Mol Cell Biochem (2015) 401:133–139
DOI 10.1007/s11010-014-2300-3

Proteomic analysis of the extracellular matrix in idiopathic *pes equinovarus*

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Received: 16 September 2014 / Accepted: 27 November 2014 / Published online: 4 December 2014

Abstract Idiopathic *pes equinovarus* is a congenital deformity of the foot and lower leg defined as a fixation of the foot in adduction, supination, and varus. Although the pathogenesis of clubfoot remains unclear, it has been suggested that fibroblasts and growth factors are involved. To directly analyze the protein composition of the extracellular matrix in contracted tissue of patients with clubfoot. A total of 13 infants with idiopathic clubfoot treated with the Ponseti method were included in the present study. Tissue samples were obtained from patients undergoing surgery for relapsed clubfeet. Contracted tissues were obtained from the medial aspect of the talonavicular joint. Protein was extracted after digestion and delipidation using zip-tip C18. Individual collagenous fractions were detected using a chemiluminescent assay. Amino acid analysis of tissue samples revealed a predominance of collagens, namely collagen types I, III, and VI. The high content of glycine and h-proline suggests a predominance of collagens I and III. A total of 19 extracellular matrix proteins were identified. The major result of the present study was the observation that the extracellular matrix in clubfoot is composed of an additional 16 proteins, including collagens V, VI, and XII, as well as the previously described collagen types I and III and transforming growth factor β. The characterization of the general protein composition of the extracellular matrix in various regions of clubfoot may help in understanding the pathogenesis of this anomaly and, thus, contribute to the development of more efficacious therapeutic approaches.

Keywords Collagens · Extracellular matrix · *Pes equinovarus* · Proteomics

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Introduction

Idiopathic *pes equinovarus*, also referred to as 'clubfoot', is an isolated congenital deformity of the foot and lower leg defined as a fixation of the foot in plantar flexion, adduction, supination, and varus, with concomitant abnormalities present at birth. The incidence among Caucasians is around 1 per 1,000 live births, in Japan 0.5 per 1,000 and among natives of the South Pacific region nearly 7 per 1,000 live births. Studies on ethnic groups, populations and families suggest a genetic component as one causative factor. This abnormality is thus one of the most common birth defects involving the musculoskeletal system [1]. Although clubfoot is recognizable at birth, the severity of the deformity can vary from mild to an extremely rigid foot that is resistant to manipulation. When untreated, children with clubfoot walk on the sides and/or tops of their feet, resulting in callus formation, potential skin and bone infections, and significant limitations in mobility and employment opportunities later in life [2]. The most severe deformities in clubfoot occur in the hind part of the foot. The talus and calcaneus are generally deformed and, in severe cases, the calcaneus is in varus angulation and

medially rotated, and the navicular is severely displaced medially and may be in contact with the medial malleolus [3]. The ligaments of the posterior aspects of the ankle and of the medial and plantar aspects of the foot are shortened and thickened. Presently, two principle methods are used for the treatment of clubfoot: physiotherapy and continuous motion without immobilization [4]; and the Ponseti method, described almost 50 years ago [5]. The Ponseti method involves serial manipulation, a specific technique of cast application and, possibly, percutaneous Achilles tenotomy. Evaluation of the success rate of the Ponseti method is difficult, and significant differences between short-term and long-term results have been observed. The number of relapses during the first 3 years of treatment for which surgical intervention was indicated was markedly less than in patients in whom treatment had been started 6–8 years previously [6].

The pathogenesis of clubfoot remains unclear. Many theories have been proposed to explain its etiology, including vascular deficiencies in the talus [7], environmental factors, in utero poisoning [8], abnormal muscle insertions [9], and genetic factors [10]. While it is becoming more obvious that clubfoot is multifactorial in origin, genetics clearly play a role, as indicated by the familial pattern of inheritance and a 32.5 % concordance between monozygotic twins [11]. Although the exact genetic mechanism of clubfoot has yet to be elucidated, a multifactorial and, possibly, polygenic causation has been suggested. The search for possible 'clubfoot susceptibility' genes is underway; two transcription factor genes, *PITX1* and *TBX4,* have attracted attention. The PITX1–TBX4 pathway is responsible for early limb development, with both playing a role in hind limb development but being minimally expressed in the forelimb, perhaps explaining why there is no equivalent of clubfoot in the upper limb [10, 12].

The maximally contracted part of the clubfoot is localized between the medial malleolus, sustentaculum tali and navicular bone. This area differs macroscopically from the surrounding tissue; due to its similarity to the intervertebral disk, it is referred to as 'disc-like tissue'.

Ultrastructural study of the contracted tissue in clubfoot has revealed cells containing microfilaments identical in nature to those described in palmar fibromatosis [13]. Cells from the contracted tissue express type III collagen at high levels [14–16]. Furthermore, cells from palmar fibromatosis and the contracted tissues in clubfoot express several growth factors including platelet-derived growth factor (PDGF) and transforming growth factor beta (TGFβ). Blockade of PDGF and TGFβ led to decreased collagen expression, proliferation, and chemotaxis, which would decrease tissue contraction [16]. It has been suggested that expression of these factors may not be the primary cause of contracture, but, rather, may be secondary to activation of another signaling pathway, such as beta-catenin-mediated signaling, which plays a central role in regulating contracture in clubfoot [17, 18].

All of these studies support the hypothesis that fibroblasts and growth factors are involved in the pathogenetic mechanisms responsible for the development of clubfoot. To obtain a more global understanding of the protein composition of the extracellular matrix, we took a direct approach focused on the proteomic analysis of contracted tissue in patients with clubfoot. We hypothesize that identification of individual proteins may help to understand the mechanisms involved in this serious anomaly and, thus, contribute to the development of more efficacious therapeutic strategies.

Materials and methods

Patients and tissue samples

A total of 13 infants (ten boys, three girls) with idiopathic clubfoot who were treated using the Ponseti method at the Department of Orthopaedics, Bulovka Hospital in Prague (Czech Republic) between 2011 and 2013 were included in the present study. Treatment started between the first and 8 week of age, and consisted of gentle manipulation of the foot and the application of five to nine (average six) plaster casts on the leg above the knee as described by Ponseti [19]. Percutaneous Achilles tenotomy was performed in 12 patients. Primary correction was achieved in all cases. Tissue samples were obtained from patients undergoing surgery for relapsed clubfeet (age range 1–7 years; average 4 years): subtalar release (Mckay procedure) was performed in eight patients, transfer of the tibialis anterior muscle with osteotomy of cuneiforme and cuboid bones in three, and dorsomedial release in two. Contracted tissues were obtained from the medial aspect of the talonavicular joint. The tissue was processed as soon as possible after the surgical procedure; the sample was cryopreserved in liquid nitrogen vapor and subsequently used for protein analysis. Institutional approval for the present study was obtained from the appropriate department of the University Hospital Bulovka, and the parents of all patients provided written, informed consent to participate.

Sample preparation

All samples were washed three times in redistilled water, lyophilized, and digested in a solution containing NH_4HCO_3 (0.05 mol/L) and trypsin (0.2 mg/mL) (Sigma, St. Louis, MO, USA) 1/100 (w/w—trypsin/sample) for 3 h at 37 °C. Following trypsin cleavage, samples were delipidated using 0.3 mL of acetone at −20 °C overnight. The

delipidation step was performed three times, and samples were centrifuged at 10,000×*g*. Lyophilized pellets were digested once again in 0.2 mL of a solution containing NH_4HCO_3 (0.05 mol/L) and trypsin (0.04 mg/mL) (1/100 w/w—trypsin/sample) at 37 °C overnight. Samples were then filtered (0.45 μm pore, Millex-HV, Japan) and proteins were extracted using zip-tip C18 (Supel-Tips C18, TPSC18-96EA, Supelco, PA, USA). Extracted solutions were lyophilized and dissolved in 24 μL of 1 % formic acid.

Analysis of collagenous fraction

Tissue samples were homogenized then digested for 20 h using limited pepsin digestion in 0.5 M CH_3COOH, at 4 °C (ratio 12 % of wet tissue). The samples were then centrifuged (15,000×*g* for 2 h) and the extract was lyophilized. Samples of the lyophilized collagenous fraction were dissolved in electrophoretic sample buffer and separated using sodium dodecyl sulfide polyacrylamide gel electrophoresis. Gels with electrophoretically separated samples were then blotted onto nitrocellulose membrane (20 h, 500 mA/52 V) and membranes were then analyzed immunochemically by Western blot. Primary polyclonal rabbit antibodies (1:500) were used to detect collagen type I (abcam—cat. no. ab 292, abcam, USA) and collagen type III (abcam cat. no. ab 7778). Peroxidase-labeled anti-rabbit IgG antibodies (1:250) (Sigma cat. no. A0545) were used as secondary antibodies. Individual collagenous fractions were detected using a chemiluminiscent assay.

Analysis of tryptic digests with LC-MS/MS

The nano-LC apparatus used for protein digests analysis was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a maXis Q-TOF (quadrupole—time of flight) mass spectrometer with ultra-high resolution (Bruker Daltonics, Bremen, Germany) by nanoelectrosprayer. The nLC-MS/MS instruments were controlled with the software packages HyStar 3.2 and micrOTOF-control 3.0. The data were collected and analyzed using the software packages ProteinScape 3.0 and DataAnalysis 4.0 (Bruker Daltonics). Peptide mixtures were injected into a NS-AC-11-C18 Biosphere C18 column (particle size: 5 μm, pore size: 12 nm, length: 150 mm, inner diameter: 75 μm), with a NS-MP-10 Biosphere C18 pre-column (particle size: 5 μm, pore size: 12 nm, length: 20 mm, inner diameter: 100 μm), both obtained from NanoSeparations (Nieuwkoop, Netherlands).

The separation of peptides was achieved via a linear gradient between mobile phase A (water) and B (acetonitrile), both containing 0.1 % (v/v) formic acid. Separation was started by running the system with 5 % mobile phase B, followed by gradient elution to 30 % B at 70 min. The next step was gradient elution to 50 % B in 10 min, and then a gradient to 100 % B in 8 min was used. Finally, the column was eluted with 100 % B for 2 min. Equilibration before the subsequent run was achieved by washing the column with 5 % mobile phase B for 10 min. The flow rate was 0.25 μ/min, with the column held at ambient temperature (25 °C).

Online nano-electrospray ionization (easy nano-ESI) in the positive mode was used. The ESI voltage was set at +4.5 kV, scan time 1.3 Hz. Operating conditions: drying gas (N_2), 1 l min^{-1}; drying gas temperature, 160 °C; nebulizer pressure, 40 kPa. Experiments were performed by scanning from 100 to 2,200 *m*/*z*. The reference ion used (internal mass lock) was a monocharged ion of $C_{24}H_{19}F_{36}N_3O_6P_3$ (1221.9906 *m*/*z*). Mass spectra corresponding to each signal from the total ion current chromatogram were averaged, enabling an accurate molecular mass determination. All LC-MS/MS analyses were performed in duplicate.

Amino acid analysis

Amino acid analyses were performed using a PICO-TAG Amino Acid Analysis System (Waters, Milford, MA, USA). The method exploits pre-column derivatization with phenylisothiocyanate (performed according to the manufacturer's instructions) followed by the separation of the resulting products by high-performance liquid chromatography on a reversed-phase column (C18; Pico-Tag column 300 × 3.9 I.D. mm; Waters, USA) using an acetate buffer (pH 6.4, 0.14 mol/L) -acetonitrile gradient (according to the manufacturer's instructions). Protein hydrolysis was performed using HCl vapors (6 mol/l HCl with 2 % phenol) for 20 h at 110 °C in a vial under vacuum (following a nitrogen flush).). The molar content of individual amino acids was compared with the molar content of tyrosine. The values were expressed as mean ± SE; for comparison was used Students *t* test, $p < 0.001$ was considered as statistically significant.

Database search

Data were processed using ProteinScape software. Proteins were identified by correlating tandem mass spectra to the IPI and SwissProt databases, using the MASCOT search engine (http://www.matrixscience.com). The taxonomy was restricted to *Homo sapiens* to avoid protein identification redundancy. Trypsin was chosen as the enzyme parameter. One missed cleavage was allowed, and an initial peptide mass tolerance of ±10.0 ppm was used for MS and ±0.05 Da for MS/MS analysis. Cysteines were assumed to be carbamidomethylated, proline and lysine to be hydroxylated, serine, threonine, and tyrosine to be phosphorylated,

and methionine was allowed to be oxidized. All of these possible modifications were set to be variable. Monoisotopic peptide charge was set at 1+, 2+, and 3+. The Peptide Decoy option was selected during the data search process to remove false-positive results. Only significant hits (MASCOT score ≥60 [http://www.matrixscience.com]) were accepted.

Results

Amino acid analysis

Amino acid analyses of tissue sample composition revealed a predominance of collagens, namely collagen types I, III, and VI. The proportion of glycine was always approximately 28 %, and prolines (i.e., proline + hydroxyproline − h-proline) 21 % (Fig. 1). The high molar content of glycine and h-proline, which were present at similar levels, suggests the predominance of collagens I and III.

Extracellular matrix proteins

A total of 19 extracellular matrix proteins were identified and are summarized in Table 1. The major constituent of all analyzed samples was fibrillar collagens type I and III, which are the primary components of the extracellular matrix. In addition, other types of collagens were detected, particularly collagens V, VI, and XII. The remaining pool of 14 proteins included protein ABI3PB; the small leucine-rich proteoglycan biglycan; the leucine-rich proteoglycans asporin and prolargin; the proteoglycan osteoglycin/mimecan; the oxidative stress-sensitive proteoglycan fibromodulin; the keratin sulfate proteoglycan lumican; membrane primary amine oxidase; the large fibroblast proteoglycan versican; the extracellular matrix glycoprotein tenascin-x, which is exclusively expressed in fibroblasts; fibronectin, a high molecular weight glycoprotein that binds collagen; periostin, a TGFβ-inducible matricellular protein; and the TGFβ-induced protein IG-H3 (TGFβIp).

Discussion

The extracellular matrix represents a complex composition of numerous proteins including collagens, glycoproteins, small leucine-rich proteoglycans, as well as other types of proteins in a stoichiometric organization that gives various extracellular matrices and associated tissues their unique structural and functional characteristics [20]. The major result of the present study was the observation that the extracellular matrix in clubfoot is composed of an additional 16 proteins, including collagens V, VI, and XII, as well as the previously described collagen types I and III and transforming growth factor β [16, 18].

Collagens represent a family of matrix molecules used by cells for structural integrity and function. The three α chains that form the triple helical part of collagen molecules are composed of repeating peptide triplets of glycine–X–Y. X and Y can be any amino acids but are often proline and hydroxyproline, respectively. From a total of 28 different collagen types described to date [21], five prevail in humans. The major components of the extracellular matrix in clubfoot consist of collagen types I and III. Type I collagen is most abundant in skin, tendon, ligament, bone, cornea, etc., where it comprises between 80 and 99 % of the total collagen. The proportion of type I collagen in a particular tissue can vary at different sites during development and pathology. Type III collagen is a normal constituent of skin (10–20 %) and it has been found in many other connective tissues. It is present in variable amounts associated with type I collagen. The ratio of types I to III collagen varies significantly during ontogenetic development: in early fetal life, type III collagen is more abundant than type I. Similarly, a preponderance of type III and later reversion to the 3:1 ratio toward normal has been observed in dermal wound repair [22].

In the present study, it was observed for the first time that the extracellular matrix in clubfoot is also composed of collagens type V, VI, and XII. Type V collagen appears to be particularly abundant in vascular tissues, where it appears to be synthesized by smooth muscle cells. In differentiated cartilage, antibodies to type V collagen localize around the pericellular matrix within the chondrocyte lacunae [23, 24]. This type may be a specific form of collagen that contributes to cell shape by localizing on the surface of the cells and to the formation of an exocytoskeleton, as well as to binding to other connective tissue components [23].

Microfibrillar collagen type VI is a unique member of the collagen family that is ubiquitously expressed throughout the extracellular matrix [21, 25]. Sabatelli et al. [26] observed a restricted and differential distribution of the novel α5 and α6 chains in skeletal muscle when compared with the widely distributed α3 chain, suggesting that these new chains play specific roles in specialized extracellular matrix structures. Collagen VI is present in human connective tissues such as those of the joint capsule ligament, tendons, and skin. In fact, the major function proposed for collagen VI is an anchoring meshwork that connects collagen fibers to the surrounding matrix [27]. Type VI collagen has a crucial role in the function of muscle, evidenced by mutations causing Bethlem myopathy and Ulrich congenital muscular dystrophy [28, 29]. Muscles lacking collagen VI are characterized by the presence of dilated sarcoplasmic reticulum and dysfunctional

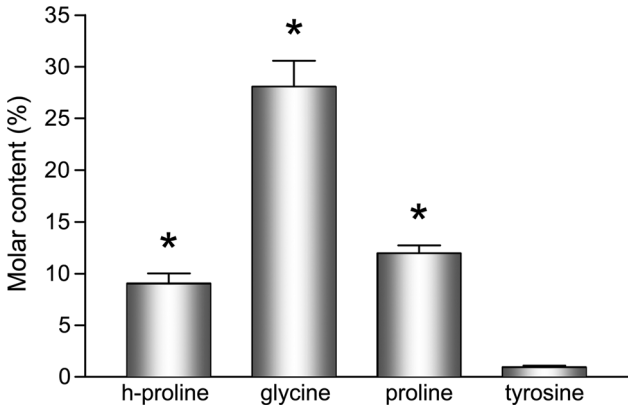

Fig. 1 Amino acid analysis of tissue samples. The molar content of individual aminoacids was compared with the molar content of tyrosine. The values are expressed as mean ± SE; $*p < 0.001$ versus tyrosine

mitochondria [30]. Latent mitochondrial dysfunction is present in both muscle cells and in fibroblasts derived from muscle cultures of patients with the above-mentioned muscular dystrophies, suggesting the important role of mitochondria in the pathogenesis of these disorders [25]. Collagen XII is a member of FACIT collagens (fibril-associated collagens with interrupted triple helices), is overexpressed in permanent human and mouse corneal scars [31] and likely plays a role in stromal architecture and fibril organization [20].

Available information regarding the function of other proteins that were observed for the first time in the extracellular matrix in clubfoot in the present study is, unfortunately, sparse. Small leucine-rich proteoglycans, such as biglycan, are involved in collagen fibril assembly and its fragmentation is likely to be associated with collagen turnover during the pathogenesis of diseases that involve deregulated extracellular matrix remodeling, such as rheumatoid arthritis and liver fibrosis [32]. Leucine-rich proteoglycans, such as asporin (which interacts with TGFβ) and prolargin (which binds the basement membrane heparin sulfate proteoglycan perlecan and collagen type I), contribute to cardiac remodeling during cardiac ischemia/reperfusion injury [33]. The proteoglycan osteoglycin/mimecan (which also interacts with TGFβ) is involved in arteriogenesis [34] and cardiac growth [35]. Fibromodulin (an oxidative stress-sensitive proteoglycan) regulates the fibrogenic response (extracellular matrix organization) to liver injury in mice [36]. Lumican (a keratin sulfate proteoglycan) promotes skin wound healing by facilitating wound fibroblast activation and contraction [37]. Membrane primary amino oxidase is involved in cell adhesion and associated with various forms of inflammation and fibrosis [38]. Uterine fibroids and keloid scars contain relatively high amounts of versican (a large fibroblast proteoglycan that binds hyaluronic acid); this affects the expansion of fibrotic process due to the effect on cell proliferation, and TGFβ and/or collagen formation [39]. Tenascin-x, an extracellular matrix glycoprotein exclusively expressed in fibroblasts, can mediate fibrosis in the presence of collagen; it potentially interacts with collagen types I, III, and V. According to observations from Jing et al. [40], tenascin-x is an initiator of myocardial fibrosis via upregulation of TGFβ. Fibronectin, a high molecular weight glycoprotein that binds collagen, is a major biosynthetic product of cultured fibroblasts and, together with TGFβ, was immunohistochemically detected in fibrotic processes of the liver and peritoneum [41]. The relatively close co-distribution of collagen VI and fibronectin observed in the extracellular matrix of normal epithelial cells appears to be consistent with the fact that the collagen VI globular domain can bind to immobilized fibronectin. This suggests that collagen VI is an important regulator of fibronectin fibrillogenesis [27]. Periostin, a TGFβ-inducible matricellular protein, supports adhesion and migration of epithelial cells and is upregulated in myocardial fibrosis [42].

Almost all connective tissue appears to be under some degree of mechanical tension, even at rest. Although it is clear that myofibroblasts can generate and maintain contractile force, the question arises as to how this translates into the tissue shortening that is observed in pathological contractures. Connective tissue contracture is a slow, permanent, and low energy shortening process that involves matrix-dispersed cells and is dominated by extracellular events including matrix remodeling. Connective tissue contracture involves incremental, anatomical shortening of the extracellular matrix material. Any remodeling process inevitably involves the removal of matrix molecules, and is largely mediated by matrix metalloproteinases. The limited understanding of the relationship between myofibroblast contraction and extracellular matrix remodeling makes it very difficult to define how this process occurs [43]. Some recent insights into the process of connective tissue contracture support the importance of the changing material properties of the collagen matrix on overall cell and tissue function [44]. Greater understanding of the protein composition of the extracellular matrix would, thus, be highly valuable.

Possible clinical relevance of our results lays, e.g., in the potential application of trypsin to the disk-like tissue between medial malleolus, sustentaculum tali and navicular bone. Extracellular matrix in this area contains increased amount of collagen VI which is better degradable than collagen I. The injection of trypsin might lead to the release of the contracture and consequently to the reduction of the number of surgical interventions, as well as to the facilitation of the conservative approach to the treatment of clubfoot.

Table 1 List of proteins detected by nLC-MS/MS

Accession no.	Protein name	Score	Peptides	SC (%)	Hits	Molecular function
IPI00304962	Collagen type I alpha-2 chain	3985.3	78	60.2	13	Extracellular matrix structural constituent
IPI00021033	Collagen type III alpha-1 chain	2114.0	51	41.5	13	Extracellular matrix structural constituent
IPI00220701	Collagen type VI alpha-3 chain	1706.7	39	18.5	13	Extracellular matrix structural constituent
IPI00291136	Collagen type VI alpha-1 chain	948.5	19	30.6	13	Extracellular matrix structural constituent
IPI00304840	Collagen type VI alpha-2 chain	778.2	17	26.1	11	Extracellular matrix structural constituent
IPI00010790	Biglycan	471.6	10	49.8	10	Extracellular matrix structural constituent
IPI00020987	Prolargin	379.4	9	30.6	11	Cytoskeletal anchoring activity
IPI00025465	Osteoglycin (Mimecan)	313.0	7	25.0	9	Growth factor activity
IPI00000860	Fibromodulin	300.6	8	25.8	8	Extracellular matrix structural constituent
IPI00018219	Transforming growth factor-β-induced protein ig-h3	294.6	8	19.2	6	Receptor binding
IPI00020986	Lumican	229.4	6	19.5	4	Extracellular matrix structural constituent
IPI00221384	Collagen alpha-1(XII) chain	241.4	5	3.8	3	Extracellular matrix structural constituent
IPI00418431	Asporin	192.5	7	17.4	7	Extracellular matrix structural constituent
IPI00004457	Membrane primary amine oxidase	183.4	4	11.7	1	Cell adhesion molecule activity
IPI00215630	Versican	179.5	5	9.0	3	Extracellular matrix structural constituent
IPI00844090	Collagen alpha-1(V) chain	172.0	2	2.5	1	Extracellular matrix structural constituent
IPI01011141	Tenascin	161.7	5	3.7	1	Cell adhesion molecule activity
IPI00440822	ABI3BP Isoform 1 of Target of Nesh-SH3	143.5	2	3.0	1	Unknown
IPI00910262	Periostin	136.2	3	11.6	1	Cell adhesion molecule activity
IPI00479723	Fibronectin	121.8	3	2.0	1	Extracellular matrix structural constituent

Accession n = indication of protein in IPI (international protein index) database (ftp://ftp.ebi.ac.uk/pub/databases/IPI), Score = Mascot Score, Peptides = number of unique peptides detected in samples, SC = Sequence coverage (%), Hits = number of samples, where the protein was significantly detected

The molecular functions of proteins in biological processes were categorized according to the classification system used in the public database available at http://www.hprd.org

One limitation of the present study was that clubfoot tissue samples were obtained from the feet of patients who underwent operative treatment that was almost always preceded by a period of corrective casting [6]. It appears, however, that changes associated with tissue contracture are not related to postnatal therapy [18]. Another potential limitation was the absence of a control group; however, obtaining normal tissues is particularly difficult and, consequently, was not included in the present study. A potential alternative for a control sample may be the use of less contracted tissue from the plantar surface of the calcaneocuboid joint [18]. Such a comparison will be the subject of a future investigation.

Conclusion

This present study was the first global proteomic analysis of the extracellular matrix in the contracted tissue of clubfoot patients. The determination of general proteomic parameters for various regions of the clubfoot may serve as a foundation for future studies characterizing extracellular matrix proteomes to provide molecular insight into various disease states and potential therapeutic interventions. The subsequent analysis of the mechanisms that regulate extracellular matrix remodeling and formation of contracture will be important for understanding the biology of this pathological process and the implementation of preventive or therapeutic strategies.

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