TECHNISCHE UNIVERSITÄT MÜNCHEN

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Minimal Height of Anastomotic Rings after Stapled Circular Colorectal Anastomosis as a Risk Factor for Anastomotic Leakage

Anastomotic rings morphology as risk factor for anastomotic leakage

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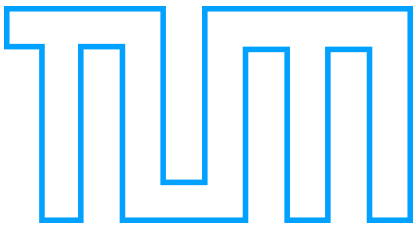

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1. INTRODUCTION

1.1 Background and Current Situation

Anastomotic leakage (AL) is currently the most common serious complication after colorectal surgery (La Regina et al., 2019; Sparreboom et al., 2018). In recent years, although the technical skills have greatly improved, the incidence of AL can be detected in up to 19% of the cases (Gessler, Eriksson, & Angenete, 2017; Iversen, Ahlberg, Lindqvist, & Buchli, 2018; Olsen, Sakkestad, Pfeffer, & Karliczek, 2019). The occurrence of postoperative AL in surgery for colorectal cancer (CRC) increases the mortality of patients and may also lead to a higher local recurrence rate and a drop of long-term survival rate (Artus et al., 2020; Bashir Mohamed et al., 2020; Crippa et al., 2020; Foppa, Ng, Montorsi, & Spinelli, 2020; Ha, Kim, & Lee, 2017). Therefore, in order to effectively improve the tumor-specific survival, early detection and timely treatment of AL following colorectal surgery are of great importance.

1.2 Established Marker of Anastomotic Leakage

Although patients with AL often present with non-specific clinical symptoms such as fever, leucocytosis, dyspnea or abdominal distension, these symptoms often occur late and cannot be used for early detection of AL after colorectal surgery (Gessler, Eriksson, & Angenete, 2017; Karliczek et al., 2009). Therefore, the use of serum inflammatory markers to early predict the occurrence of AL is established, among which the C-reactive protein (CRP) is the most commonly used (Jin & Chen, 2021). After operations with colorectal anastomoses, the patients' CRP increases

significantly on the first day after surgery, to then gradually decrease. Still, the postoperative CRP levels of patients with AL are significantly higher than that of patients with successful healing (Almeida et al., 2012). On the fourth or fifth day after surgery, CRP levels can be used as an indicator to predict AL (Bennis et al., 2012; Platt et al., 2012).

However, the heterogeneous primary disease and surgical methods of the patients enrolled in previous studies, as well as inconsistent diagnostic criteria for AL and inconsistent cut-off values of postoperative CRP have affected the application value of CRP as an early predictor of AL. Therefore, finding reliable and easily detectable predictors in prevention of AL still has high clinical significance.

1.3 Anastomotic Rings

In recent years, intraluminal stapling techniques have been widely used, especially in laparoscopic surgery (Tejedor, Sagias, Flashman, Kandala, & Khan, 2020). So far, anastomotic rings have not received considerable attention as potential risk factor for AL and exact data about the differences in ring morphology is lacking. Their integrity is usually examined qualitatively and the stapler manufacturers do not define a size range of the rings, for instance, the critical value of width and height. As mentioned above, established laboratory makers for AL, as the CRP already have a certain value in predicting postoperative AL (Italian ColoRectal Anastomotic Leakage Study, 2020; McAnena et al., 2020; Scepanovic et al., 2013). Another indicator to predict AL, the Neutrophil to Lymphocyte Ratio (NLR), has established in recent years (Al Lawati et al., 2021; Clemente-Gutierrez et al., 2021; Radulescu et al., 2020).

However, both inflammatory markers have the limitation of being only available postoperatively, while the ring measurements are already available intraoperatively and could have an important impact by triggering early countermeasures to avoid the development of AL.

2. AIM OF THE PRESENT STUDY

A flawless technical performance of stapled anastomoses is crucial to prevent leakage formation following colorectal resections. Here we aim to investigate the morphological configuration of anastomotic rings as an early available prognostic marker for anastomotic leakage and compare the prognostic value to the neutrophil to lymphocyte ratio (NLR) as established marker of anastomotic leakage formation.

3. MATERIALS AND METHODS

3.1 Patients Collective

Informed consent was obtained from all patients included in the study. Patients were included during the study period from August 10th, 2020 to August 12th, 2021. Patient data were collected during the study period, including gender, age, ASA score, Body Mass Index (BMI), operation time, intraoperative blood loss, tumour stage, CRP and NLR.

3.2 Inclusion Criteria

Patients undergoing surgical resection of pathologically confirmed CRC with subsequent stapled circular anastomosis.

3.3 Exclusion Criteria

Patients with the following confounders were excluded:

1) Patients with preoperative signs of infection;

2) Patients with hematological diseases;

3) Patients with additional diagnoses of malignant diseases.

4) Patients with preoperative radiotherapy / chemotherapy or preventive ileostomy.

3.4 Configuration of Anastomotic Rings

To measure the configuration of anastomotic rings during the operation, the surgeons first observed the integrity of the rings, and then using a sterile ruler to measure the minimal height (H_{min}), maximal height (H_{max}), minimal width (W_{min}) and

maximal width (W_{max}) of the fresh rings (as demonstrated in Figure 1). The measurement was performed by two surgeons independently. For the inconsistent results, consent was achieved by repeated measurement of the surgeons together.

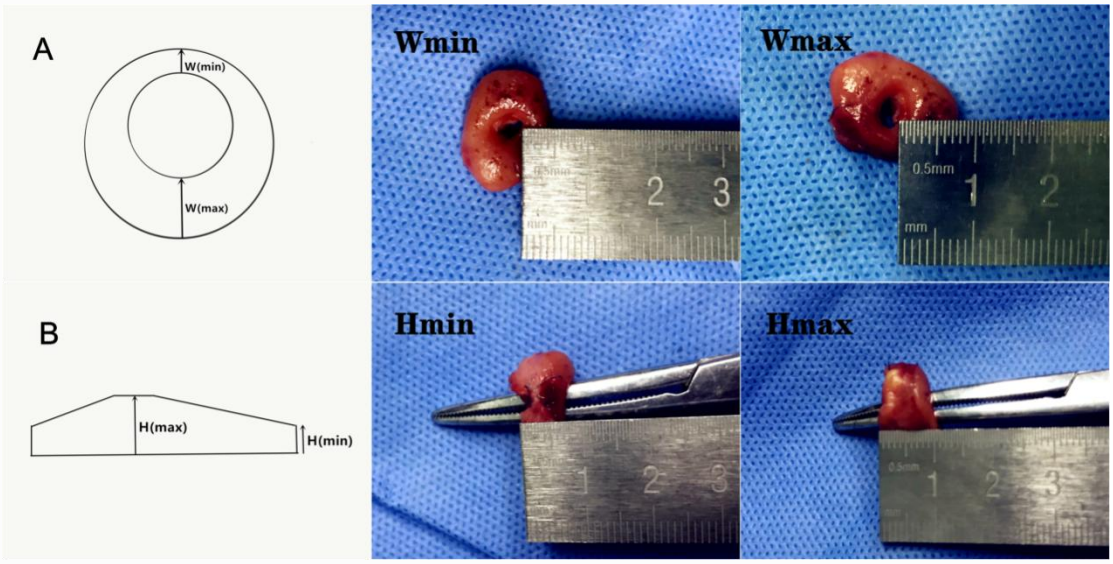


Figure 1 Measurement of anastomotic ring configuration

Minimal width (W_{min}), maximal width (W_{max}), minimal height (H_{min}) and maximal height (H_{max}) were measured after stapled circular anastomosis formation intraoperatively.

3.5 Diagnostic Criteria of Anastomotic Leakage

During the inpatient treatment time until the first follow-up 14 days postoperatively, AL was diagnosed if one or more of the following occurred in the period of the hospitalization and postoperative follow-up:

1) Proof of anastomotic defect by digital rectal exam, colonoscopy or rectoscopy;

2) Purulent or stool-like secretion of the pelvic drainage;

3) Radiologically confirmed perianastomotic abscess formation or anastomotic defect;

4) Rectovaginal or rectovesicular fistula.

3.6 Statistical Methods

SPSS 26.0 software was used for statistical analyses. Ratio data were presented as mean ± standard deviation (mean ± SD), categorical data were presented as frequencies (in %). Chi-square test or Fisher's exact test was used for the comparison of categorical data. T test was used for the comparison of ratio scaled, Gaussian distributed data. The Receiver Operating Characteristic (ROC) curve as well as Area-Under-the-Curve (AUC) analyses were used to evaluate the ring measures and the NLR in as binary classifier to predict AL postoperatively. Optimized cut-off values were determined by maximizing the Youden-index. Values of $p<0.05$ were considered statistically significant.

4. RESULTS

4.1 Patients' Clinical Characteristics

In total, 247 patients underwent resection for colorectal cancer followed by circular stapler anastomosis during the period from August 10th, 2020 to August 12th, 2021. Among these 247 patients, 43 cases were excluded for meeting the exclusion criteria. The resulting 204 patients had a mean age of 63.2 ± 11.6 years and a mean BMI of 22.8± 2.1. The collective consisted of 83 females and 121 males. The patients included were staged for stage II or III according to TNM Classification of Malignant Tumours (UICC/AJCC 8th Edition), and underwent anterior resection of the proximal rectum and the distal sigmoid colon with sigmoidorectostomy. All the operations were completed under laparoscopy with the same surgical group. All procedures were carried out laparoscopically without conversion to conventional laparotomy. The specimens were removed through left lower quadrant incision. The mean operation time was 161.4 ± 31.1minutes, the mean intraoperative blood loss was 175.8 ± 38.1ml (Table 1).

Table 1 Patients' characteristics

Items	Total (n=204)	Groups		P value
		Leakage (n=19)	Healing (n=185)	
Gender (M/F)	121/83	12/7	109/76	0.720
Age	63.2±11.6	66.2±11.8	62.9±11.5	0.242
ASA* Score	2.2±0.4	2.1±0.3	2.2±0.4	0.238
BMI**	22.8±2.1	22.5±1.9	22.9±2.1	0.463
OP Time (min)	161.4±31.1	161.1±33.5	161.4±30.9	0.963

Blood Loss (ml)	175.8±38.1	165.8±44.5	176.8±37.3	0.230
UICC/AJCC*** Stage (Ⅱ/Ⅲ)	143/61	13/6	130/55	0.867

*ASA refers to American Society of Anesthesiologists; **BMI refers to Body Mass Index; ***UICC refers to Union for International Cancer Control and AJCC refers to American Joint Committee on Cancer.

4.2 Configuration of Anastomotic Rings

To perform anastomosis, two different stapler sizes were used: CDH 25A and CDH 29A (Ethicon Endo-Surgery, LLC). Although the diameter of the rings differed between the stapler types, there were no statistical differences between the two stapler types regarding all other measurements of anastomotic rings, as shown in Table 2.

Table 2 Anastomotic ring configuration of different stapler types

Items	Mean±SD (mm)		P value
	CDH 25A (n=69)	CDH 29A (n=135)	
H_{min}*	5.213±0.820	5.091±0.771	0.297
H_{max}**	8.764±0.955	8.659±1.068	0.494
W_{min}***	4.993±0.774	4.896±0.697	0.365
W_{max}****	8.728±1.044	8.586±0.795	0.282

*H_{min} is the minimal height of the ring; **H_{max} is the maximal height of the ring; ***W_{min} is the minimal width of the ring; ****W_{max} is the maximal width of the ring.

The measurement results of the rings in 204 patients were as follows: the rings' H_{min} was statistically lower in the LEAK group compared to the HEAL group (4.4 ± 0. 5 mm

vs. 5.2 ± 0.8 mm; p<0.001). H_{max}, W_{min} and W_{max} did not show statistical differences between the LEAK group and the HEAL group (Table 3).

Table 3 Configuration of anastomotic rings

Items	Mean±SD (mm)		P value
	Leakage(n=19)	Healing(n=185)	
H_{min}*	4.421±0.454	5.205±0.779	<0.001
H_{max}**	8.568±1.033	8.708±1.032	0.576
W_{min}***	4.863±0.781	4.935±0.719	0.681
W_{max}****	8.458±0.806	8.652±0.895	0.365

*H_{min} is the minimal height of the ring; **H_{max} is the maximal height of the ring; ***W_{min} is the minimal width of the ring; ****W_{max} is the maximal width of the ring.

4.3 H_{min} as Binary Classifier to Predict Anastomotic Leakage

Anastomotic leakage occurred in 19 of 204 patients (9.3%), after a mean of 5.79±1.44 days. Compared with the 185 cases in HEAL group, there was no statistical difference in terms of gender, age, ASA score, BMI, operating time, intraoperative blood loss and tumour stage, as shown in Table 1.

To further analyze H_{min} as a potential binary classifier for anastomotic leakage, the optimal cut-off value of 4.95mm was determined by maximizing the Youden-index. At this value, the sensitivity for predicting AL following colorectal surgery was 56.8% with a specificity of 84.2% (Figure 2).

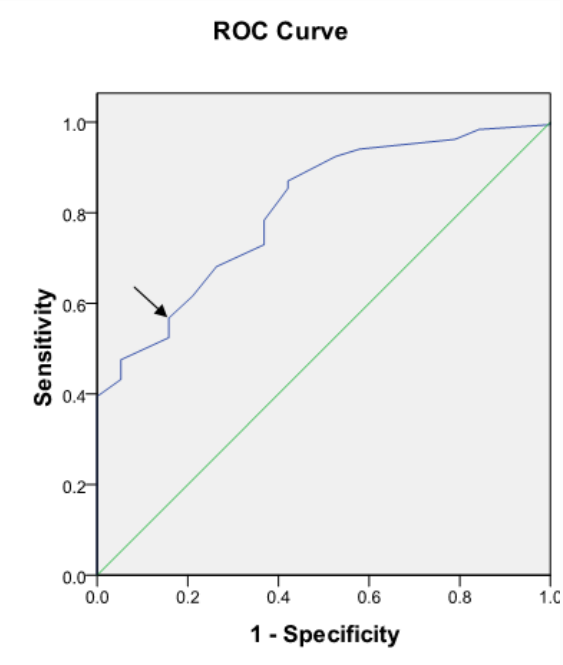


Figure 2 ROC curve for H_{min} of anastomotic rings to predict anastomotic leakage

Optimized cut-off value (Youden-index, as the arrow shows): 4.95mm;

Sensitivity: 56.8%; Specificity: 84.2%; AUC: 0.81.

4.4 Anastomotic Leakage Rate

Of the 204 cases, 108 cases had minimal anastomotic ring heights of more than 4.95mm, of which three (2.8%) developed AL. In 96 cases, minimal anastomotic ring heights were below 4.95mm, of which 16 (16.7%) developed AL. Between the two groups separated by an H_{min} value of 4.95mm, the difference in leakage rate was statistically significant (x^2=11.607, p=0.001), as shown in Table 4. Other factors such as gender, age, ASA score, BMI, operating time, intraoperative blood loss and tumour stage did not show any statistical difference between the former group (H_{min} above 4.95mm) and the latter group (H_{min} below 4.95mm), as shown in Table 5.

Table 4 Anastomotic leakage rate

Cut-off Value (4.95mm)	Total (n)	Groups		Leakage Rate
		Leakage (n)	Healing (n)	
Over 4.95mm	108	3	105	2.78%*
Below 4.95mm	96	16	80	16.67%*
Total	204	19	185	9.31%

*Chi-square value x^2=11.607. p<0.05 (p=0.001).

Table 5 Comparison of groups H_{min} above and below 4.95mm

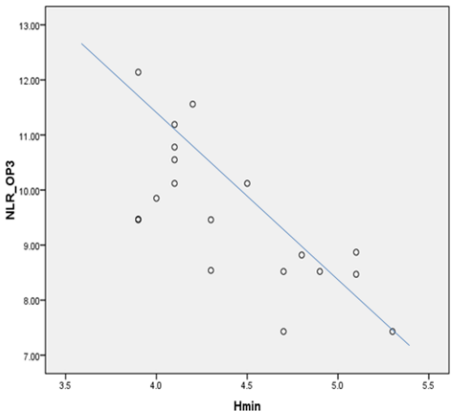
Items	Total (n=204)	Cut-off Value (4.95mm)		P value
		Above (n=108)	Below (n=96)	
Gender (M/F)	121/83	63/45	58/38	0.762
Age	63.2±11.6	62.5±11.6	63.96±11.5	0.376
ASA* Score	2.2±0.4	2.2±0.4	2.2±0.4	0.794
BMI**	22.8±2.1	22.9±2.1	22.8±2.1	0.738
OP Time (min)	161.4±31.1	161.0±33.0	161.8±29.0	0.864
Blood Loss (ml)	175.8±38.1	178.9±37.4	172.3±38.7	0.217
UICC/AJCC*** Stage (Ⅱ / Ⅲ)	143/61	79/29	64/32	0.313
Distance to Anal Verge (>10cm / <10cm)	114/90	64/44	50/46	0.303

*ASA refers to American Society of Anesthesiologists; **BMI refers to Body Mass Index; ***UICC refers to Union for International Cancer Control and AJCC refers to American Joint Committee on Cancer.

4.5 Correlation Between H_{min} and NLR

Interestingly, within the AL patients, the H_{min} and the NLR even showed a significant inverse correlation: the H_{min} and the NLR on the third day after operation highly

correlated (|r|>0.7, p=0.001), as shown in Figure 3a; the H_{min} and the NLR on the fifth day after operation correlated significantly, as well (0.4≤|r|<0.7, p=0.003) (Figure 3b).



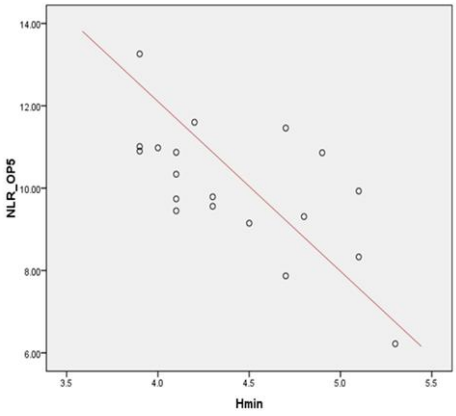


Figure 3a **Figure 3b**

Figure 3 Correlation between anastomotic ring minimal height (H_{min}) and Neutrophil to Lymphocyte Ratio (NLR) on the third (3a) and fifth (3b) postoperative day

(3a) Correlation is significant at the 0.01 level (2-tailed), r = -0.726, p=0.001;

(3b) Correlation is significant at the 0.01 level (2-tailed), r = -0.642, p=0.003.

4.6 Postoperative Development of NLR and CRP Values

In order to compare H_{min} to established inflammatory markers, the NLR and the CRP value were monitored and analyzed perioperatively. Compared to the preoperative measurements (2.8±0.5), the NLR markedly increased on POD 1 (12.7±1.0), and then stabilized (POD 3: 9.5±1.3; POD 5: 10.0±1.6) in the LEAK group. Patients in the HEAL group had NLR values of 2.6±0.4 preoperatively, followed by a gradual decrease of 12.6±1.4, 8.4±1.1 and 6.7±1.5 postoperatively. The values between the groups were statistically different at POD 3 (p=0.002) and POD 5 (p<0.001) (Figure 4).

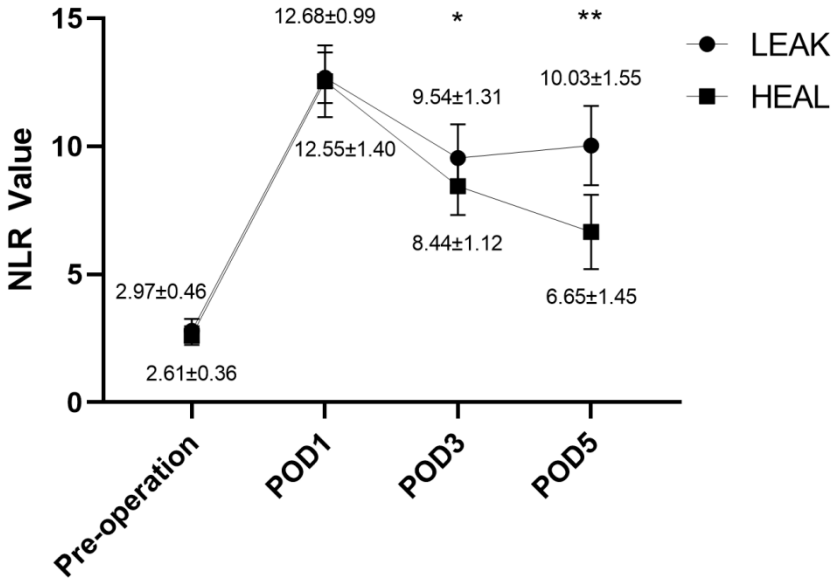


Figure 4 NLR chart pre-operation and post-operation

POD1 = first postoperative day; POD3 = third postoperative day;

POD5 = fifth postoperative day. *p=0.002; **p<0.001.

Compared to preoperative values (4.7±0.7 mg/L), CRP kept increasing postoperatively in the LEAK group (POD 1: 22.3±11.2mg/L; POD 3: 53.8±13.0mg/L; POD 5: 64.9±10.4mg/L). Patients in the HEAL group had CRP values of 5.1±0.8mg/L, followed by a less pronounced postoperative increase of 26.3±11.0mg/L, 52.0±13.3mg/L, 55.2±12.6mg/L. The values between the groups were statistically different at POD 5 (p=0.001) (Table 6).

Table 6 Comparison NLR/CRP between LEAK group and HEAL group

Items	(Mean ± SD)		P value
	Leakage (n=19)	Healing (n=185)	
NLR:			
Pre-operation	2.794±0.461	2.607±0.360	0.068
POD 1*	12.680±0.987	12.545±1.403	0.685

POD 3**	9.542±1.312	8.442±1.123	0.002
POD 5***	10.033±1.548	6.652±1.450	<0.001
CRP (mg/L):			
Pre-operation	4.737±0.678	5.071±0.802	0.081
POD 1*	22.279±11.177	26.344±10.995	0.127
POD 3**	53.763±13.044	51.960±13.296	0.573
POD 5***	64.895±10.434	55.169±12.609	0.001

*POD 1 represents the first day after operation; **POD 3 represents the third day after operation; ***POD 5 represents the fifth day after operation.

4.7 Comparison of NLR Between LEAK Group and HEAL Group

On the first day after operation, there was no statistically significant difference in the NLR between patients with anastomotic leakage (LEAK group) and those with successful healing (HEAL group) ($p>0.05$). However, on the third day and fifth postoperative day, the NLR value of LEAK group was higher than that of HEAL group, and the difference was statistically significant ($p<0.05$), as shown in Table 6. The ROC curve showed that when the NLR cut-off value was ≥8.5 on the third postoperative day, the sensitivity and specificity for predicting postoperative AL were 89.5% and 58.4% respectively, the ROC-AUC was acceptable at 0.74 (Figure 5a). When the NLR cut-off value was ≥7.8 on the fifth postoperative day, the sensitivity and specificity for predicting postoperative AL were 94.7% and 82.7% respectively, the ROC-AUC was excellent at a value of 0.93 (Figure 5b).

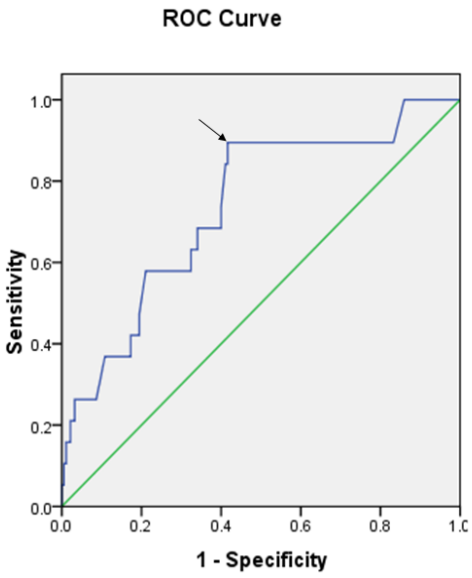


Figure 5a

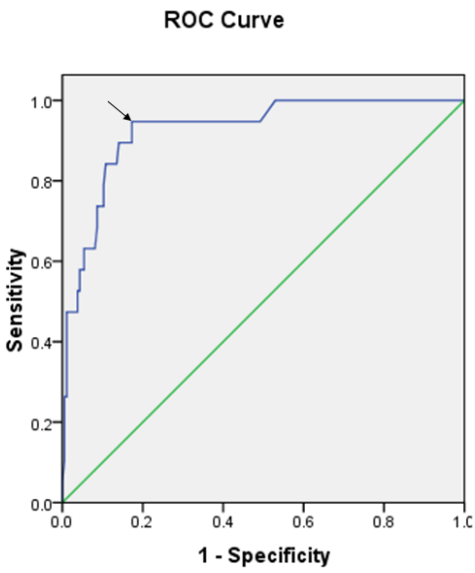


Figure 5b

Figure 5 Receiver Operating Characteristic (ROC) curve for NLR values on third (5a) and fifth (5b) postoperative day to predict anastomotic leakage

(5a) Optimized cut-off value (Youden-index, as the arrow shows): 8.5; Sensitivity: 89.5%; Specificity: 58.4%; AUC: 0.74. (5b) Optimized cut-off value (Youden-index, as the arrow shows): 7.8; Sensitivity: 94.7%; Specificity: 82.7%; AUC: 0.93.

4.8 Accuracy of H_{min} Compared to NLR and CRP as Binary Classifiers for Anastomotic Leakage

The ROC curve showed only moderate ROC-AUC at 0.74 for NLR at POD 3, while it was even poor at 0.54 for CRP at POD 3. For CRP at POD 5 the ROC-AUC was only moderate at 0.70. H_{min} outperformed all values with a good ROC-AUC value of 0.81. NLR had very good ROC-AUC of 0.93 at POD 5 (Figure 6).

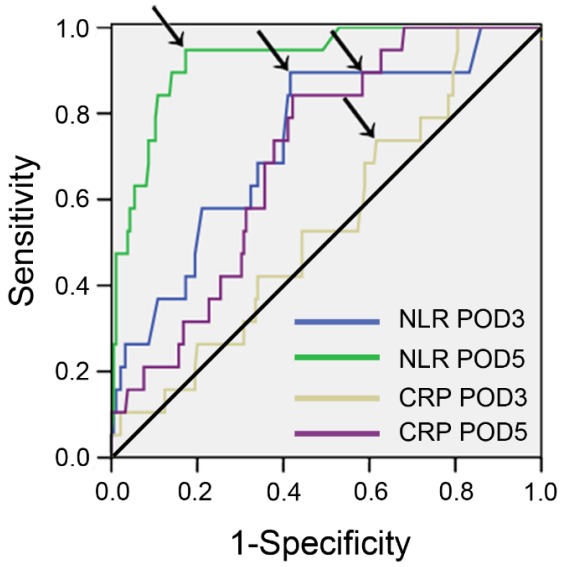


Figure 6 Comparison of ROC-analyses of the postoperative NLR and the CRP

5. DISCUSSION

Intraluminal stapling techniques for colorectal anastomosis have been widely accepted nowadays and work as a standard choice for reconstruction following colorectal surgery, particularly suitable for those who are undergoing laparoscopic operations (Tejedor, Sagias, Flashman, Kandala, & Khan, 2020). Anastomotic rings are retrieved during the colorectal anastomosis with circular staplers. This study shows that the anastomotic ring configuration, especially the H_{min} is critical for an increased risk of postoperative leakage. The H_{min} is a significant predictor for development of AL in this prospective study. Over the optimal decision point, determined by the Youden-index of 4.95mm, the leakage rate is significant lower than below. Furthermore, among these 19 leakage cases, the H_{min} and the NLR significantly correlate inversely at the third and fifth postoperative day.

In this study, the H_{min} with a ROC-AUC of 0.81 has a slightly superior predictive performance with NLR on the third postoperative day in predicting AL compared to the NLR with a ROC-AUC of 0.74. Still it is inferior to the NLR on the fifth postoperative day, with an excellent ROC-AUC of 0.93. Considering the average leakage occurrence time point is 5.79 days postoperatively, the NLR at the third/fifth postoperative day appear suitable to diagnose but not to prevent the development of AL by appropriate countermeasures. As shown above, the ring configuration is a significant real-time predictor for AL. Therefore, the H_{min} is a useful tool to identify technical problems of anastomosis formation allowing the decision for immediate intraoperative revision of the anastomosis.

Additionally, our study confirmed that compared to the CRP, the NLR as leakage marker is not only simpler and available at a lower cost, but has better efficiency as well (Paliogiannis et al., 2020). In our study, the NLR was already significantly different between the HEAL group and the LEAK group at postoperative day 3 compared to the CRP, which did not differ at postoperative day 3 but only at postoperative day 5 between the groups. Other studies have shown that NLR can be used to predict perioperative complications in patients undergoing colorectal surgery (Kubo et al., 2016; Liu, Wang, & Fu, 2020; Walker, Kunjuraman, & Bartolo, 2018). A study of Cook et al. shows that for patients undergoing colorectal resection at a NLR ≥ 9.3 on the third day after surgery, complications are more likely to occur (Cook et al., 2007). NLR can also reflect the severity of systemic inflammation, the study of Mik et al. confirms that the NLR of patients dying from AL on the fourth day after operation is higher than that of other patients with AL (Mik, Dziki, Berut, Trzcinski, & Dziki, 2018).

Although being the first prospective single center cohort study, this has some limitations that should be discussed in the following. An important limitation of our study is the limited number of included patients. To validate our findings further (multi-center) studies will be needed. Loss of follow-up cannot be excluded for patients suffering from late AL after discharge from the hospital. Still, the risk should be low, as usually AL happens early and within the postoperative in-house treatment. Although gender, age, ASA score, BMI, operation time, blood loss and tumour stage

did not differ between the groups, we cannot exclude other confounders to affect the occurrence of AL in our analysis.

However, this study was performed to evaluate the morphological configuration of anastomotic rings as an early available marker for AL and compare it with the established but delayed marker NLR. We could demonstrate a critically low H_{min} as technical flaw in colorectal surgery leading to AL.

6. SUMMARY AND CONCLUSION

In summary, our study confirms that proper configuration of the anastomotic rings is essential to prevent leakage. A low minimal height represents a technical flaw and can indicate the risk to develop leakage with a high specificity, and thus should be evaluated intraoperatively. It is a very simple and economic measure to allow decision for early application of protective measures as revision of the anastomosis, diversion by ileostomy or at least very careful postoperative surveillance. Therefore, the configuration of anastomotic rings deserves more attention and requires further studies to find its final value in anastomotic surgery.

The minimal height of the anastomotic rings as indicator for a technically insufficient anastomosis is an equivalent predictor of anastomotic leakage, compared to the NLR as established but delayed marker. Its real-time availability intraoperatively allows for early countermeasures as formation or revision of the anastomosis or at least careful postoperative surveillance of patients at risk.

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8. TABLES

8.1 Table 1 Patients' characteristics

Items	Total (n=204)	Groups		P value
		Leakage (n=19)	Healing (n=185)	
Gender (M/F)	121/83	12/7	109/76	0.720
Age	63.2±11.6	66.2±11.8	62.9±11.5	0.242
ASA* Score	2.2±0.4	2.1±0.3	2.2±0.4	0.238
BMI**	22.8±2.1	22.5±1.9	22.9±2.1	0.463
OP Time (min)	161.4±31.1	161.1±33.5	161.4±30.9	0.963
Blood Loss (ml)	175.8±38.1	165.8±44.5	176.8±37.3	0.230
UICC/AJCC*** Stage (Ⅱ/Ⅲ)	143/61	13/6	130/55	0.867

*ASA refers to American Society of Anesthesiologists; **BMI refers to Body Mass Index; ***UICC refers to Union for International Cancer Control and AJCC refers to American Joint Committee on Cancer.

8.2 Table 2 Anastomotic ring configuration of different stapler types

Items	Mean±SD (mm)		P value
	CDH 25A (n=69)	CDH 29A (n=135)	
H_{min}*	5.213±0.820	5.091±0.771	0.297
H_{max}**	8.764±0.955	8.659±1.068	0.494
W_{min}***	4.993±0.774	4.896±0.697	0.365
W_{max}****	8.728±1.044	8.586±0.795	0.282

*H_{min} is the minimal height of the ring; **H_{max} is the maximal height of the ring; ***W_{min} is the minimal width of the ring; ****W_{max} is the maximal width of the ring.

8.3 Table 3 Configuration of anastomotic rings

Items	Mean±SD (mm)		P value
	Leakage(n=19)	Healing(n=185)	
H_{min}*	4.421±0.454	5.205±0.779	<0.001
H_{max}**	8.568±1.033	8.708±1.032	0.576
W_{min}***	4.863±0.781	4.935±0.719	0.681
W_{max}****	8.458±0.806	8.652±0.895	0.365

*H_{min} is the minimal height of the ring; **H_{max} is the maximal height of the ring; ***W_{min} is the minimal width of the ring; ****W_{max} is the maximal width of the ring.

8.4 Table 4 Anastomotic leakage rate

Cut-off Value (4.95mm)	Total (n)	Groups		Leakage Rate
		Leakage (n)	Healing (n)	
Over 4.95mm	108	3	105	2.78%*
Below 4.95mm	96	16	80	16.67%*
Total	204	19	185	9.31%

*Chi-square value x^2=11.607. p<0.05 (p=0.001).

8.5 **Table 5 Comparison of groups H_{min} above and below 4.95mm**

Items	Total (n=204)	Cut-off Value (4.95mm)		P value
		Above (n=108)	Below (n=96)	
Gender (M/F)	121/83	63/45	58/38	0.762
Age	63.2±11.6	62.5±11.6	63.96±11.5	0.376
ASA* Score	2.2±0.4	2.2±0.4	2.2±0.4	0.794
BMI**	22.8±2.1	22.9±2.1	22.8±2.1	0.738
OP Time (min)	161.4±31.1	161.0±33.0	161.8±29.0	0.864
Blood Loss (ml)	175.8±38.1	178.9±37.4	172.3±38.7	0.217
UICC/AJCC*** Stage (Ⅱ / Ⅲ)	143/61	79/29	64/32	0.313
Distance to Anal Verge (>10cm / <10cm)	114/90	64/44	50/46	0.303

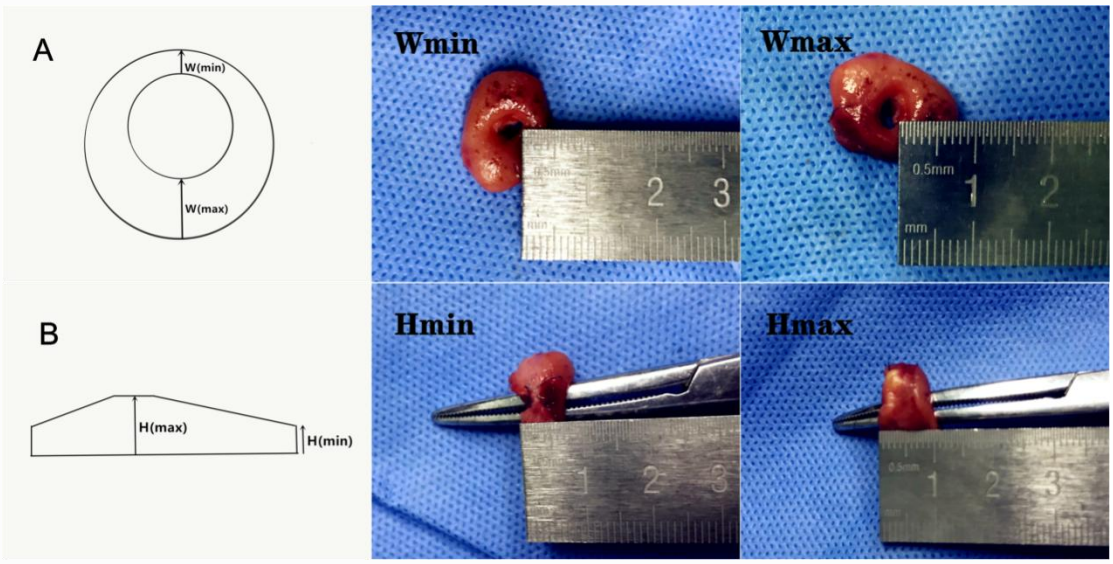
*ASA refers to American Society of Anesthesiologists; **BMI refers to Body Mass Index; ***UICC refers to Union for International Cancer Control and AJCC refers to American Joint Committee on Cancer.

8.6 Table 6 Comparison NLR/CRP between LEAK group and HEAL group

Items	(Mean ± SD)		P value
	Leakage (n=19)	Healing (n=185)	
NLR:			
Pre-operation	2.794±0.461	2.607±0.360	0.068
POD 1*	12.680±0.987	12.545±1.403	0.685
POD 3**	9.542±1.312	8.442±1.123	0.002
POD 5***	10.033±1.548	6.652±1.450	<0.001
CRP (mg/L):			
Pre-operation	4.737±0.678	5.071±0.802	0.081
POD 1*	22.279±11.177	26.344±10.995	0.127
POD 3**	53.763±13.044	51.960±13.296	0.573
POD 5***	64.895±10.434	55.169±12.609	0.001

*POD 1 represents the first day after operation; **POD 3 represents the third day after operation; ***POD 5 represents the fifth day after operation.

9. FIGURES



9.1 **Figure 1 Measurement of anastomotic ring configuration**

Minimal width (W_{min}), maximal width (W_{max}), minimal height (H_{min}) and maximal height (H_{max}) were measured after stapled circular anastomosis formation intraoperatively.

9.2

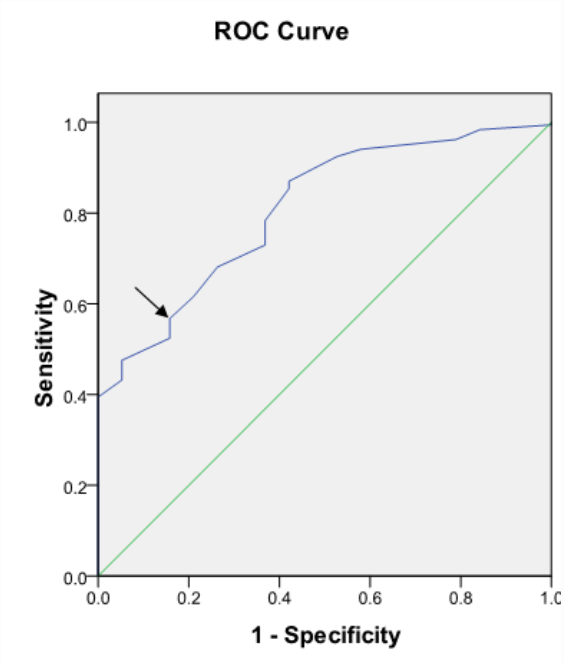
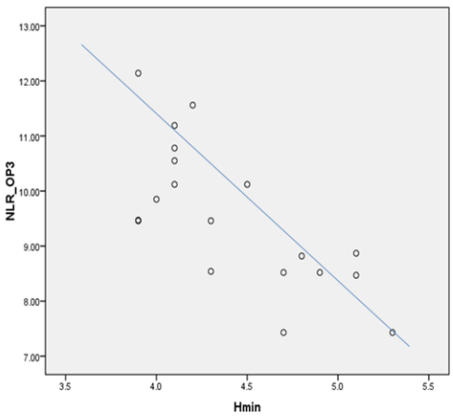


Figure 2 ROC curve for H_{min} of anastomotic rings to predict anastomotic leakage

Optimized cut-off value (Youden-index, as the arrow shows): 4.95mm;

Sensitivity: 56.8%; Specificity: 84.2%; AUC: 0.81.

9.3



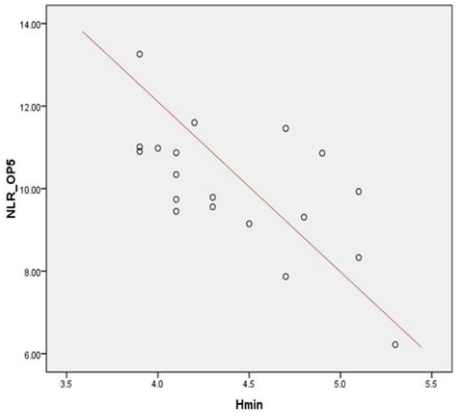


Figure 3a **Figure 3b**

Figure 3 Correlation between anastomotic ring minimal height (H_{min}) and Neutrophil to Lymphocyte Ratio (NLR) on the third (3a) and the fifth (3b) postoperative day

(3a) Correlation is significant at the 0.01 level (2-tailed), r = -0.726, p=0.001;

(3b) Correlation is significant at the 0.01 level (2-tailed), r = -0.642, p=0.003.

9.4

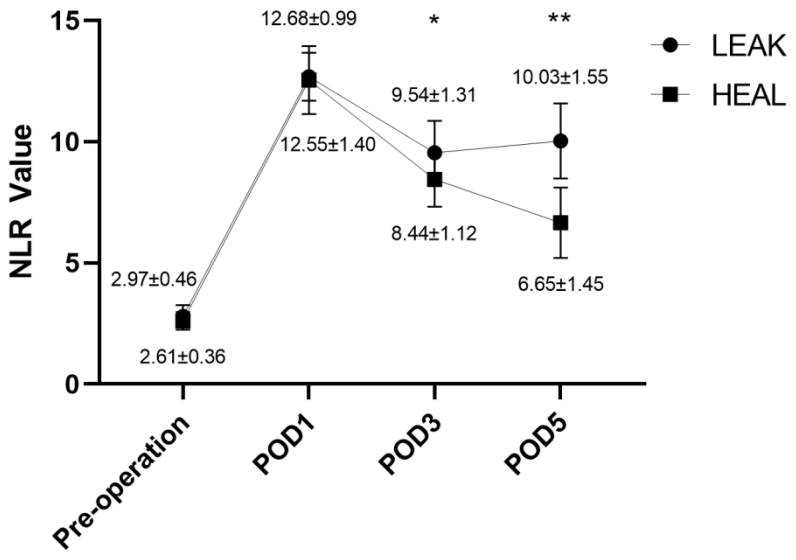


Figure 4 NLR chart pre-operation and post-operation

POD1 = first postoperative day; POD3 = third postoperative day;

POD5 = fifth postoperative day. *p=0.002; **p<0.001.

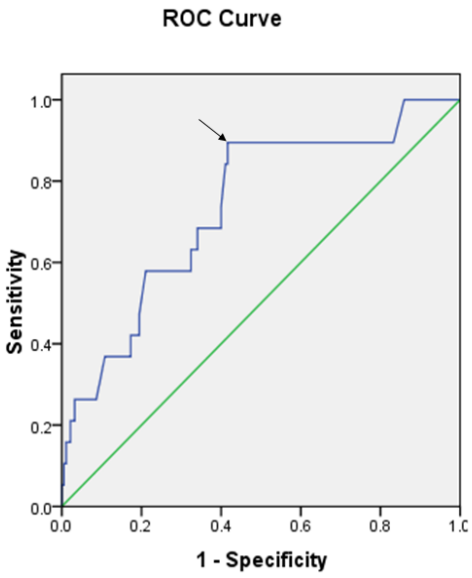


Figure 5a

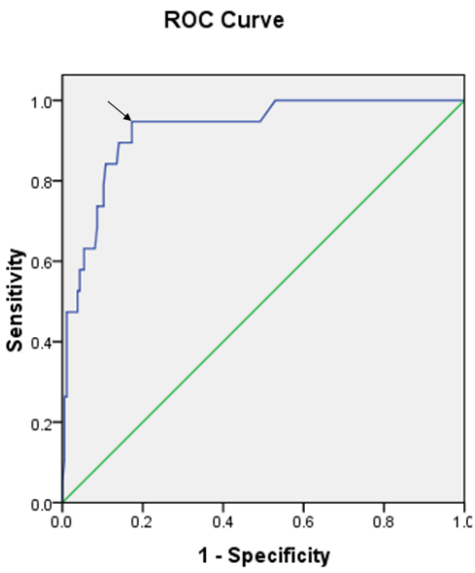


Figure 5b

9.5 **Figure 5 Receiver Operating Characteristic (ROC) curve for NLR values on the third (5a) and the fifth (5b) postoperative day to predict anastomotic leakage**

(5a) Optimized cut-off value (Youden-index, as the arrow shows): 8.5; Sensitivity: 89.5%; Specificity: 58.4%; AUC: 0.74. (5b) Optimized cut-off value (Youden-index, as the arrow shows): 7.8; Sensitivity: 94.7%; Specificity: 82.7%; AUC: 0.93.

9.6

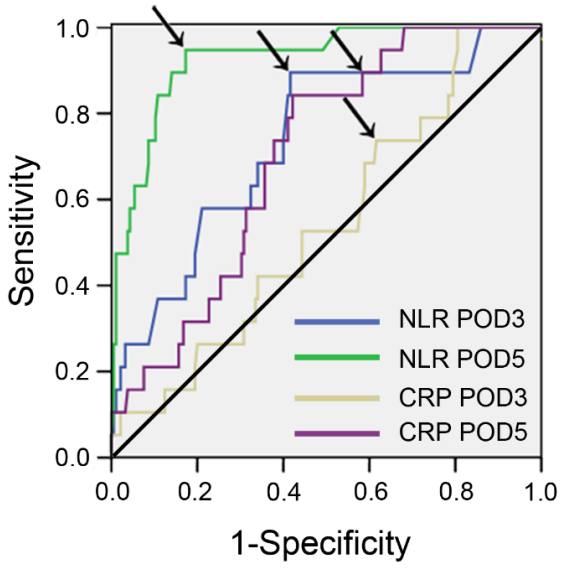


Figure 6 Comparison of ROC-analyses of the postoperative NLR and the CRP

9.7 Figure Legend

Fig. 1. Measurement of anastomotic ring configuration.

Fig. 2. Receiver Operating Characteristic (ROC) curve for minimal height of anastomotic rings to predict anastomotic leakage

Fig. 3. Correlation between anastomotic ring minimal height (H_{min}) and Neutrophil-to-Lymphocyte-Ratio (NLR) on the third (3a) and the fifth (3b) postoperative day

Fig. 4. NLR chart pre-operation and post-operation

Fig. 5. ROC curve for NLR values on the third (5a) and the fifth (5b) postoperative day to predict anastomotic leakage

Fig. 6. Comparison of ROC-analyses of the postoperative NLR and the CRP

10. INFLAMMATION AND COLORECTAL CANCER

Research Progress on the Relationship Between Inflammation and Colorectal Cancer

[Abstract] Colorectal cancer is one of the common malignant tumors. Relevant epidemiology and a large number of experimental studies have proved that chronic inflammation is highly correlated with the occurrence and development of colorectal cancer. And the inflammatory bowel disease has been proven to be an independent risk factor for colorectal cancer. Various inflammatory cells participate in the establishment of the chronic inflammatory intestinal microenvironment required for the onset of colorectal cancer. The abnormal signal pathways mediated by various inflammatory factors and inflammatory mediators promote the occurrence of tumors, which are related to colorectal cancer and pathogenesis-related inflammation mechanisms. At the gene level, miRNAs can also affect the pathogenesis of colorectal cancer by regulating mesenchymal epithelial transformation. This article reviews the relationship between inflammation and colorectal cancer as well as the related inflammatory mechanisms.

[Keywords] Colorectal Cancer, Inflammation, Intestinal Microenvironment, Cytokines, MiRNAs

Colorectal cancer (CRC), as one of the most prevalent types of cancer worldwide and the most common gastrointestinal malignant tumor, has become the third largest fatality rate of malignant tumor (Archambault et al., 2020; Jacobs, Smits,

Lardon, Pauwels, & Deschoolmeester, 2015; Thomas et al., 2020). As people change the way of lifestyle in recent years, together with the environmental pollution influence factors, the incidence of CRC is on the rise in the world. CRC is easily misdiagnosed, those diagnosed in early phase are less than 40% and most of the patients at the time of definite diagnosis, the cancer has already metastasized (van de Velde, 2015). The treatment with surgery is given priority to, combined with radiotherapy and chemotherapy, but epidemiological data show that patients with CRC mortality are still higher (Arnold et al., 2017; Ledel, Stenstedt, Hallstrom, Ragnhammar, & Edler, 2015). In the middle of the nineteenth century, Virchow observed leukocyte infiltration in tumor tissues and first proposed the possible correlation between malignant tumors and inflammation (Okada, 2014). With the deepening of such research, it has now been confirmed that chronic inflammation takes part in tumor's start, proliferation, metastasis, aging, and apoptosis at various stages. Inflammation is also known as the seventh largest biological features of malignant tumor (Desai, Prickril, & Rasooly, 2018; Mantovani, Allavena, Sica, & Balkwill, 2008; Murata, 2018; Okada, 2014).

1. Inflammation and Tumors

1.1 Tumors Associated with Chronic Inflammation

The occurrence and development of tumors include many complex physiological and pathobiological behaviors, including the activation of oncogenes, the formation of tumor microenvironment promoted by chronic inflammation, and

ultimately lead to cell proliferation and malignant transformation (Fidler, Soerjomataram, & Bray, 2016). Inflammation is a series of defense responses of the body against pathogen infection and tissue damage, and through the interaction of various cytokines in the body's microenvironment, it regulates the balance of various physiological and pathological states of the body. In a normal body, the inflammatory response will end after infection and other inflammatory factors disappear. This inflammation is called "resolving inflammation", but if the tissue cannot be separated from the continuous external stimulation, the inflammatory response persists. This inflammation is called "nonresolving inflammation" (Nathan & Ding, 2010). Uncontrollable inflammation plays an important role in inducing and promoting the formation and metastasis of malignant tumors (Elinav et al., 2013).

The occurrence of a variety of tumors can confirm the above view. For example, chronic gastritis with helicobacter pylori infection can induce gastric cancer (Tsukamoto, Nakagawa, Kiriyama, Toyoda, & Cao, 2017; Yoshida et al., 2014), inflammatory bowel disease is related to the incidence of CRC (Dulai, Sandborn, & Gupta, 2016; Keller, Windsor, Cohen, & Chand, 2019; Stidham & Higgins, 2018), and hepatitis B virus infection can induce liver cancer (El-Serag, 2012; Levrero & Zucman-Rossi, 2016). Silicosis caused by asbestos fibers or silica dust is related to lung cancer (Dreassi, 2018). Barrett's esophagitis is related to esophageal cancer (Alkhayyat, Kumar, Sanaka, & Thota, 2021; Moayyedi & El-Serag, 2021).

1.2 Evidence of the Relationship Between Inflammation and CRC

Inflammation is also closely related to the occurrence and development of CRC.

The most representative one is the relationship between inflammatory bowel disease (IBD) and CRC (Du, Kim, Shen, Chen, & Dai, 2017). IBD includes Crohn's disease (CD) and ulcerative colitis (UC). Studies have shown that IBD is an independent risk factor for CRC. And with the prolongation of the history of IBD, the incidence of CRC has increased significantly (Ananthakrishnan et al., 2014). In the study of primary CRC, it was found that there are inflammatory cell infiltrations and high expressions of related inflammatory factors in cancer tissues. Inflammation is highly related to the occurrence and outcome of CRC (Ning et al., 2011). In this review, we selected several items to present an overall picture of the relationship between inflammation and CRC, as shown in Table 1.

Table 1 Inflammation and Colorectal Cancer

Items	Contents		
Inflammatory Markers	C Reactive Protein (**CRP**)	Neutrophil to Lymphocyte Ratio (**NLR**)	Tumor-Associated Macrophages (**TAMs**)
Transcription Factors	Nuclear Factor kappa-light-chain-enhancer of activated B cells (**NF-kB**)	Signal Transducer and Activator of Transcription 3 (**STAT3**)	Reactive Oxygen Species (**ROS**) & Reactive Nitrogen Species (**RNS**)
Cytokines	Tumor Necrosis Factors-α (**TNF-α**)	Interleukin Family (**IL Family**)	Cyclo-Oxygen-Ase-2 (**COX-2**) & Nonsteroidal Antiinflammatory Drugs (**NSAIDs**)
Gene Level	micro RiboNucleic Acids (**miRNAs**)		

2. Inflammatory Microenvironment and CRC

2.1 Inflammatory Makers Participate in the Establishment of the Intestinal Microenvironment of CRC

Inflammation is associated with the progression of most malignant tumors including CRC, and the inflammatory response markers reflect the degree of the host's inflammatory response to the tumor (Park, Watt, Roxburgh, Horgan, & McMillan, 2016). Studies have found that inflammatory response markers are indicators of good predictive value in the prognostic evaluation of CRC (Coussens & Werb, 2002; Mantovani, Allavena, Sica, & Balkwill, 2008; Rossi et al., 2017), and they also have good predictive value in the prognostic evaluation of metastatic CRC as well (Riedl et al., 2017; Shibutani et al., 2015). Inflammatory response markers mainly include serum albumin, C-reactive protein (CRP), plasma fibrinogen, neutrophil to lymphocyte ratio (NLR), platelet to lymphocyte ratio (PLR) and lymphocyte to monocyte ratio (LMR) (Proctor et al., 2012; Shrotriya, Walsh, Bennani-Baiti, Thomas, & Lorton, 2015; Stotz et al., 2014; Wang et al., 2016).

2.1.1 CRP

Among these inflammatory response markers, CRP is a sensitive and most widely used marker reflecting the degree of systemic inflammation, and it has a good predictive effect on the prognosis of patients with tumors that can or cannot be surgically resected (Clarke et al., 2011; Roxburgh & McMillan, 2010). Meanwhile, it can also be used as a good indicator to predict anastomotic leakage following CRC surgery, for colorectal surgery, the patients' CRP increase significantly on the first

day after surgery, and then gradually decrease, the postoperative CRP level of patients with anastomotic leakage is significantly higher than that of patients with successful anastomotic healing; on the third or fourth day after surgery, CRP level can be used as an early indicator to predict anastomotic leakage (Bennis et al., 2012; Platt et al., 2012). The study results of Scepanovic (Scepanovic et al., 2013) show that the sensitivity of CRP>135mg/L in predicting abdominal surgery anastomotic leakage is 73% and the specificity is 73% on the third postoperative day. Almeida's (Almeida et al., 2012) study shows that on the fifth postoperative day, the sensitivity of CRP>140mg/L in predicting anastomotic leakage after colorectal surgery is 78%, and the specificity is 86%.

2.1.2 NLR

There exist neutrophil infiltrations in tissues in the inflammatory phase, which can produce cell growth factors and related proteases to promote the transformation of normal cells into tumor cells (Spicer et al., 2012). Tumor-infiltrating lymphocytes (TILs) are considered to be an important part of inducing the body to produce an anti-tumor immune response, they are a special type of lymphocytes that can kill tumor cells and reduce tumor metastasis. The number and function of TILs can reflect the level of the body's anti-tumor response (Lee & Margolin, 2012).

Elevated neutrophil levels and decreased lymphocyte levels are one of the specific signs of systemic inflammation, the significant role of NLR as a systemic inflammatory response indicator in the prognostic evaluation of CRC is recognized,

NLR can effectively predict local recurrence, complication rate, distant metastasis rate, disease-free survival rate and overall survival rate after CRC surgery (Song et al., 2017; Spicer et al., 2012).

Neutrophils are an important part of inflammatory cell infiltration, they can protect the body and eliminate pathogens when the body is infected by microorganisms, and are also widely present in the tumor microenvironment (Shibutani et al., 2015). it has been found that neutrophils can be induced by related factors in the tumor microenvironment to undergo phenotypic and functional remodeling and in the early stage of inflammation, mature neutrophils in the bone marrow are rapidly activated, and the number in the blood increases rapidly; In addition, due to the action of bacteria and various toxins, specific immune activation and non-specific damages make lymphocytes apoptosis and the number of lymphocytes in the blood decrease, during the process of inflammation, the increase in NLR can be used as a marker of inflammation (Piccard, Muschel, & Opdenakker, 2012).

Therefore, the neutrophil to lymphocyte ratio (NLR) can reflect the immune status of the body, as well as the tumor microenvironment composed of inflammatory factors and the inflammatory system, it has been shown that the NLR value is closely related to the prognosis of malignant tumors (Mallappa, Sinha, Gupta, & Chadwick, 2013). Guthrie (Guthrie, Roxburgh, Farhan-Alanie, Horgan, & McMillan, 2013) found that a low NLR value indicates a better prognosis for patients with CRC. Masatsune (Shibutani et al., 2015) mentioned that if patients with CRC still have a

high NLR value after surgery, it indicates that the patient is in a state of easy recurrence. Woo (Choi et al., 2015) proved that NLR is related to the infiltration depth of CRC, tumor node metastasis (TNM) stage and other clinical features.

2.1.3 TAMs

Tumor-Associated Macrophages (TAMs) are also involved in the occurrence of tumors. TAMs can differentiate into different types under different conditions, including anti-tumor M1 macrophages and tumor-promoting M2 macrophages (Franklin & Li, 2016). In most malignant tumors, TAMs tends to the expression of M2, through the secretion of corresponding growth factors such as Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor-β (TGF-β), Platelet Derived Growth Factor (PDGF) and more chemokines are involved in the escape of tumor cells (Yang et al., 2013). TAMs in the stroma can also participate in matrix remodeling and promote tumor infiltration. In CRC, Schafer (Schafer et al., 2013) found that STAMs can enhance the migration of intestinal epithelial cells and promote their anti-apoptotic ability, indicating that TAMs play an important role in the pathogenesis of inflammation-related CRC. Kang (Kang, Chen, Lee, Chang, & Shieh, 2010) studied pathological specimens of CRC and found that the number of TAMs in the tumor is significantly related to the TNM stage and the presence or absence of distant metastasis. Other studies have found that TAMs in CRC is not a simple M1 or M2 type, and sometimes it can exist at the same time.

2.2 The Role of Transcription Factors and Inflammatory Mediators in the

Formation of CRC

Recent studies have shown that in the occurrence and invasion of CRC, in addition to the infiltration of inflammatory cells, the activation of tumor signal pathways mediated by various inflammatory factors and inflammatory mediators is more involved. Many abnormal signal pathways are involved in the occurrence of CRC (Farooqi, de la Roche, Djamgoz, & Siddik, 2019; Qi & Ding, 2018; Tiwari, Saraf, Verma, Panda, & Jain, 2018; Zhang et al., 2018).

2.2.1 NF-kB

NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) belongs to the family of transcription factor proteins and plays a regulatory role in the process of inflammation. It has also been proven to play a role in the occurrence of inflammation-related tumors (DiDonato, Mercurio, & Karin, 2012). NF-kB can regulate the transcription of genes related to proliferation and apoptosis and induce the expression of a variety of inflammatory factors, such as TRAIL, P53, Bcl-2, and CyclinD1 which are all target gene products of NF-kB (Ji, He, Regev, & Struhl, 2019; Patel, Horgan, McMillan, & Edwards, 2018). A variety of experiments have proved that the NF-kB signaling pathway promotes the onset of CRC tumors. Clinical data has shown that (O'Leary et al., 2012) more than 50% of CRC tissues are found the activation of NF-kB. It is found in mouse experimental models that reducing the level of NF-kB in intestinal epithelial cells can significantly inhibit tumor growth (Kim et al., 2010). NF-kB is also related to the therapeutic effect of CRC. The abnormal activation

of NF-kB mediates the drug resistance of tumor cells through the anti-apoptotic pathway (Ding et al., 2019). Study has found (Saini & Sanyal, 2015) that spirulina protein and selective COX-2 inhibitors are combined with non-steroidal anti-inflammatory drugs for CRC cells, and the results show that the above-mentioned drugs have the effect of promoting apoptosis by activating P53 protein and inhibiting NF-kB activation, in this process, the Cyclin/CDK complex is inhibited to activate the expression of P53, and together with the level of NF-kB decreases.

2.2.2 STAT3

Signal Transducer and Activator of Transcription 3 (STAT3) belongs to the STAT family and is an important member of transcription activators. It exists in the cytoplasm and can be activated by extracellular signals such as epidermal growth factor (EGF), IL-6 and other cytokines (Wang, van Boxel-Dezaire, Cheon, Yang, & Stark, 2013). In colitis-related CRC, a large number of cytokines such as IL-6 and IL-11 continuously activate STAT3 to promote cell proliferation and malignant transformation (Heichler et al., 2020). It has now been confirmed that STAT3 is an independent risk factor for poor prognosis of CRC (Artas & Ozercan, 2014; Rokavec et al., 2014). The IL-6/STAT3 signaling pathway disorder plays an important role in the occurrence and metastasis of CRC. And the regulation of this pathway can inhibit the occurrence of colitis-related colon cancer (Do et al., 2016; Zhao et al., 2016).

2.2.3 ROS and RNS

The oxidative stress response often occurs in chronic inflammation. Under the stimulation of chronic inflammation, inflammatory cells produce large amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing DNA strand breaks and base mutations. Meanwhile, it is accompanied by high mutation of P53 and eventually leads to the occurrence of CRC (Cooks et al., 2013). Nitric oxide (NO), one of the RNS, produced during inflammation can cause pathological changes as the dose increases and participate in the growth of tumor cells (Lonkar & Dedon, 2011).

2.3 Cytokines and CRC

2.3.1 TNF-α

Tumor Necrosis Factors-α (TNF-α) can induce tumor cell apoptosis under normal circumstances, but under pathological conditions, it acts as an inflammatory factor to promote tumor development (De Simone et al., 2015; Stoian, State, Stoica, & Radulian, 2014). TNF-α can induce the expression of genes involved in tumor invasion and metastasis by activating NF-kB, including adhesion molecules, matrix metallopeptidase 9 (MMP9), Cyclo-oxygen-ase-2 (COX-2) and vascular endothelial growth factor (VEGF) (De Simone et al., 2015; Sun, Zhu, Liang, Yang, & Zhao, 2018). In addition, TNF-α can also trigger the activation of epithelial-mesenchymal transition (EMT) (Lee et al., 2018). These characteristics can promote the formation and metastasis of CRC neovascularization. Clinical studies have shown that high

levels of TNF-α expression are found in the bodies of a large number of CRC patients, and TNF-α levels can be seen decrease after the removal of the primary tumor, which may be due to the surgical removal of the primary tumor, thereby reducing the stimulation of immune system and reducing the production of TNF-α by lymphocytes (Shi et al., 2018). These studies prove the high correlation between TNF-α and CRC (Wang & Karin, 2015). In animal models, mice deficient in TNF receptors also have a significantly reduced incidence of CRC (Antoniou, Margonis, Angelou, Zografos, & Pikoulis, 2015).

2.3.2 Interleukin (IL) family

IL-6 and IL-10 are important cytokines related to the occurrence of CRC, and participate in the occurrence of CRC through various mechanisms. IL-6 stimulates the proliferation of CRC cells mainly by activating the STAT3 transcription factor, and also promotes tumor angiogenesis and tumor cell growth and invasion by regulating immune cell function (Guthrie, Roxburgh, Richards, Horgan, & McMillan, 2013; Moriasi, Subramaniam, Awasthi, Ramalingam, & Anant, 2012). Compared with the normal control group, IL-6 is highly expressed in CRC, and the level of IL-6 in the peripheral blood of CRC patients is highly correlated with its clinical stage and disease progression (Gamero et al., 2010). In addition to being produced in large quantities by T cells, IL-10 can also be produced by T regulatory cells (Tregs) both in normal colorectum and CRC, it is an important immune factor with a dual effect of immune suppression and immune stimulation, excessive local secretion of IL-10 will produce an immunosuppressive microenvironment around the tumor, while

systemic IL-10 increase inhibits the body's anti-tumor immune killing effect (Francescone, Hou, & Grivennikov, 2015). IL-10 in the tumor microenvironment can also enhance the activation of STAT3, thereby affecting the immune status of the body (Ibrahim et al., 2018). Studies have found that in mouse experimental models, the increased IL-10 can promote the occurrence and development of CRC (Huang et al., 2020; Zhao, Wu, Wu, Wang, & Huang, 2015).

2.3.3 Other cytokines and CRC

Cyclo-oxygen-ase-2 (COX-2) is mostly not expressed or underexpressed under normal physiological conditions, but when there is an inflammatory stimulus, it is activated through various pathways such as Protein Kinase A (PKA), and then rapidly synthesized and expressed (Yu, Lao, & Zheng, 2016). Prostaglandin E2 (PGE2) produced by COX-2 has been shown to be highly correlated with the occurrence and development of CRC (Ricciotti & FitzGerald, 2011; Wang & Dubois, 2010). After PGE2 binds to the EP2 receptor, it activates the related signaling pathways, thereby regulating inflammation and promoting tumorigenesis. The content of PGE2 in CRC tissue is closely related to the growth of tumor cells (Cahlin, Lonnroth, Arvidsson, Nordgren, & Lundholm, 2008), and the selective inhibition to PGE2 can inhibit CRC and regulate mucosal immunity (Wang, Fu, Sun, Guo, & DuBois, 2015). Another study found (Honda, Inagawa, & Yamamoto, 2011) that in the bodies of patients with liver metastases from CRC, the PGE2 value was significantly higher than that of the control group, suggesting that PGE2 may be involved in tumor metastasis and spread.

A large number of epidemiological and clinical studies have shown that the clinical application of nonsteroidal antiinflammatory drugs (NSAIDs) can reduce the incidence of CRC (Kuo, Pan, Huang, Tsai, & Chang, 2018; Seaton et al., 2019). NSAIDs such as aspirin can inhibit the formation of COX-2, reduce the synthesis of PGE2, and have a certain effect on the treatment of CRC (Burn & Sheth, 2016). Flossmann (Flossmann, Rothwell, British Doctors Aspirin, & the, 2007) found through clinical studies that long-term oral aspirin can reduce the incidence risk of CRC. All of the above indicate that COX-2/PGE2 is closely related to the occurrence and development of CRC, and also provide new ideas for the treatment of COX-2/PGE2 as the target.

3. MiRNAs Affect the Occurrence and Development of CRC by Regulating Epithelial-Mesenchymal Transition

MicroRNAs (miRNAs) are a type of non-coding single-stranded small RNAs that participate in the regulation of various physiological and pathological processes in the body (Berindan-Neagoe, Monroig Pdel, Pasculli, & Calin, 2014). MiRNAs mainly exerts biological functions by combining with the 3'non-coding region of specific target genes, thereby reducing the expression of target genes, or by inhibiting their translation, participating in the regulation of cell proliferation and differentiation, tumor metastasis and invasion and other processes (Bartel, 2009). It has been pointed out in the literature that compared with normal tissues, CRC tissues have different miRNAs expression levels (Baraniskin et al., 2012). MiRNAs have a duality in

the pathogenesis of CRC. On the one hand, it can act as an oncogene or tumor suppressor gene. On the other hand, it can play an important role in promoting the occurrence of CRC by regulating epithelial-mesenchymal transition (EMT) (Yu et al., 2016). EMT process can stimulate the production of inflammatory factors by cancer cells (Suarez-Carmona, Lesage, Cataldo, & Gilles, 2017), and the evidence of its relationship with inflammation in CRC has been fixed (Briede, Strumfa, Vanags, & Gardovskis, 2020). EMT mainly refers to the biological process in which epithelial cells lose their epithelial properties and turn into mesenchymal cells. Its main features are the reorganization of the cell skeleton and the loss of intercellular adhesion. Among them, the expression of E-cadherin, which can enhance intercellular adhesion, is reduced, and it is converted to the main vimentin of mesenchymal cell skeleton, the adhesion between cells is decreased, the connection is loose, and it is easier to invade and metastasize (Nieszporek, Skrzypek, Adamek, & Majka, 2019; Yeung & Yang, 2017). Studies have shown that in patients with CRC, the expression of E-cadherin is reduced, and the tumor is more prone to invasion and metastasis (Elzagheid et al., 2012).

Studies have shown that some miRNAs can regulate EMT to participate in the pathogenesis of CRC by regulating the expression of E-cadherin, vimentin, etc., and it has a dual nature. The miR-200 family can enhance the transcription and translation of E-cadherin by binding to the 3'non-coding regions of E-cadherin transcription inhibitors Zinc finger E-box-binding homeobox 1 (ZEB1) and Zinc finger E-box-binding homeobox 2 (ZEB2), thereby inhibiting tumor invasion (Davalos et al., 2012). When

ZEB1 is expressed in CRC, it can also increase the expression of miR-200, reverse the EMT process, and inhibit tumor cell proliferation and migration (Lu et al., 2005). This shows that miR-200 and ZEB1 regulate each other to form a negative feedback mechanism to regulate EMT and affect the progression of CRC. Researchers have also described the role of the miR-200 family in different cancers and have described the role of microRNAs in CRC (Humphries & Yang, 2015). The miR-200 family members are transactivated by p53, via the inhibition or overexpression of the miRNAs affects p53-regulated EMT by altering ZEB1 and ZEB2 expression, which means p53-regulated miRNAs are critical mediators of p53-regulated EMT (Kim et al., 2011). Stephen's group (O'Brien et al., 2018) performed a systematic review, regarding the function of the miR-200 family and EMT in CRC both in vitro and in human studies, concluded that the miR-200 family played a central role in EMT process and had potential for both prognostic and therapeutic management of CRC. Hu (Hu et al., 2016) found that miR-363-3p was closely related to the pathological stage of CRC and lymph node metastasis, and the expression loss in the group with lymph node metastasis, and in vitro experiments, found that high expression of miR-363-3p could reverse the EMT process. Sun (Sun, Gu, Chen, & Xiang, 2015) confirmed that the expression of miR-610 decreased in CRC tissues. When miR-610 was increased, the expression of E-cadherin decreased, indicating that miR-610 could inhibit the occurrence of CRC by affecting the expression of EMT-related proteins. Zhang (Zhang et al., 2016) showed that the expression of miR-187 was missing or decreased in CRC tissues compared with the control group. High

expression of miR-187 could regulate the expression of target genes SOX4, NT5E and PTK6, leading to abnormal downstream signaling pathways and inhibiting the EMT process of CRC cells.

MiR-21 has the function of proto-oncogene and has been confirmed to be highly expressed in most tumors including CRC, and affects the proliferation and invasion of cancer cells. Kang (Kang, Lee, Oh, Lee, & Jung, 2015) found that the expression of miR-21 in CRC tissue was significantly increased through case analysis, and miR-21 may negatively regulate E-cadherin by increasing the expression of Metastasis-Associated protein 1 (MTA1), the elevated miR-21 was an independent risk factor for recurrence in CRC patients. Wang's (Wang, Nie, Wu, Liu, & Guo, 2017) group convinced that miR-21 promoted TGF-β-induced EMT in CRC, they found miR-21 was upregulated and promoted TGF-β-induced EMT in CRC cells, suggesting that TGF-β-induced EMT of CRC via transactivation of miR-21. Huang (Huang et al., 2015) collected clinical cases of CRC and found that the TNM stage and depth of invasion in the high expression group of miR-19a were significantly higher than those in the low expression group, and in vitro experiments suggested that up-regulating miR-19a could promote the process of TNF-α induced EMT, the ability to cause cancer cell invasion was enhanced.

4. Closing Words

CRC is a tumor closely related to inflammation. In the process of the occurrence, metastasis, and deterioration of CRC, inflammatory cells, inflammatory mediators

and various cytokines all play important roles. With the deepening of research on the relationship between inflammation and CRC, the understanding of CRC and other immune-related tumors has become more and more profound. We also hope that through research on the inflammatory indicators and pathogenesis of CRC, the probability of inflammatory bowel disease turning into intestinal cancers can be reduced, so as to improve the diagnosis and treatment of CRC.

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11. DIFFERENT PHASES OF ANASTOMOTIC HEALING

The Immunologic Changes During Different Phases of Intestinal Anastomotic Healing

Abstract: Intestinal anatomosis is a complex and multicellular process that involving three overlapped phases: exudative phase, proliferative phase and reparative phase. Undisturbed anastomotic healings are crucial for the recovery of patients after operations but unsuccessful healings are linked with a considerable mortality. This time, we concentrate on the immunologic changes during different phases of intestinal anastomotic healing and select several major immune cells and cytokines of each phase to get a better understanding of these immunologic changes in different phases, which will be significant for more precise therapy strategies in anastomoses.

Keywords: Anastomotic Phases, Would Healing, Immune Cells, Cytokines, Intestinal Surgery

1. Introductions

Intestinal anastomosis is a complicated and cellulous procedure and the barrier function of intestine is central to health and breaking down of the barrier is involved in wide varieties of clinical conditions (Alam & Neish, 2018). Successful anastomotic healings are crucial for the recovery of patients after surgeries but failed healings would result in fatal illnesses, prolonged hospitalizations and even deaths (Gilbert et al., 2016). Despite an apparent appalling clinical need and comprehensive studies performed over past several years, basic and clinical researches targeted at knowing and improving anastomotic healing are still delaying because of multiple factorial challenges. Moreover, such complexity of this process presents challenges in development of proper animal models to study anastomotic healing and potential treatments, and further limiting translation from preclinical experiments to clinic (Lindley, Stojadinovic, Pastar, & Tomic-Canic, 2016). Therefore, finding an easy but feasible method to understand the whole procedure of anastomosis is of great significance.

As we know, intestinal anatomosis can be divided into three overlapped phases: exudative phase (other names called inflammatory phase or hemostasis phase), proliferative phase and reparative phase (another name called remodeling phase) (Rijcken, Sachs, Fuchs, Spiegel, & Neumann, 2014; Wang, Huang, Horng, Yeh, & Chen, 2018). To study the immunologic changes on each phase can help us to understand the differences among the three phases and get the whole picture of anastomosis as well. In this review, we focus on the contribution of several major immune cells and

cytokines in each phase as follows:

Anastomotic Phases and Immune Cells & Cytokines

Phases	Immune Cells and Cytokines			
Exudative Phase (Inflammatory / Hemostasis Phase): 1-4 days	Platelets	Neutrophils	Platelet-Derived Growth Factor (PDFG)	Interleukin-1 (IL-1) Family
Proliferative Phase: 2-14 days	Epithelial Cells	Macrophages	Vascular Endothelial Growth Factor (VEGF)	Interferon Gamma (IFN-γ)
Reparative Phase (Remodeling Phase): after 14 days	Fibroblasts	Lymphocytes	Basic Fibroblast Growth Factor (bFGF)	Transforming Growth Factor Beta (TGF-β)

2. Exudative Phase (Inflammatory/Hemostasis Phase)

In the exudative phase, the immediate event when an injury occurs is platelet plugs that limits the bleeding and causes the release of a wide variety of immune cells and cytokines (Portou, Baker, Abraham, & Tsui, 2015). This event begins the coagulation cascade and promotes expansion and recruitment of cells for the debridement of dead tissue that basically acts as a temporary wound closure mechanism (Shah & Amini-Nik, 2017). Within hours of the injury, neutrophils are drawn to and trapped in the platelet plugs in response to platelet-derived growth factors (PDGF). They serve originally to phagocytize nonviable tissue and bacterial particles as well as use reactive oxygen species (ROS) to create a bacteria hostility

environment (Childs & Murthy, 2017). Neutrophils also provide an important proinflammatory cytokine in interleukin-1 (IL-1), which is the first signal that warns surrounding cells to barrier damage and has double effects as a proinflammatory cytokine and a stimulus for proliferation of keratinocytes. Keratinocytes release prestored IL-1. PDGF together with proinflammatory cytokines such as IL-1, are significant in attracting neutrophils to the wound site to remove contaminating bacteria (Patel, Maheshwari, & Chandra, 2016).

2.1. Platelets

Platelets, small-sized, complex non-nucleated blood components and first described over 100-year ago, were conventionally conceived to purely play an important role in regulating hemostasis. However, there is increasing experimental and clinical evidence recognizes that platelets also have a crucial role in inflammation and immunization (Jenne & Kubes, 2015; Thomas & Storey, 2015).

Although it is not completely understood yet, the immune function of platelets is a delicate balance between its regulation of hemostatic functions and its innate and adaptive immune responses (Morrell, Aggrey, Chapman, & Modjeski, 2014).

2.1.1. The Function of Platelets

Platelets play an important role in the vessel and exist in circulation for 5-7 days, primarily act as regulators of hemostasis (Tomaiuolo, Brass, & Stalker, 2017). When vascular damage or injury, platelets become activated in the blood. Then adhere to the exposed extracellular matrix (ECM) and eventually form platelet plugs and consolidate clots (Yeung, Hawley, & Holinstat, 2017). However, in addition to

regulating hemostasis which is the well-known function of platelets, some other potential functions of platelets have been declared, including the role in innate and adaptive immunity (Holinstat, 2017).

The platelets' interaction with immune cells such as neutrophils is central to start the immune response, and this response is functioned through the Toll-like receptors (TLRs) (Koupenova et al., 2014). The TLR family is made up of 13 recognized members and 10 of them are expressed in humans. TLRs are key pattern recognition receptors of the innate immune system and are located either at the cell surface such as TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6, or in the intracellular such as TLR-3, TLR-7, TLR-8 and TLR-9 (Patel et al., 2012). TLRs are expressed by cells comprising the dermis and epidermis, such as keratinocytes. The activation and timing of specific TLRs and the presence of conditions affecting TLR expression and activation determine whether TLR activation promotes or inhibits the wound healing process, leading to chronic wounds (Portou, Baker, Abraham, & Tsui, 2015). Podoplanin is an endogenous ligand for C-type lectin-like receptor 2 (CLEC-2), which is expressed on platelets, podoplanin / CLEC-2 signaling regulates keratinocyte migration via modulating E-cadherin expression through RhoA signaling (Asai et al., 2016). Therefore, altering the regulation of keratinocyte migration by Podoplanin expressed on platelets will be a novel method to wound healing.

2.2. Neutrophils

Neutrophils are the most abundant immune cells to a new wound and they are very active during the healing of wounds (Jablonska & Granot, 2017). It is known that

neutrophils remove the debris in the early phase of anastomotic healing. Concurrent with the process of hemostasis, neutrophils represent the first cells to migrate to the wound bed, brought in by pro-inflammatory signals such as IL-1 (de Oliveira, Rosowski, & Huttenlocher, 2016). Although neutrophils have a very short half-life in blood both in mice and humans, proinflammatory cytokines such as IL-1 increase their lifespan, which may contribute to relieve the inflammation (Lahoz-Beneytez et al., 2016).

Mouse models of recent studies have shown that in non-aged, non-impaired models, neutrophils depletion does not negatively affect the wound healing, but in impaired models of wound healing, such as diabetes and inflamed gut, neutrophils are badly required (Kolaczkowska & Kubes, 2013; Pitchford, Pan, & Welch, 2017; Wilgus, Roy, & McDaniel, 2013).

2.2.1. The Function of Neutrophils

Although neutrophils are not considered as an essential cell type in non-impaired, non-aged wound healing, they do complete a variety of functions that support the process (Kolaczkowska & Kubes, 2013; Pitchford, Pan, & Welch, 2017; Wilgus, Roy, & McDaniel, 2013).

First of all, neutrophils protect from wound infection by endocytosing pathogenic agents, and killing them via releasing reactive antimicrobial proteins (Wilgus, Roy, & McDaniel, 2013). Then with the process of degranulation, antimicrobial proteins can be released into the surrounding environment to kill extracellular animate things (Borregaard, 2010).

In addition to clear pathogenic agents, neutrophils also regulate inflammation and generate immune cells to induce wound healing. In the injured environment, neutrophils have the ability to increase the expression of cytokines and chemokines as well as additional neutrophils (Eming, Martin, & Tomic-Canic, 2014). Neutrophils also show increased expression of cytokines that promote angiogenesis, such as vascular endothelial growth factor (VEGF), proliferation of fibroblasts, keratinocytes, such as IL-1, and tissue remodeling, which are essential to the wound healing (Butin-Israeli et al., 2019; Butin-Israeli et al., 2016; Chen et al., 2018).

2.3. Platelet-Derived Growth Factor（PDGF）

Platelet-derived growth factor (PDGF) is a significant factor driving wound healing of actually almost all organs (Klinkhammer, Floege, & Boor, 2018). Platelet-derived growth factor is a significant mediator in the early phase of wound healing. It was discovered nearly 40 years, and it was found to have an important role in wound healing for almost 30 years as well, but studies into its physiological roles, functions and structures are ongoing (Kaltalioglu & Coskun-Cevher, 2015; Pierce, Mustoe, Altrock, Deuel, & Thomason, 1991; Ross, 1987).

There are five known members in the PDGF family: PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD (Barrientos, Stojadinovic, Golinko, Brem, & Tomic-Canic, 2008). And PDGF-BB is believed by researchers to be the most important and related member for wound healing (Babavalian et al., 2018; Li et al., 2017).

2.3.1. The Function of Platelet-Derived Growth Factor（PDGF）

Platelet-derived growth factors stimulate the production of ROS during the exudative phase of wound healing (Yang et al., 2016). We understand that PDGF-BB is the most effective factor that drives the first phase of wound healing (Babavalian et al., 2018; Li et al., 2017). Firstly, PDGF-BB cure methods doubled the rate of complete re-epithelialization of wound healing. Secondly, increased vessel formation was also found and was a significant part of the increased granulation tissue present in PDGF-BB cured wound healing. Importantly, the remarkably increase in granulation tissue formation mediated by PDGF-BB was fully reversible (Piran et al., 2018; Younesi et al., 2017).

PDGF is also known for stimulating the chemotaxis of neutrophils and macrophages which are crucial in the inflammatory phase of wound healing and produce ROS via nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) (Aviello & Knaus, 2018). PDGF could increase ROS production by stimulating the migration of immune cells and cytokines to the wound site and stimulate macrophages to produce growth factors which are critical for wound healing (Wang & Zhang, 2017).

2.4. Interleukin-1（IL-1）Family

The innate immune system is the front line defense of our bodies. It is nondirected and unspecific, through the complement activation or the activation of innate immune receptors, the innate immune response is started (Boraschi, Italiani, Weil, & Martin, 2018; MacLeod & Mansbridge, 2016).

Cytokines of the IL-1 family play key roles in innate immunity. There are eleven

members in this family: IL-1α , IL-1β , IL-1RA, IL-18, IL-36Ra, IL-36α , IL-37, IL-36β , IL-36γ , IL-38, and IL-33. Some researchers also term them from IL-1F1 to IL-1F1 depend on the order of their discoveries (Yazdi & Ghoreschi, 2016).

Though IL-1 family is component the innate immune system, it is also influence on T-cell functions. Thus IL-1 family is an important bridge between an early innate immune response and an adaptive immune response followed. Some studies also show that the IL-1 family members (such as IL-1β , IL-33) impact the inflammation phase of wound healing as well (He et al., 2017; Oshio et al., 2017; Yan et al., 2016).

2.3.1. The Function of Interleukin-1（IL-1）Family

IL-1 family members are key inflammatory cytokines that generally act synergistically to amplify the inflammatory response. During wound healing, IL-1 is expressed majorly by neutrophils and macrophages (Abarca-Buis et al., 2018).

Decreased inflammatory cell numbers and reduced epidermal thickness and fibrosis are found in the IL-1R KO mice and wild-type mice treated with IL-1ra (Thomay et al., 2009). Decreased inflammatory cytokines, myofibroblasts and proliferating cells are found by injecting of IL-1ra (Chamberlain et al., 2014). Other members of the IL-1 family (such as IL-18 and IL-33) are also involved to regulate the inflammatory and reparative response. IL-18 participates in pro-inflammatory signaling, while IL-33 exerts cytoprotective effects (Frangogiannis, 2015). And the early use of the IL-1Ra will inhibit the efficacy of IL-1 in the inflammatory cascade and can prevent early granulation formation (Nicolli et al., 2016). These researches indicate that IL-1 family is a significant indirect mediator of wound healing and that

inhibition of IL-1 signal could contribute to wound healing.

3. Proliferative Phase

In proliferative phase, the main focus of the healing process lies in covering the wound surface, angiogenesis, and epithelialization. Though there are overlaps between the wound healing phases, the capability to transit into the next phase will decide whether a wound heals suitably (Barchitta et al., 2019; Reinke & Sorg, 2012). Epithelialization occurs early in wound repair shortly after injuring. And endothelial cells produce vascular endothelial growth factor (VEGF) which is crucial to restoring impaired angiogenesis process. Macrophages have numerous functions like hosting defense, promoting and solving inflammation, removing apoptotic cells and supporting cell proliferation and tissue restoration after injury. Macrophages also play an indispensable role in a successful healing process through the synthesis of numerous potent growth factors such as VEGF, which promote cell proliferation. And under the control of regulating cytokines such as IFN-γ , the synthesis of collagen, fibronectin and other basic substances needed for wound healing, serves for the closure of tissue gaps and the restoration and enhances the wound healing effects (Velnar, Bailey, & Smrkolj, 2009; Wilhelm, Wilhelm, & Bielfeldt, 2017).

3.1. Epithelial Cells

Intestinal epithelial cells, which line the inner face of the intestinal tract, have various significant functions (Satsu, 2017), such as absorbing food substances,

immune functions like cytokine secretion, via detoxification enzymes to work as barriers against xenobiotics and help the wound healing as well (Hegyi, Maleth, Walters, Hofmann, & Keely, 2018; Schneeberger, Roth, Nieuwenhuis, & Middendorp, 2018).

It is known that immediately after wound, coagulation and hemostasis are triggered in the injured issues. Humoral and cellular inflammatory phase follows with the formation of an immune barrier against invading microorganisms. And then the wound healing mechanisms are later turned to tissue healing (Jin, Kim, Han, & Kim, 2016). Among the different processes in the proliferative phase, the epithelialization and angiogenesis are of particular significance. The epithelial cells have a special role, they make the growth and survival of new-formed tissues possible, since all tissues are depending on blood supplies and this in turn depends on epithelial cells (Velnar & Gradisnik, 2018).

3.1.1. The Function of Epithelial Cells

Epithelial cells allow organisms to keep internal homeostasis in changes of external environment and protect against infection. Some epithelial cells, take intestine epithelial cells for example, close breaches extremely fast and effectively limiting the entry of pathogens (Arwert, Hoste, & Watt, 2012; Enyedi & Niethammer, 2015).

There are a quantity of epithelial signaling events come into play roles to mediate wound closure. Some recent studies have highlighted an important role of ROS signaling in coordinating wound healing (Thi et al., 2020). Though the oxygen

has a key role in mediating wound repair is well known, the significance of cellular oxygen understanding in healing mechanisms is still a new area (Dunnill et al., 2017).

We can also establish either animal models or epithelial cells *in vitro* culture to study their migratory behavior and their roles to play in wound healing, such as intestinal anastomotic healing and so on (Goodlad, 2017; Leoni, Neumann, Sumagin, Denning, & Nusrat, 2015; Liarte, Bernabe-Garcia, Armero-Barranco, & Nicolas, 2018; Miyashita et al., 2017).

3.2. Macrophages

Similar to neutrophils, macrophages are also a significant part of the innate immune response to intestinal wound healing, partially because of their capability to start and solve inflammation and to contact with other innate and adaptive immune cells. As intestine has the largest pool of macrophages in the body (Bain et al., 2014), macrophages' role in intestinal wound healing cannot be ignored.

Generally, macrophages can be categorized into three major subtypes: inflammatory monocytes which can be rapidly differentiate into activated macrophages (M1-like phenotypes, mainly for pro-inflammatory), tissue-resident macrophages (M2-like phenotypes, mainly for anti-inflammatory) and regulatory macrophages (Krzyszczyk, Schloss, Palmer, & Berthiaume, 2018). M2-like phenotypes are believed more desirable for effective wound healing (He et al., 2019).

3.2.1. The Function of Macrophages

In all injuries, macrophages are significant players (Smigiel & Parks, 2018). They create an inflammation environment for clearing possible pathogens; resolve the

inflammation when the pathogens are cleared; and are also participant in starting tissue remodeling (Kim & Nair, 2019). But macrophages contribute most to the proliferative phase of wound healing, especially M2-like phenotypes' role in mediating resolution of inflammation. M2-like phenotypes are alternative activated cells that regulated by T helper type 2 (Th2) (Minutti, Knipper, Allen, & Zaiss, 2017).

M2-like phenotypes have the ability to self-renew and can be long-lived in body. Following the injury, M2 macrophages express adhesion molecules that recruit and guide multiple cell types. Moreover, M2 macrophages can replicate to double or triple their numbers, which are orchestrating in this wound healing process (Snyder et al., 2016).

Recent studies also show that macrophages take into effect to promote wound healing through WNT pathways which are firstly found as necessary pathways for tissue and organ development (Burgy & Konigshoff, 2018; Houschyar et al., 2015). WNT ligands secreted by macrophages enhance the intestinal regeneration (Saha et al., 2016). Transfer of M2 macrophages will quicken the wound healing in TNBS-treated (2, 4, 6-trinitrobenzenesulfonic acid treated) mice via the activation of the WNT signaling pathway (Cosin-Roger et al., 2016).

3.3. Vascular Endothelial Growth Factor（VEGF）

Vascular endothelial growth factor (VEGF) is an effective and selective mitogen for vascular endothelial cells and plays a significant role in Angiogenesis (Zhou, Ma, & Brogan, 2015). It helps wound repair by adding the vascular permeability of local blood vessels, contributing the flow of inflammatory cells to the wound sites and

adding the proliferation of endothelial cells which plays important roles in wound healing (Dinh, Braunagel, & Rosenblum, 2015; Zarei & Soleimaninejad, 2018).

There are five members in VEGF family including VEFG-A, VEFG-B, VEFG-C, VEFG-D and placenta growth factor (PLGF) (Muratori et al., 2018). Among them, VEGF-A participates in the process of wound healing, its transcription and secretion will be elevated and reach the peak at the approximately 7th day after injury. And it mainly influences the proliferative phase (Bao et al., 2009).

3.3.1. The Function of Vascular Endothelial Growth Factor（VEGF）

It is already known that a special property of VEGF is to add vascular permeability. VEGF was named vascular permeability factor for a time before its amino acid sequence was clear (Dinh, Braunagel, & Rosenblum, 2015; Zarei & Soleimaninejad, 2018). With further researches in VEGF, we know that VEGF is also a powerful and positive mediator for endothelial cells to format new blood vessels, such as proliferation (Reddy, Yosef, & Ubogu, 2013). The blood vascular components depend on angiogenesis, in which new blood vessels appear approximately from 3rd to 7th day after injury. And then capillary growth into the injured sites subsequently provides passages for nutrients and other mediators of the wound healing (Ong & Dilley, 2018).

While generally beneficial, VEGF also acts as a chemical attractant for invading pathogens, VEGF is a crucial cytokine for angiogesis, and it is also influenced by the bacterial infections, such as *Pseudomonas aeruginosa*, which may harmful to the wound healing (Birkenhauer & Neethirajan, 2015). Some studies show a new

concept called VEGF-driven keratinocyte response (An et al., 2018; Loyd et al., 2012), although we still need to find more evidence for VEGF interaction with keratinocytes, it may give another perspective to wound healing therapies in clinic (Barrientos, Brem, Stojadinovic, & Tomic-Canic, 2014).

3.4. Interferon Gamma（IFN-γ ）

IFN-γ is a main component in immune cell signaling and is a significant mediate protein for general immune responses. Its effects on cells are remarkable and have been found to regulate the expression of thousands human genes. Although IFN-γ has functions of anti viral, it is more notable for stimulating and regulating the immune cells (Lee et al., 2018; Martin & Nunan, 2015).

The innate and adaptive immune responses depend on controlled IFN-γ expression. And present researches show that IFN-γ has a significant role in the proliferation phase of wound healing via the mediation of the immune responses at the injury sites (Beyer, Koch, Lee, Jung, & Blocki, 2018). Thus, it is of great significance to understand the pathways that mediate the expression of IFN-γ .

3.4.1. The Function of Interferon Gamma（IFN-γ ）

IFN-γ is basically secreted by CD4+ and NK cells, and it contributes mainly to the activation of immune cells, has relationships with both neutrophil recruitment and cell clearance (Fenimore & H, 2016; Siska & Rathmell, 2016). As for its role in wound healing, some studies show that it can enhance the healing procedure and effects, such as IFN-γ enhances the blood vascular regeneration and wound healing through significantly up-regulated BST2 expression in both LEPCs and ECs and

increased tube formation in LEPCs (Lee et al., 2018).

As IFN-γ is well known regarding its inhibitory effects on collagen synthesis by fibroblasts. Its role in wound healing remains controversial (Taylor et al., 2018), such as IFN-KO mice exhibited accelerated healing compared with WT mice, showing that IFN-γ makes a negative contribution to the wound healing procedure (Kanno et al., 2019).

Therefore, further investigations are necessary to illuminate the effects of IFN-γ therapy on wound healing, and it is also of great importance to clarify its optional dose (Burke & Young, 2019).

4. Reparative Phase (Remodeling Phase)

The reparative phase is where the wound achieves maximum strength as it matures. It is characterized by wound contraction and collagen remodeling and the reorganization of the distinct layers of the intestinal wall (Reinke & Sorg, 2012). An essential feature of normal wound repair is the formation of granulation tissue, for instance, tissues containing fibroblasts, collagen, and blood vessels, which means the hallmark of an established healing response. As we described above, macrophages are the key cells in proliferation phase, while fibroblasts are becoming the principle cells in remodeling phase (Bainbridge, 2013). Net collagen synthesis will continue after wounding. The added rate of collagen synthesis during wound healing is from the increase of fibroblasts (van Koppen & Hartmann, 2015). The quality and appearance of the wound healing or repairing is mostly decided by this phase.

Therefore, we should have a better management on this phase in order to get a better scar or scarless healing. In this part, we focus on four major cells and cytokines which may helpful in reparative phase.

4.1. Fibroblasts

Fibroblasts, mesenchymal cells, are present in many tissues in the body, play a major role in structural support (Darby & Hewitson, 2007). Since they have the ability to secrete and respond to cytokines, they also take part in the wound healing processes, especially in reparative phase (Udhayakumar, Shankar, Sowndarya, & Rose, 2017).

It has been more than 40 years since fibroblasts were first reported, much interest has concentrated on the control of them since that time. They have capability of changing during the wound healing processes to a contractile phenotype involved in adding ECM production and contraction in the process of wound healing (Cao, Hicks, & Standley, 2013; Law, Chowdhury, Aminuddin, & Ruszymah, 2017).

4.1.1. The Function of Fibroblasts

The activity of fibroblasts and their following differentiation is relied on the links of the action of growth factors, ECM components, and mechanical stress. Local proliferation and migration from adjacent tissues, especially near the vascular region, have generally been accepted as the mechanisms by which the tissue fibroblast numbers may increase (desJardins-Park, Foster, & Longaker, 2018; Schmidt & Horsley, 2013; Woodley, 2017).

Rinkevich and his colleagues demonstrated the discovery of a “scarring fibroblast” that responsible for depositing the very majority of scar tissue in mice. They showed that these same cells could be reliably identified through expression of the marker CD26 and that ablation of these cells would reduce the scarring, though this also might delay the wound healing (Rinkevich et al., 2015). In order to get a better understand of this “scarring fibroblast”, Plikus and his colleagues’ research illustrated that during the wound repairs, fat cells can be generated from activated fibroblasts which involved in wound contraction (Plikus et al., 2017). Some other researches also demonstrated the lineage among fibroblasts involved in wound healing (Stunova & Vistejnova, 2018; Werner, Krieg, & Smola, 2007).

However, further studies are needed to fully clarify the contributions of different fibroblast lineages to wound healing, characterize the most specific subtype both in animals and human beings.

4.2. Lymphocytes

Lymphocytes are critical components of the adaptive immune responses, originally from the bone marrow, and can be mainly divided to three directions: mature into B lymphocytes; travel to thymus and develop into T lymphocytes; and stay primitive as Natural killer cells (NKCs) (Li, Tan, Martino, & Lui, 2018). Among them, B lymphocytes develop into plasma cells which secrete antibodies. T lymphocytes can be further divided into CD4+ helper cells and CD8+ cytotoxic cells on the basis of their surface marker proteins. CD4+ cells can activate B lymphocytes in order to make B lymphocytes work properly. CD8+ cells have the ability to clear

viral-infected or dysfunctional cells (Keen, 2008).

Adaptive immunity activation requires highly specific cooperation between antigen-presenting cells and distinct antigen-specific receptors on lymphocytes. Lymphocytes, especially T lymphocytes, play a significant regulatory role in wound healing through both the modulation function of fibrosis and its adaptive immune responses pathway (Hofmann & Frantz, 2015; Vatankhah et al., 2017).

4.2.1. The Function of Lymphocytes

Lymphocytes have a regulatory role in normal wound healing through the secretion of lymphokines that are soluble protein factors produced by antigen-stimulated lymphocytes and act as chemical messengers (Keen, 2008). Some studies have shown that lymphokines influence fibroblast activities and collagen synthesis which are belong to the reparative phase or remodeling phase of wound healing (Nosbaum et al., 2016).

The process of lymphocytes activation that is stimulated by antigen-presenting cells mainly happens in the lymph nodes and spleen. Then activated-lymphocytes are transported to the periphery through the lymphatic vessels and arterial vessels. Some researchers believed that lymphocytes are crucial to competent wound healing since they perform significant regulatory functions during wound healing as well as their important roles in adaptive immune responses (Haertel, Joshi, Hiebert, Kopf, & Werner, 2018; Nunes-Silva, Frantz, & Ramos, 2017).

In order to examine lymphocytes relationship with adaptive immunity, researchers have found that T lymphocytes promote the wound healing via

endogenous vascular endothelial growth factor receptor 1 tyrosine kinase (VEGFR1-TK) pathway (Betto et al., 2019). And they also secrete the lymphokines to regulate the healing of the epithelium and protect barrier function of intestinal epithelial cells (Cook et al., 2019). Another research group even use neutrophil-lymphocyte ratio and platelet-lymphocyte ratio to predict the effect of wound healing in reconstruction (Maruyama et al., 2017).

4.3. Basic Fibroblast Growth Factor（bFGF）

Fibroblast growth factors are a very big family consisted of many homologous peptides, such as Acidic fibroblast growth factor (aFGF or FGF-1), Basic fibroblast growth factor (bFGF or FGF-2) and Keratinocyte growth factor (KGF or FGF-7) (Maddaluno, Urwyler, & Werner, 2017; Przybylski, 2009). Among FGF family, bFGF's ability to accelerate the process of both acute and chronic wound healing has been already proved and it is mainly produced by fibroblasts, macrophages and endothelial cells (El Agha, Kosanovic, Schermuly, & Bellusci, 2016).

The bFGF is a multiple potential glycoprotein that promotes various cells such as fibroblasts, keratinocytes and endothelial cells. Because of its mitogenic and angiogenic characteristics, the bFGF plays an important role in inducing tissue remodeling and wound healing (Aoki et al., 2017; Carter, 2003).

4.3.1. The Function of Basic Fibroblast Growth Factor（bFGF）

The bFGF is a powerful mitogen and chemical attractant for endothelial cells and fibroblasts and stimulates the metabolism and growth of the ECM, which are very important for wound healing. In some animal experiment, bFGF-knockout mice

showed delayed healing of skin injury (Qu et al., 2018; Zhao et al., 2008).

And bFGF is widely accepted and used in accelerating wound healing in clinical treatment. Some studies show bFGF is not only helpful for wound repair, but also improve the scar quality and regeneration (Xie et al., 2008; Zhang et al., 2018). The practice and action of bFGF in scar management is highly significant both in understanding scarless wound healing in the lab and fulfilling minimally invasive concept in the clinic (Akita, Akino, & Hirano, 2013). Accelerating wound healing improves the quality of healing and alleviates the scar (Shi et al., 2013). Those researches imply a feasible anti-scarring effect of bFGF during wound healing.

Although human recombinant bFGF is used for wound healing far and wide nowadays, the problem of its short half-life still remains to be sorted (Hayashida, Fujioka, Morooka, Saijo, & Akita, 2016; Nakamichi et al., 2016; Numata et al., 2006).

4.4. Transforming Growth Factor Beta（TGF-β ）

Transforming growth factor-β (TGF-β) is a multiple functional cytokine that play a key role in wound healing and in tissue repairing. TGF-β is found in almost all tissues in body, it is mainly produced by infiltrating cells, like platelets, macrophages and lymphocytes (Wang, Han, Owens, Siddiqui, & Li, 2006; Zaiss, Minutti, & Knipper, 2019). Thus, after the injury, these cells are becoming potential sources of TGF-β .

Generally, the production and activation of TGF-β will stimulate the production of various ECM proteins, and will decelerate the breakdown of those proteins as well. TGF-β contributes to wound healing through these actions. Under ideal circumstances, we hope the wound heals to the restoration of normal tissue

architecture or at least to a scar less tissue (Beanes, Dang, Soo, & Ting, 2003; Biancheri et al., 2014). Therefore, exploration of TGF-β activity in scarless wound healing, and understanding of TGF-β function in scarless wound healing is a very promising field which is also of great significance in improving healing qualities in clinical scenarios, such as in inflamed colons and diabetic wounds.

4.4.1. The Function of Transforming Growth Factor Beta（TGF-β ）

Several growth factors are involved in wound repair, while central to wound healing is TGF-β which is of particular significance for almost all phases of this process, especially in the remodeling phase (Kiritsi & Nystrom, 2018). TGF-β exerts multi-effects on wound healing by regulating cell proliferation, differentiation, ECM production and mediating the immune response as well. TGF-β is a cytokine which is secreted by several different cell types involved in wound healing and has different effects (Lichtman, Otero-Vinas, & Falanga, 2016).

Many different cell types are involved in wound healing, such as epithelial cells, fibroblasts and macrophages are shown to be responsive to TGF-β (Lichtman, Otero-Vinas, & Falanga, 2016). TGF-β promotes monocyte chemotaxis and growth factor production. Moreover, it promotes the regenerative maturation of keratinocytes and recruits fibroblasts into the wound bed. And its most significant function is stimulating the collagen production by fibroblasts, though the procedure of collagen production is also involves other cytokines like IFN-γ (Le et al., 2012; Lichtman, Otero-Vinas, & Falanga, 2016).

Several studies offered the evidences that TGF-β was present in the healing

wound, and suggested that TGF-β might be an important marker of the wound healing procedures. We also noted that there were an increased fibroblasts and an obvious increase in collagen deposition with the application of TGF-β. These studies might imply that TGF-β is a potential pharmacological agent to accelerate the wound healing (Hameedaldeen, Liu, Batres, Graves, & Graves, 2014; Mokoena, Dhilip Kumar, Houreld, & Abrahamse, 2018; Takzaree et al., 2016; Wang et al., 2017).

5. Closing Remarks and Outlook

Successful intestinal anastomotic healing managements require a thoroughly understanding of wound healing processes and the related factors like immune cells and cytokines that play important roles on them. This review tries to illustrate the immunologic changes in different healing phases and cover 12 significant immune cells and cytokines, average 4 immune cells and cytokines in each phase. As it is mentioned at the beginning, the three anastomotic healing phases are an overlapping process, thus an immune cell or cytokine may influence multiple or even the whole healing procedure. But we assign it in one phase according to the greatest impact where it has.

Immune cells and cytokines are critical for coordinating multiple cell types in intestinal anastomotic healing that make the wound repairing possible. Effective wound healing should be guided by strict regulation of these regulators as well as a good repair circumstance that support their actions.

Nowadays, although many promising biomarkers are used in different sample

collecting ways, we should pay attention to the consistent implementation and efficacious follow-up therapeutics as well.

Another impressive finding is that some cytokines such as basic fibroblast growth factor（bFGF） and transforming growth factor beta（TGF-β ）which have the anti-scar effect should be seen and paid attention. It may never be possible to eliminate the risk of an injury, whether artificial (like anastomotic operations) or accidental (like inflammatory or traumatic diseases), but we should try our best to repair it with minimally invasion and continue to expand our medical armamentarium that help us to get a better and more successful healing.

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12. ABBREVIATIONS

AL	Anastomotic Leakage
CRC	Colorectal Cancer
CRP	C-Reactive Protein
NLR	Neutrophil to Lymphocyte Ratio
BMI	Body Mass Index
ROC	Receiver Operating Characteristic
AUC	Area Under the Curve
ASA	American Society of Anesthesiologists
UICC	Union for International Cancer Control
AJCC	American Joint Committee on Cancer
IBD	Inflammatory Bowel Disease
CD	Crohn's Disease
UC	Ulcerative Colitis
TAMs	Tumor-Associated Macrophages
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
STAT3	Signal Transducer and Activator of Transcription 3
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
TNF-α	Tumor Necrosis Factors-α
IL	Interleukin

COX-2	Cyclo-Oxygen-Ase-2
NSAIDs	Nonsteroidal Antiinflammatory Drugs
miRNAs	micro RiboNucleic Acids
PLR	Platelet to Lymphocyte Ratio
LMR	Lymphocyte to Monocyte Ratio
TNM	Tumor Node Metastasis
VEGF	Vascular Endothelial Growth Factor
TGF-β	Transforming Growth Factor-β
PDGF	Platelet Derived Growth Factor
EGF	Epidermal Growth Factor
MMP9	Matrix Metallopeptidase 9
EMT	Epithelial-Mesenchymal Transition
Tregs	T regulatory cells
PKA	Protein Kinase A
PGE2	Prostaglandin E2
ZEB1	Zinc Finger E-box-binding homeobox 1
ZEB2	Zinc Finger E-box-binding homeobox 2
MTA1	Metastasis-Associated Protein 1
IFN-γ	Interferon Gamma
bFGF	Basic Fibroblast Growth Factor
ECM	Extracellular Matrix
TLRs	Toll-like receptors

CLEC-2	C-type Lectin-like receptor 2
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Th2	T helper type 2
PLGF	Placenta Growth Factor

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