variants presumably leading to altered interactions with ECM constituents	MS, indel
Molecularly unsolved					

Hypermobile (hEDS)	Unknown	Unknown		/	
EDS types not included in the 2017 Classification					
Classical-like type 2 (clEDS2) *[provisional]*	Adipocyte enhancer-binding protein 1 (AEBP1)	*AEBP1*	AR	Defective AEBP1, which compromises the polymerization of type I collagen, but the underlying mechanism remains unknown	NS, FS, SS, MS
COL1-related overlapping disorder (C1ROD)	Pro-α1-chain of type I collagen Pro-α2-chain of type I collagen	*COL1A1* *COL1A2*	AD	Variants located in the most N-terminal part of the helical region of either the pro-α1(I) or pro-α2(I) chain introduce a conformational change in the procollagen N-proteinase cleavage site with delayed processing of the N-propeptide	NS, FS, SS, MS, Indel SS, MS

946

947 Footnotes: [(a)] IP: inheritance pattern, AD: autosomal dominant, AR: autosomal recessive, NS: nonsense variant, FS frameshift variant, SS: splice
948 site variant, MS: missense variant, TIC: variant affecting the translation initiation codon, Indel: small in-frame insertion/deletion, Gdel: genomic
949 deletion (exon, multi-exon, gene level), Gdup: genomic duplication (exon, multi-exon, gene level), * variants causing partial or complete loss of
950 exon 6.

951

952

953 **Table 2**. **Summary of the extracellular, pericellular and intracellular alterations observed in EDS**.

954

		EXTRACELLULAR										PERICELLULAR*			INTRACELLULAR				
				In vitro deposition*															
		Biochemical *	**TEM**	**Type I collagen**	**Type III collagen**	**Type V collagen**	**Type VI collagen**	**Type XII collagen**	**Fibronectin**	**Tenascins**	**Fibrillins**	**Thrombospondin**	**MMP activity**	**α2β1 integrin**	**α5β1 integrin**	**αvβ3 integrin**	**Dilated ER (TEM)**	**UPR activation** (expression/protein)	**TGFβ signaling**	**delayed wound closure**	**Expression analysis *** (altered genes/pathways)
Control	/		collagen fibrils with uniform round contours, tightly packed	+	+	+	+		+	+				+	+	-	-	-		-	
cEDS	*COL5A1/ COL5A2*		atypical large (cauli)flower-shaped collagen fibrils	↓ [5pt]^{2}	↓ [5pt]^{2}	↓ [5pt]$_{2}$			↓ [10pt]^{3}		- [4pt]			↓ [1pt]	↓ [6pt]$_{2}$	↑ [10pt]$_{3}$			↑ [*Mm*]	+	[micro-array – 4pt]: - matrisome gene expression - TGFβ signaling - ER homeostasis and autophagy - cell cycle regulation
vEDS	*COL3A1*	qualitative or quantitative alterations in pro-α1(III) collagen chain	aberrant diameters and distribution of collagen fibrils.	↓ [1pt]	↓ [3pt]^{2}	↓ [1pt]			↓ [8pt]^{3}		↓ [3pt]		↑ [*Mm*]	↓ [1pt]	↓ [4pt]$_{2}$	↑ [8pt]^{2}	+ / -	+ / -			[micro-array – 3pt]: - ECM gene expression - ER homeostasis - increased MMP9 expression [murine aorta]

aEDS	*COL1A1/ COL1A2*	N-propeptide retention in either pro-α1(I) or pro-α2(I) collagen chain	collagen fibrils with smaller diameters and irregular contours																			
dEDS	*ADAMTS 2*	N-propeptide retention in both pro-α1(I) or pro-α2(I) collagen chain	loss of circular dimensions and typical hieroglyphic pattern of collagen fibrils																			
C1ROD	*COL1A1/ COL1A2*		decreased collagen fibril diameters and lower fibril density, some fibrils with slightly irregular contours	↓ [3pt]	↓ [3pt]	↓ [3pt]			↓ [3pt]					↓ [3pt]	↓ [3pt]	↑ [3pt]						
cvEDS	*COL1A2*	absence of pro-α2(I) collagen chain																				
kEDS	*PLOD1*	- uniform underhydroxylation of (pro-)α collagen chains - increased LP/HP crosslink ratio in urine - altered crosslinking	fibrils with variable and overall increased diameters and irregular fibril spacing, fibril contours vary from round to irregular															-				[RNAseq – 3pt]: - ECM gene expression - vascular integrity

kEDS	*FKBP14*		no overt alterations in collagen fibril contours and diameters, fragmentation of elastin	↓/+ [2/2pt]$_2$	↓/+ [2/2pt]$_2$ (IC) [2pt]		↓/+ [2/2pt]$_2$ (IC) [2pt]	↓ [2pt]	↓ [2pt]	- [2pt]		↓ [2pt]		- [2pt]	- [2pt]		+ [1pt]	- [2pt]			[RNAseq – 3pt]: - ECM gene expression - inner ear development - vascular integrity
clEDS	*TNXB*		normal contour and size of collagen fibrils with reduced collagen fibril density	↓ [1pt]	↓ [1pt]	↓ [1pt]			+ [1pt]				↑ [*Mm*]		+ [1pt]	- [1pt]			↑ [12pt]		
mEDS	*COL12A1*		reduced collagen fibril density					↓ [5pt] (IC/+) [2/3pt]													
mcEDS	*CHST14*	decreased DS levels and increased CS levels	dispersed collagen fibrils with increased interfibrillar space	↓ [5pt]^3 (IC) [1pt]	↓ [5pt]^3	↓ [5pt]$_2$	↓ [2pt]		↓/+ [2/1pt]$_2$	↓ [2pt]	↓ [1pt]			- [2pt]	- [2pt]						
mcEDS	*DSE*	decreased DS levels and increased CS levels	normal ultrastructure	↓ [2pt]	↓ [2pt]	↓ [2pt]			↓ [2pt]												
spEDS	*B4GALT7*	decreased CS/DS and HS		↓ [1pt]																+ [1pt]	

spEDS	*B3GALT6*	decreased CS/DS and HS, formation of unconventional trilinker region (in urine)	loosely packed dermal collagen fibrils with variable fibril diameters and increased interfibrillar spaces	+ [2pt]	↓ [2pt]	↓ [2pt]			+ [2pt]	+ [2pt]				↓ [2pt]	+ [2pt]				+	+	[micro-array – 2pt (sisters)]: - ECM gene expression - proteins and/or enzymes responsible for posttranslational processing, folding and ECM remodeling - GAG biosynthesis -TGFβ/BMP signaling
spEDS	*SLC39A13*																		↑ [*Mm*]		
BCS	*ZNF469*		normal fibril diameters, minor variation in fibril diameter with occasionally slightly thinner fibrils	- [1pt]	↓ [1pt]	↓ [1pt]			↓ [1pt]					↓ [1pt]	↓ [1pt]						
BCS	*PRDM5*			↓ $[4pt]^2$	↓ $[4pt]^2$	↓ $[4pt]_2$			↓ $[4pt]^2$					↓ $[4pt]_2$	↓ $[4pt]_2$						[micro-array – 2pt]: - downregulation of ECM gene expression
pEDS	*C1R*		variable collagen fibril diameters with some abnormally shaped fibrils and increased interfibrillar spacing														+ [3pt]				- type I collagen degradation [4pt]
pEDS	*C1S*																				

clEDS 2	*AEBP1*		collagen fibrils with variable diameters and irregular contours																(+) [*Mm*]			

Footnotes: Findings for different EDS types are compared to healthy control. +: present (in normal quantity and quality), -: absent/negligible amounts, ↑: increased levels, ↓: decreased levels. When no information is available, the box is empty. *: all findings are from primary dermal fibroblast cultures (unless stated otherwise). TEM: transmission electron microscopy, ER: endoplasmic reticulum, UPR: unfolded protein response, (IC): apparent intracellular retention, LP: lysyl pyridinoline, HP: hydroxylysyl pyridinoline, DS: dermatan sulfate, CS: chondroitin sulfate, HS: heparan sulfate, [Mm]: observed in *Mus musculus* (mouse) models, [x pt]^{y}: with x the number of patients (pt) studied and y the number of independent studies (only indicated when higher than 1).

OUTSTANDING QUESTIONS

Which factors contribute to the inter- and intrafamilial phenotypical variability observed in all EDS types?

What is the contribution of molecular heterogeneity on the phenotype and mechanistic underpinnings within an EDS type?

Which intracellular/signaling pathways are shared and/or are distinct between the different EDS types and what is the exact contribution of each of these pathways on the EDS phenotype?

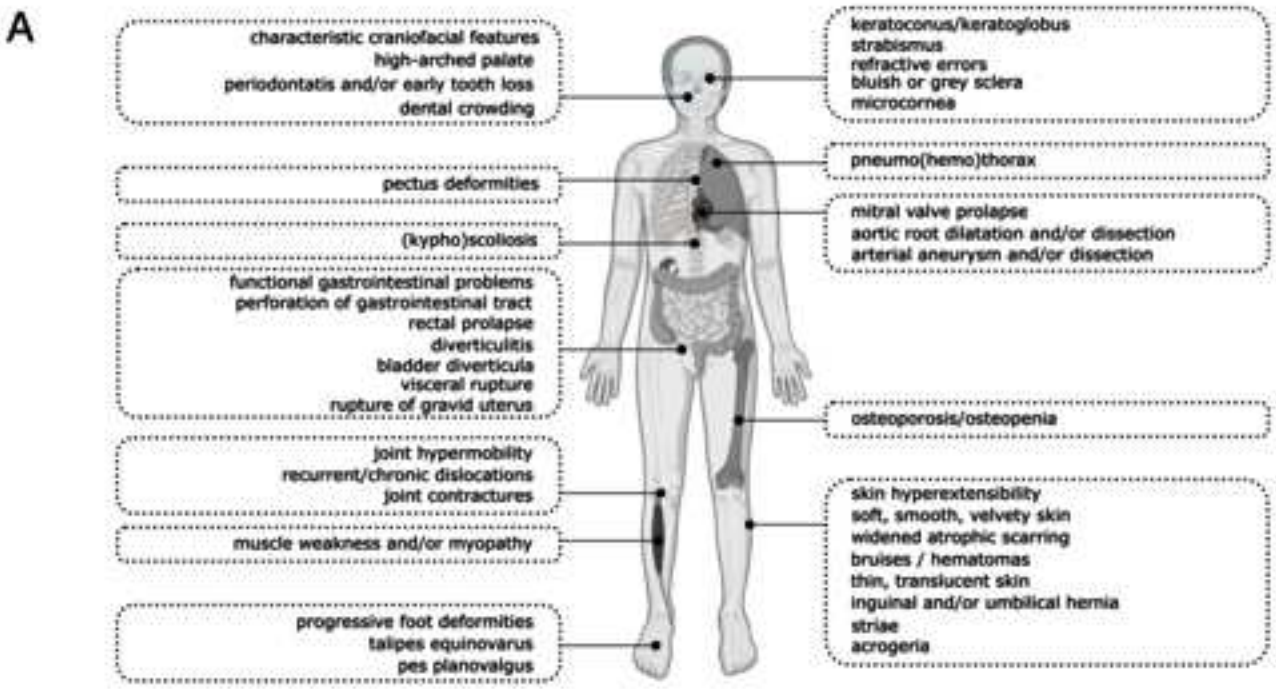
How many disease genes remain currently unknown?

What is the molecular basis of hypermobile EDS?

Which factors are driving chronic pain in EDS patients?

Figure_1

Click here to access/download;Figure;Figure_1_-_Clinical_picture_R1_FINAL.png



B

EDS type	cEDS	cEDS	vEDS	aEDS	aEDS	dEDS	C1REDS*	cvEDS	kEDS	kEDS	clEDS	mEDS	mcEDS	mcEDS	spEDS	spEDS	spEDS	BCS	BCS	pEDS	pEDS	clEDS2**
gene	*COL5A1*	*COL5A2*	*COL3A1*	*COL1A1*	*COL1A2*	*ADAMTS2*	*COL1A1/ COL1A2*	*COL1A2*	*PLOD1*	*FKBP14*	*TNXB*	*COL12A1*	*CHST14*	*DSE*	*B4GALT7*	*B3GALT6*	*SLC39A13*	*ZNF469*	*PRDM5*	*C1R*	*C1S*	*AEBP1*
craniofacial																						
skin / integument																						
tendon / ligament																						
skeletal																						
vascular																						
cardiac																						
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Figure_2

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