MMPs/TIMPs and inflammatory signalling de-regulation in human incisional hernia tissues

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Abstract

Background: Incisional hernia is a common and important complication of laparotomies. Epidemiological studies allude to an underlying biological cause, at least in a subset of population. Interest has mainly focused on abnormal collagen metabolism. However, the role played by other determinants of extracellular matrix (ECM) composition is unknown. To date, there are few laboratory studies investigating the importance of biological factors contributing to incisional hernia development. We performed a descriptive tissue-based analysis to elucidate the possible relevance of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in association with local cytokine induction in human incisional hernia tissues. The expression profiles of MMPs, TIMPs and pro-inflammatory cytokine signalling were investigated in aponeurosis and skeletal muscle specimens taken intraoperatively from incisional hernia ($n = 10$) and control ($n = 10$) patients. Semiquantitative RT-PCR, zymography and immunoblotting analyses were done. Incisional hernia samples displayed alterations in the microstructure and loss of ECM, as assessed by histological analyses. Moreover, incisional hernia tissues showed increased MMP/TIMP ratios and de-regulated inflammatory signalling (tumor necrosis factor [TNFA] and interleukin [IL]-6 tended to increase, whereas aponeurosis TNFA receptors decreased). The changes were tissue-specific and were detectable at the mRNA and/or protein level. Statistical analyses showed several associations between individual MMPs, TIMPs, interstitial collagens and inflammatory markers. The increment of MMPs in the absence of a counterbalance by TIMPs, together with an ongoing de-regulated inflammatory signalling, may contribute in inducing a functional defect of the ECM network by post-translational mechanisms, which may trigger abdominal wall tissue loss and eventual rupture. The notable TIMP3 protein down-regulation in incisional hernia fascia may be of pathophysiological significance. We conclude that this study may help to pinpoint novel hypotheses of pathogenesis that can lead to a better understanding of the disease and ultimately to improvement in current therapeutic approaches.

Keywords: incisional hernia • extracellular matrix • aponeurosis • skeletal muscle • MMP/TIMP • TNFA • TNFRSFs • IL-6

Introduction

Incisional hernia is the most common complication of laparotomies, with a cumulative incidence of about 20% [1–3]. It occurs when the abdominal wall heals incompletely and impedes the timely re-establishment of an efficient load-bearing scar at the myofascial layer. Patient- and technical-related risk factors have been identified [4]. However, the molecular mechanisms that control its formation remain ambiguous. The late appearance after laparotomy and the recurrence rate curves observed after operation strongly suggest a biological underlying dysfunction, at least in a subset of patients [5, 6]. Interest has mainly focused on abnormal collagen metabolism, and a number of studies have investigated changes in type I to type III collagen ratios [7, 8] and alterations in individual matrix metalloproteinases (MMPs) [7–9]

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and collagen-interacting proteins [10]. Nevertheless, as the majority of hernias occur in patients that have no history of a wound-healing defect (making them safe surgical candidates) and also do not express a defect at the primary surgical site [6], other triggers are feasible. Some studies provide experimental evidence relating mechanical failure and incisional hernia development (reviewed in reference 7), possibly as a consequence of altered mechanotransduction and repair fibroblast biology [7, 11]. Other mechanisms have so far received little attention. It is possible that new descriptive tissue-based research may help to establish novel hypotheses of pathogenesis. For instance, the relevance of local factors intrinsic to the target tissue, like an ongoing induction of pro-inflammatory mediators or persistent extracellular matrix (ECM) turnover, is still unknown.

The balance between MMPs and tissue inhibitors of metalloproteinase (TIMPs) is a critical determinant of ECM integrity and function. Alterations in MMPs/TIMPs ratios are implicated in multiple diseases [12]. De-regulated specific MMPs expression has been previously reported in incisional hernia [7, 9]. Because studies have usually focused on a single MMP (MMP1, MMP13 [7] and MMP2 [9]) and there are no reports concerning TIMPs measurement, there is a need to perform a more comprehensive survey of MMPs and TIMPs to study them in a more integrated fashion, because it is the concerted action of the MMPs and TIMPs that determines their biological actions.

Also, cytokines are important regulators of MMPs/TIMPs systems. In the setting of surgical stress, cytokines and other factors are released with consequent autocrine/paracrine actions [13, 14]. Initially decisive for the host recovery, the persistence of de-regulated inflammatory signalling may be detrimental. ECM is critical in fine-tuning the spatial and temporal expression patterns of cytokines at sites of inflammation and healing [15, 16].

Finally, incisional hernia affects both the skeletal muscle and the fascial layers. To date, the fascia has been focused on in most of the investigations [17], but it is probable that insights into disease pathogenesis may be gained by simultaneously analysing both tissues. Skeletal muscle is a load-bearing structure that confers abdominal wall compliance. It is highly responsive to changes in functional demands, but it has been scarcely studied in relation to incisional hernia in human cases. Because the skeletal muscle is especially sensitive to pro-inflammatory stimuli, responding with tissue loss and failure [18], we hypothesized that it may effectively contribute to incisional hernia formation if persistently challenged by a continuous exposition to increased pro-inflammatory gene products, thus being a potential therapeutic target.

The aim of this study was basically exploratory to generate new biologically plausible hypotheses into how, in a pathophysiological sense, incisional hernia formation and local tissue microenvironment can be linked. In particular, we wanted to observe whether in incisional hernia patients: (1) there is a local tissue-specific pattern of changes in the ECM proteolytic system characterized by ongoing increased MMPs/TIMPs ratios, that is, a profile known to impair ECM turnover and favour ECM degradation; and (2) the abdominal myofascial tissues (fascia and skeletal muscle) participate in the

Table 1 Patient characteristics

Variable	Incisional hernia (*n* = 10)	Non-incisional hernia controls (*n* = 10)	*P*-value*
Age ± SEM (range)	61 ± 4 (42–72)	55 ± 3 (43–69)	0.19
Male sex	7	7	1.00
Smokers	3	2	1.00
Alcoholics	0	2	0.47
Obesity (body mass index >29)	2	5	0.35
Diabetes	0	0	1.00
Hyperlipidaemia	1	1	1.00

*Student's t-test or Fisher's exact test

overproduction of cytokines and/or in the de-regulation of the inflammatory response.

Patients and methods

Tissue sources

Samples included in this study were selected from our incisional hernia tissue bank. The tissue bank stores fascia and skeletal muscle biopsies obtained from the abdominal wall tissues of incisional hernia patients and non-incisional hernia controls and data associated with them, such as demographic features, co-morbidities, etc. Specimens are taken intraoperatively at the Department of Surgery of the Hospital Universitari Vall d'Hebron, by a specialized surgeon (incisional hernia samples are collected at repair, at >8 cm of the edge of the defect). The areas of visible necrosis or granulation tissue are intentionally avoided. The biopsies are immediately divided into multiple pieces and fixed in 10% formalin for histopathological analyses or snap-frozen in liquid nitrogen until analysis. Only in relation to specific research projects are DNA, RNA and proteins isolated from the solid tissue for downstream applications.

The patient characteristics are described in Table 1. Incisional hernias were diagnosed in the outpatient clinic by physical examination. Previous operations leading to primary incisional hernia formation included resection of colon or rectal tumour (*n* = 8) and cholecystectomy (*n* = 2). To our knowledge, the patients did not experience immediate major post-operative complications. The interval between prior laparotomy and repair was 69 ± 32 months (range 13–252 months; 13 months, *n* = 4; 14 months, *n* = 3; 96 months, *n* = 1; 252 months, *n* = 2). All the hernias were in the midline. Hernia repair was performed with an open onlay technique. The size of the defect is not available. Reference tissue samples were obtained from voluntary donors undergoing elective digestive abdominal surgery. Patients with previous wound infections or those with diabetes or connective or systemic inflammatory diseases were not included in this study. The research protocol was reviewed and approved by the institutional ethics committee. Written informed consent was obtained from all participants.

Table 2 Sequences of PCR primers

Group	Gene	Primer sequence forward 5'—3' (exon)	Primer sequence reverse 5'—3' (exon)	Product size	Acc. number
Collagens	*COL1A1*	GTCTTCCTGGCCCCTCTGGTG (40)	TCGCCCTGTTCGCCTGTCTCA (45)	390	NM_000088
	COL3A1	CAGGGGCCCCAGGACTTAGAG (30)	GGGCCAGGAGGACCAATAGGT (33)	249	NM_000090
	COL4A1	CAATGCCCTTCCTGTTCTGC (51)	GTGGACGGCGTAGGCTTCTT (53~54)	451	NM_001845
MMPs	*MMP2*	ACATCAAGGGCATTCAGGAG (8)	TGAACCGGTCCTTGAAGAAG (9~10)	168	NM_004530
	MMP9	GGGAAGATGCTGCTGTTCA (11)	TCAACTCACTCCGGGAACTC (13)	202	NM_004994
	MMP14	GCAGAAGTTTTACGGCTTGC (2)	CTTGGGGGTGTAATTCTGGA (4)	199	NM_004995
	TACE/ADAM-17	TCATTGACCAGCTGAGCATC (17)	CGCAGGAAAGGGTTTGATAA (20)	233	NM_003183
TIMPs	*TIMP1*	CTGCGGATACTTCCACAGGT (3)	GTTTGCAGGGGATGGATAAA (5)	209	NM_003254
	TIMP2	GATGCACATCACCCTCTGTG (4)	GTGCCCGTTCATGTTCTTCT (5)	196	NM_003255
	TIMP3	CTGACAGGTCGCGTCTATGA (3~4)	AGTCACAAAGCAAGGCAGGT (5)	165	NM_000362
	TIMP4	CTTGGTGCAGAGGGAAAGTC (4)	GGCTGAACGATGTTCAACAAA (5)	249	NM_003256
Cytokines and receptors	*TNFA*	CAGAGGGCCTGTACCTCATC (2)	GGAAGACCCCTCCCAGATAG (4)	219	NM_000594
	TNFSFR1A	GTGCCTACCCCAGATTGAGA (7)	TGTCGATTTCCCACAAACAA (8~9)	175	NM_001065
	TNFSFR1B	GGAAACTCAAGCCTGCACTC (4)	TGCAAATATCCGTGGATGAA (5)	224	NM_001066
	IL-6	CCACACAGACAGCCACTCAC (2)	TTTCACCAGGCAAGTCTCCT (3)	217	NM_000600
Ribosomal	*RPL19*	CAATGCCAACTCCCGTCAGC (1~2)	CTTGGTCTCTTCCTCCTTGG (6)	487	NM_000981

Primers were generated with the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and were mRNA/cDNA-specific. Preliminary experiments were conducted to ensure that the number of cycles represented a linear portion for the PCR optical density (OD) curve. The specificity of each primer pair was verified by sequencing the amplified products. PCR cycles were as follows: 25 (TIMP2 aponeuroses), 27 (MMP2 and TIMP3), 28 (COL1A1 aponeuroses), 29 (RPL19), 30 (TIMP1 and COL4A1; and TIMP2 and TIMP3 muscles), 31 (TNFSFR1B and COL3A1; and COL1A1 muscles), 32 (MMP2 muscles), 35 (MMP14, TIMP4, TNFA and TNFSFR1A; and IL-6 aponeuroses), 36 (IL-6 muscles), 37 (MMP9 aponeuroses) or 40 (MMP9 muscles).
COL1A1, collagen, type I, alpha-1; COL3A1, collagen, type III, alpha-1; COL4A1, collagen, type IV, alpha 1.

Histology and immunohistochemistry

Biopsies for histology and immunohistochemistry were immediately fixed in 10% neutral buffered formaldehyde and embedded in paraffin. Consecutive sections (4-μm-thick) were cut and stained with haematoxylin and eosin to visualize the morphology, Masson's trichrome to determine collagenous components and Alcian blue (pH 2.5) to estimate glycosaminoglycans (GAGs) abundance, as performed in routine clinical practice. For immunohistochemistry, paraffin-embedded sections were deparaffinized and rehydrated with xylene and a graded alcohol series. Endogenous peroxidases were blocked by incubation with 2% H_2O_2 for 30 min. Antigen retrieval was performed in 0.01 mol/l sodium citrate buffer (pH 6.0) with an electric pressure cooker. The tissue sections were blocked in serum-blocking solution and staining was performed with anti-α-SMA (Clone 1A4, 1:100; Dako M0851, Dako Diagnósticos, Barcelona, Spain) as primary antibody [19]. Detection was completed with the avidin–biotin complex peroxidase method (ABC Elite kit; Vector, Burlingame, CA, USA) followed by 3,3'-diaminobenzidine tetrachloride staining (DAB; Sigma, Deisenhofen, Germany) and haematoxylin counterstaining. Negative controls consisted in the omission of the primary antibody.

An experienced soft-tissue pathologist from the Vall d'Hebron University Hospital Department of Pathology (P.H.), who was blinded to the clinical diagnosis, examined the biopsy specimens. The samples were characterized in detail based on haematoxylin and eosin- and Masson's trichrome-stained slides. The staining intensities of Masson's trichrome and Alcian blue stains preparations were assessed visually and graded semiquantitatively. A minimum of three sections (10 randomly chosen, non-contiguous and no overlapping high-power fields each) were examined per patient.

RNA extraction and RT-PCR

Total RNA was extracted with TRIzol reagent, using a single-step method (Invitrogen, Barcelona, Spain). The RNA quality and quantity were assessed with a 2100 Bioanalyzer (Agilent, Madrid, Spain). RNA was reverse transcribed with the use of Superscript II reverse transcriptase (Invitrogen) and random primers. The PCR protocols were performed with cDNA products, human-specific primers, and 1 U of TaqDNA polymerase (Invitrogen), as depicted in Table 2. The Multiplex PCR Kit for Human MMP

Genes Set-1 (Maxim Biotech, Inc., Madrid, Spain) was used to amplify MMP1, MMP3, MMP7, MMP13 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs. The PCR products were separated on 2% agarose gels, visualized with ethidium bromide and quantified by densitometry (Quantity One Quantitation Software™; Bio-Rad, Barcelona, Spain). The results were expressed as a ratio between signals corresponding to the gene of interest and ribosomal protein L19 (RPL19; or GAPDH for Multiplex kit genes). All samples received pooled reagents and comparisons were made only with simultaneously performed experiments. Negative controls were done with omission of retrotranscription.

Tissue extraction and immunoblotting

The muscle tissues were homogenized in Tissue-PE LB™ lysis buffer (GenoTechnology, Inc., Barcelona, Spain) containing protease inhibitors (Set III, 1:100; Calbiochem, Barcelona, Spain) and centrifuged (12,000× g, 20 min.). The supernatants were collected, and the pellets were further dissolved in RIPA buffer (50mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.25% sodium deoxycolate, 1% Triton X-100, 2% SDS, plus protease inhibitors), centrifuged and the supernatants collected. Aponeuroses were homogenized in RIPA buffer and centrifuged (12,000× g, 20 min.). The supernatants were collected and concentrated with Ultrafree®-MC devices (Millipore, Madrid, Spain). Protein levels were quantified (BCA Protein Assay; Pierce, Madrid, Spain). Equal amounts of total protein were size-fractioned in 7.5, 10, 12 or 15% SDS-PAGE under reducing conditions (20 mA/gel; RT), transferred overnight to nitrocellulose membranes (60 V, 4℃), and stained with red Ponceau. The membranes were blocked in TBST buffer (0.9% NaCl, 0.02 M Tris, pH 7.5, 0.05% Tween 20) and 10% skimmed milk (1 hr, RT) before incubation with the respective anti-human primary antibodies: Clone I-8H5 (1:40, 1:20) for collagen I, Clone III-53 (1:100) for collagen III, Clone 41–1E5 (1:50) for MMP1 and Clone 136–13H4 (1:20) for TIMP3, from Calbiochem; tumor necrosis factor-alpha converting enzyme (TACE)/ADAMT17 (catalogue no. AB19027; 1:1000) and Clone loop no. 3pAb (1:200) for TIMP4, from Chemicon Int. (Madrid, Spain); Clone T3 (1:10) for TNFA, Clone H398 (1:10) for tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A) and Clone 80M2 (1:10) for tumor necrosis factor receptor superfamily, member 1B (TNFRSF1B), from HyCult Biotech (Madrid, Spain); and Clone pAb 1:666 for interleukin (IL)-6, from R&D Systems (Madrid, Spain). Detection was performed with appropriate peroxidase-labelled secondary antibodies. An enhanced chemiluminescence kit (Amersham, Barcelona, Spain) was used to detect immunoreactive bands.

Zymography and reverse zymography analyses

Equal protein quantities diluted in denaturing non-reducing Laemmli buffer were resolved on SDS-PAGE gels co-polymerized with gelatin (7.5%, 1.5 mg/ml), casein (10%, 1.5 mg/ml) or carboxymethylated transferrin (10%, 1 mg/ml), as described previously [19]. The gels were washed, incubated (37℃, 22 hrs) in substrate buffer and stained. TIMPs activities were analysed by reverse zymography [20] under non-reducing conditions. For reverse zymography, equal protein quantities were loaded on 12% SDS-PAGE gels impregnated with both 2.5 mg/ml gelatin and gelatinase A (1.2 μg/10 ml of gel; Oncogene, Barcelona, Spain). The gels were run, washed and incubated likewise gelatin zymography, excepting incubation time (37℃, 15 hrs). Proteolytic activities were quantified by densitometry scanning (Quantity One Quantitation Software™).

Statistical analysis

Data were quantified as means ± standard error (SE). Differences between group means were analysed using the two-way unpaired Student's t-test or the Mann–Whitney U-test, after checking the homogeneity of variances. Categorical data were analysed by Fisher's exact test. Multi-variate data analyses (principal component analysis [PCA] and multiple regression analysis) were performed with data obtained from RT-PCR to unravel potential relationships between transcripts. We extracted principle components (PCs) separately from aponeurosis and muscle. PCA is a simple, non-parametric method, which was used as an exploratory tool to reduce the dimensionality of the data set [21]. Backward stepwise multiple regression analysis (with entry criteria of $P < 0.10$, adjusted by group) was performed to determine possible significant associations between individual genes. A P-value of less than 0.05 was considered significant. A P-value between 0.05 and 0.10 was considered to indicate a trend towards an association.

Results

Table 1 shows patient characteristics at biopsy collection. Of interest, age, sex and co-morbidities were not statistically different between the control and the incisional hernia groups. All patients in this study had developed a primary incisional hernia after a previous operation. Thus, samples were collected when the patients were treated for their first incisional hernia repair when meshes were not present.

Histological analysis

The fascial layer consisted of a small number of fibroblasts, vessels and a large ECM. Compared with controls (Fig. 1A), incisional hernia fascias (Fig. 1B) showed histoarchitecture damage and loss of ECM, together with a tendency towards more rounded fibroblasts and disruption of the cell–matrix contacts. Incisional hernia skeletal muscle was characterized by a higher deposition of connective tissue in the endomysium, together with a higher percentage of fibres with centrally located nuclei (Fig. 1D). No signs of infection were seen. Histological evidence of inflammatory infiltration or granulation tissue was negligible in all samples studied. Interestingly, Alcian blue staining (pH 2.5) was increased in hernia samples (data not shown).

ECM synthesis and degradation

Some degree of up-regulated interstitial collagen mRNA expression was observed in incisional hernia samples, although, at the statistical level, only a trend towards increased skeletal muscle collagen, type III, alpha-1 (COL3A1) transcripts was detected ($P = 0.093$; Fig. 2). Discordant levels were observed at the protein level, which may be ascribable to partially inefficient protein extraction and solubilization.

Fig. 1 Representative haematoxylin and eosin-stained sections. As compared with controls (**A**), incisional hernia aponeuroses (**B**) are characterized by a loss of ECM organization, tissue wasting and more rounded fibroblasts. Incisional hernia muscles (**D**) display higher endomysial connective tissue deposition and more fibres with centrally located nuclei than control muscles (**C**). Original magnification, ×400.
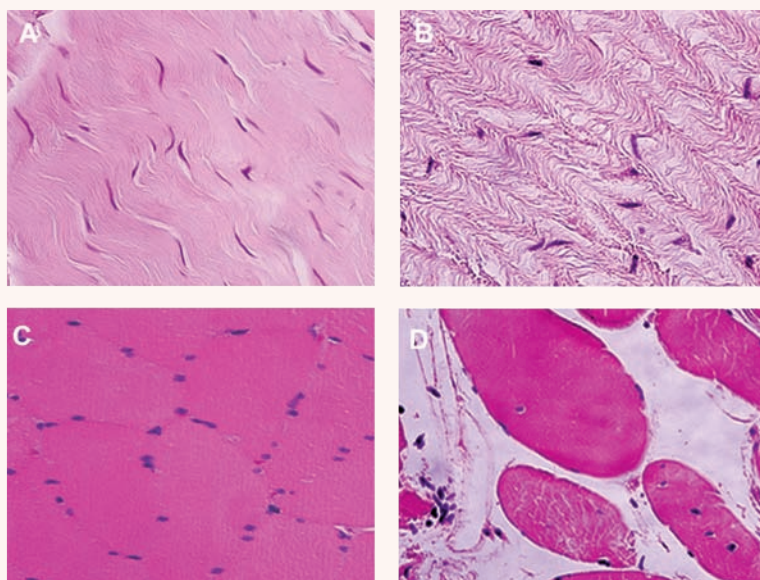
Conversely, changes in MMPs and TIMPs expression were identified in incisional hernia tissues both at the transcriptional and at the post-transcriptional levels (Figs. 2 and 3). These changes resulted in higher MMP/TIMP ratios (Fig. 4), which is consistent with a net MMP system activation. Specifically, incisional hernia specimens displayed increased RNA transcripts for MMP1 ($P = 0.055$) in aponeurosis, and for MMP2 ($P = 0.074$), MMP9 ($P = 0.045$) and MMP14 ($P = 0.016$) in skeletal muscle, and reduced RNA transcripts for MMP13 ($P < 0.001$), MMP3 ($P = 0.060$) and TIMP4 ($P = 0.036$) in aponeuroses, and for TIMP3 ($P = 0.012$) in skeletal muscle. Significant gene expression results were further corroborated by enzyme assays and immunoblotting to determine activities and amounts of MMPs and TIMPs. So, active MMP1 (43 kD) was overexpressed (muscle $P = 0.014$, aponeurosis $P = 0.031$), and TIMP3 (24 kD) was underexpressed (muscle $P = 0.080$, aponeurosis $P = 0.008$) in incisional hernia samples, as assessed by immunoblotting (Figs. 2 and 3). Zymography and reverse zymography (Figs. 2 and 3) revealed a strong induction of MMP9 (92 kD and 86 kD, $P = 0.026$) and MMP3 ($P = 0.040$) activities, together with largely decreased TIMPs activities in incisional hernia aponeuroses ($P = 0.015$). Under the conditions used (and probably due to the fact that muscle ECM protein is minimal as compared with total protein), MMPs and TIMPs activities were undetectable in skeletal muscle specimens.

Pro-inflammatory signalling

As shown in Figs. 2 and 3, TNFA and IL-6 transcripts tended to increase in incisional hernia (TNFA: aponeurosis $P = 0.064$, skeletal muscle $P = 0.077$; IL-6: skeletal muscle $P = 0.097$). TNFA receptors mRNA expression tended to decrease in incisional hernia aponeuroses (TNFRSF1A $P = 0.071$, TNFRSF1B $P = 0.063$), whereas TNFRSF1A tended to increase in skeletal muscle ($P = 0.089$). At the protein level, IL-6 ($P = 0.056$) and TNFA ($P = 0.099$) tended to be up-regulated in incisional hernia skeletal muscle. A striking down-regulation of the 40 kD highly soluble TNFRSF1B protein was observed in incisional hernia aponeuroses ($P = 0.022$). Noteworthy, only the soluble 17 kD TNFA protein (sTNFA) was detected in muscle, whereas, in the aponeurosis, exclusively low levels of 26 kD mTNFA protein were obtained. Finally, IL-6 protein was highly embedded in the ECM, and it was only rescued by strong extraction procedures.

Statistical relationships between transcripts

Complex networks of reciprocal interactions are established between MMPs, TIMPs, cytokines and growth factors, which regulate a wide variety of inflammatory and tissue remodelling mediators [22]. As it is difficult to compare the expression pattern of the 17 molecules simultaneously, PCA was performed. PCA is a powerful, exploratory non-parametric tool that has found application in several fields to extract relevant information from multidimensional data sets, that is, it filters out the noise and reveals hidden structure, allowing the selection of the most useful variables [21, 23]. By observing the correlations between the indices and the original variables (factor loadings), associations between the original variables can be identified and summarized. Factor loadings revealed the genes that provided the largest positive and negative contribution to incisional hernia (loading $> \pm 0.6$). Each correlation or loading indicates how strongly the original variable 'loads' on it. As depicted in Table 3, the PCA included in this report
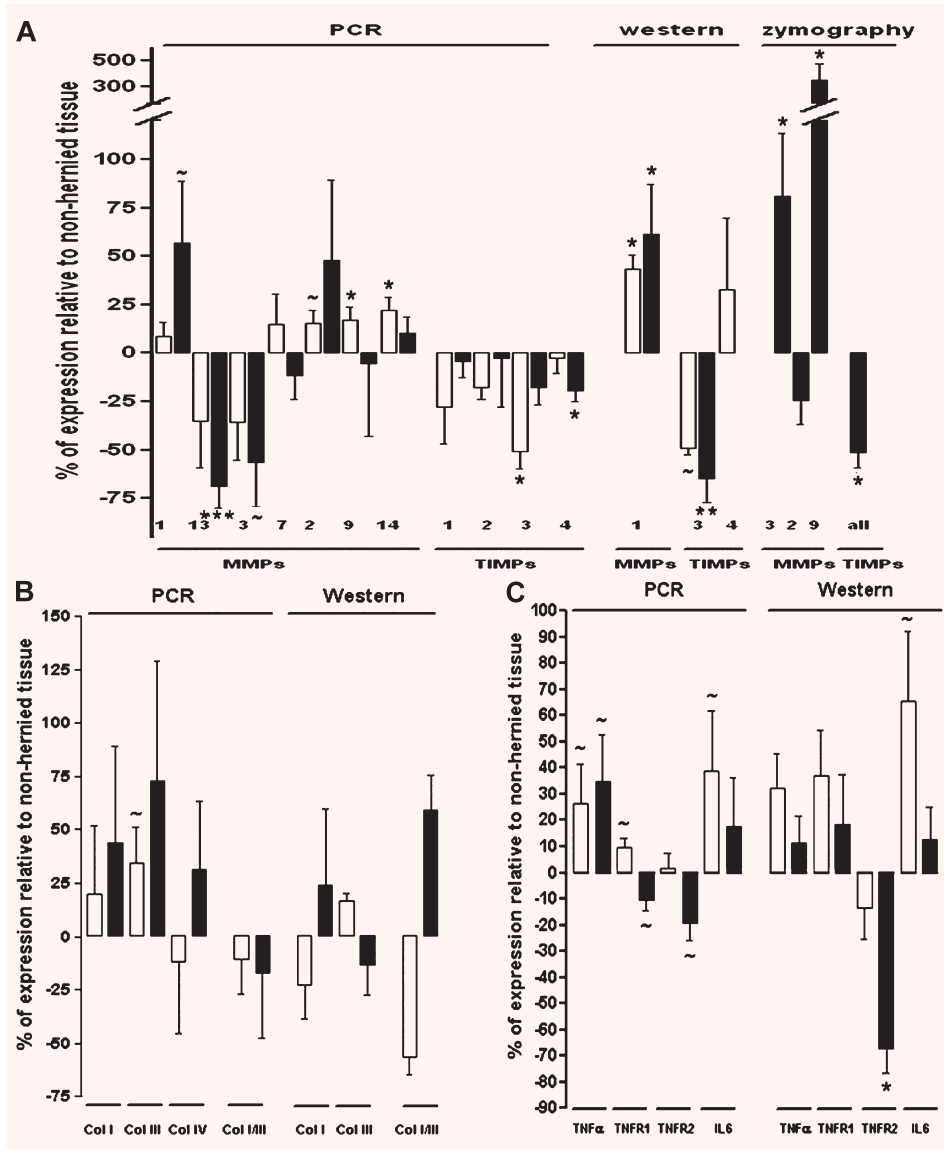
Fig. 2 Representative RT-PCR, Western blot and zymography analyses obtained in incisional hernia and control tissues. (**A**) MMPs and TIMPs, (**B**) collagens (types I, III and IV) and type I to type III collagen ratios and (**C**) inflammatory mediators (TNFA, TNFRSF1A, TNFRSF1B and IL-6). Results are displayed graphically using percentages of change of each molecule (incisional hernia *versus* non-incisional hernia controls) to facilitate the interpretation of the results. Only detectable results are represented. ~*P* value 0.05–0.10, **P value 0.05–0.01, ***P value 0.01–0.001, ****P* value < 0.001. ■, aponeurosis; □, skeletal muscle.

identifies interstitial collagens, TIMPs, MMPs (MMP1, MMP9 and MMP14) and TNF receptors as closely associated to the incisional hernia phenotype in a tissue-specific manner. Finally, several predictive models were constructed using different combinations of variables by backward multiple regression analysis. The most effective ones are described in Table 4 and yield interesting insights into the co-regulation of gene sets within the TNF and MMP marker families.

Discussion

We performed a tissue-based, exploratory molecular survey to gain a better understanding of the role of local tissue microenvi-

ronment in patients with a clinical history of incisional hernia. Our major novel findings were that in incisional hernia: (1) the constitutive baseline MMPs/TIMPs quotients are significantly higher, (2) there is a trend towards de-regulated pro-inflammatory cytokine content and signalling and (3) both the aponeurosis and the skeletal muscle are affected and exhibit multiple tissue-specific alterations. Because biopsies were obtained avoiding the hernia ring, the results point to the presence of subclinical events reflected systemically. Also, this study seems to be one of the first to explore human skeletal muscle samples in this disease.

Limited information is available on MMPs and TIMPs expression within human abdominal wall tissues. Here, we showed that, in hernia, the MMPs/TIMPs balance was altered in favour of net proteolytic activity, which may alter the ECM network by
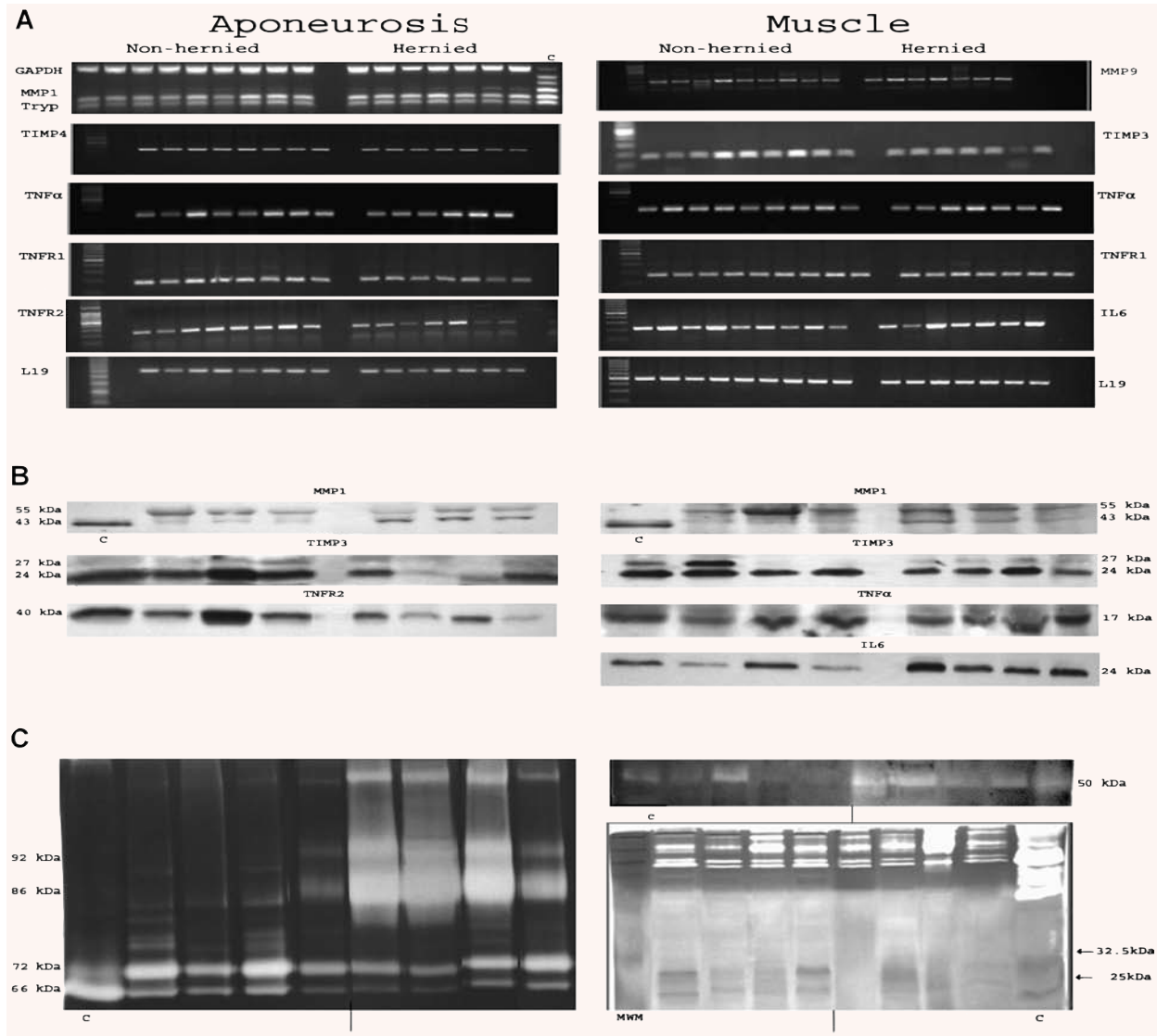
Fig. 3 (**A**) Representative RT-PCR products obtained in incisional hernia and control tissues. Only statistically significant transcripts are displayed (aponeurosis: MMP1, TIMP4, TNFA, TNFRSF1A, TNFRSF1B and RPL19; skeletal muscle: MMP9, TIMP3, TNFA, TNFRSF1A, IL-6, RPL19), c = Multiplex® PCR control, provided by the manufacturer. (**B**) Representative immunoblotting analyses obtained in incisional hernia and control specimens. Only statistically significant blots are presented (aponeurosis: MMP1, TIMP3 and soluble TNFRSF1B; skeletal muscle: MMP1, TIMP3, TNFA and IL-6), c = MMP1 control provided by Sigma. (**C**) Representative gelatinolytic (left), caseinolytic activity (right, up) and reversed zymography (right, down) in aponeurosis tissues. A concentrate of serum-free-conditioned medium (SFCM) from human skin fibroblasts treated with TPA (Sigma-Aldrich, Barcelona, Spain) was added to each gel to serve as a positive control. Only results obtained in aponeurosis are presented.

post-translational mechanisms and contribute to incisional hernia development. The apparent failure to replace connective tissue with newly synthesized material could be ascribed to inefficient collagen synthesis [24, 25]. Also, albeit not mechanistically investigated here, the lack of collagen deposition could be due to improper extracellular post-translational collagen maturation secondary to cytokine action [26].

Among the MMPs analysed, stands out the elevated expression of MMP1, MMP3 and MMP9 in incisional hernia fascia, especially at the activity level. Because tissue biopsies failed to show any inflammatory exudates, we speculate that they may be produced by native mesenchymal cells subtly stimulated by an unrelenting inflammatory signalling [27, 28]. MMP1 plays a unique role in eliminating defective pro-collagens during new collagen
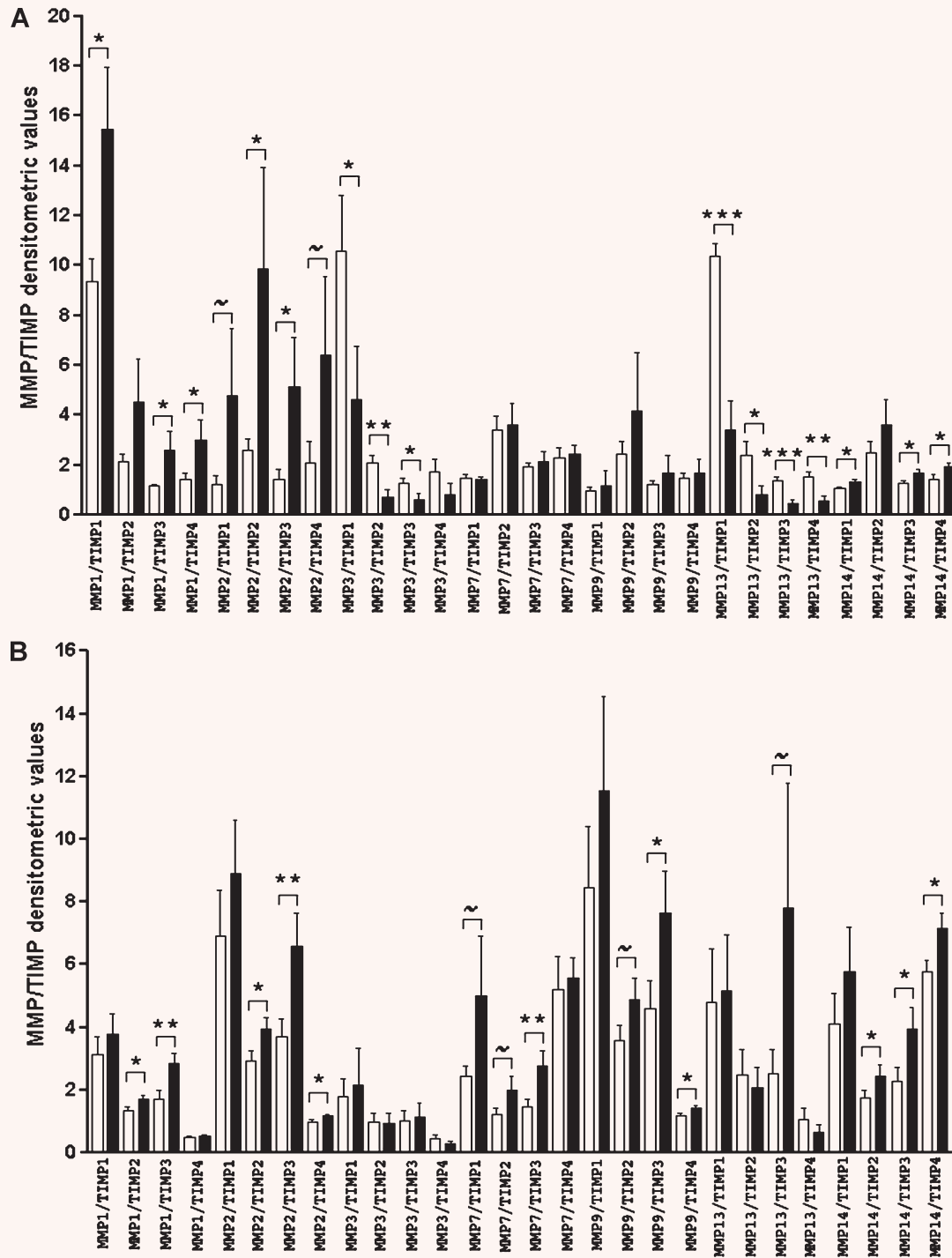
Fig. 4 MMP/TIMP mRNA ratios obtained in incisional hernia and control tissues. (**A**) aponeurosis and (**B**) skeletal muscle. ~P value 0.05–0.10, *P value 0.05–0.01, **P value 0.01–0.001, ***P value < 0.001 (incisional hernia *versus* control values). ■, incisional hernia; □, non-incisional hernia.

Table 3 Loading factors

Non-incisional hernia				Incisional hernia			
Aponeurosis				**Aponeurosis**			
PC1		**PC2**		**PC1**		**PC2**	
MMP14	0.973	TNFRSF1B	0.966	COL3A1	−0.842	COL1A1	0.880
MMP1	0.934	TIMP4	0.933	TNFRSF1A	0.835	TIMP2	0.867
TNF	0.933			MMP1	−0.814	MMP9	−0.827
TIMP3	0.926			TIMP4	0.797	TIMP1	0.754
MMP7	0.920			TIMP3	0.730	MMP14	0.740
MMP13	0.920			MMP2	0.677		
COL1A1	0.898						
TIMP1	0.889						
TIMP2	0.848						
MMP3	0.829						
COL3A1	0.796						
MMP2	0.764						
MMP9	0.691						
TNFRSF1B	0.608						
Skeletal muscle				**Skeletal muscle**			
PC1		**PC2**		**PC1**		**PC2**	
MMP3	0.900	TIMP4	0.740	MMP9	−0.978	TNF	0.979
TIMP2	0.896	MMP2	0.666	TIMP2	0.921	COL1A1	0.871
MMP7	0.893			TIMP3	0.901	COL3A1	0.846
MMP14	−0.886			MMP14	−0.871	TIMP4	0.786
TIMP1	0.869			TNFRSF1B	0.855	MMP1	0.635
MMP9	−0.832						
TNFRSF1A	0.830						
COL1A1	0.790						
TNFRSF1B	0.706						
MMP13	0.678						
MMP1	0.666						
TIMP3	0.665						

Representative loading factors (≥ 0.6) of the first two principal components (PCs) for non-incisional hernia and incisional hernia in aponeurosis and skeletal muscle.

formation [29], and its anomalous activity has been associated with progression of several diseases including congenital diaphragmatic hernia lungs [30, 31]. MMP3 acts as an activator for several MMPs, and it can directly stimulate cellular transformation [32]. MMP9 is important in the inflammatory phase of wound healing [33, 34], and its excessive production relates to tissue damage and matricryptin generation, as observed in degenerative inflammatory and/or autoimmune disorders [35, 36].

Previous studies did not measure TIMPs expression in the herniated abdominal wall, excepting a sole report describing

Table 4 Backward multiple regression analysis

Aponeurosis				Skeletal muscle			
TNF	**B**	**P**	**% change R^2**	**TNFRSF1A**	**B**	**P**	**% change R^2**
Intercept	38.67	0.007		**Intercept**	192.46	0.000	
MMP9	0.21	0.002	38	**MMP9**	−0.93	0.001	31
MMP1	0.19	0.018	17	**MMP7**	7.06	0.011	14
MMP3	2.27	0.026	15	**MMP2**	0.51	0.015	12
MMP13	−4.82	0.074	8	**MMP3**	−46.98	0.046	8
Group	2.79 × G	0.810	0	**Group**	−12.30 × G	0.089	5
Adjusted R^2 = 0.78; P = 0.003				Adjusted R^2 = 0.78; P = 0.003			
TNFRSF1A							
Intercept	54.42	0.007					
MMP14	0.57	0.010	19				
MMP1	−0.12	0.080	7				
MMP3	−1.07	0.078	7				
Group	15.94 × G	0.040	10				
Adjusted R^2 = 0.43; P = 0.046							
TNFRSF1B							
Intercept	34.07	0.000					
MMP13	3.44	0.028	26				
MMP9	0.12	0.031	24				
Group	−8.13 × G	0.421	3				
Adjusted R^2 = 0.43; P = 0.046							

Stepwise backward multiple regression was performed with an *F*-ratio probability of 0.10 as the criterion for removal or inclusion in the model. G = 0, incisional hernia (IH) group; G = 1, non-IH group.

Dependent variables: TNF, TNFRSF1A, TNFRSF1B (aponeurosis) and TNFRSF1A (skeletal muscle).

G, group; B, estimate of the change in the dependent variable attributable to a change of one unit in the independent variable; P, significance; R^2, *R*-squared.

decreased TIMP2 levels within the fascia transversalis of inguinal hernia [37]. A unique finding of the present study was TIMP3 down-regulation. TIMP3 is single among TIMPs in that it binds firmly to and is localized in the ECM, where it influences matrix remodelling and cell survival and inhibits matrix MMPs, aggrecanases and TNFA-converting enzyme (TACE, ADAM17) [38, 39]. Mice deficient in TIMP3 have elevated levels of TACE/ADAM17 and TNFA [40–42]. Not initially investigated, during manuscript preparation, we measured TACE levels. TACE mRNA expression did not differ between both groups (data not shown), but immunoblotting revealed a tendency towards increased active TACE (80 kD) in incisional hernia skeletal muscle (≈50%, *P* = 0.075), suggestive of TACE post-transcriptional activation. Although it is not clear yet

whether this TIMP3 down-regulation is a primary defect or the result of the loss of ECM content and structure, it could partially account for the transition to tissue rupture by providing a direct mechanistic link between inflammation and degradation. Future researches in this field may allow unravelling these questions.

Another interesting and novel finding reported here is the deregulated expression of pro-inflammatory cytokines and receptors within incisional hernia tissues. Inflammation is a crucial component of the host response to tissue injuries, but if unnecessarily persistent, it may be detrimental [43]. TNFA is involved in the pathogenesis of chronic inflammation *in vivo* [44, 45] and is one of the most important inducers of MMP protein production [27]. The specific effects of TNFA are signalled *via* TNFRSF1A and

TNFRSF1B. TNFRSF1B, which mediates TNFA effects in a paracrine/autocrine manner, was strongly down-regulated in incisional hernia fascia. The selective modulation of TNFRSF1B may represent a mechanism for protecting the host against the local deleterious effects of TNFA [46–48]. Interestingly, TNFA has also a role in mediating muscle loss in patients with chronic wasting conditions, inflammatory myopathies and insulin resistance [18, 49, 50]. The trend towards increased TNFA levels in hernia could also help to explain the more vigorous expression of IL-6 in incisional hernia skeletal muscle through a stimulatory mechanism for its secretion [51] (simple correlation analyses confirmed that TNFA and IL-6 expression is highly interrelated in skeletal muscle, $r = 0.595$, $P = 0.020$). IL-6 up-regulates the cathepsin and ubiquitin pathways of muscle proteolysis [52]. Collectively, these results raise the hypothesis that the tissue remodelling and destruction detected in incisional hernia may be related, at least in part, to the excessive cytokine production, which favours a state of chronic low-grade molecular inflammatory status, even in the absence of inflammatory cell exudates [43]. Also, it emphasizes the role of the skeletal muscles as a potential target in incisional hernia.

Concerning the limitations of this study, analyses were performed with an observational design and therefore inferences regarding causal pathways cannot be conclusively drawn. Investigating the aetiology of complex traits represents a major challenge, and observational research is a valuable investigative tool, providing rapid results at low cost. One major disadvantage is that descriptive studies do not give any indication of the absolute risk of the factor in question, but if the evidence found is convincing enough, resources can be then allocated to further more comprehensive studies. Also, although many hernias become manifest early, others may not be noted until many years after the index procedure. Thus, it is not easy to choose appropriate 'matched' controls. A group of patients who had not developed incisional hernia after a previous abdominal operation but were laparotomized for other medical indications could have been added as an additional control. However, even in this case, a hernia too small to be detected clinically may have been present. Also, we could not discard that these patients would develop an incisional defect in the (near) future. Finally, we cannot reject that the changes observed in gene expression are also due to a loss of mechanotransduction, secondary to connective tissue mechanical unloading [6], and/or to a loss of fibroblast–ECM attachment [24, 53].

In summary, the results of the present study are consistent with the hypothesis of a biology of hernia formation [5, 6]. Although studies with samples directly acquired from human patients usually do not achieve the level of mechanicistic depth and/or proof of causality than those using more tractable animal and *in vitro* systems, our findings indicate that, in addition to a fundamental biological disorder, such as abnormal collagen metabolism, other molecular mechanisms are implicated in the pathogenesis of incisional hernias and might be amenable to therapeutic modification (*i.e.* the control of collagen synthesis alone would not be sufficient to control progressive incisional hernia formation, if cytokine de-regulation persists beyond the short-term inflammatory host-response period). Thus, it seems likely that the ongoing changes in MMPs/TIMPs profiles and pro-inflammatory signalling seen in the present study are compartmentalized processes that occur concomitantly *in vivo* and probably play a role in the pathogenesis of the disease by jointly driving local ECM fibres disruption and mechanical rupture. In a clinical perspective, we conclude that the role of the local milieu in the development/progression of abdominal wall impairment warrants further investigation and will help in the development of more effective prevention and new treatment strategies.

Acknowledgements

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