CPQ Medicine (2021) 11:3
Research Article

Histological and Immunohistological Studies in Patients With Transsphincteric Fistulas After Sphincter-Preserving Operations: Could Done the Data Obtained Indicate Possible Causes of Relapse of the Disease?

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Received: 26 January 2021

Published: 28 January 2021

Keywords: *Rectal Fistula; Cytokines; Collagen; Investigations of Tissues; Surgery*

Abstract

Background

Modern surgical approaches to the treatment of anal fistulas involve operations that least damage the sphincter complex of the rectum. The essence of the operation is a complete excision of the anal fistula, which is often accompanied by a significant number of recurrences depending on the chosen method of operation. Failure is likely the result of inflammation that persists after surgery, and cytokines play an important role in these processes.

Methods

The tissues of the fistula tract was obtained in 90 patients of both sexes with transsphincteric fistulas in 2018- 2020.

Result

A large number of IL-6 and TNFα-producing cells has been identified not only in inflammatory infiltration but also in granulation and immature connective tissue, indicating the active role of these cytokines in the formation of rectal fistulas.

Introduction

Rectal fistula (anal fistula, fistula-in-ano, chronic paraproctitis) is a pathological course or cavity connecting the lumen of the anal canal or rectum with the perineum. According to foreign literature, the prevalence of rectal fistula in European countries is 10.4 - 23.2 persons per 100,000 population [1], with males this disease is more common in women than in women in a ratio of 1.8:1 [2]. Reticular fistulas are diagnosed in patients of different ages, but most often they occur in the age group of 30-50 years [3], that is, in the most able-bodied and socially active part of the population. In Sweden, A. Lundqvist *et al.* (2016) have conducted to evaluate the cost of rectal fistulas treatment in 362 patients and they shown that the total cost of patient treatment was amounted to an average of 5561 euros, of which in 80% it's were directly medical expenses for the treatment. The remaining of 20% were economic losses associated with the duration of the period of disability which averaged of 10.4 days [4]. It should be noted that such a high cost of treatment was due to the high number of recurrences and development of complications.

Rectal fistulas occupy the fourth place in the structure of proctological diseases. The method of treatment of this pathology is only surgical. Among all proctologic operations, from 13,6 to 25,4% of operations to eliminate fistulas are performed each year [5-7]. The most common cause of perianal fistula formation is inflammation of the anal glands in the anal crypt area, followed by the spread of infection to the anorectal tissue. The infection can also penetrate through the damaged rectal mucosa, lymphogenous and hematogenous. Chronic recurrent anal fistulas are due to the lining of the internal hole of the fistula with the glandular epithelium of the anal glands and the subsequent complete or partial epithelization of the lumen of the fistulous passage, preventing its independent healing.

There are more than one hundred methods of surgical treatment of fistulas of the rectum but there is still no single 'gold standard'. The main components of the surgical treatment of fistulas are the excision of the fistulous course, the elimination of purulent infections and the provision of wound healing [8-10]. Considering the involvement of the anal sphincter in the pathological process, the most important is the preservation of its functional ability. According to the literature, after opening the abscess without eliminating its internal hole, almost 70% of patients develop fistulas of the rectum [11]. The formation of rectal fistula depends on the type of bacteria, their virulence, the state of the patient body, their immunity and the nature of the reaction to inflammation. In the studies of some author, the relationship of some cytokines with the mechanisms of damage to the inflammation of the mucous membrane of the rectum and the migration of opportunistic flora of their own microbiomes induced by them, with the subsequent formation of fistula [12-14]. Continuation of the study of the pathogenesis of this disease demonstrates the active role of cytokines in the stimulation of collagen genesis and fistula formation in the vast majority of patients [15].

Aim

The aim of this project is to evaluate local production and determine the role of IL-6 and TNFα in the formation of rectal fistulas.

Materials and Methods

Study Design

The study was design to span through from September 2018 to February 2020 who underwent surgery (fistulectomy, modified LIFT technique, using of the technique of closing the fistula tract by bio-welding). The presence of IL-6 and TNF-α was quantified in frozen tissue samples by immunohistological examination. It was a retrospective study. The fistulas were later analyzed.

Inclusion and Exclusion Criteria

Criteria for Inclusion in the Study

The presence of uncomplicated transsphincteric fistula of the rectum.

Exclusion Criteria

Fistula associated with Crohn's disease, the presence of fistula after radiation therapy, fistula of the rectum on the background of specific flora (actinomycosis, tuberculosis, etc.), decompensated concomitant cardiovascular pathology, history of cancer. Patients who were included in the study underwent a number of laboratory and instrumental research methods according to standard methods. None of the patients received antibiotics and/or immunomodulatory agents prior to surgery.

Sample Collection and Processing

The materials for morphological examination was represented by the tissues of the anal fistula was obtained during the operations. All patients were divided into two groups, which differed in the method of excision of the anal fistula. The 1st group included 33 patients who underwent excision of the fistula by the modified LIFT technique [9,10], and the 2nd group included 30 patients who underwent bio-welding of the fistula tract [16]. With these interventions, it different amounts of material were obtained, which were conveniently divided into intrasphincteric and extrasphincteric parts of the fistula and evaluated qualitative and quantitative results of histological and immunohistological examination in each of the presented parts.

The resulting material was fixed in 10% formalin solution, followed by passing through alcohols of different concentrations and pouring into paraffin. Prepared serial sections with a thickness of 4-5x106m. Histological examination was performed according to standard methods. Micropreparations were stained with hematoxylin and eosin, picrofuxin according to van Gizon, according to Mallory. The micropreparations were studied on an Olympus VX-41 microscope. Immunohistochemical study was performed on paraffin

sections by the indirect immunofluorescence reaction with monoclonal antibodies (MCAs) according to the method of M. Brosman (1979) using monoclonal antibodies (MCAs) to collagen types I and III (IMTEK Ltd., Russia), as well as MCAs to CD18, CD16, TNF-α, IL-6 (Novocastra Laboratories Ltd) [17]. Micropreparations were examined under a microscope 'Olympus BX-41' (Japan), counted immune cells in the field of view at a magnification of 400 times.

Statistical Data

Statistical data processing was carried out using the statistical software package StatSoft Statistica 6.0. To determine the differences in the clinical picture distributed all patients to the methods of surgery and such criterias as of Student's, Mann-Whitney's and $\chi 2$ were used. In all cases, the verification of statistical hypotheses was conducted with a confidence probability of more than 95%. To assess the adequacy of the comparisons and the accuracy of the quality of the forecast, the method of analysis of the operational characteristics curves (ROC) was used. The optimal correlation between the sensitivity and the specificity of the prediction method was chosen based on the Pareto criterion. Prognostic effectiveness of the models was evaluated by discrimination on the basis of the AUC index. Model performance: was limited at AUC ≥ 0.70; was good at AUC ≥ 0.80 and was great at AUC ≥ 0.90.

Results

The characteristics of patients who were studied are presented in the 1st Table.

Table 1: *Evaluation Criteria of Fistulas of the Rectum*

EVALUATION CRITERIA	INDICATORS
Type of Rectal Fistula: n = 63	***Transsphincteric*** (100%)
Low	47 (74.6%)
High	16 (25.4%)
Male/ Female	48/15
Average age	41.5±8.79
Median BMI (kg/m^2)	27.8±2.42
Previous operations (1st/2nd)	5 (4/1)
Localization of an internal opening: - Anterior - Posterior - Lateral	 21 (33.3%) 40 (63.5%) 2 (3.2%)
Localization of an external opening: - Anterior - Posterior - Lateral	 18 (28.6%) 34 (53.9%) 11 (17.5%)

Distance between the external hole of the fistula and the anus (sm)	4.7±0.95
Duration of the disease: - up to 3 months - from 3 to 12 months - more than 12 months	 11 (11.5%) 24 (38.1%) 28 (44.4%)
Types of surgery: - Modified LIFT technique - Bio-welding of the fistula tract	 33 (52.4%) 30 (47.6%)

Among the patients, the male/female ratio was 48/15, average age and median BMI were 41.5 ± 8.79 and 27.8 ± 2.42 respectively. The distance between the external opening of the fistula and the anus was 4.7 ± 0.95cm. Indicators such as localization of an internal and an external openings, as well as duration of the disease and the types of surgery has been analyzed (Table 1).

The material obtained in the 1st group was represented by the intrasphincteric and extrasphincteric parts of the rectal fistula. In the intrasphincteric part of micropreparations the connective tissue component, muscular and vascular are defined mainly. Connective tissue was represented by both mature and granulation tissue; regardless of localization, edema, with sites of disorganization, in the form of fibrinoid swelling and necrosis, as determined by Mallory staining. Inflammatory infiltration represented by lymphocytes, macrophages and neutrophils was detected in the stroma. Granulation tissue was represented by connective tissue and immune cells, as well as microvessels, was determined during the inner part of the fistula course. The location of the cells attracted attention, as they were mainly localized in the area of granulation tissue. In the extrasphincteric part of the fistula's tract the presence of squamous multilayered epithelium with keratinization and acanthosis was determined. The basement membrane of the epidermis was thickened in places due to multiple sclerosis. Fuchsinophilic bundles of collagen fibers with signs of disorganization in the form of fibrinoid changes were determined in the dermis during Malory staining (Figure 1).

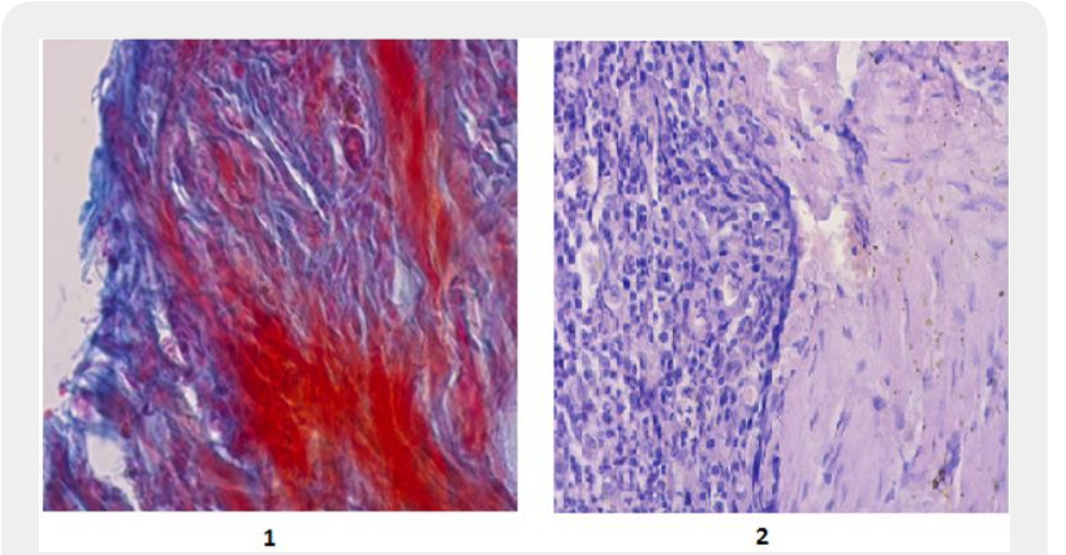

Figure 1: *1 - Edema, fibrinoid swelling and fibrinoid necrosis of connective tissue in the wall of the fistula tract: coloring by Mallory, x 400. 2 - Granulation tissue in the wall of the fistula tract in the 1st group: staining with hematoxylin and eosin, x 400.*

The connective tissue elements of the stroma was represented by fibroblasts, fibrocytes and macrophages. Massive growths of granulation tissue, as well as young connective tissue were detected.

An immunohistochemical study of the connective tissue component of the tissues of the fistula walls located near the internal fistulous opening with MCA to interstitial collagens were revealed both collagens of types I and III. At the same time, mature type I collagen predominated which was revealed in the form of a linear and focal bright glow. Less mature type III collagen was predominantly defined as focal weak or moderate luminescence intensity in the zones of localization of granulation and maturing connective tissue. Among the connective tissue fibers, as well as perivascular, focal inflammatory cell infiltration was noted. The cell infiltrate was dominated by CD18 neurophilic granulocytes (Figure 2), NK-cells (CD16), as well as IL-6 and TNFα-producing cells, were quite often detected. Just as in the tissues of the walls of the fistula canal located near the inner opening, the number of NK-cells (CD16) was large around the areas of disorganization of the connective tissue, the cells producing IL-6 and TNFα (Figure 2) were localized more around and in the granulation tissue. Immunohistochemistry revealed both interstitial collagens with a predominance collagen of I type who characteristic of mature connective tissue, whereas collagen of III type in the form of weak or moderate focal immunofluorescence was localized in granulation tissue and young maturing connective tissue.

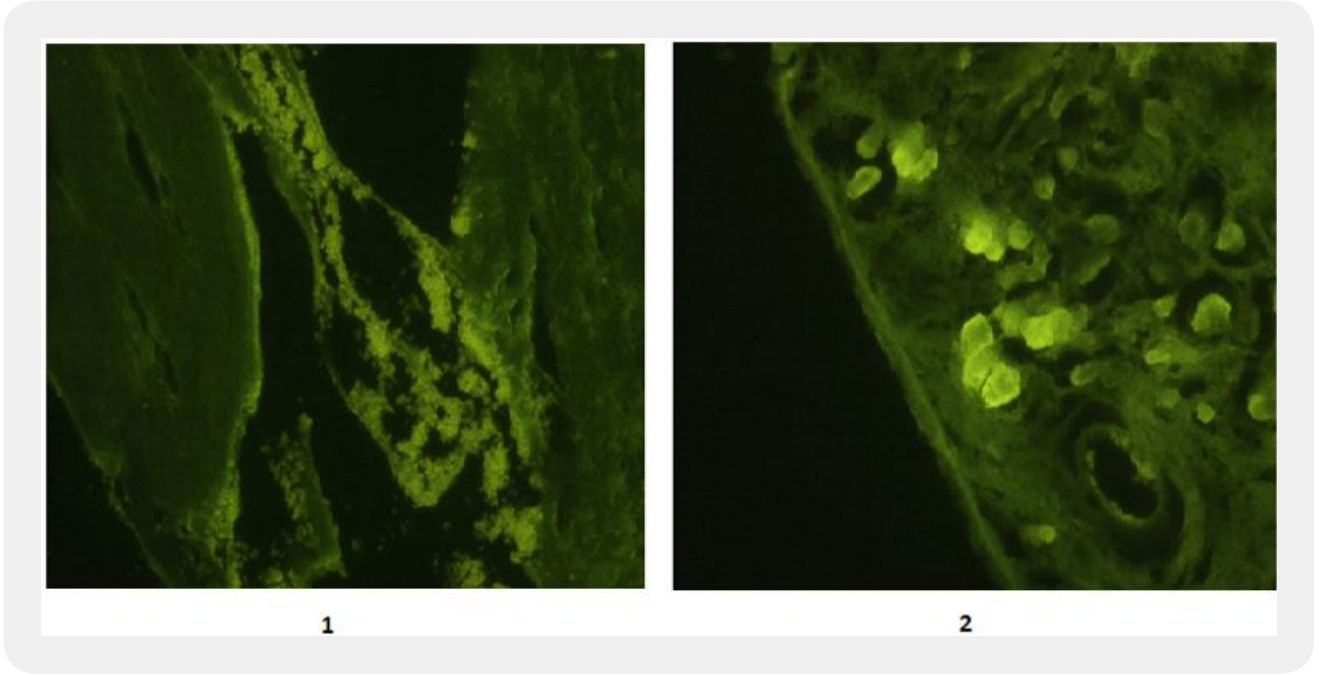

Figure 2: *1 - The abundance of neutrophilic leukocytes (CD18) in the inflammatory infiltrate of the fistula tract wall: indirect Immunofluorescence reaction with MCA to CD18, x100; 2 - Cells-producers of TNFα in the granulation tissue of the wall of the fistula tract: indirect Immunofluorescence reaction with MCA to TNF-α, x 600.*

An immunohistochemical study revealed a predominance of neutrophilic granulocytes (CD18) located both in the connective tissue and in the muscle component (Figure 2). In addition, there were cells expressing receptors for IL-6 and TNF-α and natural killer cells - CD16. The localization of these cells attracted attention. The number of CD16 - NK cells was high around the areas of disorganization of connective tissue and myocytolysis (Figure 3). Thus, the average number of these cells were 13.9 ± 1.63 and 13.73 ± 1.41, the cells producing IL-6 were 30.83 ± 1.62 and 25.63 ±1.99, TNFα were 23.7±1.83 and 18.73 ± 1.82 (Table 2) and them more often was determined in the zone of localization of granulation tissue.

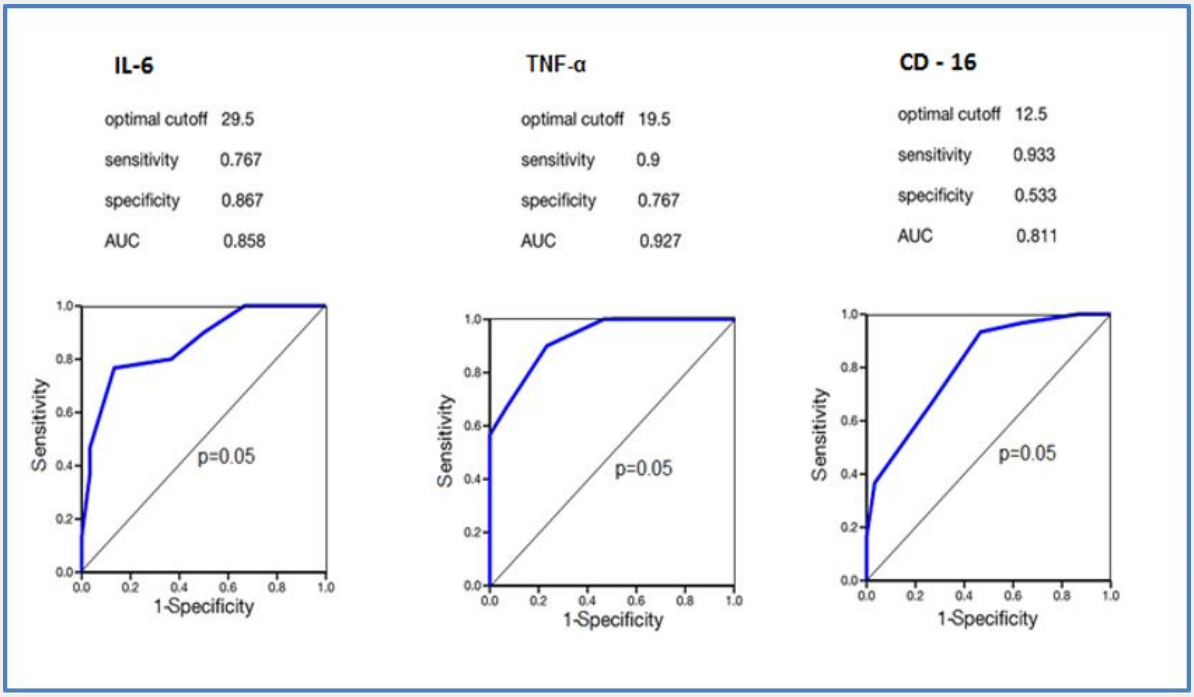

Figure 3: *ROC curves of the measured values in patients with transsphicteric fistula in the 2nd group*

Table 2: *The number of individual clones of immune cells*

GROUPS	IL-6		TNF-α		CD-16	
1st group	1	2	1	2	1	2
	30.83±1.62	25.63±1.99	23.7±1.83	18.73±1.82	13.9±1.63	13.73±1.41
	95% CI 4.3262-6.138, P = 0.000		*95% CI 0.027-5.913, P = 0.000*		*95% CI 0.6177-0.9577, P = 0.667*	
2nd group	30.3±2.84	27.53±1.89	21.93±2.03	18.4±1.35	14.17±1.39	12.27±1.48
	95% CI 1.523 -6.138, P = 0.000		*95% CI 2.639-4.421, P = 0.000*		*95% CI 1.158-2.642, P = 0.000*	

Note: 1 - intrasphincteric part of fistula; 2 - extrasphincteric part of fistula; CI – 95% confidence interval

In our study, we determined the dependence of the sensitivity of the indicators (the proportion of correctly predicted cases) on the specificity (the proportion of erroneous predictions) to determine the optimal threshold values at which the optimal ratio of sensitivity and specificity for these values was achieved. When analyzing, only indicators of the dynamics of changes in IL-6 (AUC = 0.954) and TNF-α (AUC = 0.969) showed a good result in the first group of patients: the cutoff value for assessing IL-6 was 28.5 with a sensitivity of 0.897 and a specificity of 0.931; the cutoff value for TNF-α assessment was 20.5 with a sensitivity of 1.0 and specificity (Figure 4).

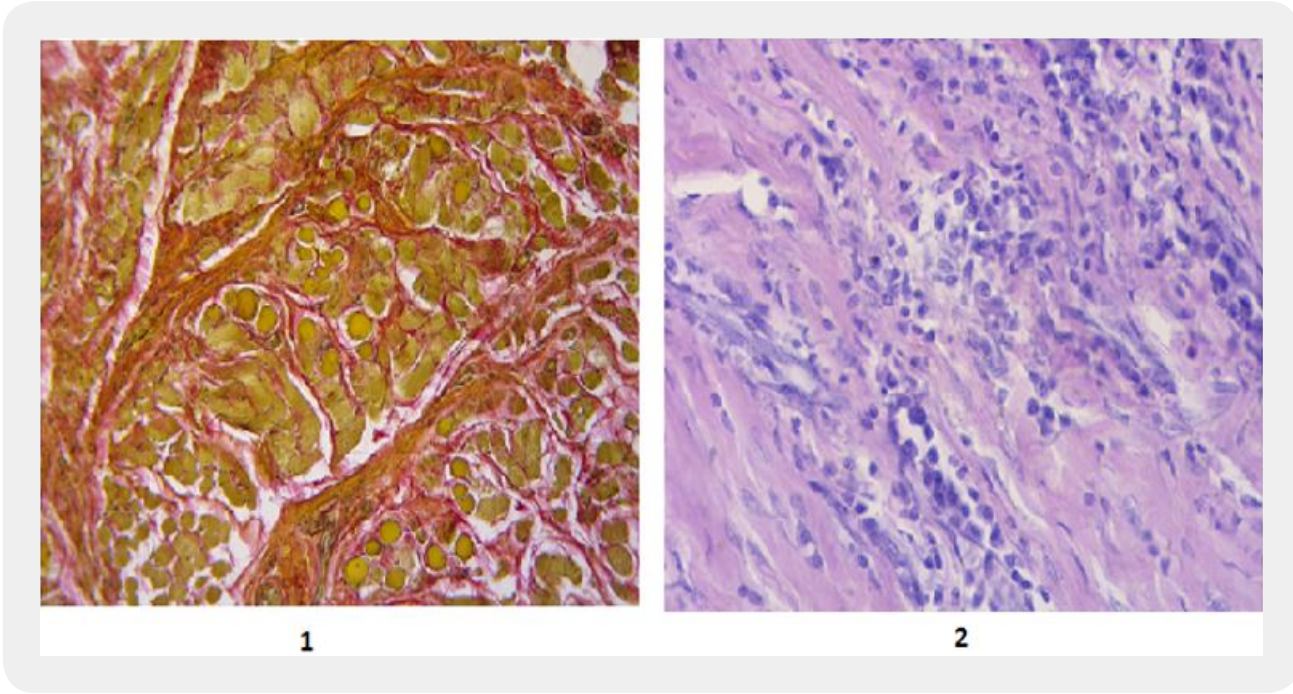

Figure 4: *1-Edema, atrophy and disorientation of muscle fibers against the background of proliferation of fibrous connective tissue: paint according to van Gieson, x 100; 2 - Diffuse inflammatory cellular infiltration between muscle and connective tissue fibers in the tissues of the fistula tract wall: staining with hematoxylin and eosin, x 400.*

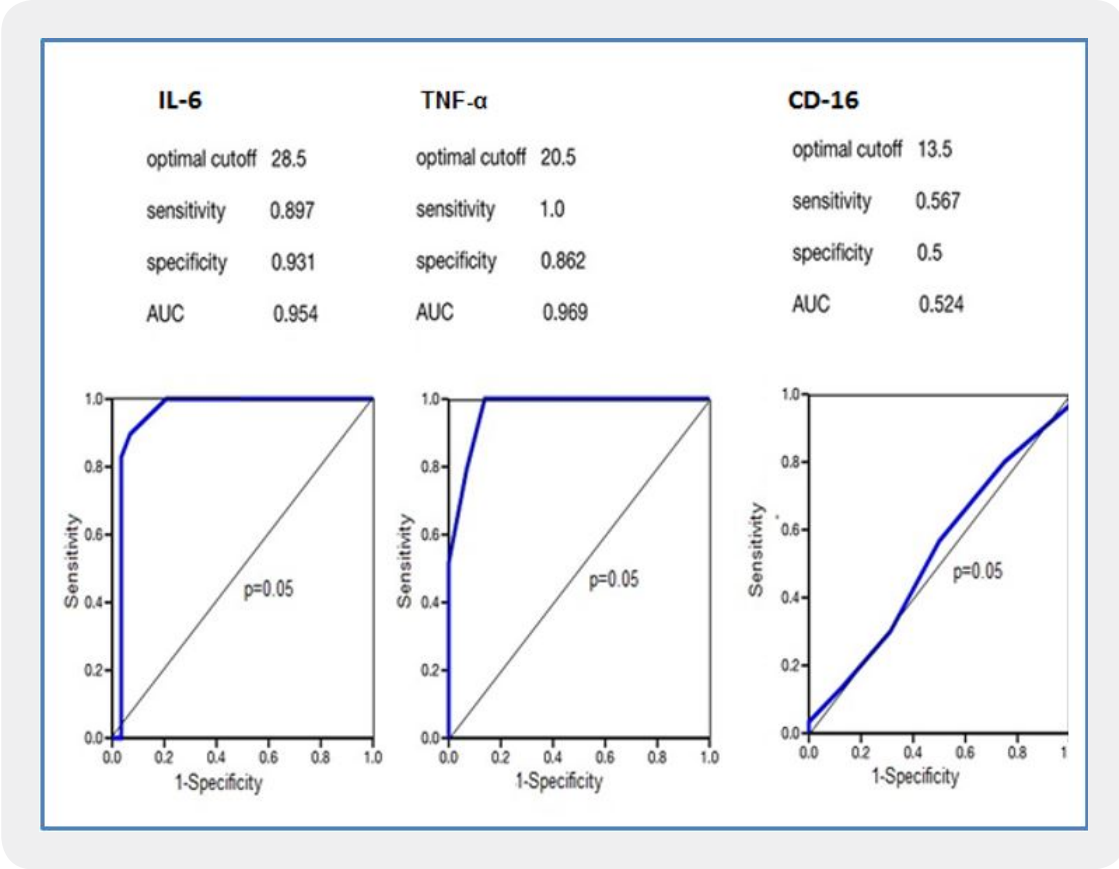

Figure 4: *ROC curves of the measured values in patients with transsphicteric fistula in the 1st group*

Between the connective tissue fibers in patients of the 2nd group there was a moderately expressed focal and in some places significantly pronounced inflammatory infiltration, represented by lymphocytes, macrophages and neutrophils. Along the inner surface of the fistulous tract, as well as in the 1st group, growths of granulation tissue were revealed, with an abundance of microvessels and cellular elements of connective tissue origin with an admixture of immune cells. Periarterially, a muff-like proliferation of connective tissue was observed, clearly visible when stained according to Malory in the form of blue. Perivascular proliferation of connective tissue led to a sharp narrowing of the lumen of the vessels, in places up to their obliteration, and this, in turn, aggravated the alterative changes due to chronic hypoxia. The muscular component was presented in the form of transversely and longitudinally oriented muscle fibers, the focal normal orientation of the muscle fibers was impaired, the phenomena of edema, fibration and atrophy of muscle fibers were revealed. Between myocytes, individual muscle fibers and their bundles, there was a proliferation of fibrous connective tissue, stained blue according to Malory, and red according to van Gieson (Figure 3).

Immunohistochemistry revealed the predominance of neutrophilic granulocytes (CD18) and cells expressing receptors for IL-6 and TNFα. As in the observations of the 1st group of patients the numbers of NK-cells (CD16) were with large areas of disorganization of connective tissue, myocytolysis and on average were 14.17 ± 1.39 and 12.27 ± 1.48, respectively (Table 2). In the same time, IL-6-producing cells (30.3 ± 2.84 and 27.53 ± 1.89, respectively) and TNF-α (21.93 ± 2.03 and 18.4 ± 1.35, respectively) were more determined in the zone of localization of granulation tissue. We determined the dependence of the sensitivity of the indicators on the specificity to determine the optimal cutoff values: all indicators of the dynamics of changes in IL-6 (AUC = 0.858), TNF-α (AUC = 0.927) and CD-16 (AUC = 0.811) showed a good result in the 2nd group of patients (Figure 3).

An immunohistochemical study in the connective tissue of the fistula walls located near the inner opening revealed the luminescence of interstitial collagens of both types I and III with a noticeable predominance of mature collagen the type of I. Less mature the collagen type of III in the form of focal weak or moderate luminescence intensity was detected in granulation and maturing connective tissue. Near the external opening of the fistula keratinizing squamous multirow epithelium with symptoms of acanthosis was noted. The epidermal basement membrane was uneven in thickness due to sclerotic changes as evidenced by focal bright fuchsinophilia when stained according to van Gieson. The vessels of the subepidermal zone are unevenly dilated and were mostly full-blooded. The dermis was represented by fibrous connective tissue with signs of disorganization in the form of fibrinoid swelling and fibrinoid necrosis, which was revealed in the form of an orange color when stained according to Malory.

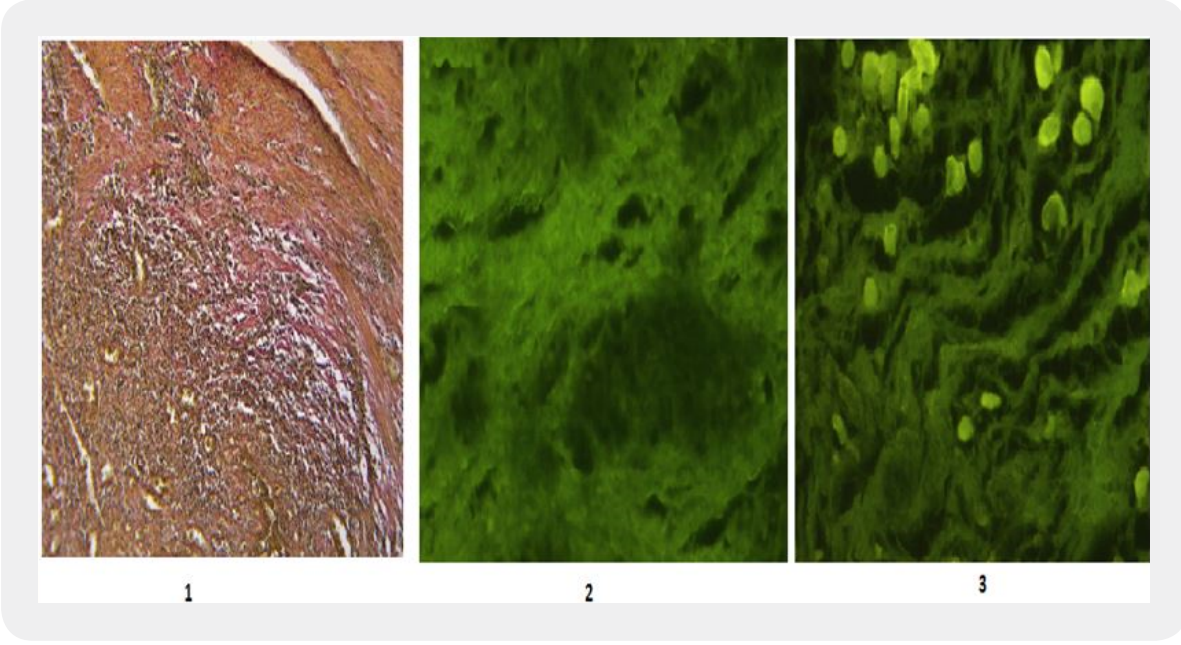

Figure 5: *1-Severe inflammatory infiltration in the tissues of the wall of the fistulous tract and pronounced fuchsinophilia of the connective tissue fibers: according by van Gieson, x 100; 2 - Bright immunofluorescence collagen of type I in the wall of the fistula tract: indirect Immunofluorescence reaction with MCA to collagen of type I, x 600; 3 - IL-6-producing cells in the inflammatory infiltration of the walls of the fistula tract: indirect Immunofluorescence reaction with MCA to IL-6, x 600*

The connective tissue fibers were intensely stained red according to van Gieson, and diffuse inflammatory infiltration was found between the fibrous structures (Figure 5-1). In the described tissues of the walls of the fistulous tract, both types of interstitial collagens were detected, collagen of type I was predominated (Figure 5-2). Focal collagen of type III luminescence was observed mainly in granulation tissue and maturing connective tissue, as well as the presence of IL-6-producing cells in the fistula tract (Figure 5-3).

Discussion

To sum up, as a result of a comparative study of both histological and immunohistochemical features of the tissue of the wall of the fistula tract in both groups of observations located near the internal fistula significant differences were found for TNF-α and IL-6. Nevertheless, intergroup comparisons of the extrasphincteric wall of the fistula tract in the 1st and 2nd groups of patients were significant for IL-6, TNF-α, CD-16, and the chronic inflammatory process in all cases was manifested by the presence of a pronounced diffuse and focal inflammatory infiltration located both between connective tissue and muscle fibers, in which lymphocytes, macrophages and plasma cells were detected. It was found that the predominance of neutrophilic granulocytes among the cellular elements of the inflammatory infiltration of CD18 indicates an exacerbation of the chronic inflammatory process. It has long been known that in the phase of attenuation of the inflammatory process in various tissues granulation tissue develops. Subsequently, against the background of sclerosis in particular perivascular due to which the vascular lumens narrow, which we noted in almost all of our observations, tissue hypoxia develops and this in turn stimulates collagen formation [18]. The greater number of cells-producing IL-6 and TNF-α in granulation tissue and in maturing connective tissue, which was shown in our study, may also be due to the active participation of these cytokines in the stimulation of collagenosis processes [19].

In 1999, A. J. Singer and R.A. Clark demonstrated [20] showed that proinflammatory cytokines such as IL-6 and TNF-α play an important role in the inflammatory phase of wound healing. It is also known, that in chronic inflammation increases the level of TNF-α [21]. The literature also contains data on the role of proinflammatory cytokines in the development and maintenance of anal fistulas in Crohn's disease [14], and TNF-α in the inflammatory infiltrate induces epithelial-mesenchymal transformation and stimulates matrix metaloproteases, which leads to tissue remodeling and fistula formation in Crohn's disease [15].

The significant content of TNFα-producing cells in the stack of the fistulous course in all our observations may indicate a significant role of this interleukin in the morphogenesis of fistula formation.

In all observation groups were pronounced sclerosis was revealed, in the form of an overgrowth of mature connective tissue and was accompanied the presence of interstitial collagens of types I and III with a significant predominance of the mature collagen of type I. Herewith, in the zones of destruction of the connective tissue and muscle components of the wall of the fistulous tract we revealed the maximum number of NK cells (CD16) - natural killers. As you know, these cells express a variety of receptors characteristic of lymphocytes and its the main function is cytotoxic action against target cells. In addition, these cells are able to secrete cytokines and some chemokines and the appearance of these cells in the zones of alteration of the connective and muscle tissue which arose under the influence of chronic hypoxia as the result of phenotypic changes caused by oxidative stress [22].

Thus, as the our studies have shown, at least two links are revealed in the morphogenesis of sclerosis of the tissues of the fistula tract wall: 1) the cytokine-stimulating function of immune cells which have been done expressing receptors for IL-6 and TNF-α and 2) chronic hypoxia which have stimulated the synthesis of interstitial fibroblasts, fibrocytes and collagens that may lead to the maturation of connective tissue. Pronounced alterative changes in the overdeveloped connective tissue component manifested by fibrinoid changes, namely fibrinoid swelling and fibrinoid necrosis, as well as the presence of foci of myocytolysis could being a consequence of both tissue chronic hypoxia, also the activiting of lysosomal enzymes which contained in neutrophilic granulocytes and as well as by the cytotoxic effects of NK-cells (CD16). We would like to point out that morphological studies were showed that in the walls of the fistula there is always an inflammatory process with exacerbation on the background of alternative changes in the connective tissue and muscle components and as well as severe sclerosis. Moreover, all processes were accompanied by the production of a large number of IL-6 and TNF-α-producing cells not only in inflammatory infiltration but also in granulation tissue and immature connective tissue which indicates the active role of these cytokines in stimulating of collagenogenesis and are possible causes of recurrence of fistulas after surgery if their surgical correction is insufficient.

It is known that the latent inflammation underlies the most dangerous human diseases, including cardiovascular diseases, atherosclerosis, type 2 diabetes, and tumors of various localizations, and the main regulators of inflammatory inflammation have been tissue macrophages which exhibit increased inflammatory activity. We assume that the increased activation of tissue macrophages is a consequence of changes in peripheral blood monocytes under the influence of such soluble factors as disorders of glucose metabolism and increased blood peroxidation, disruption of molecular mechanisms in the regulation of growth factors, modified lipoproteins, etc. Monocyte activation of these latent factors leads to increased expression of intracellular and surface biomarkers (for example, FcfRI - CD16) and as our research has shown that can be used for existing latent inflammation.

After confirming the studies of other authors and showing some of their features in our study, we also had a question about revising the cryptoglandular theory of the origin of anal fistulas [23]. As the authors noted, Park's cryptoglandular theory is currently widely accepted, although there has been very little follow-up research to support or refute it. As far back as in 1967, J.C. Goligher and co-authors generally questioned the cryptoglandular hypothesis of the formation of anif anal fistulas, showing in their study the absence of a connection between their formation and the presence of an anal crypt in about 2/3 of the patients they treated [24]. In our study was shown that pro-inflammatory cytokines such as IL-6 and TNF-a were determined in large quantities in the fistula tract. It research have showed that these indicators had positive dynamics of changes in IL-6 and TNF-α (AUROC more than 0.8 in both the intrasphincteric and extrasphincteric parts of the fistula tract). Microscopic examination in the tissues of the walls of the fistulous tract, we had revealed signs of a chronic inflammatory process in the acute stage against the background of alterative changes in the connective tissue and muscle components, as well as severe sclerosis with the predominance of 1st type of mature collagen. Among the cellular elements of inflammatory infiltration, CD18 neurophilic granulocytes predominated, which indicates an exacerbation of the chronic inflammatory process. A large number of cells producing IL-6 and TNF-α in granulation tissue and in maturing connective tissue may be due to the active role of these cytokines in stimulating collagenosis processes. The favorite localization of

NK cells (CD16) turned out to be zones of destruction of both connective tissue and muscle components of the studied material.

We are not outside experts in revising the cryptoglandular theory of anal fistula, as for these used of various surgical interventions did not lead to ideal long-term treatment results [7]. Future studies should focus on the etiology and pathogenesis of anal fistula. According to some of the findings described above, one single pathway for all perianal fistula formation seems unlikely.

Conclusion

Thus, as the our studies have shown, at least two links are revealed in the morphogenesis of sclerosis of the tissues of the fistula tract wall: 1) the cytokine-stimulating function of immune cells which have been done expressing receptors for IL-6 and TNF-α and 2) chronic hypoxia which have stimulated the synthesis of interstitial fibroblasts, fibrocytes and collagens.

Conflict of Interest

The authors declare no conflict of interest.

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