Systemic and local collagen turnover in hernia patients

Nadia A. Henriksen

This review has been accepted as a thesis together with four previously published papers by University of Copenhagen the 31st of March 2014 and defended on the 6th of June 2014

Tutors: Lars Nannestad Jørgensen, Magnus S. Ågren, Lars Tue Sørensen

Official opponents: Jacob Rosenberg, Johannes Jeekel, Agneta Montgomery

Correspondence: Digestive Disease Center, Bispebjerg Hospital, Bispebjerg Bakke 23, DK-2400 Copenhagen NV.

E-mail: nadiahenriksen@gmail.com

Dan Med J 2016;63(7):B5265

PAPERS INCLUDED IN THE THESIS

I. Henriksen NA, Yadete DH, Sorensen LT, Ågren MS, Jorgensen LN. Connective tissue alteration in abdominal wall hernia. Br J Surg 2011; 98: 210-219 [1]

II. Henriksen NA, Sorensen LT, Bay-Nielsen M, Jorgensen LN. Direct and recurrent inguinal hernias are associated with ventral hernia repair. World J Surg 2013; 37: 306-311 [2]

III. Henriksen NA, Sorensen LT, Jorgensen LN, Ågren MS. Circulating levels of matrix metalloproteinases and tissue inhibitors of metalloproteinases in patients with incisional hernia. Wound Rep Regen 2013; 21: 661-666 [3]

IV. Henriksen NA, Mortensen JH, Sorensen LT, Bay-Jensen AC, Ågren MS, Jorgensen LN, Karsdal MA. The collagen turnover profile is altered in patients with inguinal and incisional hernia. Submitted

BACKGROUND

Abdominal wall hernia repair is a common surgical procedure. In Denmark, about 10,000 and 3,000 patients are operated on annually for groin and ventral hernias, respectively [4, 5]. The groin hernias are classified as primary and recurrent hernia, and based on the anatomic location as the indirect and direct inguinal hernia and the femoral hernia. The ventral hernias are categorized as the primary hernias comprising umbilical, epigastric, Spigelian and lumbar hernias, or as the secondary hernias, which develop in a former surgical scar, i.e. incisional, trocar-site and parastomal hernias.

Throughout the years, epidemiological studies have attempted to identify risk factors for hernia formation [6-10]. Several exogenous factors such as smoking, heavy lifting, obesity and comorbidity have been suggested as factors associated with abdominal wall hernias. Smoking is a risk factor for both primary and recurrent incisional hernia formation and for inguinal hernia recurrence [11-14]. Smoking has detrimental effects on wound healing [15, 16] and may impair the initial healing process after surgery and thereby increase the risk of incisional and recurrent hernia. On the contrary, smoking appears to be negatively associated with primary inguinal hernia formation, however, the underlying pathophysiological mechanisms remain to be elucidated [7, 10, 17]. Increased intra-abdominal pressure caused by heavy lifting, chronic coughing, constipation, prostate hypertrophy or adiposity has been proposed to be a risk factor for hernia development [8, 9]. Recently, a large epidemiological study reported that years of standing increased the risk of indirect inguinal herniation slightly [18]. However, heavy workload was not significantly associated with risk of inguinal hernia, corresponding to the findings of other studies [7, 10, 18, 19]. Obesity is a risk factor for primary and recurrent incisional hernia formation [20-23]. However, obesity seems to be negatively associated with the formation of primary inguinal hernia [17, 24], whereas diverging results exist with regard to inguinal hernia recurrence [12, 25, 26].

Surgical factors related to the primary surgery are important for both incisional hernia formation and hernia recurrence. Well-documented risk factors for incisional hernia formation are re-operation, fascial closure in a suture to wound length ratio below 4, the use of quickly absorbable sutures, large stitch length, post-operative wound infection and wound dehiscence [14, 27-30]. Considering inguinal hernia recurrence, mesh size and mesh overlap of the hernia defect both seem to be significant factors, whereas surgical technique (open or laparoscopic), mesh fixation method and mesh material are of lesser importance [31-34].

The proposed endogenous factors associated with hernia development are male gender, anatomic variations, inheritance, and connective tissue disorders. Male gender is a well-known risk factor for inguinal hernia formation, most probably due to the different anatomy in the groin region of men and women [35,36]. Also, male gender seems to be a risk factor for incisional hernia development [14], though studies of patients operated on for incisional hernias reveal a larger proportion of women than men [2, 14, 37].

The patent processus vaginalis is a known risk factor for the indirect inguinal hernia, however, about 20% of men do have a patent processus vaginalis, but are not developing an inguinal hernia [6, 38, 39]. Experimentally, it was demonstrated that a patent

processus vaginalis alone only caused herniation in 20% of rats [40]. However, inhibition of the cross-link catalysing enzyme lysyl oxidase together with a patent processus vaginalis caused herniation in 90% suggesting that altered collagen quality is involved in indirect inguinal hernia formation.
Studies of families with inguinal hernias propose a genetic trait for both primary and recurrent inguinal hernias [13, 41, 42]. Furthermore, a familial susceptibility to both primary and secondary ventral hernias has been demonstrated [43]. Recently, mutations in different collagen genes have been suggested to be associated with the development of inguinal and incisional hernias in humans and scrotal hernias in pigs [44-46]. Moreover, some patients develop multiple hernias and several recurrences [47], suggesting that some patients may be more prone to herniogenesis than others. Furthermore, hernias are more prevalent in patients with rare connective tissue disorders such as Marfan's Syndrome and Ehlers-Danloss Syndrome [48-50]. Taken together, these findings lead to the hypothesis that alterations in the connective tissue contribute to hernia development.

THE CONNECTIVE TISSUE

THE EXTRACELLULAR MATRIX

The connective tissue comprises the extracellular matrix (ECM) and the cells within. The ECM may be categorized as the interstitial ECM and the basement membranes. The interstitial ECM contains proteins and proteoglycans, which together create a dense network important for tissue stability. The prominent proteins are collagens, elastin, fibrillin, fibronectin and laminin [51]. The proteoglycans consist of long chains of repeating disaccharides so-called glycosaminoglycans that are attached to core proteins [52]. The basement membrane is a thin acellular layer that separate cells from the interstitial matrix and is composed mainly of collagen type IV, laminins and perlecan [53].

COLLAGEN SYNTHESIS AND MATURATION

Collagen is the major protein of the ECM and the synthesis process is complex and involves extensive post-translational modifications before a mature collagen fibril is formed [54, 55].
Collagen is synthesised and secreted by fibroblasts [56]. On the ribosomes, pro-alpha chains of about 1000 amino acid residues are synthesised [57]. Thereafter, proline and lysine are hydroxylated into hydroxyproline and hydroxylysine in a reaction catalysed by lysyl hydroxylase that requires molecular oxygen and ascorbic acid [54]. The hydroxyproline amino acid is almost unique for the collagen molecule and is often used to quantitate collagen [58]. The next step in the collagen synthesis is a glycosylation reaction of some of the hydroxylysine residues by galactosyl- and glycosyl-transferases through an O-glycosidic linkage [55]. Both the hydroxylation and glycosylation reactions are crucial for the subsequent self-assembly and cross-linking processes [54,59, 60].
Next, the pro-alpha chains self-assemble into a triple-helical structure known as the procollagen. The procollagen molecule is soluble due to N-terminal and C-terminal extension peptides allowing the molecule to be transported within the cell [57]. The procollagen is exocytosed to the extracellular space where procollagen N- and C-proteinases remove the N- and C-terminal extension peptides. This cleavage probably occurs in the crypts of the cell membrane, as the remaining N- and C-terminals are thought to be re-entering the cell to regulate the collagen synthesis in a feed-back mechanism [57].
After removal of the extension peptides, the collagen molecules self-assemble with subsequent fibril formation. The extra-cellular self-assembly process is influenced by proteoglycans which seem to modulate the correct alignment of the fibrils [52]. The collagen fibrils are further stabilised by the formation of intra- and intermolecular covalent cross-links catalysed by the copper-dependent enzyme lysyl oxidase. The cross-linking process is extremely important as it imparts the collagen fibril with its special strength and stability [34].

TYPES OF COLLAGEN

There are 28 genetically different types of human collagens [55]. In the interstitial matrix, type I collagen is the most abundant with smaller amounts of type III and V collagens [52, 55]. Type I, III and V are all fibrillar collagens. Type I is a strong collagen, whereas type III and V collagens are weaker. The same collagen fibre can comprise both type I and III collagens or type I and V collagens [61-63]. The more type III collagen relative to type I collagen, the thinner and weaker the fibre [64]. Type V collagen seems to be essential for the initiation of fibrillogenesis [65]. As a contrast to the fibrillar collagens, type IV collagen creates a sheeth-like structure and is found in basement membrane [53]. The skin and fascia consist mainly of type I collagen with smaller amounts of type III, IV and V collagens [55].

MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are a family of 23 human zinc-dependent proteases involved in collagen remodelling. The MMPs are divided into groups based on their structure and function: the collagenases, gelatinases, stromelysins, matrilysins, membrane type MMP (MT-MMP) and other MMPs [51,66].
The classical collagenases (MMP-1, MMP-8 and MMP-13) degrade type I and III collagen in their native triple-helical form by unwinding them into gelatin [67]. Thereafter, the gelatinases (MMP-2 and MMP-9) cleave the denatured collagens [67]. Additionally, MMP-2 and MMP-14 have the ability to cleave native type I and III collagens, but at a lower rate than the collagenases [68,69]. The stromelysins (MMP-3, MMP-10 and MMP-11) and the matrilysins (MMP-7 and MMP-26) do not cleave native collagens [69]. The MMP activity is partly controlled by α2-macroglobulin in the blood [51, 66]. Furthermore, MMPs are inhibited by the tissue inhibitors of metalloproteinases (TIMPs) [51]. The ECM remodelling process is complex. Not only does the balance between MMPs and TIMPs play an important role, the MMPs also interact with several growth factors and cytokines in regulatory pathways of tissue turnover [51, 69].

COLLAGEN ALTERATIONS IN HERNIA PATIENTS

Several previous studies have evaluated the connective tissue in patients with inguinal and incisional hernias [1]. Electron microscopy studies of the anterior rectus sheath have reported thinner collagen fibrils and fibres in patients with inguinal hernia [70-72]. Histological evaluation of fascial biopsies has shown disorganized collagen, fibrohyaline degeneration of muscle fibres and chronic inflammation [71-74].
Several studies reported on an imbalance between type I collagen and type III collagen in the rectus sheath, fascia transversalis and skin of patients with both indirect and direct inguinal hernias, recurrent inguinal hernias and primary and recurrent incisional hernias as compared to controls [75-91]. In subgroup analyses, the lowest type I to III collagen ratio was observed in patients

with recurrence of both inguinal and incisional hernias [80, 81, 86-88].

Increased collagen degradation by MMPs has been suggested to be associated with hernia formation. Studies on the interstitial collagenase MMP-1 generated ambiguous results in patients with inguinal hernias [76-80, 92, 93]. On the other hand, the MMP-2 protein level was significantly higher in the fascia transversalis, skin and serum of patients with inguinal hernia. The highest levels were measured in patients younger than 50 years with direct inguinal hernia [92-98]. Two studies reported on increased MMP-9 levels in fascia of patients with inguinal hernia [93, 98]. MMP-13 was not detected in the skin, hernia sac or in fibroblasts from inguinal hernia patients [76-79]. Studies on the involvement of MMPs in incisional hernia patients reported inconclusive results [3, 80, 87, 89, 99, 100].

IN SUMMARY

Hernia formation is a multifactorial disease that involves important endogenous factors. These are probably affected by exogenous factors. Male gender and a familial history of inguinal hernia seem to be important factors for primary inguinal hernia formation. Smoking, surgical factors and a familial history of incisional hernia or hernia recurrence are factors associated with incisional hernia and recurrent inguinal hernia, respectively. Furthermore, alterations in collagen composition seem to contribute to abdominal wall hernia formation, possibly related to increased collagen breakdown. The collagen composition appears altered in fascial tissue but also in skin biopsies, suggesting that the collagen alterations are systemic. More pronounced collagen alterations are found in patients with hernia recurrences. Different risk factors for primary inguinal hernias and incisional hernias and hernia recurrences suggest various specific pathogeneses. Hypothetically, primary inguinal hernias are formed due to a systemic predisposition to altered connective tissue, whereas impaired healing affects the formation of incisional hernias and hernia recurrences.

HYPOTHESES

This thesis was undertaken to evaluate the overall hypothesis of a systemically altered collagen metabolism in hernia patients. The collagen alterations may differ between different types of hernia patients. Hypothetically, patients with direct inguinal hernias, recurrent hernias and multiple primary hernias present the most severe collagen alterations.

- Patients with a direct or recurrent inguinal hernia have a higher risk of ventral herniation than patients with indirect inguinal hernia due to systemic collagen alterations
- MMPs are systemically altered in patients with hernias
- The collagen turnover is systemically altered in patients with hernias and could be used as biomarkers for the hernia disease
- Formation of incisional hernia and recurrent hernia is associated with impaired wound healing
- The collagen content and the morphology are altered in the fascia transversalis of patients with hernias

AIMS

The overall objective of this thesis was to describe and investigate the collagen turnover systemically and locally in patients with primary inguinal hernia, multiple hernias and incisional hernia.

Specifically, the thesis addressed the:

- relevant evidence of connective tissue alterations in hernia patients
- association between inguinal hernia repair and ventral hernia repair
- systemic levels of MMPs in hernia patients
- systemic levels of biomarkers for collagen turnover in hernia patients
- total collagen deposition and levels of MMPs in granulation tissue of hernia patients
- total collagen concentration and morphology of the fascia transversalis from hernia patients

MATERIAL AND METHODS

STUDY OVERVIEW

This thesis is based on a systematic literature review (Study 1), a nationwide database study (Study 2), a clinical study of a previously enrolled study population (Study 3) and a clinical study with prospectively enrolled patients (Study 4). An overview of the studies is listed in Table 1.

The material and methods are described briefly below; please refer to the specific papers for details. Unpublished material from the studies 4.2 and 4.3 are outlined in detail below.

PATIENT POPULATION, STUDY 2

In Denmark, inguinal and ventral hernia repairs are registered prospectively in the Danish Inguinal Hernia Database, established in 1998, and in the Danish Ventral Hernia Database, established in

Table 1
Overview of studies

Paper	Study	Patients	Objective	Sample	Analyses
I	1	N/A	Evaluating existing evidence	N/A	N/A
II	2	92,283 inguinal hernia 843 ventral hernia	Association between inguinal and ventral hernia repair	N/A	Multivariable logistic regressen analysis
III	3	226 controls 79 incisional hernia	Systemic levels of MMPs and TIMPs	Serum	MMP/TIMP multiarray, MMP-9, TIMP-1 ELISA, MMP-2 and MMP-9 gelatin zymography
IV	4.1	18 controls 17 inguinal hernia 21multiple hernias 25 incisional hernia	Systemic levels of biomarkers for collagen turnover	Serum	Biomarkers for typ I, III, IV and V collagen synthesis and breakdown
	4.2		MMPs in serum, total collagen deposition and MMPs in granulation tissue	Serum ePTFE tube	MMP-2 and MMP-9 gelatin zymography, hydroxyproline assay
	4.3		Collagen concentration	Fascia transversalis	Hydroxyproline assay, histology

2007. In these databases, about 98% and 80% of all hernia repairs are recorded based on each patient's unique personal identification number. In a conjoined search in both databases, patients aged 18 years or more recorded in the Inguinal Hernia Database between January 1998 and June 2010 and in the Ventral Hernia Database between January 2007 and June 2010 were identified. Perioperative data on hernia type and surgical approach are registered in the databases.

PATIENT POPULATION, STUDY 3

Patients subjected to emergency or elective laparotomy from January 1997 to December 1998 were registered prospectively in a database. After a median of 3.7 years, a total of 310 patients were examined by an experienced surgeon, and the presence of an incisional hernia was registered. An incisional hernia was defined as a palpable defect in the laparotomy scar with protrusion of tissue during Valsalva's manoeuvre, including parastomal hernias [14]. Whole venous blood was collected at the day of clinical examination from a total of 305 patients and the serum fraction was stored at -80°C until analysis.

PATIENT POPULATION, STUDY 4

Eligible patients were included prospectively from September 2009 to September 2011.

Inclusion criteria

Males older than 40 years and postmenopausal women subjected to laparoscopic elective surgery for:

- Gallbladder stones without hernias (Group 1)
- Unilateral primary inguinal hernia (Group 2)
- Three or more different hernias (Group 3)
- Incisional hernia (Group 4)

Exclusion criteria

Patients receiving corticosteroids, cytostatics or immunosuppressive medicine during the last three months prior to surgery were excluded. Women receiving hormone replacement therapy and patients who were not able to give informed consent were not included. As for patients in group 1 and 2, previous hernia surgery, connective tissue disease and aortic aneurysm disease were exclusion criteria. Patients in group 2 were excluded if they presented with any other hernias than the primary inguinal hernia.

Sample size calculation

The optimal sample size was difficult to calculate before study initiation as some of the planned analyses had never been evaluated previously in patients with hernias. Thus the sample size calculation was based on data from a previous study on hydroxyproline levels in subcutaneously implanted expanded polytetrafluoroethylene (ePTFE) tubes [101] estimating a 5% type 1 error and a 15% type 2 error. The minimally relevant difference and standard deviation were set to 1.00 and 1.13 µg hydroxyproline per centimetre of ePTFE tube, respectively. Based on an unpaired analysis, 21 patients were required in each group. It was decided to include a total of 100 patients (25 patients in each group) in the study.

Blood samples

Whole venous blood was collected preoperatively. The blood was coagulated for 30 minutes, centrifuged for 10 minutes at 1,800x g at 4°C, and the serum fraction was stored at -80°C until analysis.

The ePTFE tube

The ePTFE tube represents a validated wound healing model [102]. The ePFTE tube has a porous surface that allows for ingrowth of cells that synthesize granulation tissue. An ePTFE tube (0.25 x 6.5 cm) was implanted during surgery (Figure 1A). The tube was placed via a cannula subcutaneously in the groin region at the level of the anterior superior iliac spine and sutured to the skin (Figure 1B). The tube was removed 10 days after implantation (Figure 1C).

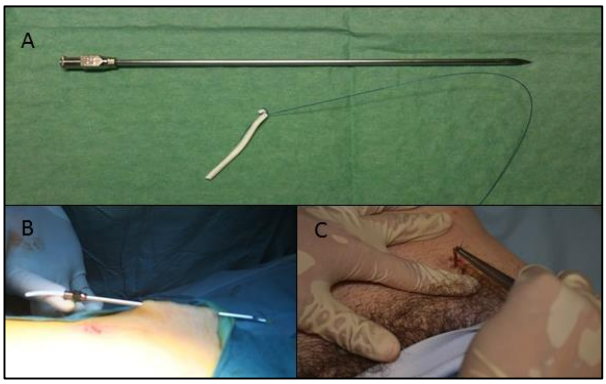

Figure 1

A) The ePTFE tube and cannula prior to implantation. B) Implantation of the ePTFE tube. C) Removal of the ePTFE tube at the 10th postoperative day.

Fascia transversalis biopsy

A fascia transversalis biopsy was excised from all included patients. It was decided to collect the biopsy from the same anatomic area in all patients so that analyses' results were comparable across groups. Furthermore, the hypothesis of a systemically altered connective tissue disease proposed that any possible alteration would also be present in the fascia transversalis of incisional hernia patients, even though the fascia was not anatomically involved in the hernia. Fascia transversalis is a part of the posterior wall of the inguinal canal and clearly visible during a transabdominal preperitoneal inguinal hernia repair.

The biopsy was collected laparoscopically, between the umbilicus and the superior spina iliac crest laterally to the epigastric vessels. The peritoneum was carefully incised and a piece of fascia (approximately 1 x 2 cm) was excised with cold laparoscopic scissors (Figure 2).

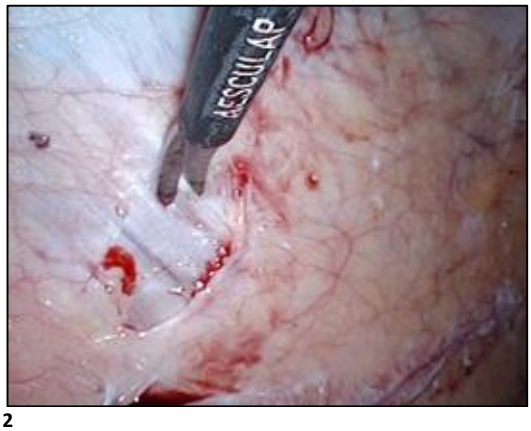

Figure 2

The peritoneum is incised, and the fascia transversalis biopsy is excised.

ETHICS

The studies were performed in agreement with the Helsinki II declaration and approved by the Committees on Biomedical Research Ethics (H-A-2009-045) and the Danish Data Protection Agency (2009-41-3864). Informed oral and written consent for participation was obtained from all included patients.

ANALYSES

MMP ANALYSES

A subset of the serum samples from patients in Study 3 were screened for MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13, TIMP-1, TIMP-2 and TIMP-4 with the ExcelArray MMP/TIMP (#82012; Pierce Biotechnology, Rockford, IL). MMP-9 (DY911; R&D Systems, Minneapolis, MN, USA) and TIMP-1 (900-K438, PeproTech, Rocky Hill, NJ, USA) was analysed in all patients from Study 3 with ELISA.

Gelatin zymography was performed for evaluation of MMP-2 and MMP-9 in serum of patients from Study 3 and in ePTFE tubes of patients from study 4. Recombinant human pro-MMP-2 (20 pg, PF037; Millipore, Billerica, MA, USA) and recombinant human MMP-9 (50 pg, 841030; R&D Systems) were used as standards. Densitometric measurements of band intensities were performed with ImageJ software (National Institute of Health, Bethesda, MD, USA) and correlated to the standards. Data are presented as geometric means of pro-MMP-2 and pro-MMP-9 in ng per mL of serum and in ng per g of ePTFE tube.

COLLAGEN BIOMARKERS

Biomarkers for collagen turnover were measured as neo-epitope fragments for collagen synthesis and degradation (Table 2). A neo-epitope is a unique fragment of a native protein, which is measurable in serum [103]. The neo-epitopes were assessed by solid-phase competitive immunoassays developed by Nordic Bioscience A/S (Herlev, Denmark). Briefly, the assays were carried out in streptavidin pre-coated 96-well plates (Roche Diagnostics, Mannheim, Germany). First, the wells were coated with respective biotinylated antigen for 30 minutes at room temperature. Then, 20 µL of the antigen standard or serum samples were incubated together with 100 µL of horseradish peroxidase-conjugated monoclonal antibodies for 1-3 hours at 4°C/20°C or for 20 hours at 4°C. Tetramethylbenzidine was added (100 µL/well) and after 15 minutes at room temperature the reaction was stopped by the addition of 100 µL 1% sulphuric acid. The optical densities were read at 450 nm and 650 nm (reference) using ELISA reader (VersaMAX; Molecular Devices, Wokingham Berkshire, UK). Standard curves were plotted using a 4-parametric mathematical fit model.

HYDROXYPROLINE ANALYSES

The amino acid hydroxyproline is almost unique for collagen and is a measure for the total collagen concentration. The hydroxyproline analysis was performed by the validated method described elsewhere [58]. The ePTFE tubes (median dry weight 8 mg) and fascia biopsies (median dry weight 1.5 mg) were delipidated in acetone, freeze-dried for 24 hours, weighed and hydrolyzed in hydrochloric acid. The hydroxyproline content of the samples was derived from linear standard curves (0-10 µg/mL) produced from 4-hydroxy-L-proline (H 1637; Sigma-Aldrich). The optical density was read at 570 nm using a microtiter plate reader (Thermo Scientific, Waltham, MA, USA). The hydroxyproline concentration was expressed as µg hydroxyproline/g of ePTFE tube and as µg hydroxyproline/mg of fascia.

Table 2
Biomarkers of collagen turnover

Collagen subtype	Neo-epitope for synthesis	Neo-epitope for degradation
Type I collagen	N-terminal pro-peptide of type I collagen (PINP)	MMP-2, MMP-9 and MMP-13 generated fragments of type I collagen (C1M)
Type III collagen	C-terminal pro-peptide of type III collagen (Pro-C3)	MMP-9 generated fragment of type III collagen (C3M)
Type IV collagen	N-terminal pro-peptide of type IV collagen (P4NP)	Pepsin and MMP-9 generated fragments of type IV collagen (C4M)
Type V collagen	C-terminal pro-peptide of type V collagen (P5CP)	MMP-2 and MMP-9 generated fragments of type V collagen (C5M

HISTOLOGY

The fascia biopsies were fixed in 4% phosphate-buffered paraformaldehyde and embedded in paraffin. Five-µm and two-µm thick sections were stained with haematoxylin and eosin and Van Gieson and Alcian Blue, respectively. The fascia morphology was graded semi-quantitatively with the Bonar score [104-106] (Table 3). The Bonar score was originally designed to assess patellar tendon morphology by light microscopy, but has been used on other tendons and fascia as well [85, 106]. There is no standardized method for describing fascia morphology, and since it resembles tendon, it was decided to use the Bonar score. The Bonar score grades collagen arrangement, the degree of ground substance, cell morphology and the level of vascularity from 0, which is normal appearance, to 3, which is abnormal appearance [105]. A total Bonar score of 12 represents the most severe abnormality of the fascia. Five randomly selected biopsies from each patient group were assessed with the Bonar score by two blinded pathologists. The mean Bonar score of the two pathologists was reported.

STATISTICS

The association between the type of inguinal hernia repair and ventral hernia repair were tested by uni- and multivariable logistic regression analyses adjusted for age, gender and surgical approach. The MMPs, TIMP, biomarkers, and hydroxyproline levels were not normally distributed and thus log transformed before statistical analyses. When testing for differences between hernia groups and controls, firstly a one-way ANOVA test was applied, and if statistically significant, pairwise comparisons between groups were performed with the two-sided t-test. Data are given as geometric means ± back-transformed standard error of the mean (SEM). The biomarkers are presented as the ratios between the marker for synthesis and the marker for breakdown, reflecting the turnover of each type of collagen. The mean Bonar score of the two pathologists were compared across groups with Kruskal-Wallis test. Unweighted kappa-statistics were applied for evaluation of agreement between pathologists. The kappa value was presented with 95% confidence intervals (CI). $P < 0.05$ was considered statistically significant.

Table 3
Overview of the Bonar score [106]

	Grade 0	Grade 1	Grade 2	Grade 3
Collagen arrangement	Collagen arranged in tightly cohesive well demarcated bundles. Homogenous polarization pattern	Separation of individual fibre bundles but with maintenance of overall bundle architecture. Non-homogeneous polarization	Separation and loss of demarcation of fibre bundles. Loss of normal polarization pattern.	Marked separation of fibre bundles with complete loss of architecture.
Ground substance	No stainable ground substance	Stainable mucin between bundles	Stainable mucin within bundles	Abundant mucin throughout the section
Cell morphology	Elongated spindle shaped nuclei with no obvious cytoplasm	Increased roundness of nuclei without conspicuous cytoplasm	Increased roundness and size of nuclei, small amount of cytoplasm is visible	Nuclei are round and large with abundant cytoplasm
Vascularity	Inconspicuous blood vessels coursing between bundles	Occasional cluster of capillaries, < 1 per 10 high power fields	1-2 clusters of capillaries per 10 high power fields	> 2 clusters of capillaries per 10 high power fields

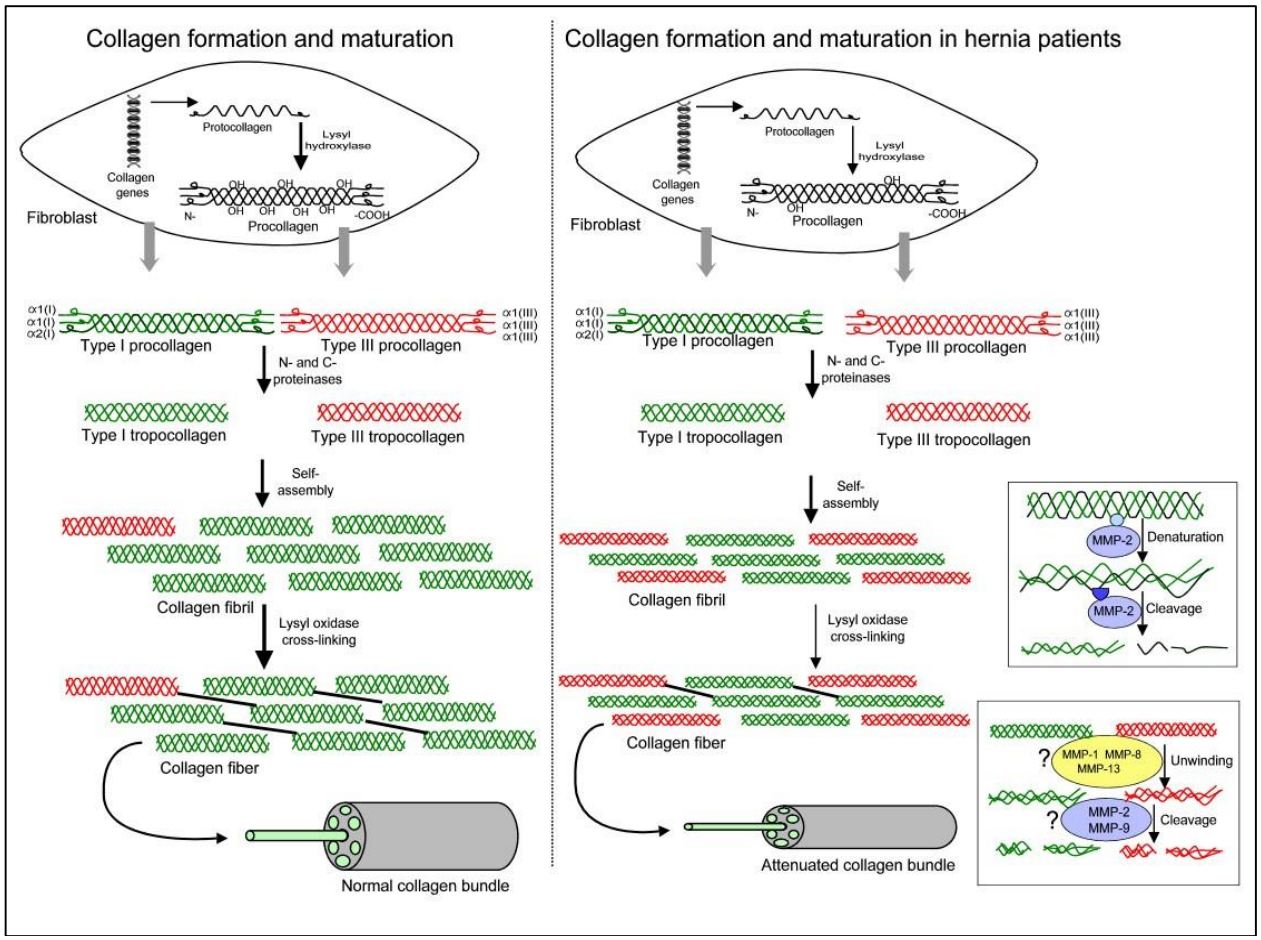

Figure 3
Overview of collagen formation and maturation in hernia patients based on a systematic literature review [1]

RESULTS

This section highlights the key results from the studies herein and unpublished results. For a more detailed description, please refer to the specific papers.

THE COLLAGEN METABOLISM OF HERNIA PATIENTS

The original systematic literature review [1] was supplemented with an updated literature search covering January 2010-October 2013. A total of 55 original articles were reviewed. The papers represented qualitative studies on connective tissue, semi-quantitative and quantitative studies on specific connective tissue proteins. A decreased type I to III collagen ratio was the main finding, probably leading to thinner collagen fibers and attenuated fascial structures as illustrated in Figure 3 [1]. Furthermore, sparse evidence suggested decreased lysyl oxidase activity and fewer cross-links in inguinal hernia patients [107, 108]. Lastly, a local and systemic MMP-2 increase was reported in younger patients with inguinal hernia [92, 94-97, 109].

THE ASSOCIATION BETWEEN INGUINAL AND VENTRAL HERNIAS

In a nationwide database study, direct and recurrent inguinal hernia repair was significantly associated with ventral hernia repair in a multivariable analysis adjusted for age, gender and type of surgery. Furthermore, female gender, age of 47-69 years and laparoscopic inguinal hernia repair was significantly associated with ventral hernia repair. In a subgroup multivariable logistic regression analysis, both direct and recurrent inguinal hernias were associated with primary ventral hernias, whereas only recurrent inguinal hernia was associated with secondary ventral hernia (Table 4).

Table 4

Subgroup multivariable logistic regression analysis of association between type of repaired inguinal hernia and primary and secondary ventral hernia surgery. Odds ratio adjusted for age, gender and surgical approach

	Primary ventral hernias, N = 620		Secondary ventral hernias, N = 223	
Inguinal hernia type	**Odds ratio (95% CI)**	**P**	**Odds ratio (95% CI)**	**P**
Unilateral, indirect	1		1	
Bilateral, indirect - indirect	1.12 (0.74-1.68)	0.603	1.12 (0.56-2.26)	0.743
Unilateral, direct	1.30 (1.06-1.58)	0.011	1.22 (0.89-1.66)	0.220
Bilateral, indirect - direct	1.89 (1.27-2.80)	0.002	1.26 (0.57-2.75)	0.569
Bilateral, direct - direct	1.53 (1.13-2.08)	<0.001	1.25 (0.70-2.24)	0.454
Uni- or bilateral recurrent	1.78 (1.35-2.32)	<0.001	1.76 (1.11-2.79)	0.016

SYSTEMIC MEASUREMENTS OF MMPS AND TIMPS

Measurement of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10 and MMP-12 and TIMP-1, TIMP-2 and TIMP-4 in a subgroup of serum samples from patients with and without incisional hernias indicated differences in MMP-9 and TIMP-1. ELISA analyses of MMP-9 and TIMP-1 of a total of 79 patients with incisional hernia and 226 controls without incisional hernias revealed no significant differences between patients with and without incisional hernias, P = 0.411 and P = 0.670, respectively [3].

Zymographic analyses of pro-MMP-2 and pro-MMP-9 revealed no differences between controls and patients with inguinal, multiple and incisional hernia (Figures 4-6). In a subgroup analysis of different hernia types, the serum pro-MMP-9 level was significantly increased in patients with recurrent incisional hernia compared with primary incisional hernia, P = 0.007 (Figure 6B). No significant differences with regard to gender and smoking status on pro-MMP-2 and pro-MMP-9 levels were found.

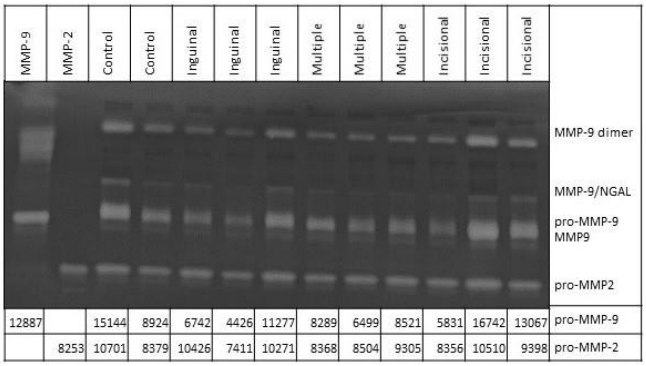

Figure 4

Zymogram of serum samples (50 nL/slot) from controls without hernia, patients with inguinal, multiple and incisional hernia. Standards are recombinant human MMP-9 (50 pg) and MMP-2 (20 pg). The positions of the four MMP-9 forms and pro-MMP-2 are indicated. Band intensities of pro-MMP-9 and pro-MMP-2 were measured by densitometry using the software ImageJ. The results of the measurements are indicated in arbitrary units below the zymogram.

SYSTEMIC BIOMARKERS OF COLLAGEN TURNOVER

PINP was significantly decreased in patients with inguinal and multiple hernias, though the ratio of PINP/C1M did not differ significantly between groups (Figure 7A). The Pro-C3/C3M ratio was significantly decreased in patients with inguinal and multiple hernias favouring decreased synthesis and increased breakdown (Figure 7B). The P4NP/C4M ratio was significantly increased in all three hernia groups, due to increased synthesis and decreased breakdown (Figure 7C). The ratio of P5CP/C5M was significantly decreased in patients with inguinal and incisional hernia, and insignificantly decreased in patients with multiple hernias (Figure 7D). There were no significant differences on biomarker levels with regard to gender, smoking, hypertension, diabetes and use of medications. In subgroup analysis, there were no significant differences between hernia subtypes (Figure 8).

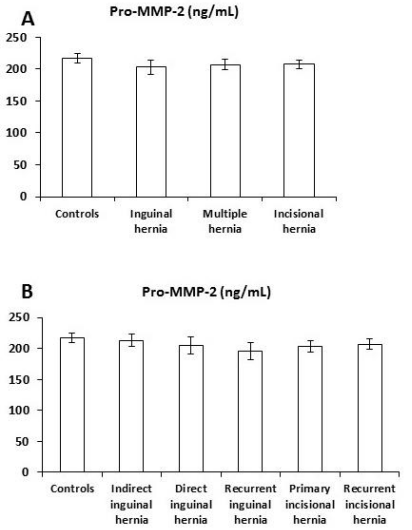

Figure 5
Zymographic analyses of pro-MMP-2 in serum of A) controls without hernia (N=18) and patients with primary unilateral inguinal hernia (N=17), multiple hernias (N=21) and incisional hernia (N=25). ANOVA, P = 0.705 and B) controls without hernia (N=18) and patients with indirect inguinal hernia (N=17), direct inguinal hernia (N=12), recurrent inguinal hernia (N=5), primary incisional hernia (N=16) and recurrent incisional hernia (N=9). ANOVA, P = 0.920. Data are geometric means ± SEM

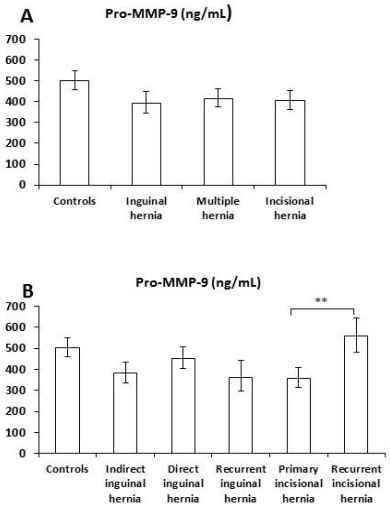

Figure 6
Zymographic analyses of pro-MMP-9 in serum of A) controls without hernia (N=18) and patients with primary unilateral inguinal hernia (N=17), multiple hernias (N=21) and incisional hernia (N=25). ANOVA, P = 0.811 and B) controls without hernia (N=18) and patients with indirect inguinal hernia (N=17), direct inguinal hernia (N=12), recurrent inguinal hernia (N=5), primary incisional hernia (N=16) and recurrent incisional hernia (N=9). ANOVA, P = 0.049. Data are geometric means ± SEM. Asterisks mark statistically significant data, **, P < 0.01

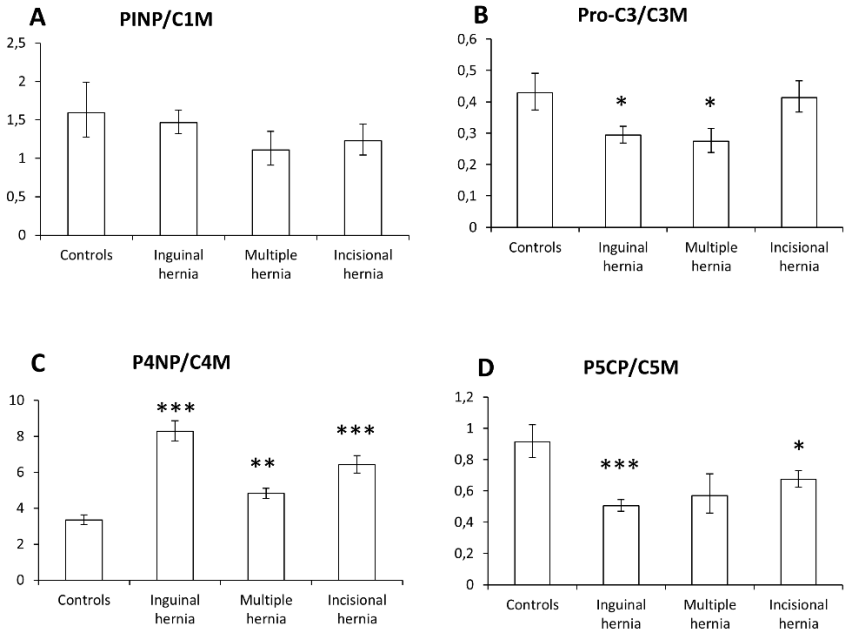

Figure 7
Ratios of collagen biomarkers in serum of controls without hernia (N=18) and patients with primary unilateral inguinal hernia (N=17), multiple hernias (N=21) and incisional hernia (N=25). A) type I collagen synthesis (PINP) and breakdown (C1M), ANOVA, P = 0.333, B) type III collagen synthesis (Pro-C3) and breakdown (C3M), ANOVA, P = 0.032, C) type IV collagen synthesis (P4NP) and breakdown (C4M), ANOVA, P < 0.001 and D) type V collagen synthesis (P5CP) and breakdown (C5M), ANOVA, P = 0.049. Data are geometric means ± SEM. Asterisks mark data statistically significant from controls, *, P < 0.05, **, P < 0.01

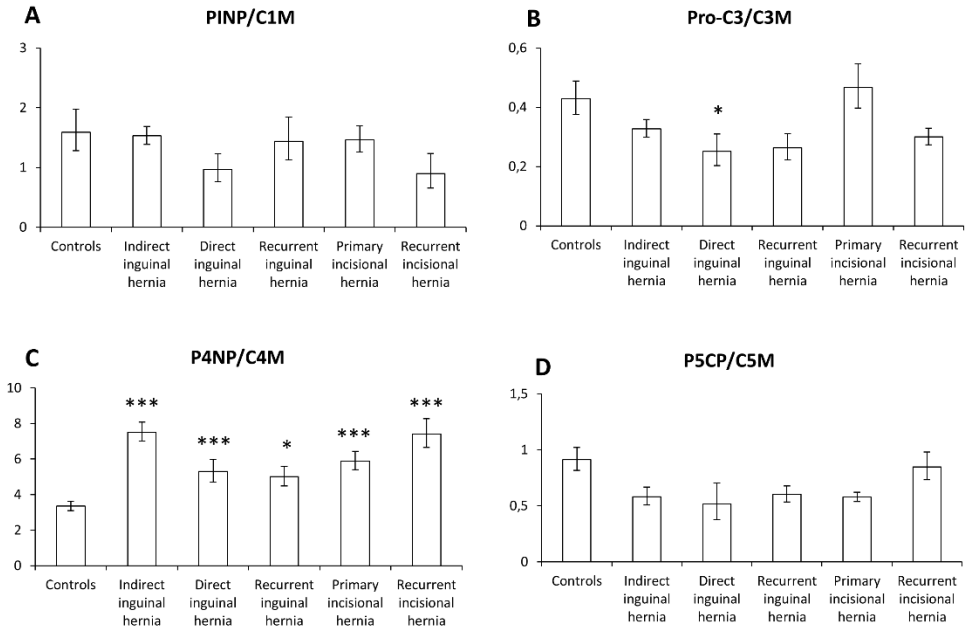

Figure 8
Ratios of collagen biomarkers in serum of controls without hernia (N=18) and patients with indirect inguinal hernia (N=17), direct inguinal hernia (N=12), recurrent inguinal hernia (N=5), primary incisional hernia (N=16) and recurrent incisional hernia (N=9). A) type I collagen synthesis (PINP) and breakdown (C1M), ANOVA, P = 0.284, B) type III collagen synthesis (Pro-C3) and breakdown (C3M), ANOVA, P = 0.040, C) type IV collagen synthesis (P4NP) and breakdown (C4M), ANOVA, P < 0.001 and D) type V collagen synthesis (P5CP) and breakdown (C5M), ANOVA, P = 0.226. Data are geometric means ± SEM. Asterisks mark data statistically significant from controls, *, P < 0.05, **, P < 0.01, ***, P < 0.001

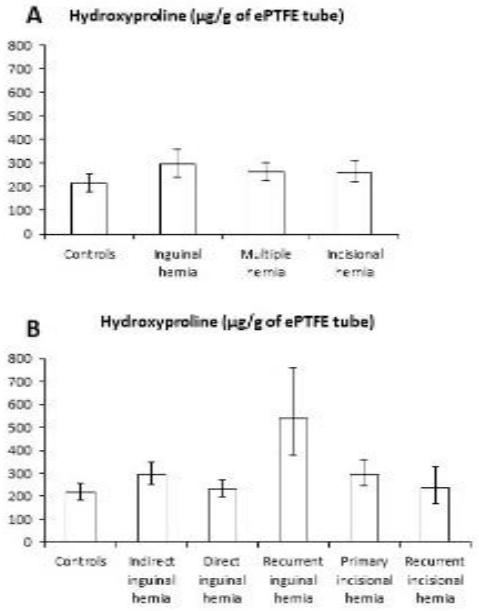

Figure 9
A) Hydroxyproline concentration in ePTFE tubes from controls without hernia (N=14) and patients with inguinal hernia (N=15), multiple hernias (N=20) and incisional hernia (N=23). ANOVA, P = 0.717.
B) Hydroxyproline concentration in ePTFE tubes from controls without hernia (N=14) and patients with indirect inguinal hernia (N=16), direct inguinal hernia (N=12), recurrent inguinal hernia (N=4), primary incisional hernia (N=14) and recurrent incisional hernia (N=9). ANOVA, P = 0.295. Data are geometric means ± back-transformed SEM

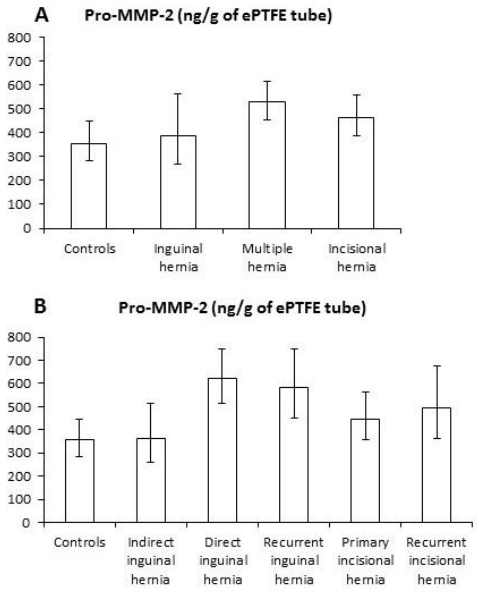

Figure 10
Pro-MMP-2 concentration in ePTFE tubes of A) controls without hernia (N=14) and patients with inguinal hernia (N=15), multiple hernias (N=20) and incisional hernia (N=23), ANOVA, P = 0.690 and B) controls without hernia (N=14) and patients with indirect inguinal hernia (N=16), direct inguinal hernia (N=12), recurrent inguinal hernia (N=4), primary incisional hernia (N=14) and recurrent incisional hernia (N=9). ANOVA, P = 0.422. Data are geometric means ± back-transformed SEM

COLLAGEN DEPOSITION AND MMP LEVELS IN EPTFE TUBES

The ePTFE tubes contained 257.6 ± 22.4 µg (geometric mean ± SEM) hydroxyproline per g of ePTFE tube. There were no significant differences on the collagen deposition in the ePFTE tubes between hernia groups and controls or between subgroups of hernia patients (Figure 9).

The pro-MMP-2 and pro-MMP-9 levels were similar in the ePTFE tubes of patients with and without hernias (Figure 10 and 11). In patients with direct inguinal hernia, there was a tendency towards increased pro-MMP-2 compared with controls, though the ANOVA test was insignificant (Figure 10B). There were no differences in collagen deposition or MMP levels with regard to gender and smoking status.

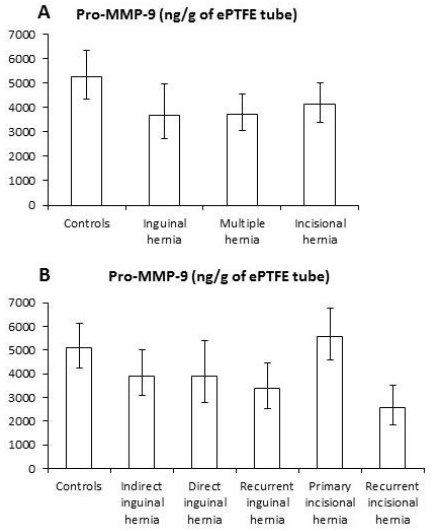

Figure 11

Pro-MMP-9 concentration in ePTFE tubes of A) controls without hernia (N=14) and patients with inguinal hernia (N=15), multiple hernias (N=20) and incisional hernia (N=23), ANOVA, P = 0.771 and B) controls without hernia (N=14) and patients with indirect inguinal hernia (N=16), direct inguinal hernia (N=12), recurrent inguinal hernia (N=4), primary incisional hernia (N=14) and recurrent incisional hernia (N=9). ANOVA, P = 0.400. Data are geometric means ± back-transformed SEM

COLLAGEN CONCENTRATION AND MORPHOLOGY OF FASCIA TRANSVERSALIS

The fascia transversalis biopsies contained 58.4 ± 1.70 µg (geometric mean ± SEM) of hydroxyproline per mg of tissue. There were no significant differences on the collagen concentration in the fascia transversalis between hernia groups and controls or between subgroups of hernia patients (Figure 12A and 12B). The fascia morphology did not differ between patients with and without hernias (Figure 13G). The mean Bonar score of the two pathologists was 3.1 and ranged from 1.5 to 5.5 (Figure 13G). The collagen arrangement ranged from tightly cohesive well-demarcated bundles to separated fibre bundles with loss of polarization (Figure 13A-13F). The pathologists agreed in 65% (13/20) of the cases, resulting in a fair agreement (kappa = 0.20 [0.0-0.54]).

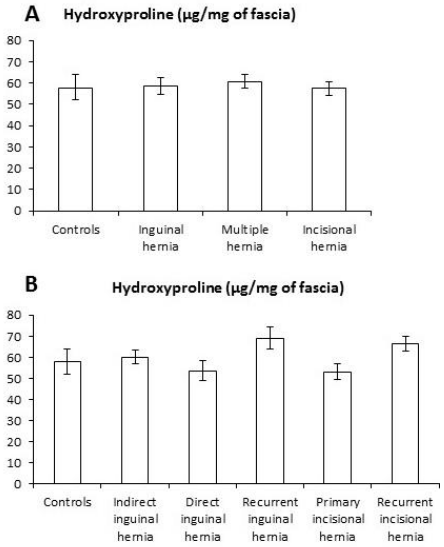

Figure 12

A) Hydroxyproline concentration of fascia transversalis from controls without hernia (N=18) and patients with inguinal hernia (N=17), multiple hernias (N=21) and incisional hernia (N=25). ANOVA, P = 0.952.

B) Hydroxyproline concentration of fascia transversalis from controls without hernia (N=18) and patients with indirect inguinal hernia (N=17), direct inguinal hernia (N=12), recurrent inguinal hernia (N=5), primary incisional hernia (N=16) and recurrent incisional hernia (N=9). ANOVA, P = 0.588. Data are geometric means ± back-transformed SEM

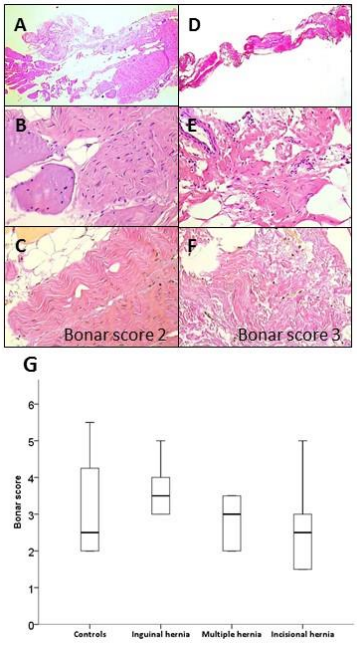

Figure 13

Morphology of fascia transversalis from a patient with incisional hernia A-C), and a patient with an inguinal hernia D-F). Original magnifications and stainings: A and D): x 20, haematoxylin and eosin; B and E): x 100, haematoxylin and eosin; C and F): x 100, Van Gieson and Alcian Blue. G) Mean Bonar score of fascia transversalis from controls without hernia (N=5), patients with inguinal hernia (N=5), multiple hernias (N=5) and incisional hernia (N=5) assessed by two pathologists independently. Horizontal line indicates median, boxes indicate interquartile range and whiskers indicate minimum and maximum Bonar score. Kruskal-Wallis test, P = 0.394.

DISCUSSION

PRINCIPAL FINDINGS

The overall aim of the studies was to evaluate the collagen turnover in patients with primary inguinal hernia, multiple hernias and incisional hernia. Firstly, a positive association between direct and recurrent inguinal hernias and ventral hernia surgery was demonstrated epidemiologically. Systemic measurement of MMPs revealed no differences between hernia patients and controls. However, an altered collagen turnover was found in serum samples from hernia patients, and biomarkers for type IV collagen turnover seemed to predict the presence of both inguinal and incisional hernias. In granulation tissue from patients with and without hernias, the collagen deposition and MMP levels were unaltered. Locally in fascia transversalis biopsies, no differences in total collagen concentration or morphology were found between hernia patients and controls.

SYSTEMIC PREDISPOSITION TO ABDOMINAL WALL HERNIA

Only a few research groups have studied patients with multiple hernias, though most general surgeons will probably recognize the cases from the clinical setting. Bilateral inguinal hernia and hernia recurrences in early childhood appear in patients with rare connective tissue diseases [110]. Further, bilateral inguinal hernia repair is associated with incisional hernia repair [111]. Moreover, inguinal hernia is associated with umbilical hernia in women [7]. In the current thesis it was demonstrated that specifically direct and recurrent inguinal hernia repair is associated with ventral hernia repair [2]. These findings suggest that some patients are predisposed to abdominal wall hernias.

Interestingly, only recurrent inguinal hernia was associated with incisional hernia repair in the current thesis [2], suggesting that hernia recurrence and incisional hernia may share common pathogenetic factors possibly related to healing after the primary surgery. Unfortunately, potential confounding variables such as smoking, overweight and comorbidity were not available from the Danish Hernia Database. In a recent study, a slightly increased spouse risk of both inguinal and incisional hernia was reported, also suggesting that exogenous factors are involved [43].

Emerging evidence suggests that inguinal hernias represent an inherited disease, however the inheritance pattern remains to be clarified [42]. Most of the literature on groin hernia inheritance included hernias in children and did not distinguish between the types of inguinal hernia [42]. Interestingly, the strongest inherited link has been found in females with groin hernias [112, 113]. As hernias in children and groin hernias in women are most often of the indirect type [38, 114], one may assume that the demonstrated inheritance patterns relate to indirect inguinal hernias. Further studies are needed to clarify whether it is the anatomic defect of a patent processus vaginalis or a collagen alteration that is inherited.

Recently, a few small-scaled genetic studies have been published. A polymorphism in the regulatory region of the COL1A1 gene and a missense point mutation in the elastin gene have been suggested in inguinal hernia patients including both indirect and direct inguinal hernias [45, 115]. Furthermore, a genome-wide association study of recurrent incisional hernia was performed, reporting on an inverse correlation between GREM1 and COL3A1 [46]. GREM1 is a regulator of tissue differentiation and seems to be related to fibrosis [46].

SYSTEMIC MMPS AND ABDOMINAL WALL HERNIA

The collagen alterations found in patients with hernias are proposedly caused by increased collagen breakdown by MMPs [116]. However, diverging results exist on MMPs and hernia. In the current thesis, there were no significant differences between the systemic MMP levels in patients with and without hernias. The measurable levels of MMPs in the circulation are affected by several factors. Active MMPs are complexed to inhibitors in the circulation such as α2-macroglobulin and TIMPs in order to protect overall tissue stability [117]. Systemically high levels of active MMPs would potentially have detrimental effects on several tissues and not only cause hernias. Furthermore, measurements of MMPs depend on which fraction of the total MMP that is measured, i.e. the complexed form, the pro-form or the active form.

Inguinal hernia

Previous studies did not report convincing results with regard to involvement of MMP-1, MMP-9 and MMP-13 in patients with inguinal hernias (Appendix 1). A total of 6 studies reported that MMP-2 was increased systemically and locally in patients with inguinal hernias, and especially increased in younger males with direct hernia [92, 94-97, 109]. On the contrary, one study reported that MMP-2 was decreased in plasma, though MMP-2 was increased in fascia from the same patients [98] indicating that the local MMP level may not be reflected systemically. An increased MMP-2 level in serum was not reproduced in the current thesis. This may be explained by the fact that patients in the current study population were older than 40 years. MMP-2 appears to be up-regulated in skin and serum of older individuals [118, 119], which may mask a systemic increase in older patients with hernias.

Incisional hernia

Only a few previous studies evaluated MMPs in incisional hernia patients and reported ambiguous results (Appendix 1). The current study is the largest to date evaluating multiple MMPs and TIMPs systemically in patients with and without incisional hernia, and no significant differences were found between patients with primary incisional hernia and controls [3].

Interestingly, zymographic analysis of serum from Study 4 demonstrated significantly increased pro-MMP-9 in patients with recurrent incisional hernia compared with primary incisional hernia, suggesting that surgical factors may be the main reasons for a primary incisional hernia, whereas increased collagen breakdown may cause recurrent incisional hernia in agreement with previous reports [80, 81, 88, 90, 91, 120]. This finding may be limited by the fact that the number of patients was small. Furthermore, the pro-MMP-9 level did not differ significantly from the controls. Further studies are needed to clarify this.

SEROLOGICAL BIOMARKERS FOR HERNIA DISEASE

MMPs do not appear suitable as circulating biomarkers for hernia disease, even though they may be involved locally in tissue turnover during hernia development. On the other hand, the collagen degradation products formed by MMPs may reflect the MMP activity indirectly. Besides, the pro-peptides that are cleaved off during collagen formation represent the level of collagen synthesis. The products of collagen synthesis and breakdown are known as neo-epitopes and mirror the systemic collagen turnover [103].

In patients with inguinal hernias, the turnover of type III, IV and V collagen was systemically altered, whereas only type IV and V collagen turnover was altered in incisional hernia patients. The synthesis of the interstitial type III and V collagen was decreased with an unaltered degradation. On the contrary, the basement membrane type IV collagen synthesis was increased with decreased breakdown, suggesting an imbalance between interstitial matrix collagen and basement membrane collagen. This matrix imbalance possibly impairs the matrix quality increasing the risk of hernia formation.

Type V collagen is important for the initiation of type I collagen fibrillogenesis [65]. A decreased type V collagen turnover may alter the quantity or quality of type I collagen fibres. This may correspond to the findings of a decreased type I to III collagen ratio in hernia patients reported by other research groups. A previous study evaluated type V collagen in skin scars of incisional hernia patients, but did not demonstrate any significant differences [80]. A slight overexpression of the genes coding type V collagen was found in skin of patients with recurrent incisional hernia together with a significant overexpression of the genes coding type IV collagen [46].

Type IV collagen seems to be associated with fibrosis in both the liver and kidney [121, 122]. Another research group attempted to measure type IV collagen in fascia of incisional hernia patients, but concluded that it was not detectable [89]. In an experimental study, fibrosis in the tissue surrounding the ventral hernia was reported [123]. Further, subserosal fibrosis of inguinal hernia sacs has been found [124]. Whether or not fibrosis is the explanation of the increased type IV collagen turnover of hernia patients needs further evaluation.

Until recently, the ECM has been considered a strong and supportive network that on the cellular basis anchors cells and proteins, and on a higher level supports tissue stability, for instance in tendon and fascia. Lately, emerging evidence suggests that the ECM may have more complex functions such as binding growth factors and cytokines and thereby regulate cell functions [125]. The post-translational breakdown products of various ECM proteins may have a function in themselves in controlling tissue turnover [125]. Possibly, the matrix imbalance demonstrated by the biomarker measurements in hernia patients should be considered as an alteration of the matrix function rather than as an altered tissue stability. Hypothetically, one of the breakdown products generated by the matrix imbalance has a special role in affection of collagen stability by indirectly affecting lysyl oxidase, MMPs or other important enzymes.

WOUND HEALING IN HERNIA PATIENTS

It is likely to believe that the formation of incisional and recurrent inguinal hernias is related to impaired wound healing. Smoking is a risk factor for both incisional and recurrent inguinal hernia [11, 14]. In smokers, the function of the fibroblasts is compromised and the collagen synthesis is decreased leading to delayed wound healing, possible wound dehiscence and ultimately hernia formation [15]. Furthermore, smokers have a higher risk of surgical site infections that also increase the risk of incisional hernia development [14].

The wound healing potential of hernia patients was evaluated with a subcutaneously implanted ePTFE tube that allows for ingrowth of cells that synthesize granulation tissue [102]. The total collagen deposition in the ePTFE tubes did not differ between hernia patients and controls or between the subtypes of hernias. This result is in accordance with one previous study evaluating specific models for wound healing in 20 patients with and 47 patients without groin hernias [126]. These findings suggest that the total amount of collagen in granulation tissue is not important for hernia formation. However, the collagen quality and turnover during wound healing may still play a role and remains to be evaluated.

MMPs have a variety of functions during wound healing such as degradation of ECM and facilitation of cell migration, however, the exact function of the individual MMPs is still not fully understood [127]. The levels of the gelatinases pro-MMP-2 and pro-MMP-9 were measured semi-quantitatively in the ePTFE tubes. There were no significant differences between hernia patients and controls. Interestingly, there was a tendency towards increased pro-MMP-2 protein level in the granulation tissue of patients with direct inguinal hernia. This suggests that the direct inguinal hernia is a systemic disease possibly related to systemic up-regulation of MMP-2, corresponding to the findings of previous studies [92, 94-97, 109].

LOCAL CHANGES IN FASCIA OF HERNIA PATIENTS

In the current study, the total collagen content of the fascia samples did not differ between hernia patients and controls or between patients with indirect, direct and recurrent inguinal hernias. Furthermore, there was no difference in the morphology of the fascia transversalis in patients with and without hernias.

Inguinal hernias

Considering the collagen quantity, there are a few previous reports on lower amounts of total collagen [70, 128-130], whereas others have described an unaltered level of total collagen [80, 108, 131] in the fascia of inguinal hernia patients (Appendix 1). The majority of the studies analysed the total collagen content semi-quantitatively by colorimetric quantification of histologic sections, which is not as accurate as hydroxyproline measurement [128-131]. In accordance with the current thesis, Pans et al.[108] reported no significant differences between inguinal hernia patients and controls on the total collagen content of the fascia measured as hydroxyproline. On the other hand, Wagh et al.[70] demonstrated a decreased amount of hydroxyproline in the rectus sheath of direct inguinal hernia patients.

One previous study assessed the collagen fibre organization in the anterior rectus sheath by the Bonar score and demonstrated no significant differences between hernia patients and controls, corresponding to the findings of the current thesis [85]. The agreement between the pathologists in the current thesis was only fair, suggesting that the Bonar score may not be the best tool for scoring the fascia transversalis. The Bonar score is designed for sections of tendons with longitudinally orientated fibres [106]. The fascia transversalis biopsy is very thin and difficult to orientate properly in the paraffin embedding.

A number of other studies described unorganized collagen fibres, increased vascularity, inflammation and fibrohyaline degeneration of muscle fibres by unstandardized evaluations of tissue biopsies from inguinal hernia patients [71, 73, 74]. These descriptive studies suggest that the fascial collagen architecture is altered in inguinal hernia patients, but due to the qualitative design they are difficult to compare with the current study findings. Patients with multiple primary hernias have never been included previously in studies on collagen alterations. Hypothetically, patients with multiple hernias would exhibit more severely altered collagen than patients with one unilateral primary inguinal hernia. Surprisingly, no differences between inguinal and multiple

hernia patients was demonstrated with regard to total collagen quantity nor systemic collagen turnover. In the group of patients with multiple hernias, there were more direct hernias than in the group of patients with primary unilateral inguinal hernia. Still, there were no signs of enhanced collagen alterations in the multiple hernia patients. Furthermore, no differences between inguinal hernia subtypes were found.

Incisional hernias

The total collagen quantity has not been evaluated previously in fascial tissue from incisional hernia patients. However, a total of 12 studies evaluated collagen subtypes in skin and fascia from incisional hernia patients. Half of the studies reported on a decreased type I/III collagen ratio in recurrent incisional hernias only [80, 81, 88, 90, 91, 120], and four studies reported on pronounced alterations in recurrent incisional hernias as compared to primary incisional hernias [85-87]. Two studies on ventral hernia patients and primary incisional hernia patients did not find a decreased type I/III collagen ratio [84, 132]. The decreased type I/III collagen ratio has mainly been found in the skin and in fascia probably surrounding the hernia defect, however, the exact anatomic position was not described [78, 87, 89]. One study did report that the decreased type I/III collagen ratio was not present in the fascia transversalis of incisional hernia patients [90]. A recent study concluded that the type I/III collagen alterations of the skin correlated significantly with the alterations in both the fascia transversalis and the anterior rectus sheath in both primary and recurrent inguinal and incisional hernia patients [85], suggesting a systemic collagen alteration in both inguinal and incisional hernia patients.

Collagen alterations of the fascia and skin have been demonstrated in patients with both inguinal and incisional hernias, suggesting that both types of hernia share pathogenic factors. Incisional hernia recurrence may be associated to a compromised healing potential reflected as altered collagen turnover in fascia and skin.

METHODOLOGICAL CONSIDERATIONS

Due to the strength and stability of the collagen protein, the protein level may be difficult to analyse. In vitro, it is necessary to degrade the cross-links in order to measure the collagen. Furthermore, identification of the collagen subtypes is useful. The hydroxyproline amino acid is specific for collagen, but it lacks the specificity to differentiate between the collagen subtypes [133]. Besides, elastin contains small amounts of hydroxyproline, also contributing to the total hydroxyproline content [58]. The factor for defining the total collagen content from hydroxyproline depends on the distribution of collagen subtypes, which vary between tissues. To be certain that the true conversion factor is used, an internal control of specific tissue is necessary. Alternatively, data must be presented as the amount of hydroxyproline per tissue.

Collagen analyses depend on the extraction protocol. The number of extraction processes with pepsin is crucial for the collagen cross-links to be broken down. Variations between extraction methods may cause bias in analysis results, rendering the results incomparable. Pepsin extraction of collagen was performed in a few studies, but the number or length of extraction was either not described or only performed for 24 hours [76, 86, 87, 108]. Whether 24 hours of pepsin extraction is enough to degrade collagen cross-links in fascial tissue is questionable.

Previous studies analysing collagen in hernia patients collected biopsies from both linea alba, the anterior rectus sheath, fascia transversalis and skin. No standardized method for biopsy collection with regard to hernias has been described. The total collagen content of fascia transversalis seems to be lower than the collagen content of the anterior rectus sheath [108]. Furthermore, emerging evidence suggests that the collagen alterations of the rectus sheath correlate with the collagen alteration in the skin of hernia patients, indicating that the collection of skin biopsies may be sufficient in future studies [85].

In the current study, biopsies from the fascia transversalis were collected in all patients based on the hypothesis of a systemic collagen alteration in hernia patients. The fascia transversalis is anatomically involved in groin hernias, but not in incisional hernias. One disadvantage with fascia transversalis biopsies is that the fascia may be difficult to reach and locate during other procedures than the transabdominal pre-peritoneal inguinal hernia repair. Besides, the fascia transversalis is very thin and the biopsy crumples after removal making it difficult to orientate properly in a paraffin embedding. Furthermore, due to the thinness, a fascia transversalis biopsy is easily contaminated with underlying muscle tissue, which may affect some analyses. Biopsies from the linea alba or anterior rectus sheath would probably have been easier to collect in a uniform manner from the umbilical trocar site.

Selection of a matched non-hernia control group may be difficult as it is unknown whether the controls will develop a hernia later in life and actually possess systemically connective tissue alterations at the moment of inclusion. Furthermore, there are ethical considerations with regard to biopsy collection and in the current study implantation of the ePTFE tube. There is always a risk of bleeding and infection when performing a surgical procedure, though none of that was encountered in the present experiments.

STRENGTHS AND LIMITATIONS

The current thesis evaluates the association between hernias and collagen alterations extensively in a thorough literature review, epidemiologically with a nationwide database study and experimentally with collagen analyses of patients with inguinal, multiple and incisional hernias.

STUDY 1

A thorough review of the literature concerning connective tissue alterations in hernia patients was completed, giving an overview of the current knowledge for the general surgeon. It was decided to include all English papers evaluating connective tissue alterations in humans with abdominal wall hernias. A standardized methodology checklist for selection of included studies was not used, and the quality of the studies was not assessed systematically. A part of the studies included quantitative evaluations of the connective tissue, which may be difficult to assess and report in a systematic review, increasing the risk of reporting bias. Very few studies reported insignificant results, suggesting that publication bias may be a significant limitation to the review. Many of the studies included few patients and some lacked a control group. Furthermore, the included studies were inhomogeneous with regard to techniques for biopsy collection, sample handling and types of analyses, and it is unknown whether these methods were reproducible and comparable.

STUDY 2

The epidemiological study was strengthened by the fact that data was retrieved from the nationwide Danish Hernia Database in

which data on hernia repairs are registered prospectively. The type of inguinal hernia is available leading to the unique possibility to evaluate differences between patients undergoing indirect, direct and recurrent inguinal hernia repair. Nearly all (98%) inguinal hernia repairs in Denmark are registered in the database, leading to a low risk of selection bias. Unfortunately, the registration rate of the ventral hernia database is only 80%, possibly leading to an underestimation of the ventral hernia repairs.
The ventral hernia database only dates back to 2007, hence the time overlap between the two databases is only 3 years. Only patients operated on for hernias are registered in the databases, and the real hernia prevalence may be much higher. Potential confounders such as smoking, BMI and comorbidity are not available from the database, which is a significant limitation to the study, as smoking is a known risk factor for both recurrent inguinal hernia and incisional hernia. Furthermore, laparoscopic surgery appeared as a confounder for the repair of a ventral hernia, as patients undergoing laparoscopic surgery for bilateral primary or recurrent inguinal hernia may easily have an umbilical hernia repaired during the same procedure.

STUDY 3
This is the first study to analyse multiple MMPs and TIMPs in a large cohort of patients examined for incisional hernia after laparotomy. The patients included in the subgroups were randomly selected based on gender, smoking status and incisional hernia. Multiple MMPs and TIMPs were only analysed in the subgroups, and the results may be biased by the subgroup selection. As only 9 patients were included in each subgroup, a single sample with an outlier in the protein level could have affected the entire group. Local MMP or TIMP alterations possibly involved in incisional hernia formation may change over time, and may hypothetically only be measurable in serum during wound healing in the first days after the primary laparotomy, and not in blood collected at the day of clinical examination years after incisional hernia formation. Last but not least, the incisional hernia group comprises a heterogenic patient population undergoing different types of primary laparotomies ranging from open elective cholecystectomy to colorectal cancer surgery to emergency laparotomy. Besides, known risk factors for incisional hernia formation, such as re-laparotomy, wound dehiscence and suturing technique were not accounted for. Possibly, some incisional hernias are caused by these known risk factors, whereas others are caused by increased collagen breakdown.

STUDY 4
Four well-defined patient groups were enrolled prospectively, and serum, ePTFE tubes and fascia biopsies were collected from the patients in order to assess collagen alterations systemically and locally. No pilot study was carried out, and unexpected problems were encountered during the process. The recruitment of patients was more difficult than expected, and was terminated after two years despite inclusion of fewer patients than planned. Patients with a unilateral primary inguinal hernia were difficult to recruit, as many of them presented with an asymptomatic contralateral inguinal hernia or an umbilical hernia upon clinical examination. Furthermore, there were fewer males in the control group than in the inguinal hernia and multiple hernia groups. The study design with three hernia groups and one control group included a risk of type I error due to multiple testing. This was accounted for in the statistical analyses by initial testing for differences with one-way ANOVA test. Pairwise comparisons between groups were only performed if the ANOVA test was statistically significant.
The sample size calculation was based on data from a previous study evaluating hydroxyproline levels in ePTFE tubes [101]. Fewer patients had an ePTFE tube implanted than estimated by the sample size calculation. A retrospective power calculation was carried out leading to a power of 50% suggesting that the risk of type II error was highly increased. This means that a potential alteration of collagen deposition in granulation tissue of hernia patients may not be detected due to the small number of patients.
Serological biomarkers of collagen turnover have never been evaluated previously in hernia patients. The biomarkers are not tissue-specific and alterations in biomarker levels may arise from other factors than the hernia itself. Also, it is unknown whether the biomarker alterations are a cause or a consequence of the hernia disease.
There is no standardized scoring system for evaluation of fascia morphology. The Bonar score may not be optimal for fascia transversalis morphology, as the agreement between pathologists was only fair. Furthermore, only a subgroup of 20 randomly selected fascia transversalis biopsies was scored increasing a risk of selection bias.

PERSPECTIVES

IMPLICATIONS FOR RESEARCH
A large epidemiological study on unselected material re-evaluating the extrinsic factors associated with inguinal and ventral hernia formation could be relevant. The optimal setting would include clinical examination of all patients to evaluate the accurate hernia prevalence. It seems that smoking, obesity and diabetes are negatively associated with primary inguinal hernia, but on the contrary, some are risk factors for incisional hernia formation and hernia recurrence. This divergence needs further evaluation in order to properly understand the pathogenesis of inguinal and incisional hernia formation.
Emerging evidence suggests that inguinal hernia represents an inherited disease. However, the inheritance pattern has not yet been determined. Furthermore, a large genome-wide association study including patients with familial susceptibility for hernia is still lacking.
The present study suggests that serological biomarkers for type IV and V collagen turnover are altered in hernia patients. We intend to evaluate if the findings are reproducible in a larger patient cohort. Furthermore, it would be interesting to evaluate if the biomarkers predict hernia formation, though such a study should include a long follow-up and may be difficult to complete. Besides, local alterations of type IV and V collagens in fascial tissue remain to be evaluated.
Collagen fibril assembly requires several critical steps, and a missing step will affect the overall quality of the collagen and possibly the composition of collagen fibre. The cross-linking process imparts the collagen molecule with its special strength. For a proper cross-linking process to succeed, the glycosylation reaction and self-assembly with the aid of glycosaminglycans are important together with proper functioning of lysyl oxidase. Several steps of the collagen formation process remain to be evaluated with regard to the hernia disease. Further research on collagen alterations in hernia patients should focus on the collagen quality both during wound healing and locally in fascia. Analyses of lysyl oxi-

dase activity and the covalent collagen cross-links are lacking and may play a role in hernia formation.

CLINICAL IMPLICATIONS

In international guidelines for inguinal hernia repair it is recommended that women should be operated on laparoscopically. Furthermore, a recurrent inguinal hernia primarily repaired with open technique should be scheduled for a laparoscopic repair and vice versa [134]. However, the preferred surgical technique for a man with a primary inguinal hernia is a mesh repair either open or laparoscopic depending on the surgeon's expertise. If a serological biomarker could predict the risk of recurrence or the risk of developing a contralateral inguinal hernia, a tailored surgical strategy for the individual patient with a groin hernia could be a future option.

The subgroup of incisional hernia patients of particular interest are those who undergo primary laparotomy under clinically perfect circumstances and despite correct fascial closure and no post-operative complications still develop an incisional hernia. These patients may have a connective tissue disease solely leading to hernia development. If they could be identified preoperatively with a serological biomarker, prophylactic precautions could be considered such as prophylactic mesh augmentation. This surgical principle has been evaluated in patients at higher risk of developing incisional hernia [135-137]. Larger randomized controls are lacking, but it seems that prophylactic mesh augmentation does decrease the incisional hernia rate without a rise in post-operative complications [138].

CONCLUSIONS

Direct and recurrent inguinal hernia repair are associated with ventral hernia repair, suggesting a systemic predisposition to hernia disease in these patients. MMPs are not suitable as serum biomarkers for inguinal or incisional hernia disease. Though MMPs may be involved in local tissue processes, the systemic levels may not reflect the local environment. Serological biomarkers of type III, IV and V collagen turnover are altered in patients with inguinal and incisional hernia. Specifically, markers for type IV collagen turnover seem to predict the presence of hernias. This novel finding suggests that type IV and V collagen may be involved in hernia development. During wound healing, the total collagen deposition and MMP levels seems to be the same in patients with and without hernias. Besides, the total collagen content and morphology of fascia transversalis appears unaltered in patients with hernias. There are no signs of more severe connective tissue affection in patients with multiple hernias and no difference in collagen turnover between patients with indirect or direct inguinal hernia.

Taken together, the findings of this thesis suggest that abdominal wall hernia is a clinical manifestation of a systemic disease, which is not associated with the local collagen quantity, but rather with the systemic collagen turnover. Further studies are needed to evaluate if markers of collagen turnover can predict the formation of hernias. A systemic biomarker predicting hernia disease would be useful to plan a tailored surgical strategy for the individual patient.

SUMMARY

BACKGROUND

Hernia formation is a multifactorial disease involving important endogenous factors possibly affected by exogenous factors. Alterations in collagen composition seem to contribute to abdominal wall hernia formation, possibly related to increased collagen breakdown. The collagen composition appears altered in fascial tissue but also in skin biopsies, suggesting that the collagen alterations are systemic. More pronounced collagen alterations are found in patients with hernia recurrences. Hypothetically, primary inguinal hernias are formed due to a systemic predisposition to altered connective tissue, whereas impaired healing influences on the development of incisional hernias and hernia recurrences. The overall objective of this thesis was to investigate the collagen turnover systemically and locally in patients with primary inguinal hernia, multiple hernias and incisional hernia.

METHODS AND RESULTS

In a systematic literature review, a total of 55 original articles were reviewed evaluating connective tissue alterations in patients with abdominal wall hernias. Patients with inguinal and incisional hernias exhibit a decreased type I to III collagen ratio in fascia and skin biopsies with the most pronounced alterations found in patients with direct inguinal hernia and hernia recurrence. An increased level of MMP-2 was reported in patients with inguinal hernias.

In a nationwide study from the Danish Hernia Database, 92,283 patients with an inguinal hernia repair were identified from January 1998 until June 2010. A total of 843 patients were also registered with a ventral hernia repair. Direct (OR = 1.28 [95% C.I. 1.08-1.51]) and recurrent (OR = 1.76 [95% C.I. 1.39-2.23]) inguinal hernia repairs were significantly associated with ventral hernia repair compared to indirect inguinal hernia repair after adjustment for gender, age and surgical approach. In a multivariable subgroup analysis, direct and recurrent inguinal hernia repair were associated with primary ventral hernia surgery, whereas only recurrent inguinal hernia repair was associated with secondary ventral hernia surgery.

In a cohort of 305 patients followed up a median of 3.7 years after emergency or elective laparotomy, a total of 79 patients were identified with an incisional hernia. Patients were subgrouped based on the identified risk factors male gender and smoking in eight groups with nine patients in each. Pooled serum samples were screened for MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13, TIMP-1, TIMP-2, and TIMP-4 with a multiarray and zymography. The screening indicated differences in MMP-9 and TIMP-1, which were measured in serum samples of the whole patient cohort with ELISA. There were no differences in systemic MMP-9 and TIMP-1 levels between patients with and without incisional hernia.

Patients were enrolled consecutively in four groups; 1) patients undergoing elective laparoscopic cholecystectomy without hernias (N = 18), patients operated on for 2) primary unilateral inguinal hernia
(N = 17), 3) multiple hernias defined as three or more primary hernias (N = 21) and 4) incisional hernia
(N = 25). Venous blood was collected preoperatively. Pro-MMP-2 and pro-MMP-9 were measured in serum by gelatine zymography, and there were no significant differences between hernia patients and controls. Furthermore, serological biomarkers for

type I, III, IV and V collagen turnover were measured in serum by solid-phase competitive immunoassays. In patients with inguinal hernia, type III and V collagen turnover were significantly decreased, whereas type IV collagen turnover was significantly increased. In incisional hernia patients, type V collagen turnover was significantly decreased, whereas type IV collagen turnover was significantly increased. Type IV collagen turnover seem to predict the presence of both inguinal and incisional hernia.
An ePTFE tube was implanted perioperatively in all four patient groups and explanted on the 10th postoperative day. Newly synthesized granulation tissue in the ePFTE tube represents the patients' wound healing potential. Hydroxyproline levels were measured as a marker for total collagen deposition and were unaltered in hernia patients compared to controls. Pro-MMP-2 and pro-MMP-9 levels in the PTFE tubes did not differ between hernia patients and controls. A fascia transversalis biopsy was excised perioperatively in all four patient groups. There were no significant differences between hernia patients and controls in total collagen concentration or morphology of the fascia transversalis.

CONCLUSIONS
Direct and recurrent inguinal hernia repair are associated with ventral hernia repair, suggesting a systemic predisposition to the hernia disease. MMPs are not suitable as serum biomarkers for inguinal or incisional hernia disease. Serum biomarkers for collagen turnover are altered in both inguinal and incisional hernia patients; specifically markers for type IV collagen turnover seem to predict the presence of hernias. A systemic biomarker predicting hernia disease would be useful to plan a tailored surgical strategy for the individual patient.

REFERENCES

1. Henriksen NA, Yadete DH, Sorensen LT et al. Connective tissue alteration in abdominal wall hernia. Br J Surg 2011;98:210-219.

2. Henriksen NA, Sorensen LT, Bay-Nielsen M et al. Direct and recurrent inguinal hernias are associated with ventral hernia repair: a database study. World J Surg 2013;37:306-311.

3. Henriksen NA, Sorensen LT, Jorgensen LN et al. Circulating levels of matrix metalloproteinases and tissue inhibitors of metalloproteinases in patients with incisional hernia. Wound Repair Regen 2013;21:661-666.

4. Kehlet H, Bay-Nielsen M. Nationwide quality improvement of groin hernia repair from the Danish Hernia Database of 87,840 patients from 1998 to 2005. Hernia 2008;12:1-7.

5. Helgstrand F, Rosenberg J, Bay-Nielsen M et al. Establishment and initial experiences from the Danish Ventral Hernia Database. Hernia 2010;14:131-135.

6. Abrahamson J. Etiology and pathophysiology of primary and recurrent groin hernia formation. Surg Clin North Am 1998;78: 953-972.

7. Ruhl CE, Everhart JE. Risk factors for inguinal hernia among adults in the US population. Am J Epidemiol 2007;165:1154-1161.

8. Carbonell J, Sanchez J, Peris R et al. Risk factors associated with inguinal hernias: A case control study. Eur J Surg Acta Chir 1993;159:481-486.

9. Flich J, Alfonso JL, Delgado F et al. Inguinal hernia and certain risk factors. Eur J Epidemiol 1992;8:277-282.

10. Liem MS, van der Graaf Y, Zwart RC et al. Risk factors for inguinal hernia in women: a case-control study. The Coala Trial Group. Am J Epidemiol 1997;146:721-726.

11. Sorensen L, Friis E, Jorgensen T et al. Smoking is a risk factor for recurrence of groin hernia. World J Surg 2002;26:397-400.

12. Junge K, Rosch R, Klinge U et al. Risk factors related to recurrence in inguinal hernia repair: a retrospective analysis. Hernia 2006;10:309-315.

13. Jansen P, Klinge U, Jansen M et al. Risk factors for early recurrence after inguinal hernia repair. BMC Surg 2009;9:18.

14. Sorensen L, Hemmingsen U, Kirkeby L et al. Smoking is a risk factor for incisional hernia. Arch Surg 2005;140:119-123.

15. Sorensen LT. Wound healing and infection in surgery: the pathophysiological impact of smoking, smoking cessation, and nicotine replacement therapy: a systematic review. Ann Surg 2012;255:1069-1079.

16. Sorensen LT. Wound healing and infection in surgery. The clinical impact of smoking and smoking cessation: a systematic review and meta-analysis. Arch Surg 2012;147:373-383.

17. Rosemar A, Angeras U, Rosengren A. Body mass index and groin hernia: a 34-year follow-up study in Swedish men. Ann Surg 2008;247:1064-1068.

18. Vad MV, Frost P, Bay-Nielsen M et al. Impact of occupational mechanical exposures on risk of lateral and medial inguinal hernia requiring surgical repair. Occup Environ Med 2012;69:802-809.

19. Abramson JH, Gofin J, Hopp C et al. The epidemiology of inguinal hernia. A survey in western Jerusalem. J Epidemiol Comm Health 1978;32:59-67.

20. Bucknall TE, Cox PJ, Ellis H. Burst abdomen and incisional hernia: a prospective study of 1129 major laparotomies. Br Med J 1982;284:931-933.

21. Sugerman HJ, Kellum JM, Jr., Reines HD et al. Greater risk of incisional hernia with morbidly obese than steroid-dependent patients and low recurrence with prefascial polypropylene mesh. Am J Surg 1996;17:80-84.

22. Veljkovic R, Protic M, Gluhovic A et al. Prospective clinical trial of factors predicting the early development of incisional hernia after midline laparotomy. J Am Coll Surg 2010;210:210-219.

23. Sauerland S, Korenkov M, Kleinen T et al. Obesity is a risk factor for recurrence after incisional hernia repair. Hernia 2004;8: 42-46.

24. Zendejas B, Hernandez-Irizarry R, Ramirez T et al. Relationship between body mass index and the incidence of inguinal hernia repairs: a population-based study in Olmsted County, MN. Hernia 2014;18:283-88.

25. Matthews RD, Anthony T, Kim LT et al. Factors associated with postoperative complications and hernia recurrence for patients undergoing inguinal hernia repair: a report from the VA Cooperative Hernia Study Group. Am J Surg 2007;194:611-617.

26. Rosemar A, Angeras U, Rosengren A et al. Effect of body mass index on groin hernia surgery. Ann Surg 2010;252:397-401.

27. Israelsson LA, Jonsson T. Suture length to wound length ratio and healing of midline laparotomy incisions. Br J Surg 1993;80:1284-1286.

28. Israelsson LA, Jonsson T, Knutsson A. Suture technique and wound healing in midline laparotomy incisions. Eur J Surg 1996;162:605-609.

29. van Ramshorst GH, Eker HH, van der Voet JA et al. Long-term outcome study in patients with abdominal wound dehiscence: a comparative study on quality of life, body image, and incisional hernia. J Gastrointest Surg 2013;17:1477-1484.

30. Meijer EJ, Timmermans L, Jeekel J et al. The principles of abdominal wound closure. Acta Chir Belg 2013;113:239-244.

31. Neumayer L, Giobbie-Hurder A, Jonasson O et al. Open mesh versus laparoscopic mesh repair of inguinal hernia. N Engl J Med 2004;350:1819-1827.

32. Lowham AS, Filipi CJ, Fitzgibbons RJ, Jr. et al. Mechanisms of hernia recurrence after preperitoneal mesh repair. Traditional and laparoscopic. Ann Surg 1997;225:422-431.

33. Arvidsson D, Berndsen FH, Larsson LG et al. Randomized clinical trial comparing 5-year recurrence rate after laparoscopic versus Shouldice repair of primary inguinal hernia. Br J Surg 2005;92:1085-1091.

34. Eklund AS, Montgomery AK, Rasmussen IC et al. Low recurrence rate after laparoscopic (TEP) and open (Lichtenstein) inguinal hernia repair: a randomized, multicenter trial with 5-year follow-up. Ann Surg 2009;249:33-38.

35. Rosen A, Nathan H, Luciansky E et al. The inguinal region: anatomic differences in men and women with reference to hernia formation. Acta Anat (Basel) 1989;136:306-310.

36. Condon RE, Carilli S. The Biology and Anatomy of Inguinofemoral Hernia. Sem Laparosc Surg 1994;1:75-85.

37. Helgstrand F, Rosenberg J, Kehlet H et al. Nationwide prospective study of outcomes after elective incisional hernia repair. J Am Coll Surg 2013;216:217-228.

38. Rowe MI, Copelson LW, Clatworthy HW. The patent processus vaginalis and the inguinal hernia. J Pediatr Surg 1969;4: 102-107.

39. Van Veen R, Van Wessem K, Halm J et al. Patent processus vaginalis in the adult as a risk factor for the occurrence of indirect inguinal hernia. Surg Endosc Interv Tech 2007;21:202-205.

40. Conner WT, Peacock EE, Jr. Some studies on the etiology of inguinal hernia. Am J Surg 1973;126:732-735.

41. Lau H, Fang C, Yuen W et al. Risk factors for inguinal hernia in adult males: A case-control study. Surgery 2007;141:262-266.

42. Burcharth J, Pommergaard H, Rosenberg J. The inheritance of groin hernia: A systematic review. Hernia 2013;17:183-189.

43. Zoller B, Ji J, Sundquist J, Sundquist K. Shared and nonshared familial susceptibility to surgically treated inguinal hernia, femoral hernia, incisional hernia, epigastric hernia, and umbilical hernia. J Am Coll Surg 2013;217:289-299.

44. Du Z, Zhao X, Vukasinovic N et al. Association and haplotype analyses of positional candidate genes in five genomic regions linked to scrotal hernia in commercial pig lines. PLoS One 2009;4:e4837.

45. Sezer S, Simsek N, Celik HT et al. Association of collagen type I alpha 1 gene polymorphism with inguinal hernia. Hernia 2014;18:507-512.

46. Calaluce R, Davis JW, Bachman SL et al. Incisional hernia recurrence through genomic profiling: a pilot study. Hernia 2013;17:193-202.

47. Sevonius D, Gunnarsson U, Nordin P et al. Repeated groin hernia recurrences. Ann Surg 2009;249:516-518.

48. Thomas GP, Purkayastha S, Athanasiou T et al. General surgical manifestations of Marfan's syndrome. Br J Hosp Med 2008;69:270-274.

49. Girotto JA, Malaisrie SC, Bulkely G et al. Recurrent ventral herniation in Ehlers-Danlos syndrome. Plast Reconstruct Surg 2000;106:1520-1526.

50. Liem MS, van der Graaf Y, Beemer FA et al. Increased risk for inguinal hernia in patients with Ehlers-Danlos syndrome. Surgery 1997;122:114-115.

51. Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol 2003;200:448-464.

52. Thorpe CT, Birch HL, Clegg PD et al. The role of the non-collagenous matrix in tendon function. Internat J Experiment Pathol 2013;94:248-259.

53. Kruegel J, Miosge N. Basement membrane components are key players in specialized extracellular matrices. Cell Mol Life Sci 2010;67:2879-2895.

54. Shoulders MD, Raines RT. Collagen structure and stability. Annu Rev Biochem 2009;78:929-958.

55. Kadler KE, Baldock C, Bella J et al. Collagens at a glance. J Cell Sci 2007;120:1955-1958.

56. Canty EG, Kadler KE. Procollagen trafficking, processing and fibrillogenesis. J Cell Sci 2005;118:1341-1353.

57. Fleischmajer R. Collagen fibrillogenesis: a mechanism of structural biology. J Invest Dermatol 1986;87:553-554.

58. Edwards CA, O'Brien WD, Jr. Modified assay for determination of hydroxyproline in a tissue hydrolyzate. Clin Chim Acta 1980;104:161-167.

59. Kivirikko KI, Myllyla R. Collagen glycosyltransferases. Int Rev Connect Tissue Res 1979;8:23-72.

60. Robins SP. Biochemistry and functional significance of collagen cross-linking. Biochem Soc Trans 2007;35:849-852.

61. Henkel W, Glanville RW. Covalent crosslinking between molecules of type I and type III collagen. The involvement of the N-terminal, nonhelical regions of the alpha 1 (I) and alpha 1 (III) chains in the formation of intermolecular crosslinks. Eur J Biochem 1982;122:205-213.

62. Kadler KE, Holmes DF, Trotter JA et al. Collagen fibril formation. Biochem J 1996;316:1-11.

63. Fleischmajer R, Perlish JS, Burgeson RE et al. Type I and type III collagen interactions during fibrillogenesis. Ann N Y Acad Sci 1990;580:161-175.

64. Romanic AM, Adachi E, Kadler KE et al. Copolymerization of pNcollagen III and collagen I. pNcollagen III decreases the rate of incorporation of collagen I into fibrils, the amount of collagen I incorporated, and the diameter of the fibrils formed. J Biol Chem 1991;266:12703-12709.

65. Wenstrup RJ, Florer JB, Brunskill EW et al. Type V collagen controls the initiation of collagen fibril assembly. J Biol Chem 2004;279:53331-53337.

66. Murphy G, Nagase H. Progress in matrix metalloproteinase research. Mol Aspects Med 2008;29:290-308.

67. Somerville RP, Oblander SA, Apte SS. Matrix metalloproteinases: old dogs with new tricks. Genome Biol 2003;4:216.

68. Aimes RT, Quigley JP. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. J Biol Chem 1995;270: 5872-5876.

69. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 2006;69:562-573.

70. Wagh PV, Leverich AP, Sun CN et al. Direct inguinal herniation in men: a disease of collagen. J Surg Res 1974;17:425-433.

71. Szczesny W, Cerkaska K, Tretyn A et al. Etiology of inguinal hernia: ultrastructure of rectus sheath revisited. Hernia 2006;10:266-271.

72. Szczesny W, Glowacka K, Marszalek A et al. The ultrastructure of the fascia lata in hernia patients and healthy controls. J Surg Res 2012;172:33-37.

73. Amato G, Marasa L, Sciacchitano T et al. Histological findings of the internal inguinal ring in patients having indirect inguinal hernia. Hernia 2009;13:259-262.

74. Amato G, Agrusa A, Romano G et al. Muscle degeneration in inguinal hernia specimens. Hernia 2012;16:327-331.

75. Friedman DW, Boyd CD, Norton P et al. Increases in type III collagen gene expression and protein synthesis in patients with inguinal hernias. Ann Surg 1993;218:754-760.

76. Klinge U, Zheng H, Si Z et al. Expression of the extracellular matrix proteins collagen I, collagen III and fibronectin and matrix metalloproteinase-1 and -13 in the skin of patients with inguinal hernia. Eur Surg Res 1999;31:480-490.

77. Klinge U, Zheng H, Si ZY et al. Synthesis of type I and III collagen, expression of fibronectin and matrix metalloproteinases-1 and -13 in hernial sac of patients with inguinal hernia. Int J Surg Investig 1999;1:219-227.

78. Rosch R, Klinge U, Si Z et al. A role for the collagen I/III and MMP-1/-13 genes in primary inguinal hernia? BMC Med Genet 2002;3:2.

79. Zheng H, Si Z, Kasperk R et al. Recurrent inguinal hernia: disease of the collagen matrix? World J Surg 2002;26:401-408.

80. Rosch R, Junge K, Knops M et al. Analysis of collagen-interacting proteins in patients with incisional hernias. Langenbecks Arch Surg 2003;387:427-432.

81. Junge K, Klinge U, Rosch R et al. Decreased collagen type I/III ratio in patients with recurring hernia after implantation of alloplastic prostheses. Langenbecks Arch Surg 2004;389:17-22.

82. Taniguchi S, Ueda K, Inoue T et al. Impact of collagen subtype proportions in peritoneal tissues on inguinal hernia formation in adults and infants. Pediatr Surg Int 2006;22:600-604.

83. Zhang R, Che X, Liu H et al. Preliminary study on the content of Type I and Type III collagen in posterior rectus sheath in patients with inguinal hernia. J Xi'An Jiaotong Univ Med Sci 2007;28:308-310.

84. Zimin JI, Chichevatov DA, Ponomareva EE. Impact of the content of collagens I and III and their ratio in cancer patients for the formation of postoperative ventral hernia. Bull Exp Biol Med 2010;149:663-665.

85. Peeters E, De Hertogh G, Junge K et al. Skin as marker for collagen type I/III ratio in abdominal wall fascia. Hernia 2014;18:519-525.

86. Klinge U, Si ZY, Zheng H et al. Abnormal collagen I to III distribution in the skin of patients with incisional hernia. Eur Surg Res 2000;32:43-48.

87. Klinge U, Si Z, Zheng H et al. Collagen I/III and matrix metalloproteinases (MMP) 1 and 13 in the fascia of patients with incisional hernias. J Invest Surg 2001;14:47-54.

88. Si Z, Bhardwaj R, Rosch R et al. Impaired balance of type I and

type III procollagen mRNA in cultured fibroblasts of patients with incisional hernia. Surgery 2002;131:324-331.

89. Salameh JR, Talbott LM, May W et al. Role of biomarkers in incisional hernias. Am Surg 2007;73:561-568.

90. White B, Osier C, Gletsu N et al. Abnormal primary tissue collagen composition in the skin of recurrent incisional hernia patients. Am Surg 2007;73:1254-1258.

91. Rosch R, Binnebosel M, Junge K et al. Analysis of c-myc, PAI-1 and uPAR in patients with incisional hernias. Hernia 2008;12:285-288.

92. Bellon JM, Bujan J, Honduvilla NG et al. Study of biochemical substrate and role of metalloproteinases in fascia transversalis from hernial processes. Eur J Clin Invest 1997;27:510-516.

93. Aren A, Gokce AH, Gokce FS et al. Roles of matrix metalloproteinases in the etiology of inguinal hernia. Hernia 2011;15:667-671.

94. Bellon J, Bajo A, Ga-Honduvilla N et al. Fibroblasts from the transversalis fascia of young patients with direct inguinal hernias show constitutive MMP-2 overexpression. Ann Surg 2001;233: 287-291.

95. Smigielski J, Kolomecki K, Ziemniak P et al. Degradation of collagen by metalloproteinase 2 in patients with abdominal hernias. Eur Surg Res 2009;42:118-121.

96. Pascual G, Rodriguez M, Gomez-Gil V et al. Active matrix metalloproteinase-2 upregulation in the abdominal skin of patients with direct inguinal hernia. Eur J Clin Invest 2010;40:1113-1121.

97. Smigielski J, Brocki M, Kuzdak K et al. Serum MMP 2 and TIMP 2 in patients with inguinal hernias. Eur J Clin Invest 2011;41:584-588.

98. Antoniou GA, Tentes IK, Antoniou SA et al. Matrix metalloproteinase imbalance in inguinal hernia formation. J Invest Surg 2011;24:145-150.

99. Rosch R, Lynen-Jansen P, Junge K et al. Biomaterial-dependent MMP-2 expression in fibroblasts from patients with recurrent incisional hernias. Hernia 2006;10:125-130.

100. Guillen-Marti J, Diaz R, Quiles MT et al. MMPs/TIMPs and inflammatory signalling de-regulation in human incisional hernia tissues. J Cell Mol Med 2009;13:4432-4443.

101. Jorgensen LN, Agren MS, Madsen SM et al. Dose-dependent impairment of collagen deposition by topical granulocyte-macrophage colony-stimulating factor in human experimental wounds. Ann Surg 2002;236:684-692.

102. Jorgensen LN. Collagen deposition in the subcutaneous tissue during wound healing in humans: a model evaluation. APMIS Suppl 2003:1-56.

103. Karsdal MA, Henriksen K, Leeming DJ et al. Novel combinations of Post-Translational Modification (PTM) neo-epitopes provide tissue-specific biochemical markers--are they the cause or the consequence of the disease? Clin Biochem 2010;43:793-804.

104. Cook JL, Feller JA, Bonar SF et al. Abnormal tenocyte morphology is more prevalent than collagen disruption in asymptomatic athletes' patellar tendons. J Orthop Res 2004;22:334-338.

105. Maffulli N, Longo UG, Franceschi F et al. Movin and Bonar scores assess the same characteristics of tendon histology. Clin Orthop Rel Res 2008;466:1605-1611.

106. Fearon A, Dahlstrom JE, Twin J et al. The Bonar score revisited: Region of evaluation significantly influences the standardized assessment of tendon degeneration. J Sci Med Sport 2014;17:346-350.

107. Pascual G, Rodriguez M, Mecham RP et al. Lysyl oxidase like-1 dysregulation and its contribution to direct inguinal hernia. Eur J Clin Invest 2009;39:328-337.

108. Pans A, Albert A, Lapiere CM et al. Biochemical study of collagen in adult groin hernias. J Surg Res 2001;95:107-113.

109. Jain V, Srivastava R, Jha S et al. Study of matrix metalloproteinase-2 in inguinal hernia. J Clin Med Res 2009;1:285-289.

110. Barnett C, Langer JC, Hinek A et al. Looking past the lump: genetic aspects of inguinal hernia in children. J Pediatr Surg 2009;44:1423-1431.

111. Alnassar S, Bawahab M, Abdoh A et al. Incisional hernia postrepair of abdominal aortic occlusive and aneurysmal disease: five-year incidence. Vascular 2012;20:273-277.

112. Jones ME, Swerdlow AJ, Griffith M et al. Risk of congenital inguinal hernia in siblings: a record linkage study. Paediatr Perinat Epidemiol 1998;12:288-296.

113. Sawaguchi S, Matsunaga E, Honna T. A genetic study on indirect inguinal hernia. Japan J Hum Genet 1975;20:187-195.

114. Koch A, Edwards A, Haapaniemi S et al. Prospective evaluation of 6895 groin hernia repairs in women. Br J Surg 2005;92: 1553-1558.

115. Rodrigues C, Yoo J, Rodrigues, Jr. Elastin (ELN) gene point mutation in patients with inguinal hernia. Genet Mol Biol 2006;29:45-46.

116. Antoniou S, Antoniou G, Granderath F et al. The role of matrix metalloproteinases in the pathogenesis of abdominal wall hernias. Eur J Clin Invest 2009;39:953-959.

117. Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci 2002;115:3719-3727.

118. Ashcroft GS, Horan MA, Herrick SE et al. Age-related differences in the temporal and spatial regulation of matrix metalloproteinases (MMPs) in normal skin and acute cutaneous wounds of healthy humans. Cell Tissue Res 1997;290:581-591.

119. Guo LJ, Luo XH, Wu XP et al. Serum concentrations of MMP-

1, MMP-2, and TIMP-1 in Chinese women: age-related changes, and the relationships with bone biochemical markers, bone mineral density. Clin Chim Acta 2006;371:137-142.

120. Lynen Jansen P, Rosch R, Rezvani M et al. Hernia fibroblasts lack beta-estradiol-induced alterations of collagen gene expression. BMC Cell Biol 2006;7:36.

121. Veidal SS, Karsdal MA, Vassiliadis E et al. MMP mediated degradation of type VI collagen is highly associated with liver fibrosis--identification and validation of a novel biochemical marker assay. PLoS One 2011;6:e24753.

122. Kuo DS, Labelle-Dumais C, Gould DB. COL4A1 and COL4A2 mutations and disease: insights into pathogenic mechanisms and potential therapeutic targets. Hum Mol Genet 2012;21:R97-110.

123. DuBay DA, Choi W, Urbanchek MG et al. Incisional herniation induces decreased abdominal wall compliance via oblique muscle atrophy and fibrosis. Ann Surg 2007;245:140-146.

124. Baradi AF, Parry BR, Heslop JH. Peritoneal fine structure of inguinal hernia: a transmission electron microscope study. Histol Histopathol 1992;7:251-257.

125. Karsdal MA, Nielsen MJ, Sand JM et al. Extracellular matrix remodeling: the common denominator in connective tissue diseases. Possibilities for evaluation and current understanding of the matrix as more than a passive architecture, but a key player in tissue failure. Assay Drug Dev Technol 2013;11:70-92.

126. Jorgensen LN, Sorensen LT, Kallehave F et al. Increased collagen deposition in an uncomplicated surgical wound compared to a minimal subcutaneous test wound. Wound Repair Regen 2001;9:194-199.

127. Chen P, Parks WC. Role of matrix metalloproteinases in epithelial migration. J Cell Biochem 2009;108:1233-1243.

128. Rodrigues Junior AJ, Rodrigues CJ, da Cunha AC et al. Quantitative analysis of collagen and elastic fibers in the transversalis fascia in direct and indirect inguinal hernia. Rev Hosp Clin Fac Med Sao Paulo 2002;57:265-270.

129. Ozdogan M, Yildiz F, Gurer A et al. Changes in collagen and elastic fiber contents of the skin, rectus sheath, transversalis fascia and peritoneum in primary inguinal hernia patients. Bratisl Lek Listy 2006;107:235-238.

130. Casanova AB, Trindade EN, Trindade MR. Collagen in the transversalis fascia of patients with indirect inguinal hernia: a case-control study. Am J Surg 2009;198:1-5.

131. Meyer AL, Berger E, Monteiro Jr O et al. Quantitative and qualitative analysis of collagen types in the fascia transversalis of inguinal hernia patients. Arq Gastroenterol 2007;44:230-234.

132. Fachinelli A, Maciel Trindade MR. Qualitative and quantitative evaluation of total and types I and III collagens in patients with ventral hernias. Langenbecks Arch Surg 2007;392:459-464.

133. Chen CZ, Raghunath M. Focus on collagen: in vitro systems to study fibrogenesis and antifibrosis state of the art. Fibrogen Tissue Rep 2009;2:7.

134. Simons MP, Aufenacker T, Bay-Nielsen M et al. European Hernia Society guidelines on the treatment of inguinal hernia in adult patients. Hernia 2009;13:343-403.

135. Takagi H, Sugimoto M, Kato T et al. Postoperative incision hernia in patients with abdominal aortic aneurysm and aortoiliac occlusive disease: a systematic review. Eur J Vasc Endovasc Surg 2007;33:177-181.

136. El-Khadrawy OH, Moussa G, Mansour O et al. Prophylactic prosthetic reinforcement of midline abdominal incisions in high-risk patients. Hernia 2009;13:267-274.

137. Bevis PM, Windhaber RA, Lear PA et al. Randomized clinical trial of mesh versus sutured wound closure after open abdominal aortic aneurysm surgery. Br J Surg 2010;97:1497-1502.

138. Timmermans L, de Goede B, Eker HH, van Kempen BJ, Jeekel J, Lange JF. Meta-Analysis of Primary Mesh Augmentation as Prophylactic Measure to Prevent Incisional Hernia. Dig Surg 2013;30:401-409.