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Immunohistochemical evaluation of cellular composition of the immune system of lymph nodes in acute appendicitis

Jakub Żurawski*[1, a], Patrycja Talarska[1, b], Stanisław Łazowski[2], Marcin Grochowalski[3, c], Jacek Karoń[3, d]

[1] Department of Immunobiochemistry, Poznan University of Medical Sciences, Poland
[2] Specialised City Hospital of Jozef Strus Hospital, Laboratory of Pathological Anatomy, Poznań, Poland
[3] Clinic of General Surgery, Poznan University of Medical Sciences, Poland

* *Corresponding Autor:* Jakub Żurawski, 8 Rokietnicka Street, 60-806 Poznań, Poland, email: zurawski@ump.edu.pl

[a] https://orcid.org/0000-0002-5838-0451
[b] https://orcid.org/0000-0003-1305-0990
[c] https://orcid.org/0000-0002-0249-8434
[d] https://orcid.org/0000-0003-4330-7224

ABSTRACT

Introduction. There is not much data about the composition of populations of the immune system in acute appendicitis. The basic histopathological criterion for the diagnosis of acute appendicitis is neutrophil infiltration of the muscle membrane.
Aim. The subject of this publication is a semi-quantitative evaluation of B lymphocytes (CD20+), T lymphocytes (CD3+) and macrophages (CD68+), and the determination of the number of active lymph nodes during the course of inflammation.
Material and Methods. The study material was obtained from 79 patients who had an appendectomy due to acute appendicitis. In this group, the tissue was obtained from: 34 women (aged 20 to 91) and 45 men (aged 20 to 72).
Results. In the course of acute appendicitis, there is involvement of lymph node B lymphocytes, T lymphocytes and macrophages. Independent of the type of inflammation, the cellular make-up of the nodes is similar. The number of lymph nodes decreases with age and is gender dependent.
Conclusions. In the course of acute appendicitis, there is involvement of lymph node B lymphocytes, T lymphocytes and macrophages. The number of lymph nodes decreases with age and is gender dependent. A statistically significant number of the examined cells of the immunological system in the lymph nodes changed due to inflammation ($p < 0.001$). B and T lymphocytes in the lymph nodes and in the mucous membrane of the appendix differed depending on the sex, and the presence of B lymphocytes in the mucous membrane was significantly higher in the group of 20–40 years of age. T lymphocytes were predominant in the centres of the lymph nodes in groups 20–40 and 61–91 years of age, and in the peripheral zones in the group of 41–60 years of age.

Keywords: appendicitis, lymph nodes, histomorphometry.

Introduction

Periumbilical pain is a frequent early symptom of appendicitis, which later migrates to the abdominal right lower quadrant. The pain is characteristically accompanied by nausea, vomiting, and fever. Sharp pain at McBurney's point (two-thirds of the distance from the navel and the anterior superior iliac spine) is pathognomonic of appendicitis. In most cases, an appendectomy is the best treatment choice.

The anatomic location, as well as intestinal stasis and the abundance of lymphoid tissue in the intestinal wall create favourable conditions for the development of inflammation.

The disease may be caused by bacterial, viral and parasitic infections. The inflammation is facilitated by anatomy, such as the length, flexion and close location to the colon. Acute appendicitis occurs as a result of increased pressure in its lumen, and consequently obstruction of venous outflow. In most cases, it is also associated with occlusion by faecal mass, or stercolith, with the proliferation of bacteria, which are a direct cause of inflammation [1].

Inflammatory changes can be seen as hyperplasia of the lymph nodes (appendicitis follicularis). Neutrophil infiltration (appendicitis purulenta) may also occur. When inflammation crosses the appendiceal wall, necrotic foci may appear (appendicitis gangrenosa).

Lymph nodes are round or ovoid structures of stroma and reticular fibres, filled with B lymphocytes, dendritic cells and macrophages.

There are two types of lymph nodes: primary and secondary. Primary lymph nodes have a homogeneous structure and contain small, inactive B lymphocytes (naïve lymphocytes).

Secondary lymph nodes have a more differentiated structure. The centre, the so-called reactive centre, is less dense due to the loose pattern of chromatin in the proliferative nuclei of the centroblasts. B lymphocytes in germinal centres differentiate into plasma cells that secrete antibodies, or into memory lymphocytes. The peripheral zone of the lymph node is a significantly darker zone, called the mantle zone [2].

The presence of the reactive centre and the mantle zone is the result of a response to stimulation by antigens. Hyperplasia of the lymph nodes is caused mainly by the activity of centroblasts in germinal centres.

There is not much data about the composition of populations of the immune system in acute appendicitis, which is the focus of this paper.

Aim

The subject of this publication is a semi-quantitative evaluation of B lymphocytes (CD20+), T lymphocytes (CD3+) and macrophages (CD68+), and the determination of the number of active lymph nodes during the course of inflammation.

Material and Methods

The study material was obtained from 79 patients who had an appendectomy due to acute appendicitis. In this group, the tissue was obtained from: 34 women (aged 20 to 91) and 45 men (aged 20 to 72). The average and median age was higher for the women than than for the men. The difference was statistically significant according to the Mann-Whitney test; $p = 0.0427$

For antigen retrieval, antibodies against: CD3 (Dako A0452), CD20 (Dako M0755) and CD68 (Dako M0814) were used. Preparations were incubated in a water bath at 96°C in a citrate buffer (pH 6.0) for 50 minutes. Endogenous peroxidase was inhibited by 3% H_2O_2. The tissue samples were incubated with antigen for 60 minutes at room temperature. After that, they were rinsed for 10 minutes in TBS followed by incubation in an EnVision system (DakoCytomation, K5007) for 30 minutes.

In all preparations, the DAB-3,3 chromogen was used, which helped to locate the antigen. Following that, preparations were stained with Mayer's hematoxylin and dehydrated in a series of alcohols to xylene.

For immunohistochemical determination, a negative control was performed by omission of the primary antibody.

Photographs of all lymph nodes present in the examined samples were taken with an Olympus BX43 light microscope and XC 30 digital camera, at 100x magnification. Based on photographic documentation, semi-quantitative evaluation of the immunohistochemical reactions was performed, using the cellSens commercial software by Olympus (**Figures 1**, **2**). Statistical calculations were performed with the STATISCTICA 10 statistical package.

Results

Lymph nodes

In all cases, activated lymph nodes were observed (on average 18) (**Figure 3**). Their number depended on the patient's gender. For men, it was 21, and

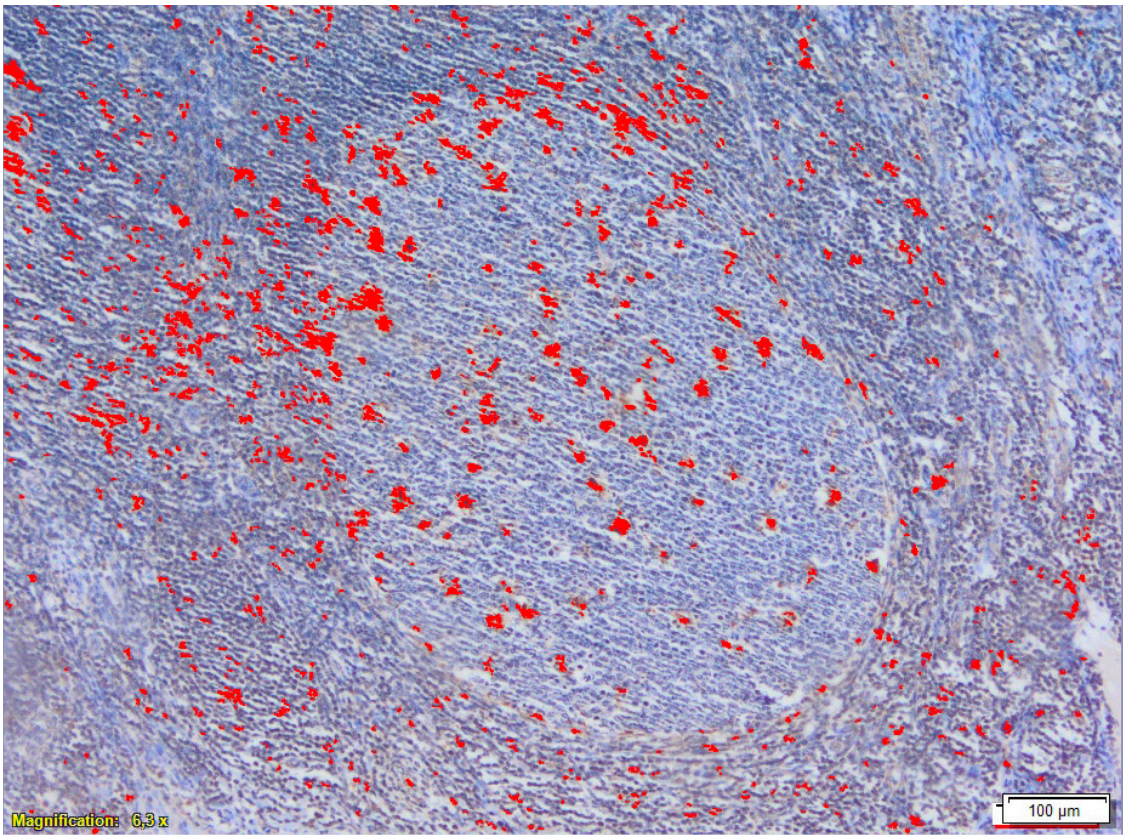

Figure 1. Print screens of cellSens dimension software by Olympus — before image analysis

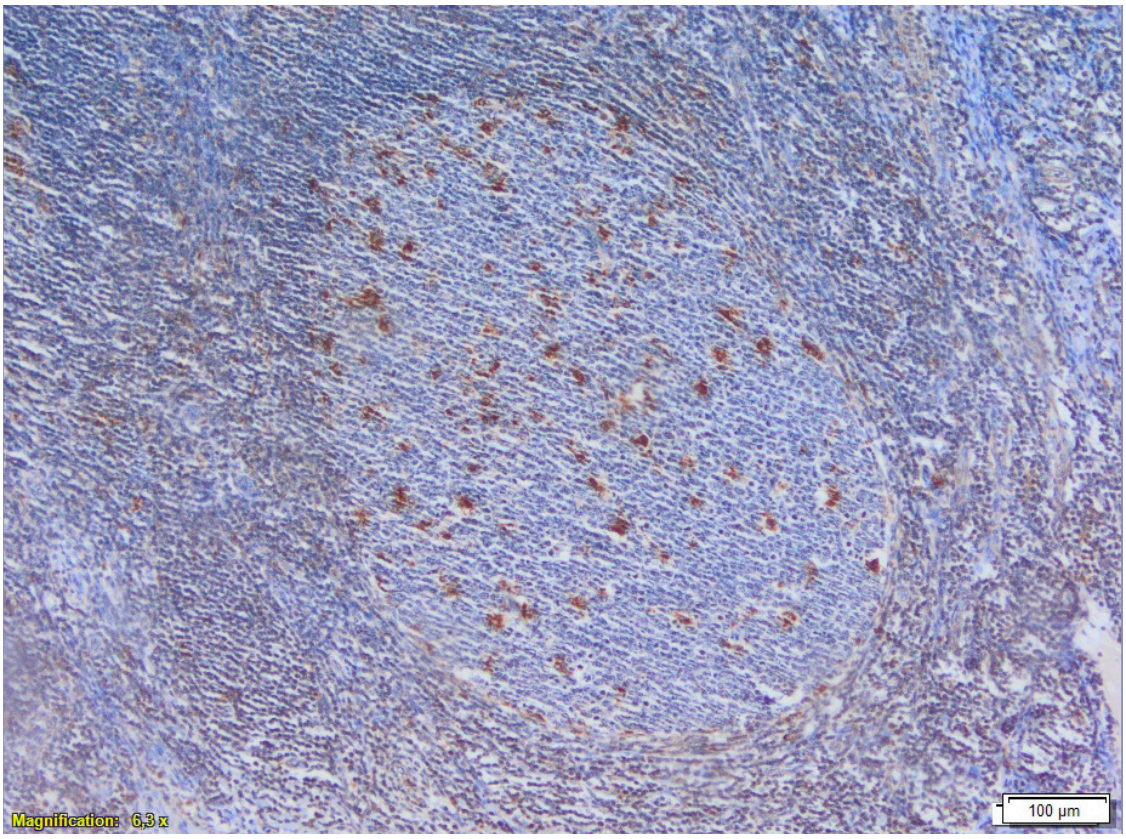

Figure 2. Print screens of cellSens dimension software by Olympus — after image analysis

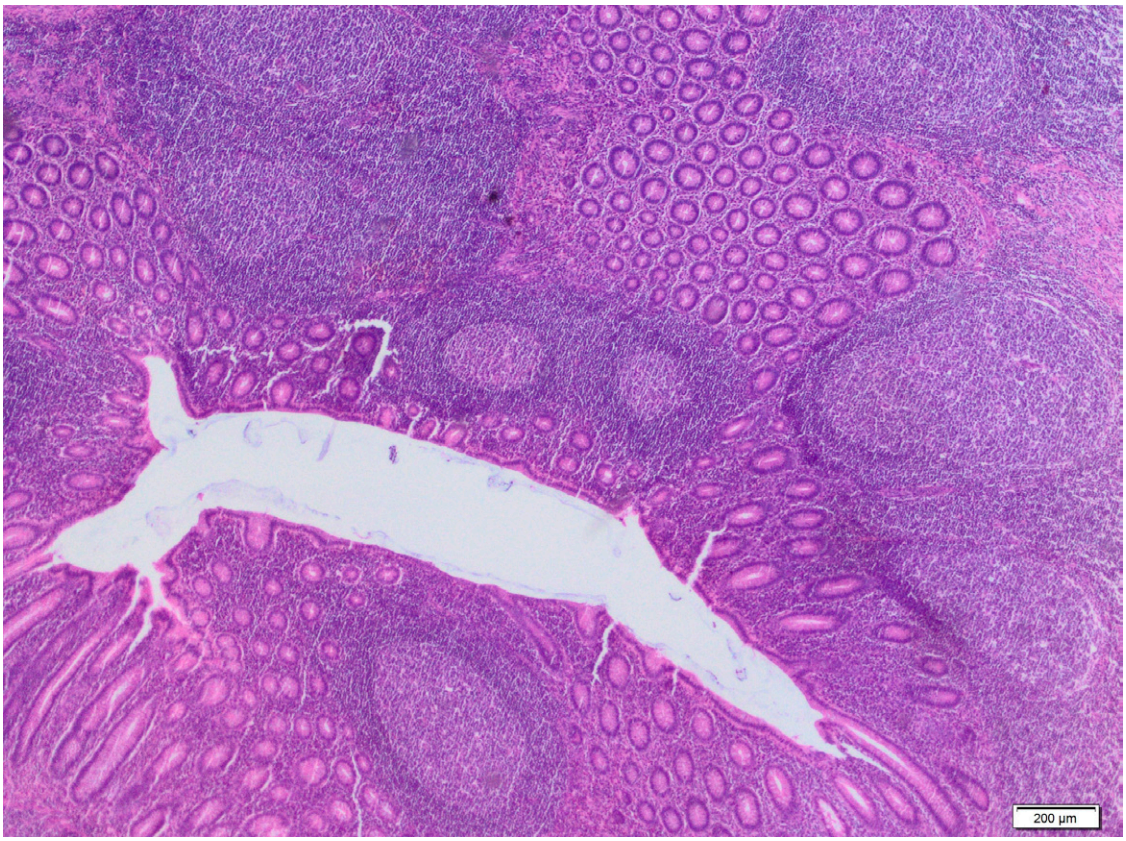

Figure 3. Lymph nodes. HE staining. Magnification 40x

for women, 15, on average. The Mann-Whitney test did not show statistically significant differences (p = 0.2669).

The number of lymph nodes decreased with age. In patients aged 20–40, there were on average 20 nodes, with patients aged 41–60, there were 17, whereas in patients aged 61–91, there were only 10 nodes. The Kruskal-Wallis test did not show a statistically significant difference in the number of lymph nodes in age groups (p > 0.05).

Sex and location of T and B lymphocytes and macrophages

In men, T lymphocytes were more frequently found in the centre (C), and simultaneously in the peripheral zone of the lymph node and in the mucous membrane (PZ/MM) (p = 0.0280).

In women, T lymphocytes were more frequently found in the peripheral zone (PZ), in the mucous membrane (MM), simultaneously in the centre and in the peripheral zone (C/PZ), or simultaneously in the centre of the lymph node and in the mucous membrane (C/MM) (**Table 1**).

In men, there were more B lymphocytes in the centre of the lymph node (C), in the mucous membrane (MM), simultaneously in the centre and in the peripheral zone (C/PZ), or simultaneously in the centre, in the peripheral zone and in the mucous membrane (C/PZ/MM).

In women, on the other hand, B lymphocytes were more frequently observed in the peripheral zone (PZ), and simultaneously in the centre and

Table 1. Location of T lymphocytes in women and men. PZ – peripheral zone of the lymph node, C – centre of the lymph node, MM – mucous membrane, PZ/MM – in the peripheral zone of the lymph node and in the mucous membrane, C/MM – in the centre of the lymph node and in the mucous membrane

Location	Men n = 45		Women n = 34		p
	n	%	n	%	
PZ	12	26.7	13	38.2	0.2765
C	13	28.9	8	23.5	0.9493
MM	2	4.4	2	5.9	0.7630
C/PZ	3	6.7	6	17.6	0.1311
PZ/MM	11	24.4	2	5.9	**0.0280***
C/MM	3	6.7	3	8.8	0.7273

* statistically significant, p < 0.05

in the mucous membrane (C/MM). The test of significance in this case did not show any significant differences in the frequency of B lymphocytes in women and men ($p > 0.05$) (**Table 2**).

Table 2. Location of B lymphocytes in women and men. PZ – the peripheral zone of the lymph node, C – the centre of the lymph node, MM – the mucous membrane, C/PZ – the centre and peripheral zone of the lymph node, C/MM – the centre of the lymph node and the mucous membrane, C/PZ/MM – the centre, the peripheral zone of the lymph node, and the mucous membrane

Location	Men n = 45		Women n = 34		p
	n	%	n	%	
PZ	0	0.0	1	2.9	0.2503
C	30	66.7	21	61.8	0.6521
MM	2	4.4	1	2.9	0.7284
C/PZ	5	11.1	3	8.8	0.7371
C/MM	5	11.1	8	23.5	0.1409
C/PZ/MM	1	2.2	0	0.0	0.3841

No statistically significant differences in the location of macrophages in women and men were noticed, either in the mucous membrane (MM), in the submucosa (SM) or in the lymph nodes ($p > 0.05$) (**Table 3**).

Table 3. Location of macrophages in women and men. MM- mucous membrane, MM/SM/lymph node – mucous membrane, submucosa, and whole lymph node

Location	Men n = 45		Women n = 34		p
	n	%	n	%	
MM	1	2.2	1	2.9	0.8436
MM/SM/LN	44	97.8	33	97.1	0.8436

Frequency of T lymphocytes, B lymphocytes and macrophages in age groups

Based on results of the Fisher-Snedecor distribution, statistically significant differences in the frequency of T lymphocytes were visible in the centre of the lymph nodes (C) ($p = 0.0401$). These cells were more frequent in persons aged 20–40 and 61–91 than in persons aged 41–60. The statistically significant difference was for the location both in the centre and in the peripheral zone (C/PZ) ($p = 0.0399$) in the group 41–60 years of age, compared to persons aged 20–40. In patients aged 61–91, T lymphocytes were not present in both locations simultaneously.

The significant difference in the frequency of B lymphocytes was confirmed with the Fisher-Snedecor F-test, observed in the mucous membrane (MM) ($p = 0.0443$) in the group 20–40 years of age (**Table 4**).

No statistically significant differences were observed with the help of the Fisher-Snedecor F-test in the number of macrophages, both in the mucous membrane (MM) and in the mucous membrane, submucosa and lymph nodes (MM/SM/LN) in any age groups ($p > 0.05$) (**Table 5**).

Table 4. Location of B lymphocytes in persons in various age groups. PZ- peripheral zone of the lymph node, C – centre of the lymph node, MM – mucous membrane, C/PZ – centre and peripheral zone, C/MM – centre of the lymph node, and mucous membrane, C/PZ/MM – centre, peripheral zone, and mucous membrane

Location	20–40 years of age n = 50		41–60 years of age n = 20		61–91 years of age n = 9		p
	n	%	n	%	n	%	
PZ	1	2.0	0	0.0	0	0.0	0.3589
C	32	64.0	12	60.0	7	77.8	0.5361
MM	3	6.0	0	0.0	0	0.0	**0.0443***
C/PZ	5	10.0	3	15.0	0	0.0	0.0670
C/MM	9	18.0	3	15.0	1	11.1	0.7679
C/PZ/MM	0	0.0	1	5.0	0	0.0	0.2079

* – statistically significant, $p < 0.05$

Table 5. Location of macrophages in various age groups and the Fisher-Snedecor F test for the k index of the structure. MM- the mucous membrane, MM/SM/LN – in the mucous membrane, submucosa and in the lymph node

Location	20–40 years of age n = 50		41–60 years of age n = 20		61–91 years of age n = 9		p
	n	%	n	%	n	%	
MM	1	2.0	1	5.0	0	0.0	0.4807
MM/SM/LN	49	98.0	19	95.0	9	100.0	0.4807

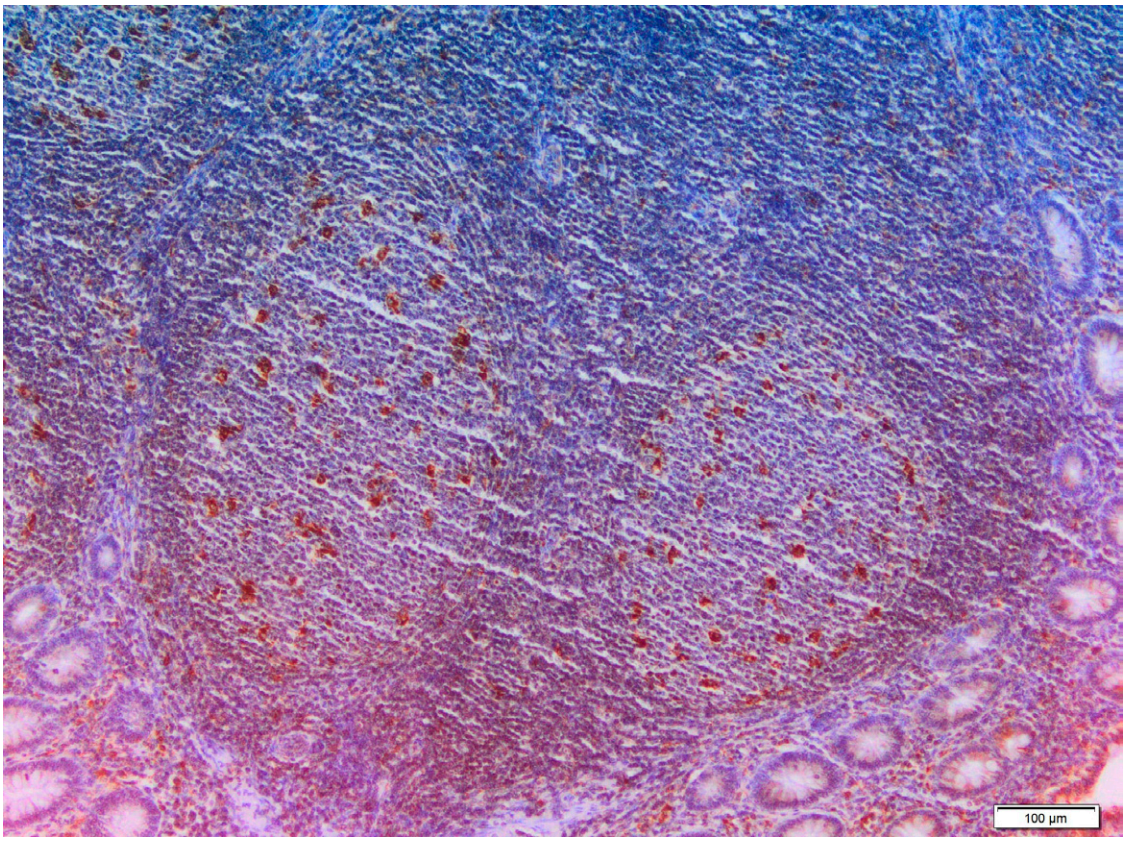

Figure 4. Macrophages in lymph nodes (CD68). Immunohistochemical staining. Magnification 100x

Number of cells of the immune system in lymph nodes

Based on results of the chi-squared test, a significant difference in the frequency of cells of the immune system in the lymph nodes ($p < 0.0001$) was observed. The most frequent were B lymphocytes (on average 3,441 cells per lymph node) — 59.3% of all cells. The second most frequent were T lymphocytes (on average 2,116 cells per lymph node), which constituted 36.5%. The least numerous were macrophages (on average 247 cells per lymph node) — 4.3% (**Figure 4**).

Location of B lymphocytes, T lymphocytes and macrophages

B lymphocytes were observed in the centre of the lymph node in 64.4% of cases (**Figure 5**). The chi-squared test showed a significant difference in the frequency of B lymphocytes ($p < 0.0001$). In 51 patients, they were observed in the centre of the lymph node (C). The lowest number was noted in the peripheral zone (PZ), and simultaneously in the centre, peripheral zone and in the mucous membrane (C/PZ/MM) (**Table 6**).

Table 6. Location of B lymphocytes. PZ — peripheral zone of the lymph node, C — centre, MM — mucous membrane, C/PZ — centre and peripheral zone, C/MM — centre of the lymph node, and mucous membrane, C/PZ/MM — centre, peripheral zone, and mucous membrane

Location	n	%	chi-squared test		
			χ^2	df	p
PZ	1	1.3	173.44	6	**< 0.0001***
C	51	64.6			
MM	3	3.8			
C/PZ	8	10.1			
C/MM	13	16.5			
C/PZ/MM	1	1.3			
No reaction	2	2.5			

* **statistically** significant, $p < 0.05$

Based on the chi-squared test, a statistically significant difference in the frequency of T lymphocytes ($p < 0.0001$) was observed. These cells were most frequently found in the peripheral zone (PZ) and in the centre of the lymph node (C): in 25 and 21 persons, respectively. T lymphocytes in the peripheral zone of the lymph node were observed in 31.6% of cases (**Figure 6**). They were least numerous in the mucous membrane (MM),

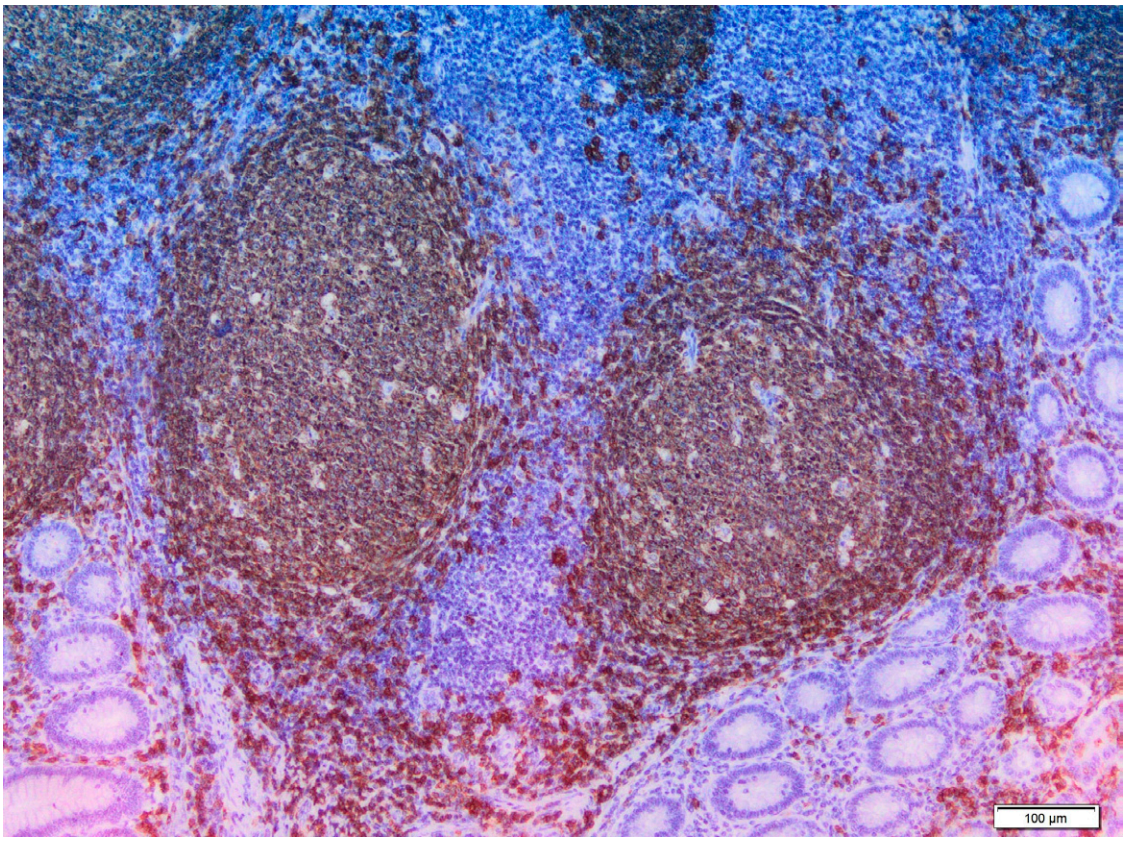

Figure 5. B lymphocytes in lymph nodes (CD20). Immunohistochemical staining. Magnification 100x

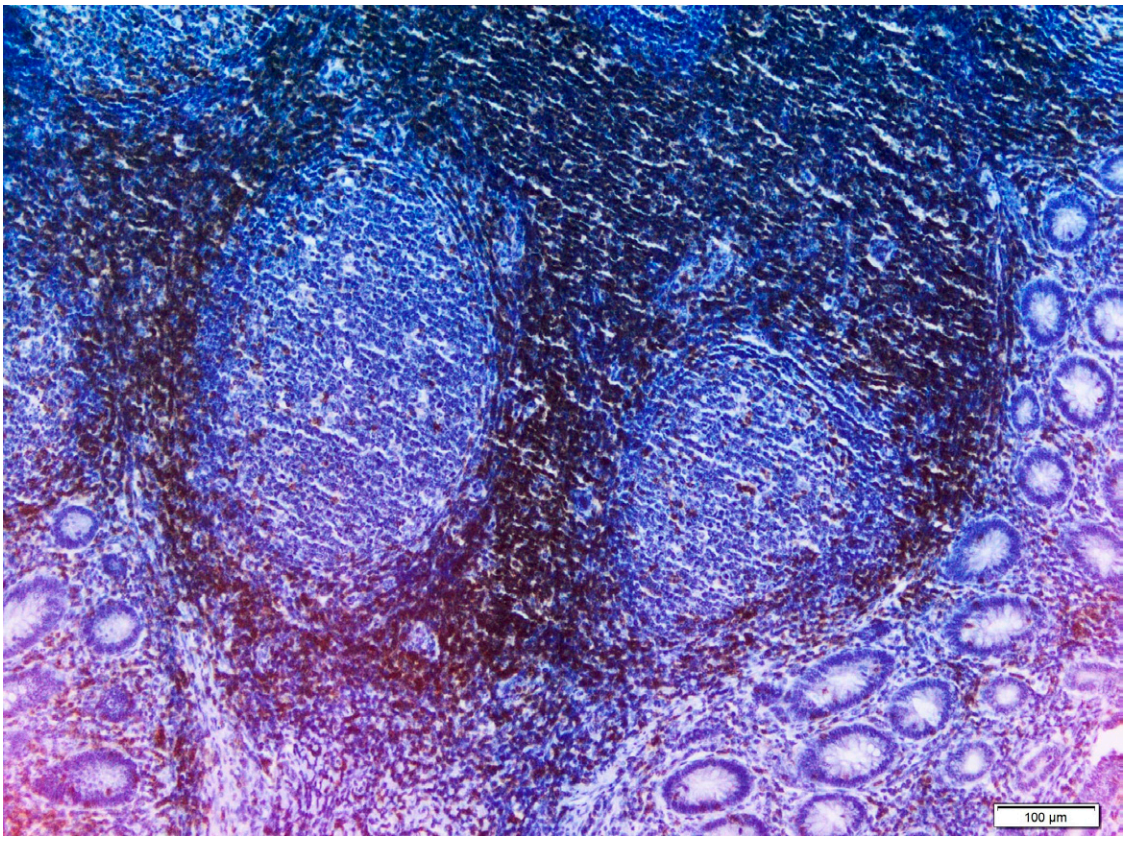

Figure 6. T lymphocytes in lymph nodes (CD3). Immunohistochemical staining. Magnification 100x

Table 7. Location of T lymphocytes. PZ — peripheral zone of the lymph node, C — centre, MM — mucous membrane, C/PZ — centre and peripheral zone, C/MM — centre of the lymph node, and in the mucous membrane

Location	n	%	chi-squared test		
			χ^2	df	p
PZ	25	31.6	42.30	6	<0.0001*
C	21	26.6			
MM	4	5.1			
C/PZ	9	11.4			
PZ/MM	13	16.5			
C/BMM	6	7.6			
No reaction	1	1.3			

* — statistically significant, $p < 0.05$

Table 8. Location of macrophages. MM — mucous membrane, MM/SM/LN — mucous membrane, submucosa and the whole lymph node

Location	n	%	chi-squared test		
			χ^2	df	p
MM	2	2.5	71.20	1	<0.0001*
MM/SM/LN	77	97.5			

* — statistically significant, $p < 0.05$

in four persons. In one case, no reaction with the CD 3 antibody was observed (**Table 7**).

The chi-squared test showed a significant difference in the frequency of their occurrence ($p < 0.0001$). In 77 patients, they were predominantly in the mucous membrane, submucosa and the whole surface of the lymph node simultaneously (MM/SM/LN). In two patients only, they were present solely in the mucous membrane (MM) (**Table 8**).

Discussion

The basic histopathological criterion for the diagnosis of acute appendicitis is neutrophil infiltration in the muscle membrane. In the samples examined, acute purulent appendicitis and acute gangrenous appendicitis were diagnosed histopathologically. Immunological reaction takes place in the lymph nodes located in the lamina propria of the mucous membrane and in the submucosa. It seems that the number of affected lymph nodes is individually variable. There are no published reliable data that would estimate an average number of activated lymph nodes per inflamed appendix. In the samples presented here, the number varied from 6 to 78. It is not, however, the total number of lymph nodes in the appendix, but only in the histological section. Independently of their number, in all cases, active nodes with a reactive centre and mantle zone were observed. Their size was dependent on the activity of centroblasts. The number of nodes was correlated with the age and gender of the patients, whereby the number decreased with age. The group examined comprised adults aged of 20 to 91 years. Our samples did not include children. Based on the data available in the literature for the children and on our own observations, we can safely assume that their number is much higher in that age group. For women, the number was smaller than for men. In the future, it would be of interest to correlate the higher number of activated lymph nodes found in men and the higher prevalence of the disease in this group.

In the cases of acute appendicitis, B lymphocytes, T lymphocytes and macrophages were observed in all tissue samples. Independently of the type of inflammation diagnosed, the cell composition of the lymph nodes was similar.

The pathogenesis of acute appendicitis is heterogeneous, and clinical symptoms are non-characteristic [3] without clear, unequivocal diagnostic criteria. The currently available literature is insufficient to determine the participation of immune cell populations during the course of acute appendicitis. Another problem is the subjectivity of the establishing of the final diagnosis by pathologists [4, 5]. Some authors consider appendectomy to be necessary. It is of significant importance in the case of neurogenic appendicitis. In the appendix, there may be an increased proliferation of cells, accompanied by secretions of vasoactive intestinal peptide and substance P. As a consequence, strong pain is present in the right lower iliac fossa. In these cases, no macroscopic or microscopic changes characteristic of acute inflammation are observed [6, 7]. Other authors advise against an appendectomy. According to them, the preservation of large quantities of lymphoid tissues in the appendix is important to the function of the immune system. [8]. After the appendectomy of a pain-free appendix, the patient may suffer from numerous post-operative complications including: wound infection, a hernia, bowel obstruction caused by post-operative adhesions, and even death [9].

A more thorough understanding of the course and location of immunological responses and the role of individual cells and their populations in relation to clinical presentation may help to establish reliable diagnostic criteria.

Conclusions

In the course of acute appendicitis, there is involvement of lymph node B lymphocytes, T lymphocytes and macrophages. The number of lymph nodes decreases with age and is gender dependent. A statistically significant difference in the number of the examined cells of the immunological system of the lymph nodes changed due to inflammation ($p < 0.001$) was observed. B and T lymphocytes in the lymph nodes and in the mucous membrane of the appendix differed depending on the sex. The presence of B lymphocytes in the mucous membrane was significantly higher in the group of 20–40 years of age. T lymphocytes were predominant in the centre of the lymph nodes in groups 20–40 and 61–91 years of age, and in the peripheral zone in the group of 41–60 years of age.

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Conflict of interest statement
The authors declare no conflict of interest.

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