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ORIGINAL ARTICLE

Morphology, quality, and composition in mature human peritoneal adhesions

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Abstract
Background and aim Peritoneal adhesions are caused by intra-abdominal surgery and can lead to relevant complications. Adhesions are supposed to consist of avascular scar tissue. The aim of the present study was to analyze whether mature postsurgical adhesions even after years still reveal a dynamic remodeling process.
Materials and methods In a prospective analysis, we investigated tissue specimen of peritoneal adhesions in 40 patients after abdominal surgery. Expression of five parameters representing wound healing and remodeling were examined (MMP-2, Ki-67, apoptosis, collagen/protein ratio, and collagen type I/III ratio).
Results Gender, age, and the number of previous operations had no impact on the parameters measured. Adhesion specimens were cell rich, containing mononuclear round cells, fibroblasts, adipose cells, and vascular endothelial cells. There was a positive expression of MMP-2 and apoptosis, whereas Ki-67 was marginal irrespective of adhesion maturity or quality. Adhesions classified as dense showed a significant increase in total collagen (118.2± 4.9 μg/mg) and collagen type I/III ratios (3.9±0.2), whereas there were no significant differences regarding the adhesion maturity.
Conclusion The distinct composition of cellular components as well as of extracellular matrix proteins may reflect an interactive cross-talk between adhesion- and stroma-derived cells even in mature adhesions. Our findings support the hypothesis that the disabilities of appropriate repair of the peritoneal surface leading to persistent adhesions are a consequence of a permanent process of disturbed remodeling.

Keywords Peritoneal adhesion · Collagen · Wound healing · Tissue remodeling

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Introduction

After intra-abdominal surgery, more than 90% of patients develop peritoneal adhesions [8, 17, 20]. Adhesions create a lifetime risk for the development of potentially relevant complications as small bowel obstruction, chronic abdominal pain or female infertility [11, 17, 20, 21]. Intestinal obstruction as the most life-threatening adhesion-related disease is associated with mortality rates of up to 15% [8, 30–33]. As a consequence, adhesions generate a remarkable socio-economic relevance with an annual cost of more than 1.2 billion dollars in USA [24].

However, the specific pathophysiology of adhesiogenesis remains elusive [27]. Currently, the formation of peritoneal adhesions is supposed to be the consequence of a peritoneal defect with local ischemia and an erroneous peritoneal regeneration [11]. Findings concerning the vascularization of adhesions are contradictory: Whereas some authors assumed adhesions to consist mainly of fibrous and avascular scar tissue, others described adhesions as highly vascularized tissue, containing well-developed arterioles, venules, and capillaries [8, 9, 11, 14].

It has been reported that young fibrinous adhesions soon become organized to persisting fibrous and vascular tissue within 10 days to 2 weeks after laparotomy [17, 19, 29]. Although spontaneous regressions of adhesions have been reported, a considerable number of patients reveal peritoneal adhesions even years after the previous operative procedure [1, 3, 13]. It may be hypothesized that a disability of appropriate repair of the peritoneal surface is a consequence of either a persistence of areactive fibrous tissue or of a permanent process of disturbed remodeling. Therefore, we analyzed quality and morphology of 40 adhesion specimens, prospectively obtained 0.5 to 240 months after the previous operation. As pivotal representatives of wound healing and remodeling, immunohistological analyses of MMP-2, Ki-67, and apoptosis as well as the ratios of collagen per protein and collagen types I/III were performed [12, 15, 23].

Materials and methods

Patient information

Adhesions were collected from 40 patients (17 female and 23 male patients) undergoing laparotomy at the Surgical Department of the RWTH Aachen University Hospital, Germany. The study was approved by the local ethics committee, and patients gave written informed consent for participation in the trial. Clinical parameters included age, gender, diagnosis, and surgical and medical history. The estimated maturity of the adhesions was calculated from the date of the last prior abdominal surgery according to Herrick et al. [9, 11]. Intraoperatively, the surgeon classified the adhesion tissue either as soft, cobweb like, and easy to dissect or as dense bands, only sharp to dissect. Samples of 5–10 mm were excised from serosal surfaces between opposing visceral organs and the parietal peritoneum. Tissue specimens were immediately fixed in 4% paraformaldehyde and embedded in paraffin wax.

Histological assessment

Histological and immunohistochemical investigations were performed on paraffin embedded 3-μm sections using peroxidase-conjugated, affinity-isolated immunoglobulins. All sections were routinely stained with hematoxylin and eosin (H and E) and were processed at the same time to reduce internal staining variations. Briefly, immunohistochemistry was done by the avidin–biotin complex method and according to the instructions of the manufacturer.

For the detection of MMP-2, we used rabbit polyclonal, 1:1000 from Biomol (Hamburg, Germany) as primary antibody and goat anti-rabbit, 1:500, Dako (Glostrup, Denmark) as the secondary antibody. Ki-67 expression was investigated with a mouse monoclonal antibody MIB-1, 1:10 from Dako and rabbit anti-mouse antibody, 1:300 from Dako as the secondary antibody. Apoptotic cells were detected by the Apop Tag Peroxidase detection kit of Q-Biogene (Carlsbad).

The expression of immunohistochemical parameters was classified by two independent blinded observers using a semiquantitative immunoreactivity score (IRS) according to the method of Remmele and Stegner [25]. Intensity of staining was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (intensive). Extend of staining was scored as 0 (0%), 1 (1–20%), 2 (21–50%), 3 (51–80%), and 4 (81–100%), indicating the percentage of positive staining in adhesion tissue. Multiplication of intensity score (0–3) and extend score (0–4) resulted in the IRS, which ranges between 0 and 12. Sections were examined by standard light microscopy (Olympus BX51, Hamburg, Germany); for each sample, six regions (400×, area 100× 100 μm) were captured by a digital camera (Olympus C-3030, Hamburg, Germany). Investigating the H and E-stained sections, two independent blinded observers scored the following histological features on a semiquantitative scale: mononuclear round cells, fibroblasts, adipose cells, and vascular endothelial cells. The number of cells was scored as 0 (0%), 1 (1–20%), 2 (21–50%), 3 (51–80%), and 4 (81–100%).

Collagen per protein ratio

Ten-micrometer-thick specimens of paraffin embedded tissue samples were obtained from each group and placed in test tubes. After deparaffination, the slices were stained with Sirius red and Fast green (Polysciences, Warrington, PA) according to Lopez-De Leon [18]. The specimens were rinsed several times with distilled water until the supernatant was colorless. Subsequently, the dyes were eluted from the sections by incubation with 0.1 N NaOH in absolute methanol. The fluid was read immediately in a spectrophotometer at the wavelengths corresponding to the maximal absorbance of Sirius red (535 nm) and Fast green (605 nm). Results are expressed as the ratio of collagen (μg) to noncollagenous protein (mg) to level out the differences of weight of the slices and were performed with five samples in each specimen.

Collagen type I/III ratio by Cross polarization microscopy

For cross-polarization microscopy (CPM), 5-μm sections were stained for 1 h in Picrosirius solution (0.1% solution of Sirius Red F3BA in saturated aqueous picric acid, pH 2) according to Junqueira et al. [16]. The sections were washed for 2 min in 0.01 N HCl, dehydrated, cleared, and

mounted in synthetic resin. To analyze collagen type I/III ratios, tissue samples were evaluated using CPM evaluated by two independent blinded observers. Thicker collagen type I fibers were stained in red-orange shades, whereas thinner collagen type III appeared as pale green shades. For each sample, ten regions within the interface (400×, area 100×100 μm) were captured by a digital camera (Olympus C-3030). Collagen I/III ratios were obtained by analysis of the amount area of collagen type I and III using a digital image analyzing software (Image-Pro Plus® 4.5, Media Cybernetics, Silver Spring, MD). Results are expressed as ratio of area of collagen type I to type III.

Statistical analysis

Statistical analysis has been carried out using the Statistical Package for Social Sciences software (SPSS®, Ver. 14.0). Specimens of adhesion tissue were configured to both adhesions younger than 12 months (n=14) and those with a maturity of 1 year or older (n=26). Furthermore, specimens were grouped to either patients with soft (n=10) or dense adhesions (n=30). Differences between study groups were analyzed by Mann–Whitney tests. For normally distributed numeric data, differences were analyzed by analysis of variance and in case of significance confirmed by t test. In case of nonparametric data, the Spearmen's correlation coefficient was calculated, and in case of numeric data, we calculated the Pearson's correlation coefficient. p values less than 0.05 were considered to be significant and p values less than 0.001 as highly significant. Data are presented as mean±standard deviation or as median values with 25th and 75th percentiles and error bars denoting the 10th and 90th percentiles.

Results

The mean age of the 40 patients was 55±19. The estimated maturity of the adhesions ranged from 0.5 months to 20 years with a median of 18 months. Eighteen of the 40 patients had one, four patients had two, and 18 patients had more than two previous abdominal and/or pelvic surgical interventions. Gender, age, and the number of previous operations had no impact on the morphology of the adhesions.

Histological findings

The investigation of the H and E-stained sections revealed a distinct predominance of either mononuclear round cells, bundles of fibroblasts, adipose cells, or vascular endothelial cells (Fig. 1a–d, Table 1). In 15 patients, adhesions were completely riddled by extended infiltrates of mononuclear round cells, and no regular structure of connective tissue bundles could be identified. In 18 patients, specimens were characterized by abundant adipose tissue squired by few collagen bonds forming either parallel arranged bundles or a diffuse and disordered collagen network. A marked vascularization could be identified in 30 of the adhesion tissues. In comparison to adhesions with a maturity of less than 12 months, the investigation of adhesions greater than 12 months revealed significantly diminished incidence of fibroblasts ($p<0.05$). In contrast, there were no significant differences in the appearance of mononuclear round cells, adipose cells, and vascular endothelial cells depending on the maturity of the adhesion specimens ($p>0.05$; Table 1). Furthermore, there were no significant differences comparing adhesion specimen grouped as soft with those grouped as dense regarding the parameters measured, respectively ($p>0.05$; Table 1).

MMP-2

All investigated samples demonstrated positive staining of MMP-2. Expression of MMP-2 could be verified cytoplasmatically, mainly in fibroblasts and in between adipose cells (Fig. 2c). The expression of MMP-2 did not differ between the specimen center and the periphery of the adhesion tissue. The MMP-2-expression was slightly reduced in mature adhesions, whereas there were no significant differences between adhesion specimen with a maturity of less than 12 months and those with a maturity greater than 12 months ($p>0.05$; Fig. 3a). There were no significant differences comparing adhesion specimens grouped as soft with those grouped as dense ($p>0.05$; Fig. 3b).

Ki-67 and apoptosis

Ki-67 was expressed rarely but cumulatively pronounced in four samples that revealed both inflammation and connective tissue bundles in close aggregation (Fig. 2b). The median expression of Ki-67 was scored as 1 (range, 1–6). No significant differences could be generated comparing the groups ($p>0.05$).

Apoptotic cells (TUNEL) were abundantly perceived, mainly surrounding vessels (Fig. 2a). Generally, the appearance of apoptotic cells did not differ between the specimen center and the periphery of the adhesion tissue. No significantly different score of apoptotic cells was detected between adhesion specimen with a maturity of less than 12 months and those with a maturity greater than 12 months ($p>0.05$; Fig. 3c). Furthermore, there were no significant differences in adhesion specimens intraoperatively grouped as soft vs those grouped as dense ($p>0.05$; Fig. 3d).

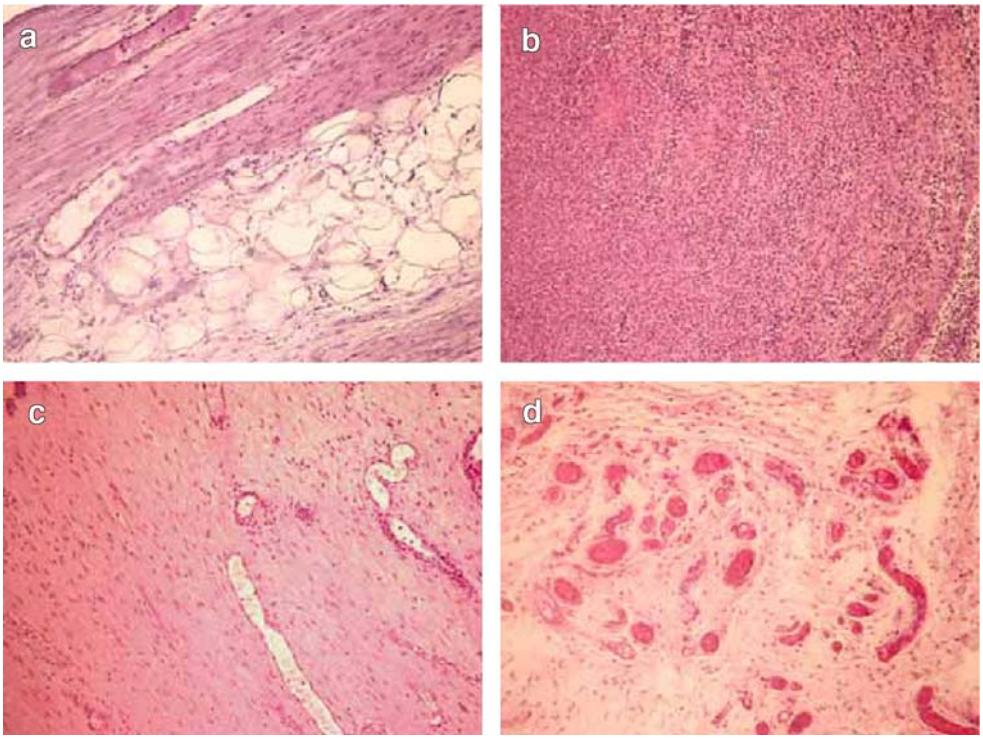

Fig. 1 Histological features of peritoneal adhesions, staining with H and E, representing abundant adipose tissue in between the two mesothel layers of adhesions tissue (**a**), predominantly mononuclear round cells (**b**), bundles of connective tissue mainly containing of collagen types I and III (**c**), and highly vascularized adhesion tissue (**d**) (original magnification, 400× in **a**–**d**)

Collagen per protein ratio and collagen type I/III ratio

The overall collagen content and the collagen I/III ratio did not differ between adhesion specimen younger than 12 months and those with a maturity of 1 year or older ($p>0.05$; Fig. 3e and g). In contrast, the mean overall collagen content was significantly lower in adhesion specimens grouped as soft compared with adhesion specimens grouped as dense ($p<0.001$; Fig. 3f). Cross-polarization microscopy revealed a mean collagen type I/III ratio of 3.7±1.2 and a significantly decreased collagen type I/III ratio in adhesion specimen grouped as soft compared with adhesion specimen grouped as dense ($p<0.05$; Fig. 3h). Collagen type I/III ratio and the overall collagen content significantly correlated with each other ($r=0.442$, $p<0.05$).

Discussion

During surgery, injuries to the peritoneum are inevitable and followed by a healing process that frequently results in the attachment of adjacent organs by a fibrous mass, referred commonly as adhesions [17, 26]. It is common experience that the extent of adhesion formation is unpredictable. Thus, adhesions may appear either circumscriptive or almost covering the entire peritoneal surface, either as dense bands

Table 1 Histopathology of peritoneal adhesions

	Adhesion quality		Adhesion maturity		All adhesions
	Soft	Dense	<12months	>12months	
Number (*n*)	10	30	14	26	40
Mononuclear round cells	3.3±0.9	3.1±0.8	3.4±0.9	3.0±0.8	3.1±0.9
Fibroblasts	2.8±0.8	2.9±0.9	3.1±0.7	2.7±1.0	2.9±0.9
Adipose cells	3.3±1.3	2.9±1.3	2.6±1.3	3.2±1.3	3.0±1.3
Vascular endothelial cells	3.1±1.0	3.1±0.8	2.9±1.1	3.2±0.7	3.1±0.8

Data are represented as mean score±standard deviation. The number of cells was scored as 0 (0%), 1 (1–20%), 2 (21–50%), 3 (51–80%), and 4 (81–100%). The estimated maturity of the resected adhesions is quoted in months.

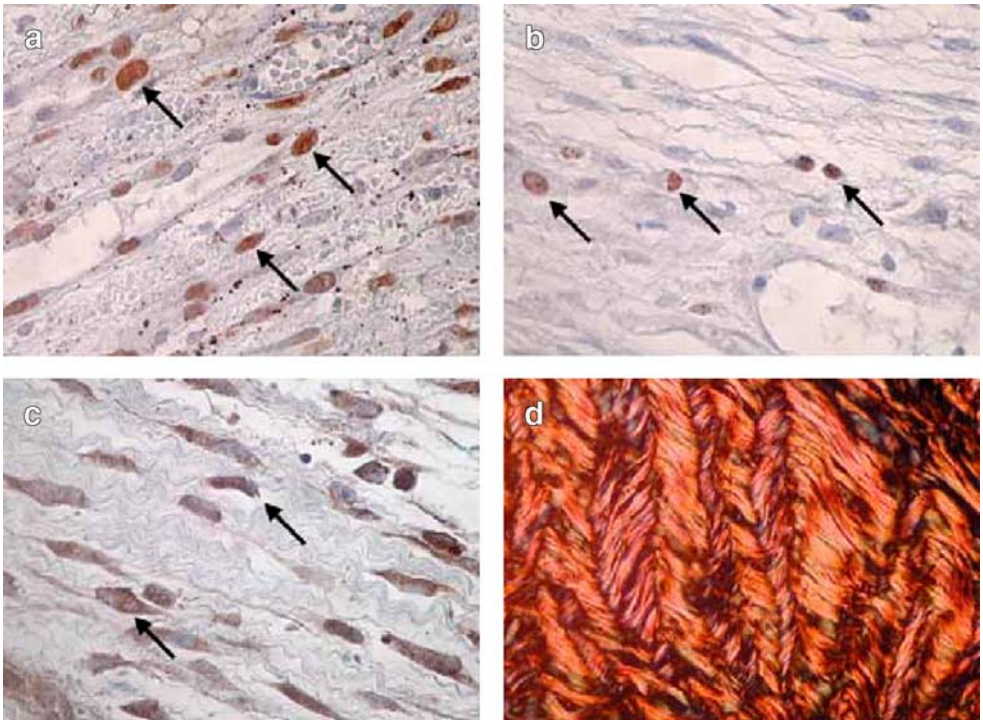

Fig. 2 Immunohistochemical and cross-polarization microscopical (CPM) features of mature adhesions (positive stained cells marked with *black arrows*). High activity levels of TUNEL (**a**) surrounding blood vessels, whereas Ki-67 (**b**) showed marginal activity levels. Positive expression of MMP-2 intracytoplasmatic in fibroblasts adjacent to collagen bundles (**c**). CPM of Sirius red-stained section of a dense adhesion with a collagen type I/III ratio of 6.26 (**d**) (original magnification, 400× in **a**–**d**)

or as cobweb-like tissues [22]. Although sometimes early adhesions may resolve spontaneously, frequently, they persist even over years. The aim of the present study was to analyze whether intraoperatively observed differences in quality and morphology of human peritoneal adhesions are reflected in a distinct composition of the adhesion tissues.

Our study revealed no association between the morphology of peritoneal adhesions and the number of previous abdominal surgery. This is in conflict with the finding of DeCherney and diZerega [4], who postulated that the extent of adhesions strictly correlates to the number of operations. Furthermore, the examination of 40 adhesion specimens did not confirm previous publications describing peritoneal adhesions as mainly avascular scar tissue forming fibrous bands [5–7, 14]. In accordance to Herrick et al. and Epstein et al., peritoneal adhesions were highly cellular and well vascularized, containing tortuous collagen bands and abundant adipose tissue [9, 11]. Interestingly, the appearance of the histological parameters measured did not differ in adhesion specimen regarding maturity and adhesion quality. Simply, elder and mature adhesions reveal less collagen bundles than younger adhesions, whereas the amount of adipose tissue increases over time.

The age and maturity of the adhesions were estimated from the date of the latest previous abdominal surgery. In mature human peritoneal adhesions persisting over years, a reduction in remodeling activity similar to changes in scar tissue was described [5]. In contrast, in our study, in particular, the mature adhesions were cell rich, consisting of mononuclear round cell infiltrates, fibroblasts, adipose cells, and vascular endothelial cells.

Mononuclear cells are capable of producing a variety of growth factors important for deposition of connective tissue and vascularization [23, 28]. As MMP-2 and apoptosis are crucial mediators in wound healing and remodeling, our findings of a positive expression of these two parameters reflect an intensive and distinguished tissue remodeling and cell turnover even after several years [2, 10, 12, 23]. The discrepancy of the high number of TUNEL-positive apoptotic cells and the marginal expression of Ki67-positive proliferating cells should result in tissue reduction. This may indicate a considerable immigration of differentiated cell lineages, e.g., from the abdominal cavity, into the adhesion tissue. However, these findings encourage our assumption that mature peritoneal adhesions do not represent just an inert fibrous scar tissue but rather represent a dynamic process in between wound healing and remodeling.

Adhesions, which were classified intraoperatively as dense by the surgeon, revealed increased total collagen contents. Considering the increased collagen type I/III ratio, these findings may indicate that the organization of fibrinous adhesions by fibroblasts lead to deposition of predominant highly cross-linked collagen type I in permanent adhesions [22, 29]. Therefore, we hypothesize that the deposition of collagen type I even in long-lasting adhesions is a consequence of a dynamic but erroneous remodeling and therefore might be an integral causation for the persistence of postoperative peritoneal adhesions.

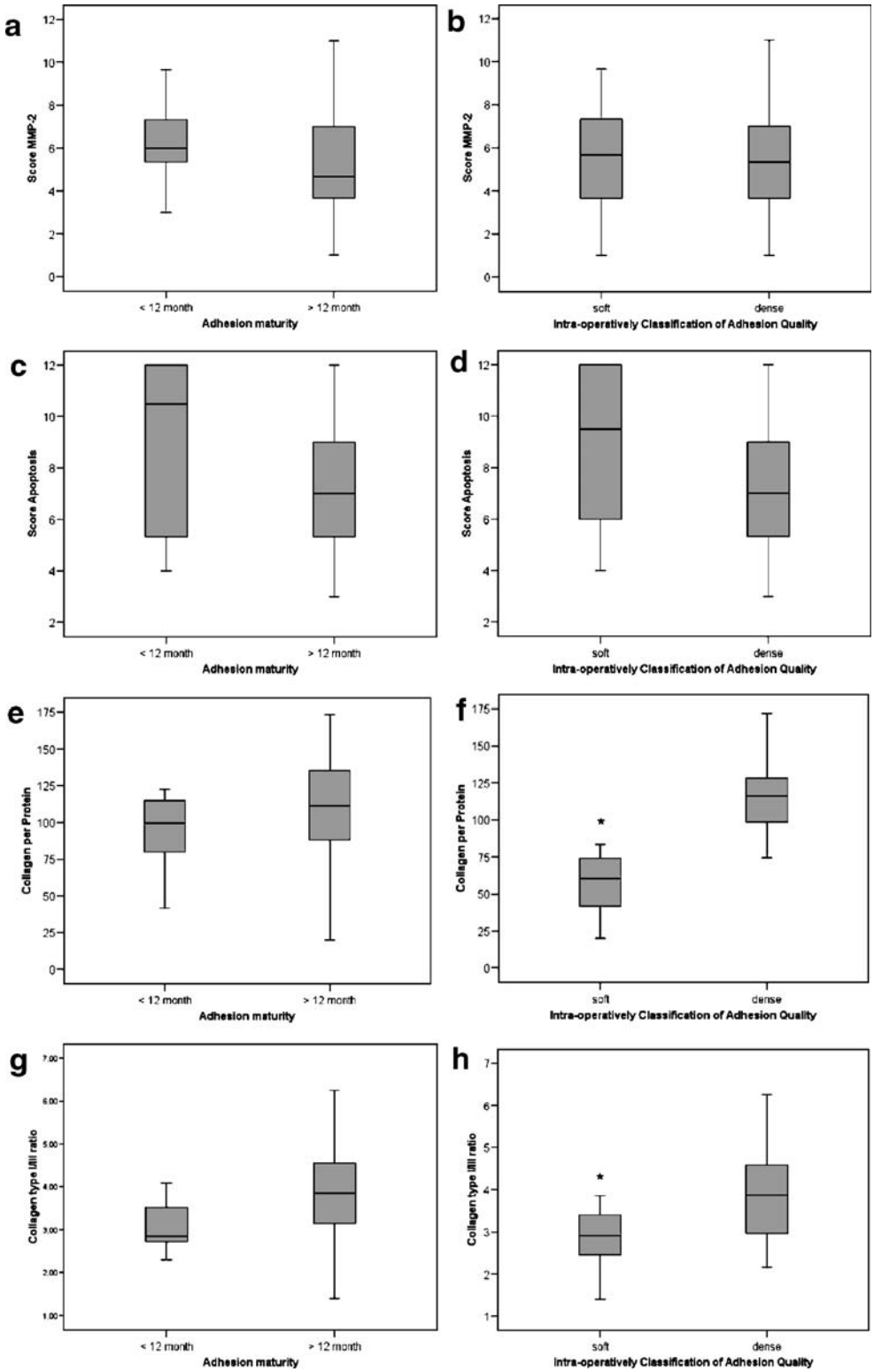

Fig. 3 Boxplots representing analyzes of peritoneal adhesions as median values with 25th and 75th percentiles and error bars denoting the 10th and 90th percentiles. **a** Expression of MMP-2, adhesion specimen with a maturity of less than 12 months (median, 6; range, 2–9.7) and those with a maturity greater than 12 months (median, 4.7; range, 1–11). Adhesion specimen grouped as soft (median, 5.7; range, 1–9.7) and those grouped as dense (median, 5.3; range, 1–11) are illustrated in **b**. Whereas, **c** illustrates Apoptotic cells (TUNEL) in adhesion specimen with a maturity of less than 12 months (median, 10.5; range, 4–12) and those with a maturity greater than 12 months (median, 7; range, 3–12; $p>0.05$). **d** Adhesion specimen intraoperatively grouped as soft (median, 9.5; range, 4–12) vs those grouped as dense (median, 7; range, 3–12). The overall collagen content in adhesion specimen less than 12 months (93.8± 6.9 μg/mg) and in specimen with a maturity greater than 12 months (108.±8.2 μg/mg) is given in **e**. The mean overall collagen content in adhesion specimen grouped as soft (56.7±7.4 μg/mg) compared with adhesion specimen grouped as dense (118.2±4.9 μg/mg) is demonstrated in **f**. In contrast, **g** represents the collagen type I/III ratio in adhesion specimen less than 12 months (3.3±0.3) compared to specimen greater than 12 months (3.9±0.2). The mean collagen type I/III ratio in adhesion specimen grouped as soft (2.9±0.2) compared with adhesion specimen grouped as dense (3.9±0.2; **h**)

Conclusion

Long-lasting adhesions containing a considerable amount of adipose tissue become increasingly dense. The distinct composition of extracellular matrix proteins as well as of cellular components including fibroblasts, mononuclear cells, and blood vessel cells may reflect an interactive cross-talk between adhesion- and stroma-derived cells. In summary, our findings support the hypothesis that the disabilities of appropriate repair of the peritoneal surface leading to persistent adhesions are a consequence of a permanent process of disturbed remodeling. Further research is needed to elucidate the pathogenesis of adhesion formation on a more molecular level, in particular, the functional impact of the numerous mononuclear round cells.

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