Novel Contribution to Clubfoot Pathogenesis: The Possible Role of Extracellular Matrix Proteins

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Received 27 September 2018; accepted 11 December 2018
Published online 7 January 2019 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.24211

ABSTRACT: Idiopathic *pes equinovarus* (clubfoot) is a congenital deformity of the feet and lower legs. Clubfoot belongs to a group of fibro-proliferative disorders but its origin remains unknown. Our study aimed to achieve the first complex proteomic comparison of clubfoot contracted tissue of the foot (medial side; $n = 16$), with non-contracted tissue (lateral side; $n = 13$). We used label-free mass spectrometry quantification and immunohistochemistry. Seven proteins were observed to be significantly upregulated in the medial side (asporin, collagen type III, V, and VI, versican, tenascin-C, and transforming growth factor beta induced protein) and four in the lateral side (collagen types XII and XIV, fibromodulin, and cartilage intermediate layer protein 2) of the clubfoot. Comparison of control samples from cadavers brought only two different protein concentrations (collagen types I and VI). We also revealed pathological calcification and intracellular positivity of transforming growth factor beta only in the contracted tissue of clubfoot. Most of the 11 differently expressed proteins are strongly related to the extracellular matrix architecture and we assume that they may play specific roles in the pathogenesis of this deformity. These proteins seem to be promising targets for future investigations and treatment of this disease. © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 37:769–778, 2019.

Keywords: clubfoot; fibrosis; extracellular matrix; proteomics; collagen

Idiopathic *pes equinovarus* (clubfoot) belongs to a group of fibroproliferative disorders involving the musculoskeletal system and its origin remains unknown. This defect occurs approximately once per 1000 births.[1] The guideline on the diagnosis and treatment of this disease was recently published by Besselaar et al.[2] These methods, although successful, are of a long-term nature and very demanding for the child patients and their parents alike because a number of plasters need to be applied starting at a very young age.

To explain the aetiology of clubfoot, a number of hypotheses have been proposed: Vascular insufficiencies in the talus,[3] environmental factors, abnormal muscle insertions,[4] and genetics.[5] Recently, it was agreed that clubfoot is probably multifactorial in origin and thus the aetiology of the disease is still not clear.

Ostadal et al.[6] reported a significant difference between the evaluation of the short-term and long-term results of the Ponseti method treatment of clubfoot. The number of relapses indicated for surgical intervention significantly increases with the time of follow up. The surgery of relapsed clubfoot has relieved that the structure of the connective tissues macroscopically differs: The medial part of the tarsus (a disc-like mass of fibrotic tissue localized between the *medial malleolus*, *sustentaculum tali*, and navicular bone) is much more rigid than the lateral part.[7] This observation supports the hypothesis that extracellular matrix (ECM) proteins, particularly fibroblasts, and growth factors are involved in the pathogenetic mechanisms responsible for the development of clubfoot,[8] and we started our research with these assumptions. In our previous proteomic study of the medial (contracted) side clubfoot tissue, we identified 18 ECM proteins.[9] The study presented here is the logical continuation of that research. This time we compared the protein composition of the medial (contracted) and lateral sides of clubfoot tissue together with corresponding tissues obtained from healthy adult cadaver controls, by means of label-free mass spectrometry. This method allowed us to compare the most abundant proteins (about 70) in these tissues for the first time. These analyses are necessary for determination of clubfoot biomarkers, better understanding of their roles in the pathological processes, and for their possible future therapeutic use.

[Correction added on March 15, 2019, after first online publication: Affiliations updated].
Conflict of Interest: The authors declare no conflict of interest.
All procedures have been performed in accordance with the ethical standards in the 1964 Declaration of Helsinki.
Institutional approval for the present study was obtained from the ethical committee of the Institute of Physiology of the Czech Academy of Sciences, v.v.i., and the parents of all patients provided written, informed consent to participate.
Grant sponsor: The Ministry of Health Department Program for Research and Development; Grant number: 17-31564A; Grant sponsor: Charles University in Prague, projects GA UK; Grant number: n. 336218; Grant sponsor: Charles University in Prague; Grant numbers: RVO:67985823, n. SVV 260440; Grant sponsor: The KZCR, a.s., Czech Republic, IGP KZ; Grant number: n. 217116002.
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METHODS

This study is analytical, prospective, level of evidence IIB.

Biological Material

A total of 13 patients (9 boys, 4 girls, mean age 58.4 months (SD = 20.2); details in Supplement Table S1) with idiopathic clubfoot were treated by the Ponseti method at the Department of Orthopaedics, Bulovka Hospital in Prague (The Czech Republic). Treatment consisted of gentle manipulation of the foot and the application of 5 to 15 (average 8) long-leg plaster casts (toes to thigh) as described by Ponseti et al.[10] Tissue samples were obtained from patients undergoing surgery for relapsed clubfeet: Contracted tissue was obtained between the ***medial*** malleolus, *sustentaculum tali,* and navicular bone (contracted side, "disc-like tissue"—**M-side**); non-contracted tissue was obtained from the **lateral** surface of the calcaneocuboid joint (non-contracted side—**L-side**) (Figure 1).[8,11] Tissue samples were taken from the feet of the aforementioned 13 patients. In three cases, samples from patients' both feet were taken, but an insufficient amount of tissue was obtained from the L-side, resulting in an uneven number of samples ($n = 16$ for M-side, $n = 13$ for L-side) (Supplement Table S1). The samples (ca 0.3 cm^3) were stored frozen at −80°C.

Control samples for mass spectrometry were taken from 10 human cadavers (5 male, 5 female; age 70 ± 10 years) in the Department of Pathology, the Municipal Hospital Litomerice, (The Czech Republic). The cause of death was not associated with any bone or joint disorder, metabolic disease (with the exception of diabetes) or systemic inflammation. No evidence of such diseases was documented during post-mortem pathological examination and autopsy.[12] The routine autopsies were performed less than 24 h after death. Tissue samples were obtained from the deltoid ligament of the talocalcaneal joint (M-side) and from the lateral surface of the calcaneocuboid joint (L-side). The samples (ca 0.3 cm^3) were stored frozen at −80°C.

Institutional approval for the present study was obtained (ethical committee), and the parents of all patients provided informed consent to participation. All procedures were performed in accordance with the 1964 Helsinki declaration and the laws of the Czech Republic.

Sample Preparation and Mass Spectrometry Quantification

All the obtained samples ($n = 16$ for M-side, $n = 13$ for L-side) were about 10 mg dry weight. Control samples (taken from cadavers) were also about 10 mg dry weight ($n = 10$ for M-side, $n = 10$ for L-side). All samples were processed as described in our previous study.[9] Sample purification was improved by extracting tryptic peptides using Stage Tips.[13] Nano-liquid chromatography procedure, mass spectrometry (MS), and tandem MS (MS/MS) analyses were performed as described in our previous studies,[14] with upgraded software. Our MS device (Maxis quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany)) allowed us to compare up to 100 most abundant proteins in two data sets using label-free quantification.

Comparisons of M-side to L-side samples were conducted separately in clubfoot patients and in cadaver controls. Samples within cadaver controls were compared to determine if there were any differences between M-side and L-side tissues in healthy feet.

Database searches were performed as described in Eckhardt et al. with the taxonomy restricted to *Homo sapiens*.[15] Only significant hits (MASCOT score ≥80 for proteins; ≥30 for peptides, http://www.matrixscience.com) were accepted.

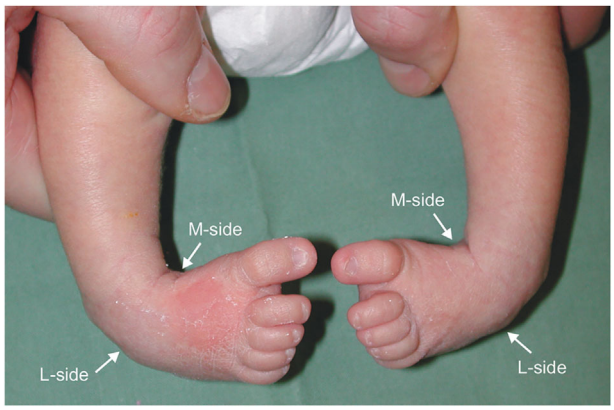

Figure 1. The medial (M-side) and the lateral (L-side) side of foot are marked with arrows.

Histopathological Investigation

Clubfoot samples from ten patients ($n = 10$ for M-side, $n = 10$ for L-side) were fixed with Baker's fluid and embedded in paraffin. Von Kossa staining was performed on longitudinal sections (5 μm) for basic histological assessment and detection of calcified structures. Subsequently, the sections were processed by immunohistochemistry (IHC). All special IHC reagents were purchased from Abcam, Cambridge, UK. Primary antibodies anti-collagen VI (ab6588, 1:200, 30 min at room temperature), anti-transforming growth factor beta (anti-TGFβ) (ab66043, 1:200, overnight at 4°C), and anti-transforming growth factor beta-induced protein (anti-TGFβip) (ab170874, 1:250, 1 h at room temperature) were used. Antigen retrieval was performed with the citrate buffer at 96°C for 15 min. Hydrogen peroxide block, protein block, secondary antibody reaction, and visualization were performed according to the Abcam protocol using the EXPOSE rabbit specific HRP/DAB detection IHC kit. Slides were counterstained with hematoxylin. Extracellular positivity of anti-collagen VI and anti-TGFβip IHC intensity were evaluated using an image analyzer (NIS Elements 3.0 AR, Laboratory Imaging Ltd., Prague, The Czech Republic) signal thresholding. Signal intensity from 10 independent parts of each sample was detected and the percentage of positive area was calculated.

Statistics

The software, Profile Analysis (version 2.1, Bruker Daltonik GmbH), was used to evaluate differences in the protein composition of the L-side and the M-side by means of label-free quantification. The peptides under consideration had to be found in at least 50% of all samples, regardless of the group, and they had to be found in at least one of the two groups (L or M) as well as in at least 50% of the group. The p-value returned by two-sample t-tests was corrected for multiple-testing by false discovery rate (FDR) based on frequency histogram (FDR adjusted p-value threshold 0.05).[16]

The IHC positive percentage area data from the L- and M-sides were checked for normality (Q-Q plot) and compared by two-sample t-test. The significance level to reject the null hypothesis was set to 0.05.[16]

RESULTS

MS Label-Free Quantification

Eleven differently expressed proteins were identified between the M-side and non-contracted L-side clubfoot tissue by MS label-free quantification. Seven proteins were significantly over-expressed in the M-side, and four proteins in the L-side of clubfoot (Table 1).

Apart from the aforementioned proteins, we detected a significantly higher concentration of three blood proteins in the M-side: Apolipoprotein A-I, hemoglobin subunit alpha, and hemoglobin subunit beta. These plasma proteins are not in direct connection with ECM and therefore are not relevant in the context of this study (data not shown).

In contrast, only two differently expressed proteins were identified between the M-side and the L-side tissue of control cadavers by MS label-free quantification. Both proteins were significantly over-expressed in the M-side (Table 2).

IHC Staining

Anti-Collagen VI IHC

The percentage of collagen VI positive area was significantly increased (p-value = 0.0417) in the M-side (Figure 2A) in comparison with the L-side (Figure 2B).

Anti-Transforming Growth Factor Beta (TGFβ) IHC

TGFβ intracellular positivity of fibroblast-like cells was observed in the M-side exclusively (Figure 3A). No intracellular positivity for TGFβ was observed in the L-side (Figure 3B).

Anti-Transforming Growth Factor Beta Induced Protein (TGFβip) IHC

TGFβip positivity was significantly higher (p-value = 0.0001) in the M-side (Figure 4A) as compared to low positivity in the L-side (Figure 4B).

Calcification Analysis

Calcified structures, which diffused along the collagen fibres of the tissue, were detected only in the M-side (Figure 5). No calcifications were detected in the L-side.

DISCUSSION

To date, only a few studies have compared the protein compositions of clubfoot tissue with healthy controls,[17,18] and some authors compared the tissue of the medial (contracted) and lateral (non-contracted) sides of clubfoot.[8,11] However, all these studies were concerned with only one or several proteins. Label-free mass spectrometry allowed us to compare the most abundant proteins (about 70) of both medial and lateral sides of the clubfoot as well as both sides of cadaveric control tissue for the first time in one study.

The Selection of a proper control for the clubfoot samples presented an ethical dilemma, however. Getting a necessary parental consent for children participation in research is extremely hard to accomplish, so obtaining control samples from healthy pediatric donors proved to be nearly impossible. This also applies to pediatric cadavers. Moreover, such samples are frequently affected by a long-term medical treat-

Table 1. List of Proteins With Significantly Different Concentrations Between the Medial (M-Side; Contracted) and Lateral (L-Side; Non-Contracted) of Clubfoot Patients

Accession number	Protein	Total number of peptides	Number of signif. different peptides	$p<$	FDR adjusted p-value	M/L (fold)
	Upregulated in M-side					
P12111	Collagen VI alpha-3 chain	89	30	0.0001	0.0004	1.246
P12109	Collagen VI alpha-1 chain	20	10	0.0001	0.0004	1.468
P12110	CollagenVI alpha-2 chain	23	7	0.0001	0.0004	1.236
P20908	Collagen V alpha-1 chain	6	6	0.0001	0.0004	1.587
P02461	Collagen III alpha-1 chain	83	9	0.0001	0.0004	1.152
P13611	Versican core protein	3	0	0.0030	0.0084	1.522
Q9BXN1	Asporin protein	6	2	0.0030	0.0084	1.401
Q15582	TGFBI Transforming growth factor-beta-induced protein	10	3	0.0040	0.0102	1.418
P24821	Tenascin-C	8	1	0.0180	0.0388	1.240
	Upregulated in L-side					
Q05707	Collagen XIV alpha-1 chain	23	19	0.0001	0.0004	0.417
Q06828	Fibromodulin	14	1	0.0001	0.0004	0.813
Q99715	Collagen XII alpha-1 chain	13	8	0.0005	0.0018	0.669
Q8IUL8	CILP-2 Cartilage intermediate layer protein 2	7	1	0.0080	0.0187	0.630

Proteins with significantly different concentrations between the medial (M-side) and lateral (L-side) are lined up by their "p" (significance). The total number of peptides indicates the number of successfully compared tryptic peptides by label-free MS detected at least in 50% of all samples, specific accession numbers of proteins are used from the UNIPROT database (www.uniprot.org) and the M/L (fold) symbolized value of protein concentration fold change (M-side/L-side).

Table 2. List of Proteins with Significantly Different Concentrations Between the Medial (M-side) and Lateral (L-side) of Controls (Cadavers Without Clubfoot)

Accession number	Protein	Total number of peptides	Number of signif. different peptides	$p<$	FDR adjusted p-value	M/L (fold)
	Upregulated in M-side					
P02452	Collagen I alpha-1 chain	177	10	0.0001	0.0007	1.154
A0A087WTA8	Collagen I alpha-2 chain	124	14	0.0001	0.0007	1.152
P12111	Collagen VI alpha-3 chain	63	12	0.0001	0.0007	1.426
P12110	Collagen VI alpha-2 chain	19	4	0.0005	0.025	1.926
P12109	Collagen VI alpha-1 chain	24	5	0.0083	0.033	1.619
	Upregulated in L-side					
	None					

Proteins with significantly different concentrations between the medial (M-side) and lateral (L-side) are lined up by their "p" (significance). The total number of peptides indicates the number of successfully compared tryptic peptides by label-free MS detected at least in 50% of all samples, specific accession numbers of proteins are used from the UNIPROT database (www.uniprot.org) and the M/L (fold) symbolized value of protein concentration fold change (M-side/L-side).

ment applied to children in intensive care units before their death. Therefore, we had to use more accessible adult cadavers as controls.

The examination of the ECM composition of contracted tissues is important in the context of better understanding the fibrotic processes, in clubfoot as well. The major original findings of our study are (i) the significant quantitative differences of protein compositions between contracted (M-side) and non-contracted (L-side) clubfoot tissues; (ii) pathological calcifications located only in the M-side tissue; and (iii) different intracellular concentrations of TGFβ in fibroblast-like cells from different sides of the clubfoot (M-side, L-side).

We are considering our research as an effort to deepen and confirm the previous findings, as well as a continuation thereof. We have brought new proofs that comparison of clubfoot samples from the M- and L-side are an appropriate model for studying this disease. The calcifications detected in the M-side (but not in the L-side) are clear signs of a pathological condition of that tissue, since there is no soft tissue which would contain such calcifications in physiological conditions.[19] Our other finding shows different intracellular concentrations of TGFβ, which for the first time prove different state and/or behavior of fibroblast-like cells in clubfoot M- and L-side. As in another fibroproliferative pathologies (Dupuytren's dissease, Peyronie's disease), TGFβ is regarded to be a key stimulator of fibroblast-like cells activity. This stimulation pathway leads to production of excessive levels of extracellular matrix proteins.[20,21]

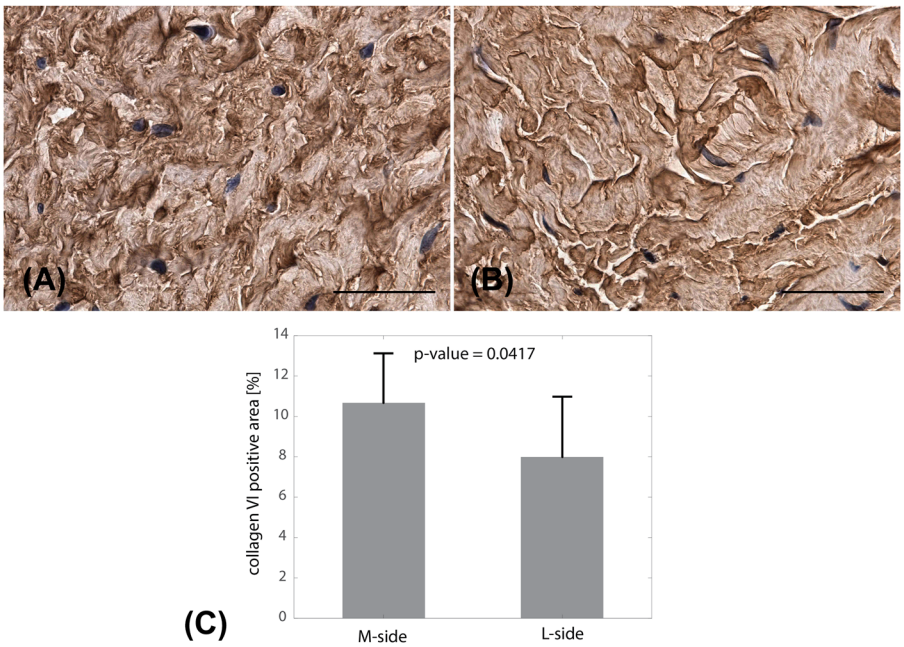

Figure 2. Immunohistochemical staining by anti-collagen-VI antibody detected a positive area in the M-side (A), and the L-side (B) (difference in intensity of brown colour areas, bar = 20 μm). The significant difference in percentages of collagen VI positive area in the M-side and the L-side are described in graph (C); $p < 0.0417$.

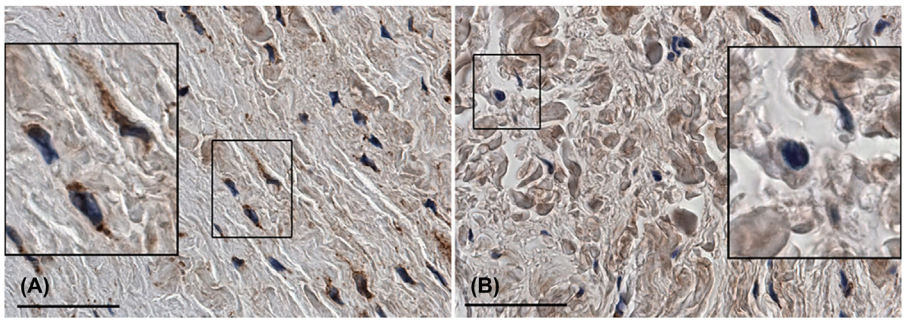

Figure 3. The staining by anti-TGFβ IHC detected (A) significant intracellular positivity in cytoplasm of fibroblast-like cells in the M-side, in comparison with (B) no intracellular positivity in cytoplasm of fibroblast-like cells in the L-side (bar = 20 μm).

Whereas the quality of protein compositions of both clubfoot sides was comparable, the quantity of 11 proteins differs. The concentrations of seven proteins were significantly increased in the M-side and four in the L-side (Table 1). Control samples had only two protein upregulations in the M-side and none protein was detected as upregulated in the L-side (Table 2). The only protein upregulated in both M-side of clubfoot patients and M-side of cadaver controls was collagen VI.

Proteins With Higher Concentration in the M-Side

The presented study has revealed seven ECM related proteins upregulated in the M-side (Table 1) (Figures 2A-C, 4A-C).

Collagen III

Observed over-expression of collagen III confirmed the results of several groups[8,11,17] which showed the protein significantly increased in the M-side (Figure 6). This observation also correlates with the higher ratio of collagen III to/collagen I in wound repair.[22] Poon et al. state that collagen III is more abundant in contracted tissue (also in Dupuytren's disease) due to elevated beta-catenin signaling pathway.[11]

Collagen V and Collagen VI

Collagen V and collagen VI are significantly upregulated in the M-side of clubfoot (Table 1), and moreover upregulated collagen VI was proved by IHC, too (Figure 2A–C). Type V collagen is particularly abundant in vascular tissues and contributes to binding to other connective tissue cells.[23] This collagen type was detected as upregulated in human skin keloid formation.[24] The major function of collagen VI is probably as an anchoring meshwork connecting collagen fibers to the surrounding matrix, but the precise role of collagen VI in cartilage has not been clearly defined.[25] Veidal et al. suggested that collagen VI turnover is a central player in liver fibrogenesis.[26] Moreover, the C-

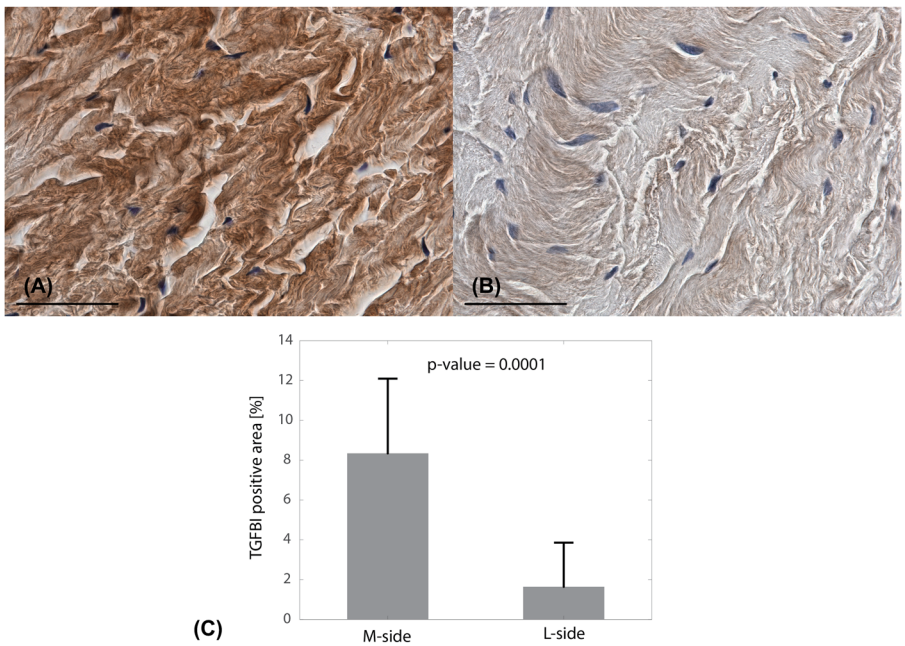

Figure 4. The staining by anti-TGFβip (TGFBI) IHC detected an increase in the percentage of TGFβip positive area in the M-side (A) in comparison with the L-side (B); (bar = 20 μm). The significant difference in percentages of TGFβip positive area in the M-side and the L-side are described in graph (C); $p < 0.0001$.

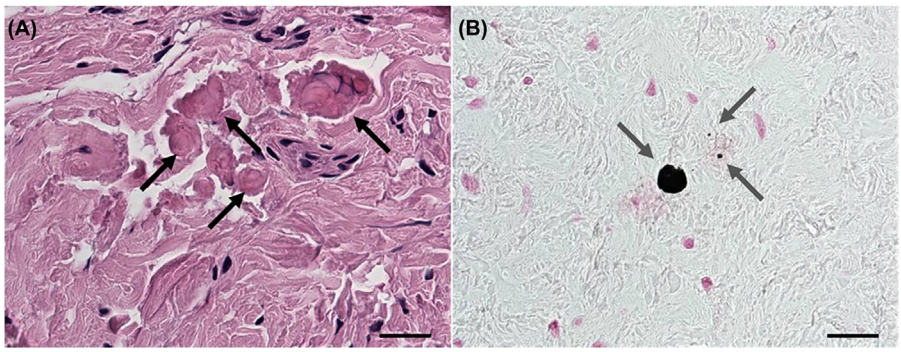

Figure 5. The staining detected calcium deposits in the M-side samples (arrows) (A—hematoxylin-eosin stain, black arrows; B—von Kossa stain, gray arrows; bar = 20 μm). No calcifications were detected in the L-side (picture not displayed).

terminate peptide of collagen VI chain alpha 3 (endotrophin) is known to promote phenotypic modulation and proliferation of cells.[27]

Both these types of collagens could be important molecules in clubfoot pathogenesis (Figure 6). However collagen VI was observed in significantly higher concentrations also in the M-side of controls (Table 2), therefore we do not know if its concentration in clubfoot M-side is higher naturally or due to clubfoot disease.

TGFβ and TGFβip

There is ample evidence that TGFβ signaling is a key regulator of myofibroblast biology in fibrotic tissues (reviewed in Piersma et al.[28]). TGFβ and platelet-derived growth factor (PDGF) were expressed at high levels in the contracted tissues of clubfoot. The blockade of TGFβ and PDGF in cells isolated from clubfoot led to decreased collagen expression and fibroblast proliferation.[8] In the presented study we did not detect TGFβ or PDGF by MS label-free quantification probably due to the low concentration of these growth factors (we were able to compare to ca 100 most abundant proteins due to our MS device).

TGFβip, also known as βig-H3, TGFBI and keratoepithelin, is a protein with the ability to bind collagen. TGFβip plays a role in the early development of cartilage (enhances the growth and adhesion of the pre-chondrogenic cells).[29] In addition, Lorda-Diez et al. documented that TGFβ may also function as a profibrogenic factor for embryonic limb mesoderm. They

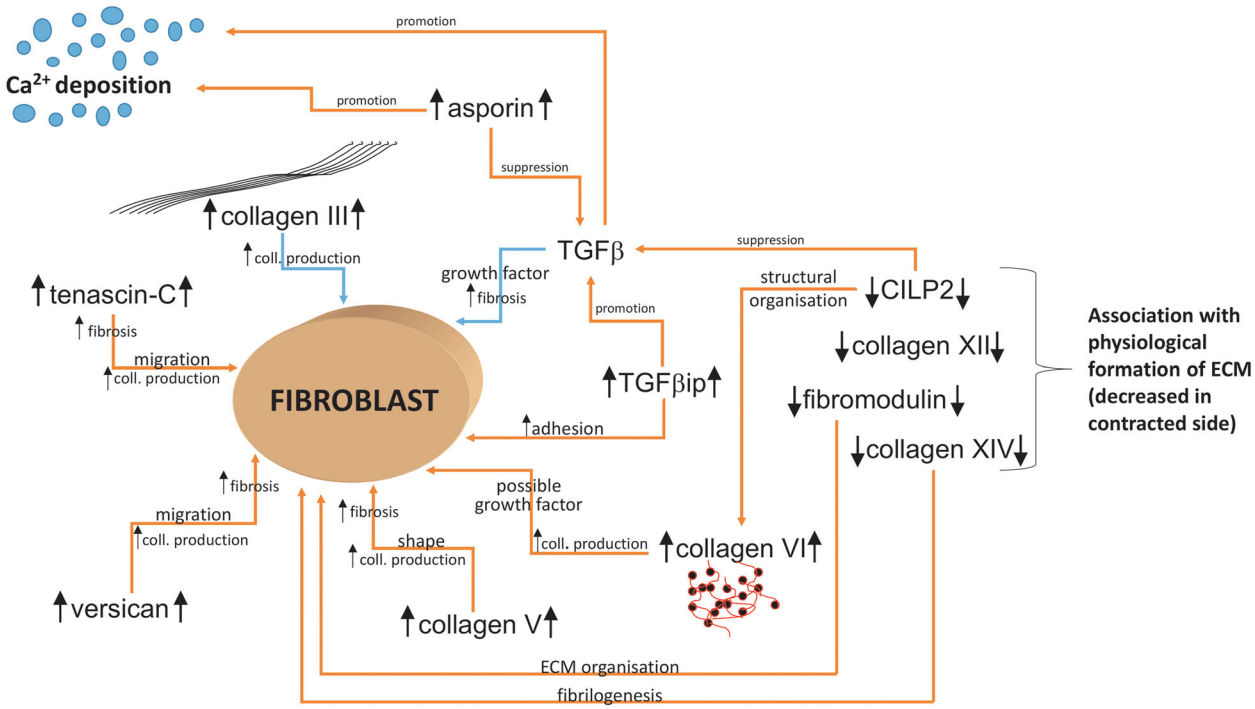

Figure 6. Doubled arrows express upregulation (↑protein↑) or downregulation (↓protein↓) of proteins to the M-side. All upregulated ECM proteins have some relationship to fibroblasts. Some upregulated proteins are implicated in fibrosis (↑fibrosis) and/or take part in migration (migration) and/or enhanced collagen production (↑coll. production) in dermal fibroblasts as reviewed in Tracy et al.[33] Orange arrows designate predicted interactions based on the upregulated proteins in the contracted side. The upregulation of these proteins was detected in this type of fibrotic tissue for the first time. Already described possible pathways are expressed in blue lines. All observed downregulated proteins associate with the physiological formation of ECM.

identified TGFβip as a factor downstream to TGFβ signaling (regulated by Smad 2 and 3), which is highly expressed in joint capsules and the differentiating tendons. Their findings indicated that TGFβip promotes the fibrogenic influence of TGFβ signaling in the pro-fibrogenic pathway.[30]

We observed intracellular presence of TGFβ, detected by IHC in fibroblast-like cells of the M-side (and no intracellular TGFβ in the L-side) (Figure 3A and B). This result suggested that fibroblast-like cells are in a different state in the M-side in comparison with the L-side. We also detected a higher concentration of TGFβip by means of label-free MS (Table 1) and IHC (Figure 4A–C) in the M-side. Our observations suggest that both of these proteins play specific roles in clubfoot pathology (Figure 6). In addition, TGFβ has direct relationships to collagen calcification, and also to another two proteins significantly changed in the presented study—asporin and CILP2 (Figure 6).[31,32]

Versican

The effects of versican (a large fibroblast proteoglycan binding hyaluronic acid) to fibrosis (wound healing) has been reviewed by Tracy et al.[33] Compared to the non-contracted tissues, uterine fibroids and keloid scars contain higher relative amounts of versican.[34] These results correlate with our observation.

Asporin

This protein interacts with TGFβ, binds collagen, and may be one of the key proteins involved in the calcification of blood vessels, tumors, or osteoarthritis. The role of asporin in collagen fibrillogenesis and biomineralization was reviewed by Kalamajski.[35] Possible mechanisms for the involvement of asporin in osteoarthritis pathology are the inhibition of TGFβ function and induction of collagen mineralization.[32,36]

These observations, together with our results (upregulated asporin (Table 1) and the presence of histologically confirmed calcium deposits in the M-side (not in the L-side) (Figure 5)) strongly support the hypothesis that asporin is an important player in clubfoot pathogenesis (Figure 6).

Tenascin-C

This ECM glycoprotein is over-expressed in fibrotic disease, including keloids.[37] The effects of tenascin-C on fibrosis (wound healing) are reviewed in Tracy et al.[33] This protein also plays a central role in maintaining inflammation in arthritic diseases.[38] By in situ detection of protein and mRNA expression, Mikic et al. demonstrated that the morphological abnormalities that result from embryonic immobilization are associated with an altered expression of tenascin-C.[39] Tenascin-C also has an influence on fibroblast migration (Figure 6).[33,40]

Three Plasma Proteins

Apart from changes in concentrations of ECM-related proteins, we detected a significantly increasing expression of three blood proteins in the M-side of clubfoot—apolipoprotein and two globulins. We assume that this may be a consequence of tissue stiffness in the M-side, due to which the blood was enclosed inside the veins in this tissue and could not be completely washed away.

Proteins With Higher Concentration in the Non-Contracted Side (L-side) of Clubfoot

The presented study has revealed that four ECM related proteins were upregulated in the L-side (Table 1).

Collagen XII and Collagen XIV

Both these collagens are members of the FACIT collagens (Fibril Associated Collagens with Interrupted Triple helices). Collagen XII was over-expressed in human corneal scars[41] and could play a role in fibril organization and the stromal architecture.[42] Collagen XII is associated with collagen I fibrils in human tendons, and by analogy, to collagen XIV, in connective tissues enriched in collagen I.[43] Agarwal et al. supposed that anchoring plaques are interconnected to the interstitial network in the papillary dermis by the collagens XII and XIV.[44] Characterization of collagen XIV-deficient mouse tendons showed a shift toward large-diameter fibrils and, thus regulated fibrilogenesis.[45]

These results indicate that both these types of collagen are important for the fully functional (supra-) structure of ECM (Figure 6).

Fibromodulin

This protein regulates the fibrogenic response (ECM organization) to liver injury in mice.[46] An insufficient concentration of fibromodulin in the M-side could be a factor in ECM disorganization.

Cartilage Intermediate Layer Protein 2 (CILP2)

CILP2 mediates interactions between components of the articular cartilage matrix, and may be associated with collagen VI.[47] Seki et al. concluded that CILP suppresses TGFβ signaling and this regulation plays a crucial role in the aetiology of lumbal disc disease.[31,48] This hypothesis could also fit into the complex aetiology of clubfoot. Both observations, mentioned above,[47,48] support our results showing that CILP2 was decreased in the M-side of clubfoot tissue samples.

All four proteins over-expressed in the L-side (and downregulated in the M-side) have strong relationships to the ECM architecture, and we assume that their scarcity in the M-side could play an important role in the development of clubfoot disease (Figure 6).

Possible Novel Pathogenetic Mechanisms in Clubfoot

The presented study revealed significantly different concentrations of 11 proteins (between the M-side and the L-side of clubfoot), which are important for physiological development of the ECM. We assume

that these proteins may play a substantial role in clubfoot pathogenesis. For example, collagens XII and XIV which have been detected in significantly lower concentration in contracted tissue, probably represent a partial absence of the structural components of the ECM physiological structure (Figure 6). These collagens could play a role in fibril organization (type XIV also in fibrilogenesis[45]), and also it is supposed that they interconnect anchoring plaques to the interstitial network.[42–44] Collagens type XII and XIV (downregulated in the M-side) could likely be replaced in prenatal development with collagen V and/or collagen VI (upregulated in the M-side and collagen VI C-terminate peptide, endotropin, is a potential growth factor).[27] The structure of the contracted ECM could thus be completely changed.

Moreover collagen XII together with tenascin-C could play a role in the developing of joint structures.[39] It was demonstrated that the morphological abnormalities (absence of menisci, disorganized cellularity in the fused region of joints) resulting from embryonic immobilization are associated with an altered expression of collagen XII and tenascin-C.[39] Mikic et al. also suggested that tenascin-C expression is sensitive to external changes in the mechanical loading environment within developing joint structures.[39] These observations could bear some relationship to clubfoot aetiology, because its pathology starts in the perinatal period (Figure 6).

Native full-length tenascin-C also arrests fibroblast cell-cycle progression in the G1 phase and promotes fibroblast migration along the fibrin-fibronectin matrices, characteristic of early wounds.[40] Conversely, fragmented tenascin-C was shown to almost completely inhibit fibroblast migration as a part of physiological wound healing. The persistence of the full-length tenascin-C in fibrotic disease may thus contribute to pathological healing.[33] This mechanism could be a part of a new hypothesis for clubfoot aetiology (Figure 6).

Tenascin-C is not the only protein with influence on cell migration. In the M-side of clubfoot we also found upregulated protein versican (highly present in keloid scars[34]), so alteration in tissue concentration of these proteins could be relevant in clubfoot pathology (Figure 6).

There are also other proteins whose concentration in the M-side was significantly higher, and which are moreover involved in the process of collagen mineralization. We observed specific calcified structures in the M-side of clubfoot for the first time (Figure 5). This could bring a direct connection with overexpression of asporin (which plays a role in biomineralization[32,49]), TGFβip (essential for TGFβ signaling[30]), and the intracellular pool of TGFβ (relationships to collagen calcification[32]) in the M-side. Moreover Seki et al. declared that overexpressed CILP suppressed TFGβ signaling in lumbal disc disease.[31] In our study, CILP2 in the M-side has a significantly lower concentration, and therefore TGFβ signaling could be disregulated. That assumes an important role for CILP2 in ECM. The connection of these four proteins (asporin, TGFβ, TGFβip, and CILP2) with pathological calcifications in clubfoot needs future research (Figure 6).

The current state of knowledge of the clubfoot disease is still limited, and we are not able to strictly determine what the causes and effects are in this pathological process. Only with a detailed description of the clubfoot contracted tissue, it may be possible to accelerate treatment of this disease with adjunctive therapeutic agents. The present study has revealed 11 significant quantitative differences in the protein composition of the contracted and non-contracted tissues of clubfoot, and suggests a new hypothesis about clubfoot pathogenesis. All these proteins may serve as potential biomarkers of the clubfoot disease. Their further analysis might bring new insights into the mechanisms of morphogenetic development of this serious congenital malformation. Chemical treatment is already clinically used in cases of some other fibroproliferative diseases, for example, Dupuytren's conctracture, where collagenase clostridium histolyticum is frequently injected.[50] However, there is no alternative or additional chemical treatment for idiopathic clubfoot to date.

AUTHORS' CONTRIBUTION

AE, MO, TN responsible for conception and design of study. MO responsible for acquisition of samples. AE, LK, DV, JK, JL, TN, JU responsible for acquisition of data. SP, TN, MD, MP, LV, IM: data analysis. DH, LH, MZ, TN responsible for statistics. AE, MO, MD, TN, JU, LV, LK interpreted the data analyses and prepared the manuscript. All authors have contributed to this research, revised it critically, and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The study was supported by the Ministry of Health of the Czech Republic, Department Program for Research and Development (n. 17-31564A), the KZCR, a.s., IGP KZ (n. 217116002), and Charles University, project GA UK (n. 336218), as part of the Specific University Research (SVV 260440), and long-term conceptual development of research organization (RVO:67985823).

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