ANALYSIS

24(106), November - December, 2020

ISSN 2321–7359
EISSN 2321–7367

Medical Science

# Genetic and morphological aspects of intestinal anastomotic leak development

**Yaroslav Y. Voitiv[1]✉, Oleksandr Y. Usenko[2], Viktor Y. Dosenko[3], Olena O. Dyadyk[4], Kateryna G.Valikhnovska[5], Ali Dzhemiliev[6]**

[1]Associate Professor, PhD (Med), Department of Surgery and Transplantology, Shupyk National Medical Academy of Postgraduate Education, Kyiv, Ukraine
[2]Professor, DSci (Med), Director of Shalimov National Institute of Surgery and Transplantology, National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine
[3]Professor, DSci (Med), Head of General and Molecular Pathophysiology Department of Bogomoletz Institute of Physiology, Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine, Kyiv, Ukraine
[4]Professor, MD, DSci (Med), Head of department Pathologic and Topographic Anatomy department of the Shupyk National Medical Academy of Postgraduate Education, Kyiv, Ukraine
[5]Assistent Professor, PhD (Med), Department of Surgery and Transplantology, Shupyk National Medical Academy of Postgraduate Education, Kyiv, Ukraine
[6]General surgery resident, Shalimov National Institute of Surgery and Transplantology, Kyiv, Ukraine

✉**Corresponding author**
Associate Professor, PhD (Med), Department of Surgery and Transplantology, Shupyk National Medical Academy of Postgraduate Education, Kyiv, Ukraine;
Email: voitiv.yaroslav@gmail.com

**Citation**
Yaroslav Y. Voitiv, Oleksandr Y. Usenko, Viktor Y. Dosenko, Olena O. Dyadyk, Kateryna G.Valikhnovska, Ali Dzhemiliev. Genetic and morphological aspects of intestinal anastomotic leak development. *Medical Science*, 2020, 24(106), 4278-4285

## ABSTRACT

*Aim:* To analyze the frequency of polymorphic variants of genes MMP-2 ($C^{-1306}$→T) and TIMP-2 ($G^{303}$→ A) in patients with intestinal anastomotic leak and establish the correlation with morphological changes. *Materials and methods*: The object of the study comprises 17 patients with anastomotic leak, who were treated in the Shalimov National Institute of Surgery and Transplantology during 2017-2019. Laboratory, genetic, histological, immunohistochemical studies and statistical analysis were performed. *Results*: As a result of genetic and statistical analysis of matrix metalloproteinase-2 ($C^{-1306}$→ T) and tissue inhibitors of metalloproteinase-2 ($G^{303}$→ A) genetic polymorphisms, genotype variants have been identified that are associated with the risk of intestinal anastomotic leak development. Significant differences in the distribution of genotypes in the studied groups were revealed. In immunohistochemical study of tissues with monoclonal antibodies to α-smooth muscle actin revealed uneven focal expression in smooth muscle cells and fibroblast; with monoclonal antibodies to Collagen IV there is a moderate positive expression in the basement membrane of blood vessels, in smooth muscle cells of the muscular layer of the vascular wall, in areas of connective tissue. *Conclusions*: Intestinal anastomotic leak is 1.36 times more common in carriers of homozygous CC genotype of the matrix

metalloproteinase-2 gene and twice less common in minor homozygotes of TT. It is statistically significant that in the group of patients with intestinal anastomotic leak the GG variant of the tissue inhibitors of metalloproteinase-2 gene was detected 1.6 times more often. Carriers of minor homozygotes of AA genotype in the group with anastomotic leak were not detected, while a similar genotype in the control group was found in 10% ($p<0.05$). Immunohistochemical examination of small and large intestinal tissues with monoclonal antibodies to Collagen IV and α-smooth muscle actin revealed signs of pathological connective tissue remodeling in the areas of anastomotic leak.

**Keywords**: Anastomotic leak, matrix metalloproteinase-2, tissue inhibitor of matrix metalloproteinase-2, genetic polymorphism, connective tissue remodeling.

## 1. INTRODUCTION

One of the most urgent problems of abdominal surgery is intestinal an astomotic leak. The incidence of such complications, according to various authors, ranges from 2-8.1% in small intestinal anastomosis, to 3.8-14.6% in operations on the colon (Eto et al., 2018; Kang et al., 2013). Anastomotic leak is accompanied by the mortality rate of 14-21.7% (Pitel et al., 2013), with the development of disseminated peritonitis, abdominal sepsis, mortality increases up to 43-82.9% (Sharonne et al., 2012; Melnyk & Poida, 2016). So far, there is no single point of view in the surgical community regarding the causes of anastomotic leak development and surgical tactics in the development of these complications. Although there is no doubt about the role of regenerative processes in the formation of intestinal anastomosis (Marjanovic & Hopt, 2011), scientific publications and research at the current methodological level on this topic are not enough. An in-depth study of the mechanisms of reparative regeneration in the area of anastomosis and possibilities of regenerative processes stimulation, adequate restoration of morpho-functional characteristics of digestive organs that have been anastomosed is necessary.

Given the almost unexplored role of genetic predisposition in the development of postoperative complications, namely the anastomotic leak, our goal is to study the polymorphism of genes encoding matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2). The choice of these genes was not accidental – we were guided by the main known pathophysiological mechanisms involved in the formation of the intestinal anastomosis (Rijcken et al., 2018). There are almost no data on the role of MMPs in the development of anastomotic leak. Having analyzed the specialized literature, we found a small number of publications on the study of MMP expression in the colorectal anastomotic leak (Stumpp et al., 2009; Agren et al., 2006), postoperative peritonitis (Hästbacka et al., 2007). However, we have not found publications on the study of genetic polymorphism of matrix metalloproteinases and their regulators in terms of the development of anastomotic leak.

### Aim

To analyze the frequency of polymorphic variants of genes MMP-2 ($C^{-1306}\rightarrow T$) and TIMP-2 ($G^{303}\rightarrow A$) in patients with intestinal anastomotic leak and establish the correlation with morphological changes.

## 2. MATERIALS AND METHODS

A prospective trial was based on data from 17 patients, who were treated in the Shalimov National Institute of Surgery and Transplantology during 2017-2019. All patients suffered intestinal anastomotic leak. For the assessment of genetic polymorphism in the population, 80 practically healthy people matched by gender and age with the experimental group were examined. Genetic studies were performed in the laboratory of the department of general and molecular pathophysiology at the Bogomoletz Institute of Physiology NAS of Ukraine. Buccal epithelium was collected using buccal brushes followed by freezing of the samples and storing them at -20° C. DNA for the genotyping was extracted from the samples using DiatomTM Prep 200 ("Isogen Laboratory", RF) according to the manufacturer's protocol.

The following polymorphisms were studied by real-time PCR: $C^{-1306}\rightarrow T$ (MMP2), rs243865 and $G^{303}\rightarrow A$ (TIMP2), rs9900972. Amplification reactions were performed using the Fast Real-time PCR System (Applied Biosystems, USA) in a final reaction volume of 20 µl containing 2X TaqMan Universal Master Mix (Applied Biosystems, USA), assay C_1792560_10 and template DNA. Amplification of gene fragments consisted of a denaturation step at 95° C for 20 sec, followed by 40 cycles of amplification at 95° C for 3 sec and 60° C for 30 sec. Data analysis was performed with 7500 Fast Real-Time PCR Software (Applied Biosystems, Foster City, USA).

Morphological studies were performed at the department of pathological and topographic anatomy at Shupyk National Medical Academy of Postgraduate Education.

Histological and immunohistochemical studies (IHS) were performed to assess the features and properties of connective tissue. Histological sections 3-4 μm thick were stained with hematoxylin, eosin, and picrofuxin according to Van Gieson's and Masson's methods (Masson Trichrome Kit 87019), PAS reaction was performed (Rosai and Ackerman's Surgical Pathology Seven edition / edited by J.Rosai- Elsevier Inc. Vol 1, Ch. 2,3-p.25-95). IHS studies were performed with the following markers (Thermo Scientific, USA): monoclonal antibodies to the type IV collagen (clone CIV22), α-smooth muscle actin α-SMA (clone 1A4 (asm-1)).

The main part of the statistical analysis was performed using the program "Statistica 7.0" (SPSS) and Excel 2000. Nominal data were presented in the form of quantitative and percentage values. The significance of differences in mean values in groups with different genotypes was determined using the method of one-way analysis of variance (URL: http://www.dgmp.kyiv.ua/index.php/snip-ka). The correspondence of genotype distribution was checked using the Hardy-Weinberg test. Pearson's χ2 test was used to compare the distribution of genotypes in the experimental and control groups.

## 3. RESULTS

To identify the possible association of polymorphic variants of the MMP-2 ($C^{-1306}$→T) and TIMP2 ($G^{303}$→A) genes with the risk of anastomotic leak, we performed a one-way analysis of variance of the frequency of genotypes in the studied groups of patients (table 1).

**Table 1** The distribution of polymorphic variants of genes MMP-2 ($C^{-1306}$→T), and TIMP-2 ($G^{303}$→A), in the studied groups

| The studied gene | | Control group n=80 (%) | Experimental group n=17 (%) |
|---|---|---|---|
| MMP2 ($C^{-1306}$ →T) | CC | 38 (47,5%) | 11 (64,7%) |
| | CT | 34 (42,5%) | 5 (29,4%) |
| | TT | 8 (10%) | 1 (5,9%) |
| Hardy-Weinberg test ($\chi^2$, p) | | $\chi^2$=0,01,p>0,05 | $\chi^2$=0,17,p>0,05 |
| χ2 test, ($\chi^2$,p) | | - | $\chi^2$=0,206,p>0,05 |
| TIMP2 ($G^{303}$→A), | GG | 50 (50%) | 14 (82,4%) |
| | GA | 32 (40%) | 3 (17,6%) |
| | AA | 8 (10%) | 0 (%) |
| Hardy-Weinberg test ($\chi^2$, p) | | $\chi^2$=0,18,p>0,05 | $\chi^2$=0,15,p>0,05 |
| χ2 test, ($\chi^2$,p) | | - | $\chi^2$=6.278,p=0,043 |

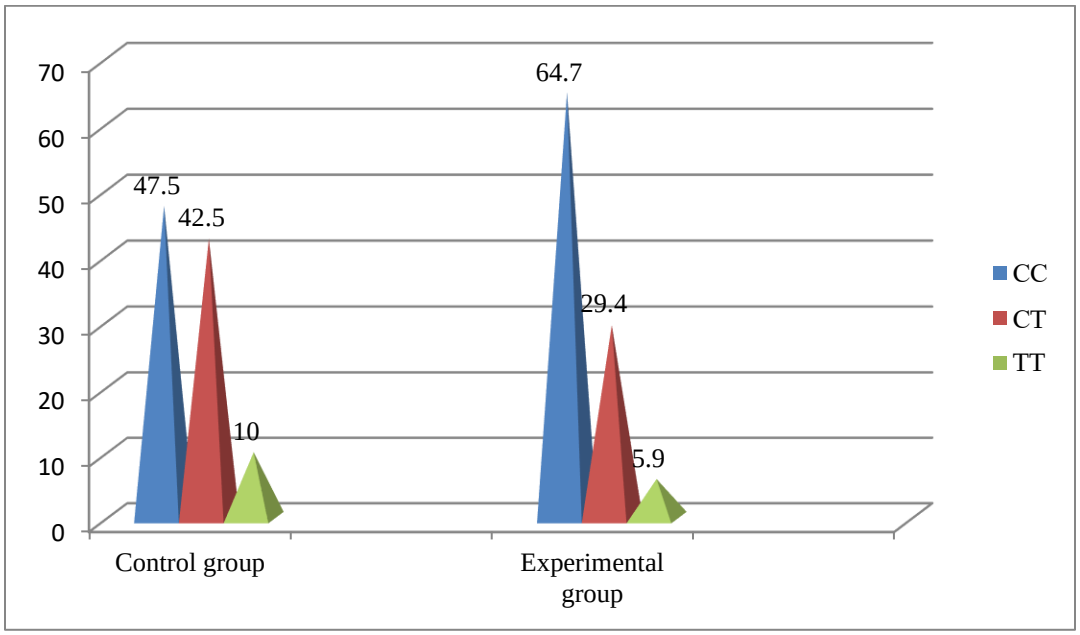



**Chart 1** Distribution frequency of allelic polymorphism (%) of the promoter ($C^{-1306}$→T) of MMP2 gene

Analysis of the multiplicative model of inheritance of the MMP-2 gene ($C^{-1306}$→T), comparison of the control (n=80) and experimental groups with anastomotic leak (n=17) showed compliance with the distribution of genotypes according to Hardy-Weinberg's law (p>0.05), which was tested in the control group using the test χ2 with 1 degree of freedom, without Yates correction. Using the test χ2 with 2 degrees of freedom, we did not find statistically significant differences in the distribution of genotypes in the group of sick people and the group of practically healthy people (p>0.05).

It is noteworthy that in the experimental group there were half as many carriers of the homozygous TT genotype as compared with the control group: 5.9% versus 10% (p>0.05), respectively. However, the number of carriers of the CC genotype dominant in all groups was greater in the group with anastomotic leak (experimental group): 64.7% versus 47.5% (p>0.05) in the control group (Chart 1).

We were able to find statistically significant differences in the distribution of genotypes (p<0.05) in the analysis of TIMP-2 inheritance models ($G^{303}$→A), in the control group, (n=80) and experimental group with anastomotic leak (n=17). In the examined population in the control group and experimental group, the distribution of carriers of GG, GA, and AA genotypes was significantly similar (chart 2). However, in the group of patients with anastomotic leak the distribution of genotype carriers was significantly different. Thus, the dominant GG variant almost twice significantly exceeded the indicators of control and experimental groups (82.4% vs.50%, p<0.05). Heterozygous GA genotype in the experimental group was more than twice as rare as in the control group (17.6% vs. 40%). Carriers of homozygous AA genotype in the group with anastomotic leak were not detected, while a similar variant in the control group was found in 10% (Chart 2).

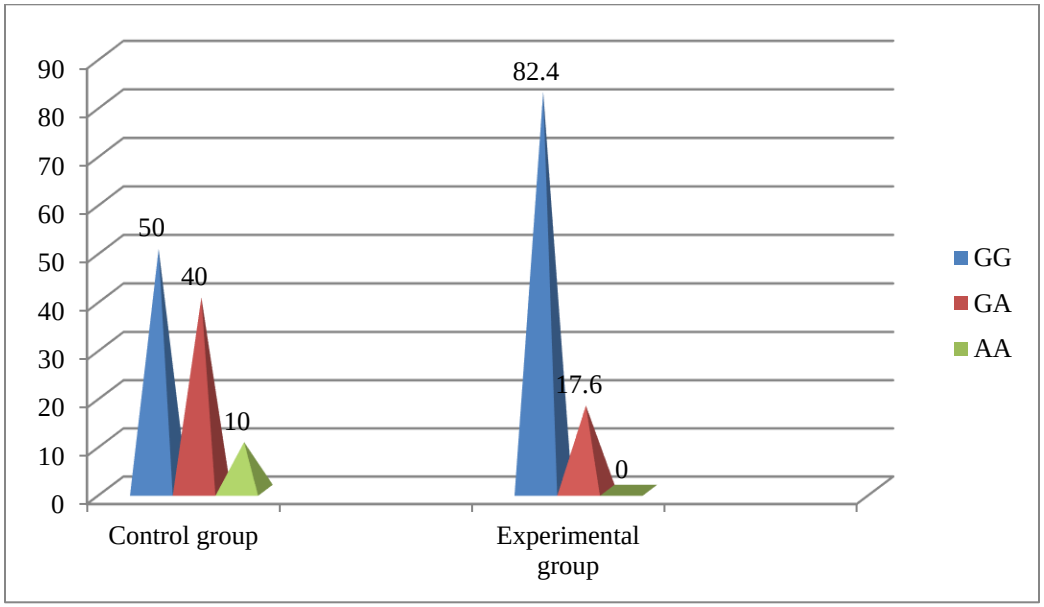
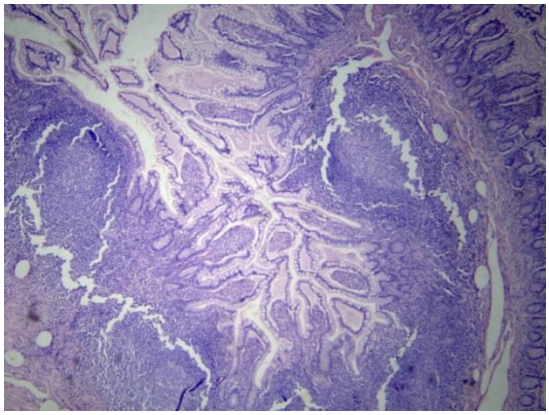



**Chart 2** Distribution frequency of allelic polymorphism (%) of the promoter ($G^{303}$→A) of the TIMP2 gene

During the morphological examination of small and large intestinal tissues fragmets from the areas of anastomotic leak (Fig. 1-2), the following histological changes were observed: uneven growth of connective tissue, diffuse and focal lymphoplasmacytic infiltration, areas of angiomatosis, vascular congestion and hyalinosis of their walls.



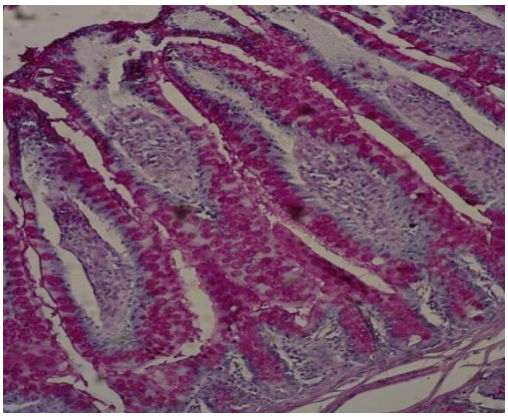


**Figure 1-2** Small intestinal tissue from the area of anastomotic leak. Staining with hematoxylin and eosin (1). Magnification x40; PAS stain (2). Magnification x100.

When performing IHS using monoclonal antibodies to α-SMA, there was a minor, mostly focal expression in the walls of blood vessels, in the area of laminapropria, areas of sclerosis with an intensity of up to 1 point in areas of tissue with anastomotic leak (Fig. 3).

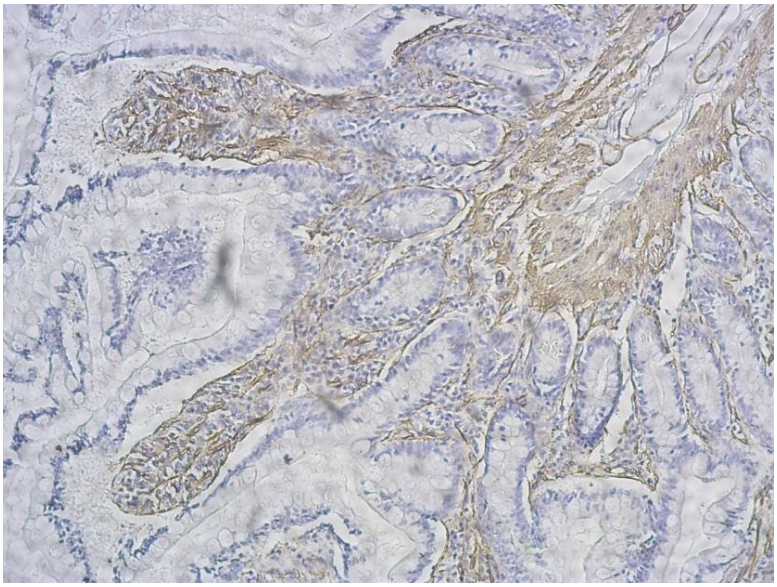


**Figure 3** The tissue of the small intestine from the area of anastomotic leak. HIS using monoclonal antibodies to α-SMA. Magnification x100.

When performing HIS using monoclonal antibodies to Collagen IV in the areas of anastomotic leak (Fig. 4) there is mainly a weak positive expression in the basement membrane of blood vessels, in smooth muscle cells of the muscular layer of the vascular wall, in areas of connective tissue with color intensity up to +, focal up to ++.

## 4. DISCUSSION

As a result of genetic and statistical analysis of the polymorphism of the MMP-2 ($C^{-1306}$→T) and TIMP-2 ($G^{303}$→A) genes, variants of genotypes associated with the risk of development of intestinal anastomotic leak were determined. Thus, in the experimental group with anastomotic leak, carriers of the homozygous CC genotype of the MMP2 gene were found to be 1.36 times more frequent than in the control group. At the same time, the minor TT homozygotes in the group of patients with anastomotic leak were almost half of those in the control group (5.9% versus 10% ($p>0.05$)). In the analysis of carriers of TIMP-2 genotypes, we obtained statistically reliable data: in the group of patients with anastomotic leak GG variant was 82.4%, which is 1.6 times higher than in the control

group (82.4% vs. 54.4%, p<0.05). Carriers of minor homozygotes of AA genotype in the group of patients with anastomotic leak were not detected, while a similar genotype in the control group was found in 10%.

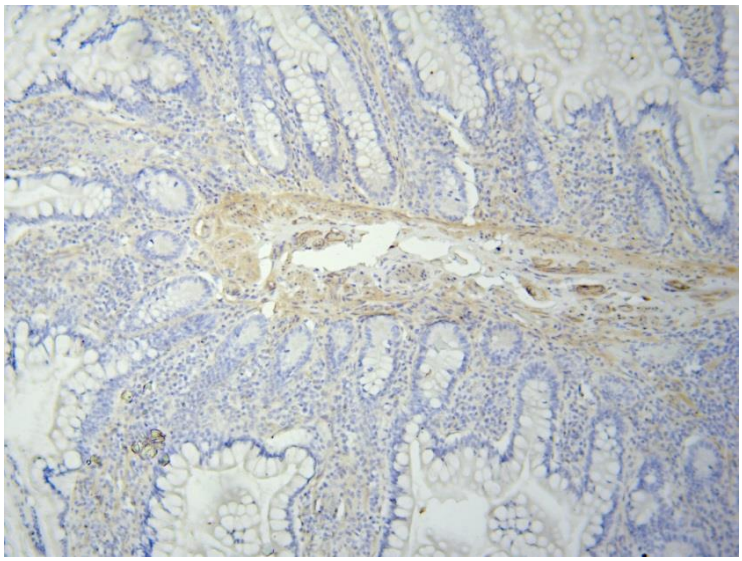


**Figure 4** The tissue of the small intestine from the area of anastomotic leak. HIS using monoclonal antibodies to Collagen IV. Magnification x100

Given the role of matrix metalloproteinases and their inhibitors in the processes of synthesis and proteolysis, connective tissue remodeling, connective tissue protein metabolism, the ability to affect vascular permeability and angiogenesis, the relevance of their study in the context of the pathogenesis of intestinal anastomotic leak is undoubted. In immunohistochemical study of tissues with monoclonal antibodies to α-smooth muscle actin revealed uneven focal expression in smooth muscle cells and fibroblast; with monoclonal antibodies to Collagen IV there is a moderate positive expression in the basement membrane of blood vessels, in smooth muscle cells of the muscular layer of the vascular wall, in areas of connective tissue, which are signs of pathological remodeling of connective tissue.

Understanding the pathogenetic processes underlying the formation of the anastomosis and possible "weaknesses" is no less important than the surgical technique. Morphological changes in the tissues of the small and large intestines are confirmed by our histological and immunohistochemical studies. In our view, the focus of future research on the pathogenetic factors of abdominal postoperative complications should be shifted to the cellular and molecular levels. Thus, a better understanding of the mechanisms of the formation of intestinal anastomosis will contribute to the development of new diagnostic, prognostic, and therapeutic techniques. The identified genetic and morphological changes in the groups with intestinal anastomotic leak are the basis for further study and research of molecular genetic markers that encode the main links in the pathogenesis of anastomotic leak.

## 5. CONCLUSION

Intestinal anastomotic leak is 1.36 times more common in carriers of homozygous CC genotype of the MMP-2 geneand twice less common in minor homozygotes of TT (5.9% vs. 10% (p>0.05)). In the group of patients with the intestinal anastomotic leak it is statistically significant that the GG variant of the TIMP-2 gene was detected 1.6 times more frequent. Carriers of minor homozygotes of AA genotype in the group with anastomotic leak were not detected, while a similar genotype in the control group was found in 10% (p<0.05). IHS study of tissues in the group of patients with anastomotic leak using monoclonal antibodies to α-SMA revealed un even, focal expression in smooth muscle differentiation cells and fibroblasts. IHS study with monoclonal antibodies to Collagen IV revealed a moderate positive expression in the basement membrane of blood vessels, in the smooth muscle cells of the muscular layer of the vascular wall, in areas of connective tissue.

**Abbreviation**

α-SMA - α-smooth muscle actin;
AIC- Akaike information criterion;
IHS- immunohistochemical studies;
MMP-2 - matrix metalloproteinase-2;

MMPs - matrix metalloproteinases;
TIMP-2 - tissue inhibitor of matrix metalloproteinase-2;

**Declarations**

The research highlighted in the article became a fragment of the research work of the Department of Surgery and Transplantology of the Shupyk National Medical Academy of Postgraduate Education on the topic: "Undifferentiated connective tissue dysplasia as a risk factor in abdominal surgery" State Registration Number 0118U001239, deadline 2018-2022.

**Ethics approval and consent to participate**

This cohort study was conducted with the Ethics Committee of the Shupyk National Medical Academy of Postgraduate Education and is compliant with the terms of the Helsinki Declaration (prot. N4, 02.04.2018). All participants completed a written consent.

**Competing interests**

The authors declare that they have no competing interest

**Funding**

This research did not receive any grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Authors' contributions**

Yaroslav Y. Voitiv ORCID 0000-0003-2638-9352 - A, B, D
Oleksandr Y. Usenko ORCID 0000-0003-4957-4104 - F
Viktor Y. Dosenko ORCID 0000-0002-6919-7724 – E
Olena O. Dyadyk ORCID 0000-0002-9912-4286 - E
Kateryna G.Valikhnovska ORCID 0000-0001-8287-0736 - C
Ali Dzhemiliev ORCID 0000-0002-0529-7902 – C
All authors read and approved the final manuscript.
A - Work concept and design
B – Data collection and analysis
C – Responsibility for statistical analysis
D – Writing the article
E – Critical review
F – Final approval of the article

## REFERENCES AND NOTES

1. Agren MS, Andersen TL, Mirastschijski et al., Action of matrix metalloproteinases at restricted sites in colon anastomosis repair: an immunohistochemical and biochemical study. Surgery. 2006 Jul; 140(1):72-82.
2. Emile Rijcken, Larissa Sachs, Thomas Fuchs et al., Growth factors and gastrointestinal anastomotic healing. J Surg Res. 2014 Mar;187(1):202-10.
3. Eto K, Urashima M, Kosuge M, et al. Standardization of surgical procedures to reduce risk of anastomotic leakage, reoperation, and surgical site infection in colorectal cancer surgery: a retrospective cohort study of 1189 patients. Int J Colorectal Dis 2018;33:755-62.
4. HästbackaJ, Hynninen M, Kolho E, et al., Collagenase 2/matrix metalloproteinase 8 in critically ill patients with secondary peritonitis. Shock. 2007 Feb;27(2):145-50.
5. Kang CY, Halabi WJ, Chaudhry OO, et al. Risk factors for anastomotic leakage after anterior resection for rectal cancer. JAMA Surg 2013;148:65-71.
6. Marjanovic, G., Hopt, U. Physiologie der Anastomosenheilung. Chirurg 2011: 82, 41–47.
7. Melnyk VM, Poida OI. Khirurhichna taktyka pry nespromozhnosti shviv mizhkyshkovykh anastomoziv. Klinichna khirurhiia. 2016; 6:8-12.
8. Pitel S,Lefèvre JH, Tiret E. Redo coloanal anastomosis: aretrospective study of 66 patients. Ann Surg. 2012; 256 (5):806-811.
9. Sharonne de Z, Usama AA, Rogier AR. Update of complications and functional outcome of the ileo-pouch analanastomosis: overview of evidence and meta-analysis of 96 observational studies. Int. J. Colorectal Dis. 2012;27:843-853.

10. Stumpf M, Junge K, Wendlandt M, et al.,Risk factors for anastomotic leakage after colorectal surgery. Chir. 2009 Jun; 134(3):242-8.

**Data and materials Availability**

All data associated with this study are present in the paper.

**Peer-review**

External peer-review was done through double-blind method.

**Article History**

Received: 04 October 2020

Reviewed & Revised: 05/October/2020 *to* 12/November/2020

Accepted: 13 November 2020

E-publication: 20 November 2020

P-Publication: November - December 2020



**General Note**

We recommended authors to print article as color digital version in recycled paper. Discovery Scientific Society will not provide any prints for subscription.