Archives of Dermatological Research (2019) 311:265–275
https://doi.org/10.1007/s00403-019-01894-0

REVIEW

# Understanding the basis of Ehlers–Danlos syndrome in the era of the next-generation sequencing

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Received: 29 July 2018 / Revised: 26 November 2018 / Accepted: 12 February 2019 / Published online: 2 March 2019


## Abstract

Ehlers–Danlos syndrome (EDS) is a clinically and genetically heterogeneous group of heritable connective tissue disorders (HCTDs) defined by joint laxity, skin alterations, and joint hypermobility. The latest EDS classification recognized 13 subtypes in which the clinical and genetic phenotypes are often overlapping, making the diagnosis rather difficult and strengthening the importance of the molecular diagnostic confirmation. New genetic techniques such as next-generation sequencing (NGS) gave the opportunity to identify the genetic bases of unresolved EDS types and support clinical counseling. To date, the molecular defects have been identified in 19 genes, mainly in those encoding collagen, its modifying enzymes or other constituents of the extracellular matrix (ECM). In this review we summarize the contribution of NGS technologies to the current knowledge of the genetic background in different EDS subtypes.

**Keywords** Ehlers–Danlos syndrome · Heterogeneity · Heritable connective tissue disorders

## Introduction

Ehlers–Danlos syndrome (EDS) comprises a clinically and heterogeneous group of heritable connective tissue disorders (HDCTs). The principal clinical features involve skin fragility, easy bruising, and joint hypermobility [13]. The incidence of EDS is estimated to be 1:5000 births and the frequency is independent of sex and ethnic background.

To date, different EDS classifications exist due to its clinical and genetic heterogeneity. The "Berlin nosology", in 1988, represents the first attempt to classify EDS, recognizing 11 EDS subtypes [4], defined by Roman numerals and classified according to clinical findings and the inheritance pattern. With the elucidation of the clinical and molecular basis of many of these EDS subtypes, a revised classification, the "Villefranche classification", was proposed in 1997 [5]. This nosology recognizes six EDS subtypes, for which major and minor criteria were identified, with the inclusion of biochemical and molecular basis, when known. A descriptive name, which captures the main characteristics of each EDS phenotype, substituted the Roman numerals. However, the recent advent of advanced genetic techniques such as next-generation sequencing (NGS) gives the opportunity to identify new EDS subtypes, leading thus to the revision of the Villefranche classification. The latest 2017 EDS nosology delineates 13 subtypes, as outlined in Table 1 [32], in which the clinical classification conserves the descriptive names that are widely used in the medical, scientific, and patient community. For the new EDS subtypes, a novel descriptor that captures the characteristic manifestations of the phenotype is proposed.

Molecular mechanisms explaining EDS subtypes are mainly characterized by causal mutations in genes encoding collagen, its modifying enzymes or other proteins involved in the biology of the extracellular matrix (ECM) [5, 13].

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**Table 1** The 2017 EDS classification (Malfait et al., 2017)

| EDS subtypes | Inheritance | Gene |
|---|---|---|
| Classical EDS (cEDS) | AD | *COL5A1, COL5A2* |
| Classical-like EDS (clEDS) | AR | *TNXB* |
| Cardiac valvular EDS (cvEDS) | AR | *COL1A2* (biallelic mutations that lead to *COL1A2* NMD and the absence of pro α2(I) collagen chains) |
| Vascular EDS (vEDS) | AD | *COL3A1* (rare: *COL1A1*, c.934C > T, p. Arg312Cys, c.1720C > T, p.Arg574Cys, c.3227C > T, p.Arg1093Cys) |
| Hypermobility EDS (hEDS) | AD | Unknown |
| Arthrochalasia EDS (aEDS) | AD | *COL1A1, COL1A2* |
| Dermatosparaxis EDS (dEDS) | AR | *ADAMTS2* |
| Kyphoscoliotic EDS (kEDS) | AR | *PLOD1, FKBP14* |
| Brittle cornea syndrome (BCS) | AR | *ZNF469, PRMD5* |
| Spondylodysplastic EDS (spEDS) | AR | *B4GALT7, B3GALT6, SLC39A13* |
| Muscolocontractural EDS (mcEDS) | AR | *CHST14, DSE* |
| Myopathic EDS (mEDS) | AD/AR | *COL12A1* |
| Periodontal EDS (pEDS) | AD | *C1R, C1S* |

*EDS* Ehlers–Danlos syndrome, *AD* autosomal dominant, *AR* autosomal recessive, *NMD* nonsense-mediated mRNA decay

Given a great phenotypic and genetic EDS variability and the clinical overlap with the EDS subtypes or other HCTDs, a final diagnosis requires molecular confirmation with the identification of causative genetic variant. This overlap makes the diagnosis difficult for clinicians, so the use of NGS can be very useful in this process. To date, the only genetically unsolved EDS form remains the hypermobile subtype (hEDS), diagnosed only by clinical finding, that have extensively been revised in the latest classification. The clinical criteria are based on the co-presence of generalized joint hypermobility, systemic manifestations of more generalized connective tissue fragility, positive family history, and/or musculoskeletal complications [32]. Dissecting the genetic etiology in patients affected by hEDS is quite challenging, probably due to clinical variability, genetic heterogeneity, and seeming sex-related penetrance. As well as the previously unrecognized EDS forms have been solved at the molecular level, it is likely that a better understanding of matrix biology will continue to expand.

In this review, we describe the genetic and molecular features of EDS, focusing on the recent genetic breakthroughs after the introduction of NGS techniques. The genetic analysis is mandatory to confirm or modify the clinical diagnosis and is also essential for evaluating prognosis, making decisions on management and treatment strategies.

## Next-generation sequencing: an overview

The advent of high-throughput NGS technologies has changed molecular approach in clinical laboratories by accelerating the rate of sequence generation and reducing the cost, as compared to the Sanger sequencing.

There are different NGS applications for the DNA analysis: whole-exome sequencing (WES) amplifies only protein-coding regions, whole-genome sequencing (WGS) amplifies the entire genome, and custom targeted sequencing in which regions including only particular group of genes (targeted approach) are amplified. WES [55] and custom targeted sequencing [17] become standard in clinical setting. To choose the correct strategy, clinicians should take into account: (a) disease model, (b) the region of interest, and (c) depth of coverage (the average number of times that a particular nucleotide is present in a determined position in a collection of random of sequences). WES is the appropriate strategy for the identification of molecular defects in patients with suspected genetic disorders [55]. Moreover, it represents the best NGS method in case of conventional Sanger sequencing or custom panel which are not able to identify the causal gene due to the genetic heterogeneity, atypical clinical presentation or lack

of causal gene [1]. Custom targeted resequencing is the best NGS application to be used when there are only a few known disease-causing genes. The major advantage is the possibility to personalize the design (i.e., inclusion of complete gene sequence or specific intronic sequences). Moreover, it is also faster and cheaper than WES and WGS [17]. On the other hand, when nothing has been detected in the exome, WGS should be useful to deliver a comprehensive view of the entire genome, with coding as well as non-coding sequences, which can be relied in regulatory regions. However, the limitations of its use in the clinical setting are related to a limited speed, high costs, and a complex data analysis as compared with WES.

Up to date, there are different platforms, such as MiSeq, NextSeq (Illumina), Ion Torrent (Thermo Scientific) to load NGS genomic libraries, generating millions of reads that are processed bioinformatically. Raw data, FastQ files (file storing biological sequence and its quality score) are processed through their quality scores, then aligned to reference genome and each base that composed reads is filtered according to statistical parameters and other kinds of information to generate VCF files (Variant Calling Format files, storing genetic variation data) [11]. The big challenge of clinical laboratories is to manage a huge amount of data produced by NGS, so it is important to standardize bioinformatic analysis and to make more easily accessible the NGS data interpretation.

## The extracellular matrix (ECM): collagen, elastic fibers, and glycoproteins

The extracellular matrix has relevant functions in regulating the development and homeostasis of all eukaryotic cells. The ECM is composed of different classes of macromolecules: fibrous proteins (including collagens and elastin), glycoproteins, and glycosaminoglycans (GAGs) [45] (Fig. 1).

Collagens are the major component of the ECM and the most abundant proteins in the body, constituting approximatively 25–30% of the total protein mass. To date, 29 types of collagen are described, each composed of a domain constituted of three α-chains intertwined as a triple helix. The α-chains consist of a triplet repeat Gly-Xaa-Yaa, where Xaa and Yaa are often hydroxyproline and proline, respectively. The role of glycine (the smallest amino acid, found at every third position) allows for tight helix formation into the center of the helix, whereas proline and hydroxyproline are essential for helical conformation [35]. Each α-chain is flanked at the N- and C-terminal ends by non-collagenous regions of variable sequence, size, and shape. The assembly process of the three α-chains into trimeric collagen monomers starts with the alignment of the C-terminal domains. Three α-chains then assemble in a C- to N-terminal directions. The process ends with post-translational modifications, such as hydroxylation of specific residues of proline or lysine and proteolytic cleavage of the N- and C-terminal propeptides [50].

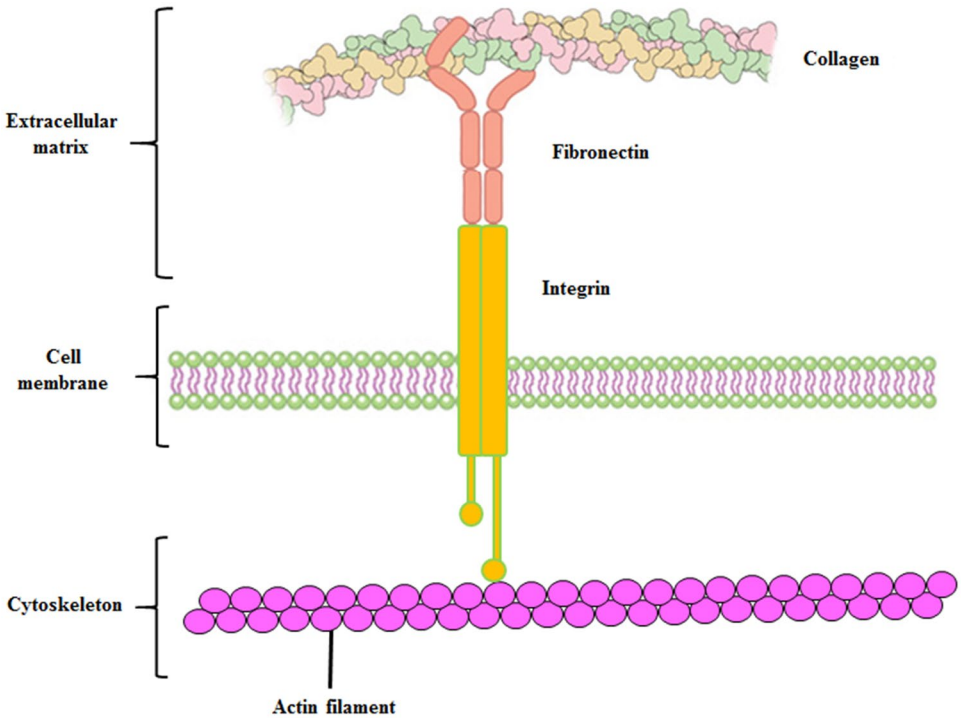


**Fig. 1** Schematic representation of the extracellular matrix (ECM). Collagen represents the major protein comprising the ECM. Fibronectin anchors cell surface to the basal lamina. The cell to ECM adhesion is regulated by specific proteins, known as integrins, that transmit the mechanical stimulus from the ECM to the cytoskeleton

Elastic fibers are abundant in connective tissue such as arteries, lungs, skin, and ligaments. Their principal functions are to endow tissues with elastic recoil and resilience, and they contribute to tissue homeostasis by regulating cytokine signaling [27]. Elastogenesis (elastic fiber assembly) is a complex and incompletely understood process that depends on spatio-temporal regulation and growth factor signaling. Elastic fibers are composed of parallel oriented microfibrillar scaffolds on which elastin is deposited [2]. Microfibrils are composed of other non-fibrillar components, that are integral part of microfibrillar or may associate with them. Non-fibrillary components have a structural role in microfibrillar assembly, elastin deposition, interactions with the other ECM proteins, including fibronectin and collagen, anchoring to basement membranes and cell surfaces [23, 26].

Glycoproteins form a considerable group of non-collagenous proteins that display a variety of functions in tissue morphogenesis and remodeling. GAGs mostly aggregate into proteoglycans by linking to a linear core protein. There are several types of proteoglycans that differ in their core protein structures and in the number, types, and lengths of the GAGs. Proteoglycans display different properties as well as elastic process and compressibility.

The ECM plays an important role in architecture of the cell and mutations in the ECM components implicate dramatic changes that can lead to HDCTs, including EDS.

## Pathological role of the ECM components (collagens, elastin, and glycoproteins) in Ehlers–Danlos syndrome

### Collagens and EDS

As stated before, collagens form fibrillary structure that gives the architecture to the ECM of all tissues and organs in the body. Mutations in collagen genes determine an abnormal collagen proteins assembly, that causes typical and widespread clinical problems in EDS [9]. Defects in type III or type V collagen represent the best example to explain knowledge of collagen mutations. The mature type III collagen is homotrimer and heterozygous mutations negatively influence the protein expression [40]. It is remarkable that some *COL3A1* mutations result in complete inability of fibroblasts to secrete type III procollagen with accumulation of the protein in the rough endoplasmic reticulum (RER) [9]. To date, the mechanism by which these molecules are retained in the RER remains unclear.

Type V collagen represents a minor component of the connective tissue and its role in the ECM is not clear. In 2004, Wenstrup et al. demonstrated the relevant role of type V collagen in nucleation of the large fibrils in skin and in other tissues. In fact, homozygous *COL5A1* knockout mice fail to survive embryogenesis and no large collagen fibrils were assembled in any tissues [54].

### Elastin and EDS

Elastin is a major component of soft tissues such as arterial walls and ligaments. It is the principal element of elastic fibers and gives elastic recoil and resilience to a variety of connective tissues (e.g., aorta and ligaments). Moreover, elastin fibers play a role in cell adhesion, migration, and participating in cell signaling.

The absence of elastin in the body is fatal. In 2012, Wagenseil et al. [53] demonstrated that elastin knockout mice die shortly after birth with subendothelial cells accumulation blocking blood flow and with markedly increased arterial. Moreover, literature data evidence that EDS subtypes have not been shown elastin abnormalities. It seems likely that elastic fiber abnormalities are a direct result of the other ECM family proteins deficiency as tenascin-X (TNX) and not a secondary consequence of altered collagen metabolism.

### Glycoproteins and EDS

Glycosaminoglycans are linear polysaccharides that form the side chain of proteoglycans. On the basis of their structural units, GAGs are divided into chondroitin sulfate, dermatan sulfate, and heparin/heparan sulfate. Their functions are mainly triggered by interactions with a wide range of proteins. Mutations in the genes encoding the enzymes responsible for the biosynthesis of GAGs cause several connective tissue disorders, such as chondrodysplasia, spondyloepiphyseal dysplasia, and EDS.

## Mutations in the collagen genes cause defects in the biosynthesis of fibrillar collagens

The recognition of abnormalities of collagen fibers confirms that EDS is a disorder of collagen metabolism. Collagen type I, III, and V mutations, identified in classical EDS (cEDS) and vascular EDS (vEDS), reflect this mechanism. Most of EDS subtypes (Table 1) show mutations in the collagen genes that cause defects in the biosynthesis of fibrillar collagens.

The cEDS, inherited in an autosomal dominant pattern, is clinically characterized by the combination of significant skin hyperextensibility with widened atrophic scars and generalized joint hypermobility. Easy bruising is observed in the vast majority of patients and may also be the presenting symptom in children. More than 90% of patients show

mutations in *COL5A1* or *COL5A2* genes, encoding the pro-α1 and pro-α2 chains of type V procollagen (COLV), respectively [43, 51]. Only rare cases of cEDS patients are caused by mutations in *COL1A1*, coding type I collagen (COLI) [29]. COLV plays an important role in collagen fibrillogenesis and coassembles with COLI to form heterotypic fibrils through the detention of N-terminal domain of the pro-α1(V) collagen chain [7]. Whereas *COL5A1* mutations are spread throughout the gene, all *COL5A2* mutations are located within the triple-helix domain, except for one mutation found in the C-propeptide. Defects in *COL5A1* are most frequently null mutations, consisting of nonsense, frameshift or splice-site mutations, which result in nonsense-mediated decay (NMD) of mutant *COL5A1* mRNA and *COL5A1* haploinsufficiency. Conversely, the majority of *COL5A2* mutations are missense, in-frame exon-skipping splice mutations, resulting in the production of pro-α2(V) chains that are expected to be incorporated into collagen molecules and probably interfere with formation of heterotrimers [43, 47, 51].

The vEDS represents an autosomal dominant condition which is generally considered the most severe form of EDS and is clinically characterized by thin, translucent skin, easy bruising, characteristic facial appearance, fragile arteries, muscles, and internal organs [32]. Unlike the other EDS subtypes, the skin is usually not hyperextensible. The vEDS is caused by defects or deficiency of pro-α1 chain of type III procollagen encoded by *COL3A1* gene, resulting in qualitative and quantitative abnormalities of mature protein [18, 38]. A wide spectrum of *COL3A1* mutations have been identified; the most typical are glycine substitutions in the triple helical region of the collagen protein. These mutations are in heterozygous state and the dominant negative effect causes a decreased production of type III collagen proteins [36]. Rarely, the vEDS is caused by mutations in *COL1A1*, including c.934C > T, p.Arg312Cys; c.1720C > T, p.Arg574Cys and c.3227C > T, p.Arg1093Cys, identified in the exons 14, 25 and 46, respectively [29]. They consist in the replacement of an arginine with a cysteine in the triple helical domain, leading to the production of α-1(I) dimers. Arginine-cysteine is a substitution with several adverse effects on collagen metabolism and stabilization. First, arginine residues in the Yaa position have a stabilizing effect on triple helical conformation, since it is involved in hydrophobic interactions contributing to collagen assembly [39]. Second, arginine substitutions cause a local destabilizing effect on the triple helix. Genetic data confirmed the relevant role of *COL1A1* c.934 C > T, p.Arg312Cys in the pathogenesis of cEDS with vascular episodes. In 2007 Malfait et al. described this mutation in three unrelated patients with clinical characteristics of EDS and propensity to arterial rupture in young adulthood [29]. On the other hand, in 2017, Colombi et al. characterized the same mutation in three-generation Italian family with six affected adult individuals with clinical diagnosis of cEDS without vascular episodes [10]. These arginine–cysteine substitution mutations have been associated with propensity of vascular episodes, mimicking *COL3A1*-vEDS. Indeed, in literature, EDS individuals carrying *COL1A1* Arg-Cys mutations are referred as "(classic) vascular like" EDS patients [43]. Given these considerations, it is very important that all EDS patients, harboring *COL1A1* Arg-Cys mutations, should be monitored annually by specific exams as computed tomography (CT) angiography and color Doppler, due to the increasing risk for developing vascular events with aging. Another group of mutations in *COL1A2* gene are represented by biallelic loss-of-function mutations (nonsense or splice-site), which result in NMD with a complete absence of pro-α2(I) chains. This leads to a rare autosomal recessive condition, namely cardiac valvular EDS (cvEDS), characterized by joint hypermobility, skin hyperextensibility, and cardiac valvular defects [32, 48].

Moreover, other defects interfering with the processing of the N-terminal propeptide of type I procollagen exist. First, splice-site mutations or deletions in exon 6 of *COL1A1* and *COL1A2* genes result in loss of the type I procollagen N-proteinase cleavage site, leading to the arthrochalasia type of EDS (aEDS). This is a rare autosomal dominant form of EDS, mainly characterized by extreme joint hypermobility, short stature, congenital bilateral hip dislocation, mild skin involvement, and distinct facial features [8, 19]. Conversely, biallellic loss-of-function mutations in *ADAMTS2* gene, coding the enzyme responsible for the cleavage of type I procollagen N-proteinase itself, cause the recessive dermatosparaxis type of EDS (dEDS), that differs from aEDS by the absence of congenital bilateral hip dislocation and the presence of markedly less severe joint hypermobility, an extremely fragile, bruisable and redundant, almost cutis laxa-like skin [52].

In a total of eight individuals from four families, Zou et al. [56] and Hicks et al. [22]. showed that mutations in *COL12A1* gene are responsible for myopathic EDS (mEDS) (Table 1), a rare EDS form characterized by muscle weakness, hypotonia, and myopathy [32]. Its clinical spectrum often overlaps with collagen type VI-related myopathies as Bethlem myopathy and Ullrich congenital dystrophy [8]. So, targeted resequencing of a gene panel including not only *COL12A1* genes but also the *COL6A1, COL6A2, COL6A3* genes is necessary to exclude clinical phenotypes overlapping with mEDS. Since then, one additional patient with mEDS has been described, carrying a missense mutation in *COL12A1* detected with a custom targeted resequencing panel [41].

## Defects in the synthesis of proteoglycans

In the ECM, collagens interact with different types of macromolecules, among which proteoglycans are the most relevant. They consist of a central core protein that is modified by the addition of a tetrasaccharide linker to target-specific serine residues in the peptide backbone. Once the linker region is completed, specific enzymes add GAG disaccharides of heparan sulfate, chondroitin sulfate, and dermatan sulfate (Fig. 2).

Defects in the biosynthesis of GAGs are associated with rare forms of EDS, including spondylodysplastic (spEDS) (previously known as EDS progeroid type) (Fig. 3) and musculocontractural type (mcEDS) (Fig. 4) (Table 1). EDS progeroid type was proposed in 1980, in which clinical characteristic of early aging is the result of defective GAG addition to several proteoglycan core proteins [21, 42]. The new EDS nosology, in consideration of the reliable clinical overlap, merged EDS progeroid type into spEDS, a rare autosomal recessive connective tissue disorder with unknown frequency and prevalence, caused by biallelic mutations in *B4GALT7, B3GALT6*, and *SLC39A13* genes [32, 44]. The criteria suggestive for spEDS are two major criteria, i.e, short stature and muscle hypotonia, plus characteristic radiographic abnormalities and at least three other minor criteria [8, 32]. Molecular testing is mandatory to reach a final diagnosis [47]. *B4GALT7* gene encodes galactosyltransferase I (or β4GalT7) (Fig. 3a), an enzyme responsible for

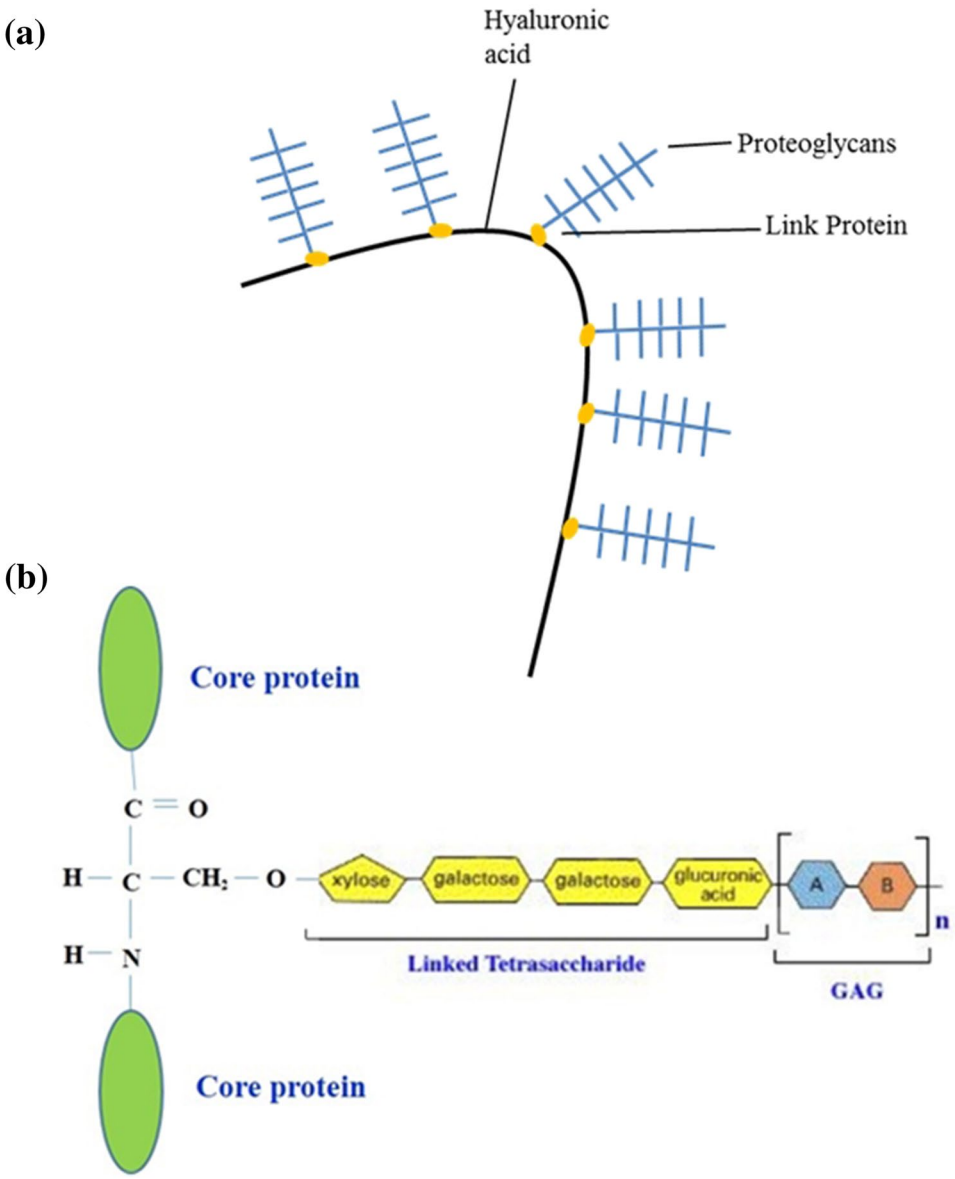


**Fig. 2** Proteoglycans: **a** schematic representation of their aggregations in the ECM. They present a common structure and connect to the ECM by linker protein; **b** Proteoglycans structure is composed of two fundamental molecules: core protein and GAGs. Core protein (contained serine residue) represents a point of attachment to different GAGs attach. It happens perpendicularly and gives a brush like-structure

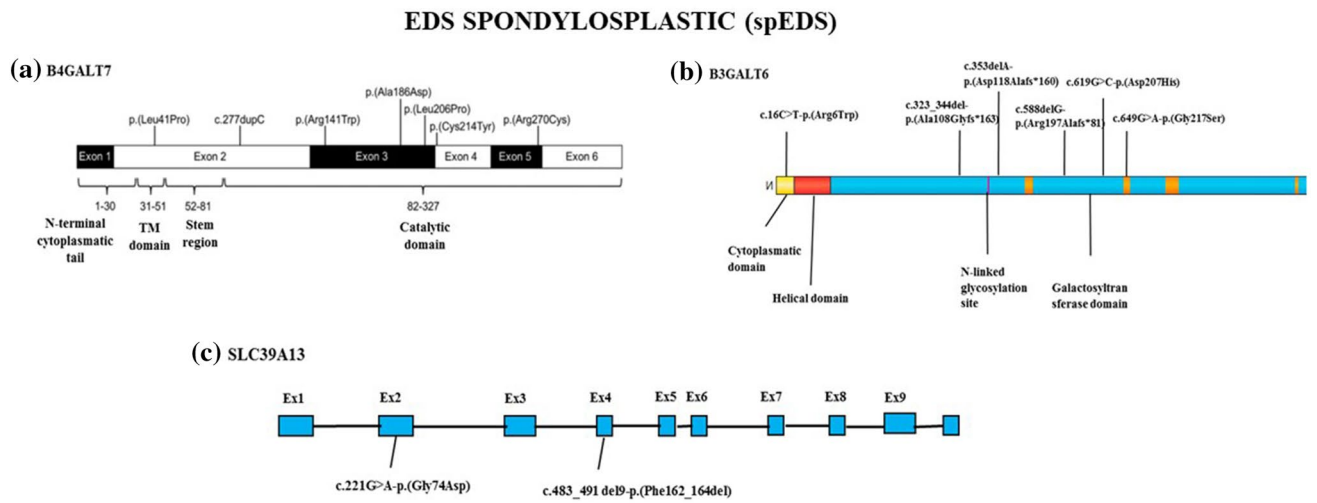


**Fig. 3** EDS spondylodysplastic (spEDS) forms schematic representation of known mutations in: **a** *B4GALT7* (beta-1, 4-galactosyltransferase 7) structure with indication of different exons and domains; **b** *B3GALT6* (beta-1, 3-galactosyltransferase 6) structure with indication different domains and known mutations, conservative motifs (I-IV) are shown in orange and predicted N-linked glycosylation site is pink; **c** *SLC39A13* (ZIP13) gene structure with known mutations

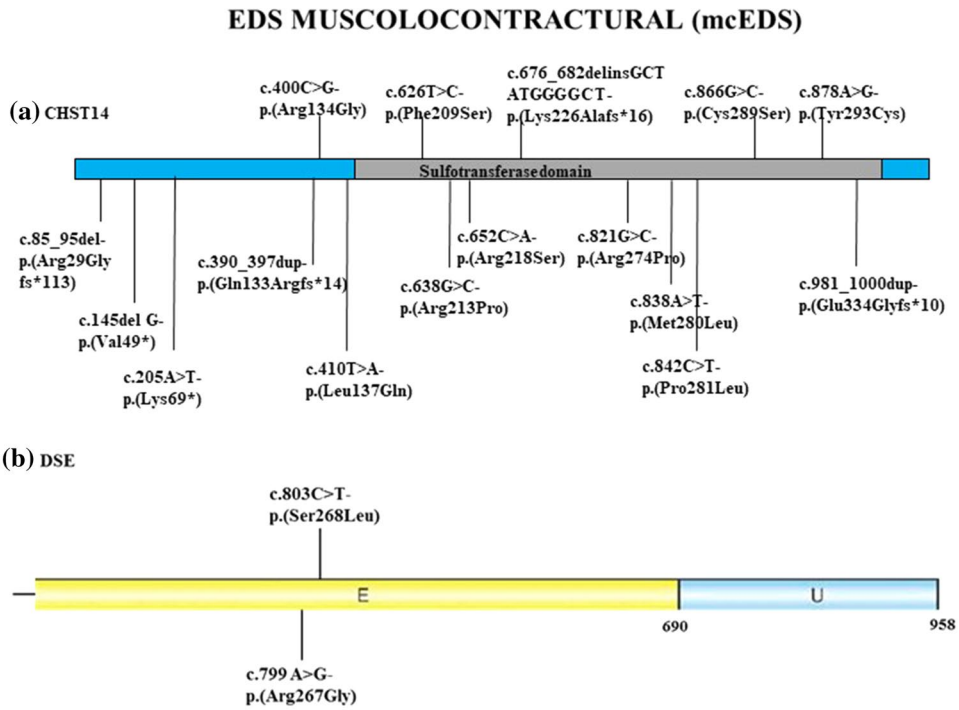


**Fig. 4** EDS muscolocontractural (mcEDS) forms: **a** *CHST14* (carbohydrate sulfotransferase14) structure, sulfotransferase domain is shown in gray and list of known mutations; **b** *DSE* (dermatan sulfate epimerase) structure with reported the two currently reported mutations in the epimerase (E) domain; (U) is unknown domain

the addition of a galactose residue to the O-linked xylose on the proteoglycan core protein [15, 35].

A WES study in seven Japanese families identified B3GALT6 as cause of spEDS (Table 1). *B3GALT6* (β3GalT6) encodes for galactosyltransferase II (Fig. 3b), the third enzyme involved in the biosynthesis of the tetrasaccharide linker region (Fig. 2) [36]. Coincidently, Malfait and collaborators identified *B3GALT6* mutations in three unrelated families with a pleiotropic EDS-like connective tissue disorder [31]. Homozygous mutations in *B3GALT6* are detected in EDS patients featuring hypotonia, bone fragility and severe kyphoscoliosis, in addition to progressive contractures [31, 36]. Deficiency of galactosyltransferase I and II affects the enzyme function, thus the target proteins are aberrantly glycosylated.

The mcEDS type results from homozygous mutations in carbohydrate sulfotransferase14 (*CHST14*) and dermatan sulfate epimerase (DSE) genes (Fig. 4). They encode two enzymes that are necessary for dermatan sulfate biosynthesis [33]. *CHST14* (Fig. 4a) encodes the dermatan 4-*O*-sulfotransferase 1 (D4ST1) [30, 33] which transfers active sulfate to the *N*-acetyl-D-galactosamine (GalNAc) residues of dermatan sulfate. *CHST14* mutations lead to no functional enzyme mostly through mRNA instability. The clinical features are skin, joints, internal organs fragility, and congenital malformations of multiple organ systems [49]. Recently, Muller et al. found a new homozygous missense mutation c.803C > T, p.Ser268Leu in *DSE* gene (Fig. 4b), encoding DS epimerase 1 (DS-epi1) without *CHST14* mutation [34]. This variant has been found in a few patients with a phenotype overlapping with mEDS. The consequence of *DSE* missense mutations is reducing the activity of dermatan sulfate that compromises GAG chains, the ECM organization, wound repair, anticoagulant process, and cell adhesion.

## Defects in other proteins

Tenascin-X, the first non-collagenous molecule implicated in EDS, is a member of the ECM family proteins and contains cysteine-rich segment at the N-terminal, followed by epidermal growth factor (EGF)-like repeats, fibronectin type III (FNIII)-like repeats and a fibrinogen-like domain at the C-terminal [24]. TNX interacts with the other ECM components including fibrillar collagens (type I, III, and V), fibril-associated collagens (type XII and IV), decorin and many others, playing thus an important role in the architecture of the ECM. Deficiency of TNX caused by biallelic mutations in *TNXB* gene is implicated in an autosomal recessive EDS form, namely classical-like EDS (clEDS), different from cEDS for the inheritance transmission pattern and clinical features such as normal scarring, profound joint hypermobility, and striking bruising [46]. Homozygous or compound heterozygous mutations in this gene lead to NMD of *TNXB*, resulting in a complete lack of TNX or, occasionally, in protein misfolding [12, 46]. One study proposed *TNXB* as candidate gene in hEDS, but its genetic screening revealed that only a small subset (2.5%) of hEDS patients carry *TNXB* mutations [57], so the molecular defect remains still unknown.

As stated above, in addition to *B4GALT7* and *B3GALT6*, spEDS grouped patients also with mutations in *SLC39A13* gene (Fig. 3). It encodes the homodimeric transmembrane Zrt/irt-like protein 13 (ZIP13) protein, a member of the SLC39A/ZIP family that controls the zinc ($Zn^{2+}$) concentration into the cytosol [6]. ZIP13 is a member of the subfamily of ZIP zinc transporters (LZT), a conserved group of eight transmembrane domain proteins that transport zinc and/or other metal ions from the extracellular space or from the organelle lumen into the cytoplasm [14]. ZIP13 loss-of-function mutations cause a change in hydroxylation of lysyl and prolyl residues in collagen α chains. Giunta et al. showed that an increased $Zn^{2+}$ concentration inside the ER competes with $Fe^{2+}$, a necessary cofactor for hydroxylation of lysyl and prolyl residues [20].

Another rare recessive form of EDS, the kyphoscoliosis type (kEDS), is clinically characterized by kyphoscoliosis, myopathy, and hearing impairment. The majority of patients were affected by kEDS harbor biallelic loss-of-function mutations in *PLOD1* gene, encoding the lysyl hydroxylase 1 (LH1), an enzyme that specifically hydroxylates the helical lysyl-residues in Gly-Xaa-Lys collagen, playing an important role in the formation of intra- and intermolecular collagen crosslinks [28]. Baumann et al. reported five families displaying a phenotype that clinically largely overlaps with kEDS_*PLOD1* patients, in which biallelic mutations in *KFPB14* have been found [3]. This gene encodes FKBP22, a protein member of the peptidyl-prolyl cis–trans isomerase family, involved in accelerating protein folding, particularly of procollagens [16]. Genetic defects in this protein result in an altered assembly of the ECM and enlarged ER cisterns in dermal fibroblasts [3].

A recent study resolves the genetic cause of periodontal EDS type (pEDS) (Table 1), a rare EDS form, clinically characterized by early-onset periodontitis leading to premature loss of teeth, joint hypermobility, and mild skin findings. Kapferer-Seebacher et al. performed a combined genetic approach of linkage analysis and WES on 19 independent families to characterize the genetic locus of pEDS, identifying heterozygous missense or insertion/deletion mutations in *C1R* or *C1S* genes, component of the classical complement pathway [23]. Pathogenic variants are associated with intracellular retention and mild endoplasmic reticulum enlargement. These findings establish a connection between the inflammatory classical complement pathway and connective tissue homeostasis in a monogenic disorder, such as EDS.

## Diagnostic molecular strategy for EDS

Although clinical findings can offer a suggestion for the diagnosis, the confirmation of the underlying molecular defect is extremely important to reach the genotype–phenotype correlation, because of the overlap among different EDS subtypes. Nowadays, molecular diagnostic strategies of EDS rely on NGS technologies, which offer the potential for parallel sequencing of multiple genes. Targeted resequencing of a panel that covers genes associated with each EDS form is a time- and cost-effective approach. It is being used to improve the speed of finding genetic defects in already

known genes. When no mutation is identified by a NGS technology and/or Sanger sequencing, this approach should be complemented with a copy number variant (CNV) identification method, including multiplex ligation-dependent probe amplification (MPLA) or array comparative genomic hybridization (aCGH) to detect large gene deletions or duplications. Moreover, WES platform not only allows to detect defects in known EDS-related genes, but it should also be useful to identify novel disease variants in other genes.

In keeping with the American College of Medical Genetics and Genomics (ACMG) guidelines, a variant can be considered as "likely pathogenic" if it is supported by some evidences of pathogenicity (e.g., the presence in a functionally active domain or high in silico prediction score). On the other hand, a variant of uncertain significance (VUS) represents ambiguous or unknown mutations, whose pathogenicity has neither been demonstrated nor excluded in literature or mutation databases. So, the clinical interpretation of such variants is challenging, making rather difficult the genetic counseling. For patients in whom a VUS is detected in one of EDS-related gene and fulfill the minimal clinical requirements for a specific EDS subtype, a provisional diagnosis of this disorder can be made and they should be followed clinically [32]. Additionally, the patient may be monitored by specific exams, such as color Doppler or CT angiography to exclude vascular complications. Over time, as more patients are reported, the clinical significance of a VUS may be revised to an informative result.

According to the current EDS diagnostic criteria [32], in case of unavailability of genetic testing, skin biopsy for transmission electron microscopy (TEM) examination of collagen flowers and biochemical test performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of collagen secretion can support the clinical diagnosis, but cannot confirm it. In particular, skin histology with TEM examination of collagen fibrils can contribute to the diagnosis that, however, remains primarily clinical for the cEDS, aEDS, dEDS, and kEDS subtypes. Moreover, biochemical analyses should be useful in support of the diagnosis of some EDS forms, including cvEDS, aEDS, dEDS, kEDS, and spEDS. SDS–PAGE is able to demonstrate the presence/absence of collagen chains or their precursors, mainly in case of loss-of-function mutations (nonsense or splice-site).

## Limitations of whole-exome sequencing as a diagnostic modality

Although WES has significantly improved the diagnostic efficiency in genetically heterogeneous disorders, its routine clinical practice as a diagnostic tool has some limitations. First, it is necessary to have efficient and fast filtering techniques that allow clinicians and non-specialists to analyze, manage, and store the huge amount of generated WES data. Indeed, the need of faster technologies and data processing methods are required for a prompt diagnosis to begin targeted therapy, manly in patients with inherited skin disorders. Furthermore, the accuracy of WES must be improved if compared with other diagnostic tests; given its relatively high variant-calling rate, Sanger sequencing is necessary to confirm the causal mutation identified by WES, making thus its use in the clinical setting time-consuming and expensive. Additionally, this platform also requires a refinement of technical guidelines and regulations. Parameters such as the sequencing depth, exon coverage or alignment calling need to be normalized and uniformed to avoid the dependence of WES on Sanger sequencing for the mutation validation. It also has to take into account the variability in DNA sequences, since GC-rich regions may be difficult to capture or not captured at all. Finally, there are number of complex ethical issues correlated to the information that should be provided to the patient. Indeed, WES could detect genetic variants which are not related to the disorder under diagnosis, but represent risk factors or might be causative of other diseases.

## Conclusion

The NGS era promises to lend new data on unsolved EDS types. As described above, NGS technologies have already successfully been used for the molecular diagnosis of EDS patients and allowed the detection of novel disease-causing genes. In particular, it played an important role in the identification of *COL12A1, B3GALT6, C1R*, and *C1S* as EDS causal genes and in the phenotypical description of their related subtypes [25, 36, 56].

Understanding and identifying the clinical characteristics, pathophysiological mechanisms and genetic etiology of different EDS forms will continue to expand the past 30-year-work towards comprehending the biological role of the ECM and macromolecules (e.g., non-collagenous proteins) in EDS. Additionally, a better knowledge of the underlying pathogenic mechanisms will stimulate further research into the clinical description of novel EDS subtypes and their causes.

**Acknowledgements** Thanks to AISED for support and Dr. Bice Strumbo and Dr. Agostino Seresini for excellent technical assistance.

**Funding** There is no funding source.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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