Human Pathology (2019) **85**, 228–234

ELSEVIER

Human PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

Characterization of esophageal inflammation in patients with achalasia. A retrospective immunohistochemical study☆,☆☆

Manuel Döhla MD[a,*], Katharina Leichauer MSc[a], Ines Gockel MD, PhD, MBA[b], Stefan Niebisch MD, PhD[b], René Thieme PhD[b], Lars Lundell MD, PhD[c,d], Johannes Schumacher MD, PhD[e], Jessica Becker PhD[e], Ralf Joachim Rieker MD, PhD, MBA[f], Arndt Hartmann MD, PhD[f], Michael Vieth MD, PhD[a], Lothar Veits MD, PhD[a]

[a]*Institute of Pathology, Klinikum Bayreuth, 95445 Bayreuth, Germany*
[b]*Department of Visceral, Transplantation, Thoracic and Vascular Surgery, University Medical Center Leipzig, 04103 Leipzig, Germany*
[c]*Department of Clinical Sciences, Intervention and Technology (CLINTEC), Karolinska Institutet, S14186 Stockholm, Sweden*
[d]*Department of Surgery, Odense University Hospital, 5000 Odense, Denmark*
[e]*Institute of Human Genetics, Biomedical Center, University of Bonn, 53127 Bonn, Germany*
[f]*Institute of Pathology, Medical University of Erlangen, 91054 Erlangen, Germany*

Received 22 August 2018; accepted 1 November 2018

Keywords:
Achalasia;
Esophagus;
Inflammation;
Regulatory T cells;
Cytotoxic T cells

Summary Autoimmunologic phenomena triggered by CD8-positive cytotoxic T cells may be involved in the pathogenesis of achalasia, but the pathogenic mechanisms generally remain unknown, and the concomitant histopathologic changes in the esophageal epithelium in achalasia are incompletely described. This study was designed to characterize the inflammation within the esophageal epithelium of these patients. We collected 72 esophageal specimens from 38 consecutive patients with achalasia (16 men, 22 women) representing different disease stages. The inflammatory cells were characterized immunohistochemically. Seven end-stage disease esophagectomy specimens were also included. Our data show a T-cell–rich inflammatory response predominantly composed of CD4-positive T cells with an overall CD4/CD8 ratio of 1.82 and only few CD4-CD25-FoxP3–positive regulatory T cells or CD20-positive B cells. Signs of inflammation were most pronounced in the middle esophagus, followed by upper and lower esophagus. Between different stages of disease, there were statistically significant differences among CD25-positive lymphocytes in the upper esophagus and CD4-, CD8-, and CD25-positive lymphocytes in the middle esophagus. The esoph-

Abbreviations: HE, hematoxylin and eosin staining; HPFs, high-power fields; HRM, high-resolution manometry.

☆ Competing interests: The authors state that there are no conflicts of interest.

☆☆ Funding/Support: The authors acknowledge funding from Wissenschaftsförderung der Klinikum Bayreuth GmbH (project number 50002, funding sum 10,000 €).

* Corresponding author at: Institute of Pathology, Klinikum Bayreuth, Preuschwitzerstraße 101, 95445 Bayreuth, Germany
E-mail addresses: manuel.winfried.doehla@gmail.com (M. Döhla), katharina.leichauer@gmail.com (K. Leichauer), ines.gockel@medizin.uni-leipzig.de (I. Gockel), stefan.niebisch@medizin.uni-leipzig.de (S. Niebisch), rene.thieme@medizin.uni-leipzig.de (R. Thieme), lars.lundell@ki.se (L. Lundell), johannes.schumacher@uni-bonn.de (J. Schumacher), jbecker@uni-bonn.de (J. Becker), ralf.rieker@uk-erlangen.de (R. J. Rieker), arndt.hartmann@uk-erlangen.de (A. Hartmann), vieth.lkpathol@klinikum-bayreuth.de (M. Vieth), lothar.veits@klinikum-bayreuth.de (L. Veits).

https://doi.org/10.1016/j.humpath.2018.11.006

ageal epithelial inflammation in achalasia seems to be of reactive and unspecific nature and does not reflect the composition of the CD8-dominant inflammatory infiltrate within the muscular esophageal wall in active disease. The statistically significant differences in unspecific inflammation between different stages of disease are more likely due to effects of the dysmotility and cannot be assumed pathognomic for the disease. In conclusion, we assume that it is not possible to diagnose or confirm achalasia by means of esophageal biopsy alone.

1. Introduction

Idiopathic achalasia is a primary esophageal motility disorder characterized by loss of esophageal ganglion cells with clinical symptoms such as dysphagia, regurgitation, chest pain, and weight loss [1,2]. The standard diagnostic procedure includes upper endoscopy, HRM to confirm the diagnosis, and a barium meal examination to assess the degree of esophageal dilatation, the axis of the esophagus, and the presence of an associated epiphrenic diverticulum [3]. HRM findings define the type of achalasia by using the Chicago classification, where achalasia with minimal esophageal pressurization is defined as type I or the classic type, achalasia with esophageal compression as type II, and achalasia with spasm as type III [4,5]. Treatment depends on the HRM type and predicted surgical risk, and it can be done by laparoscopic myotomy, pneumatic dilation, botulinum toxin injections, and esophagectomy [1,3,6]. The latest therapy option is per oral endoscopic myotomy [7].

Esophageal biopsies are taken during upper endoscopy to rule out other causes for the patients' symptoms, such as severe reflux disease with concomitant destruction of the motor function of the organ with or without Barrett esophagus [8], esophageal cancer [9], fungal infection [10], or eosinophilic esophagitis [11,12]. The etiology of idiopathic achalasia remains unknown, but a variety of possible pathogenetic mechanisms have been proposed including genetic disposition, neurodegeneration triggered by viral infection, or other related degenerative factors including autoimmune processes [2,13-15]. In this context, it is interesting to note that the local accumulation of CD8-positive T cells may be linked to the loss of the ganglion cells in the plexus of Auerbach [16,17]. Our study attempts to characterize the morphologic features and inflammatory infiltrates in mucosal specimens obtained from patients with clinical confirmed achalasia.

Lehman et al [18] showed a diffuse squamous epithelium hyperplasia with basal cell layer hyperplasia and elongated papillae in end-stage achalasia without providing numerical analysis. Our literature search did not reveal valid data concerning normal epithelial thickness in the esophagus either; however, the proportion of the length of the papilla in the squamous epithelium compared with overall thickness of the mucosa was considered normal by the ESOHISTO project if the length of the papilla did not exceed 50% of total epithelial diameter [19,20]. Thickness of basal cell layer was defined as normal when the thickness did not exceed 15% of total epithelial thickness.

2. Materials and methods

We performed a retrospective study using biopsies of 38 patients (16 men and 22 women [42.1% and 57.9%, respectively]) with a clinical confirmed diagnosis of achalasia. The mean (SD) age of these patients was 58.9 (20.6) years. The number of biopsies varied between patients: 1 biopsy was captured from 16 patients (42.1%), 2 biopsies were captured from 10 patients (26.3%), and 3 specimens were obtained from 12 (31.5%) patients. In summary, 72 mucosal samples were eligible for further investigation. Depending on stage of disease, patients were divided into 3 groups. Group 1 "without surgery" contained 42 specimens of 25 patients who had no therapy, only pharmacological therapy, or pneumatic dilatation therapy alone. In group 2, 19 biopsy specimens were obtained from 7 patients who had already undergone surgical myotomy. Group 3 represented "end-stage disease" where complete esophagectomy specimens were retrieved. This group served as a comparison for the ultimate changes occurring as a consequence of long-standing disease relevant to the inflammation within the squamous epithelium as well as the plexus myentericus. Biopsies were grouped according to their site of retrieval (upper, middle, or lower esophagus). In Table 1, their exact distributions are given. Morphologic analysis was completed after routine HE staining, and the thickness of the basal cell layer, length of papillae, and overall epithelial thickness were measured (see Fig. 1). These measurements were used to grade density of inflammatory infiltrates as described by Purdy et al [21]. In addition, slides were scanned for the presence of spongiosis (enlarged intercellular spaces) and apoptosis as well as formation of a stratum granulare.

Immunohistochemistry was performed on 3-μm tissue sections on poly-L-lysine–coated slides after drying in an oven at 60°C for 30 minutes. The immunoreactions were performed by using the automated stainer BenchMark ULTRA (Ventana Roche Diagnostics, Rotkreuz, Switzerland) with the following

Table 1 Distributed specimens by group and location

	Group 1	Group 2	Group 3
Upper esophagus	8	6	4
Middle esophagus	10	6	5
Lower esophagus	24	7	2
Sum	42	19	11

NOTE. Group 1, without surgery; group 2, with surgery; group 3, end-stage disease.

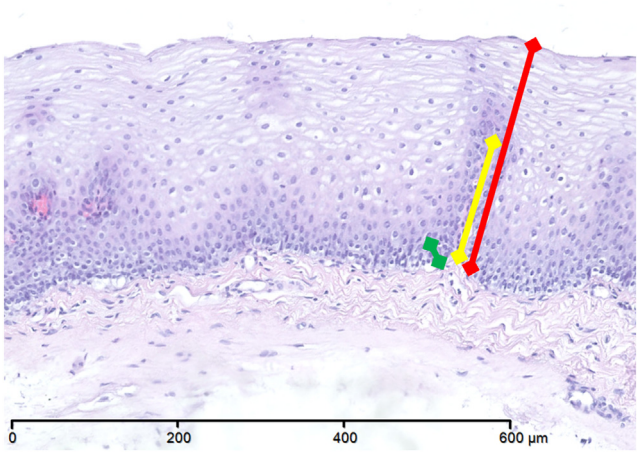

Fig. 1 Morphologic measurement (squamous epithelium, HE staining, original magnification ×100). Green arrow indicates basal cell layer; red arrow, epithelium thickness; yellow arrow, papilla length.

antibodies: CD4, clone BC/1F6 (Zytomed; Biocare Medical; RTU); CD8, clone SP16 (Zytomed; Biocare Medical, Zytomed Systems, Berlin, Germany; RTU); CD20, clone L26 (Zytomed; RTU); CD25, clone S176, (Zytomed; 1:50); and FoxP3, clone SP97 (Zytomed; RTU). Staining was regarded positive when a red-brown staining could be demonstrated. In all cases, the total number of myenteric lymphocytes was calculated in respective HE-stained slides. The number of cells that stained positive was counted per 10 HPFs by using an Olympus (Olympus, Hamburg, Germany) BX53 with 10× ocular (Wit10X/22) and 40× objective (PlanC N 40×/0.65 ∞/0.17/FN22) reaching a 400× magnification.

Hamamatsu's NanoZoomer Viewer (NDP.view, version 1.2.2) (314-5, Shimokanzo, Iwata City, Shizuoka Pref., 438-0193, Japan) was used for respective measurements. IBM SPSS Statistics version 23 for Windows was used for statistical analysis (IBM, Armonk, NY). In the statistical analyses of the data, we applied the Kolmogorov-Smirnov test for testing data distribution and Kruskal-Wallis test, Wilcoxon rank sum test, and Bonferroni correction for analyses of differences between the study groups. Friedman test was used for statistical analysis between the 3 esophageal levels.

3. Results

3.1. Morphologic data

On average, we found 170.67 (±121.727), 221.67 (±155.340), and 146.39 (±106.192) inflammatory cells per 10 HPFs within the squamous epithelium of the upper, middle, and lower third of the esophagus, respectively. There was no significant difference between the study groups (P = .686, P = .452 and P = .311). Average epithelium thicknesses were 446.83 µm (±136.668 µm; upper esophagus), 434.48 µm (±112.118 µm; middle esophagus), and 453.53 µm (±155.194 µm; lower esophagus), also with no significant differences between the study groups (P = .769, P = .814, and P = .650). The basal cell layer measured 28.07 µm (±10.925 µm) in the upper esophagus, 26.27 µm (±9.496 µm) in the middle esophagus, and 29.48 µm (±13.067 µm) in the lower esophagus. Again, no significant differences between the study groups were detected (P = .649, P = .293, and P = .795). The proportion of the thickness of the basal cell layer compared with total epithelial thickness was 6.70%, 6.44%, and 6.95% in the upper, middle, and lower esophagus. Measurements of average length of papillae were 184.22 µm (±56.990 µm; upper esophagus), 191.10 µm (±68.040 µm; middle esophagus), and 192.56 µm (±89.703 µm; lower esophagus) with no significant differences between the study groups (P = 1.130, P = .794, and P = .710). The proportions of the length of papillae compared with total epithelial thickness in the upper, middle, and lower esophageal third were 41.22%, 43.98%, and 42.25%, respectively. Table 2 gives an overview of the morphologic findings.

The grading according to Purdy et al [21] revealed 3 severe cases (7.14%) in group 1 (n = 42), 2 cases (10.53%) in group 2 (n = 19), and 5 cases (45.45%) in group 3 (n = 11). Spongiosis was seen in 29 specimens (69.05%) of group 1, 15 specimens (78.95%) of group 2, and 11 (100.00%) specimens of group 3. Formations of a stratum granulare or visible apoptotic bodies were not detected. In study group 3, we were unable to find any neural structures within the muscularis propria.

3.2. Immunohistochemical data

On average, measurement of CD4-positive lymphocytes in the upper, middle, and lower thirds of the esophagus revealed 288.94 (±234.692), 383.00 (±351.122), and 212.52 (±192.547) T cells per 10 HPFs. Statistically, no correlation among the 3 groups and localization of the inflammatory infiltrate in the upper and lower esophagus was found (P = .878 and P = .236, respectively). In the middle esophagus, we were able to show statistically significant differences between the different study groups (P = .010). By using post hoc testing with Mann-Whitney U test, we stratified data and found a significant difference between groups 2 and 3 (P = .011) and between groups 1 and 3 (P = .004) in the middle third of the esophagus. No significant difference could be shown between groups 1 and 2 (P = .880).

Measurement of CD25-positive lymphocytes in the upper, middle, and lower thirds of the esophagus revealed 9.44 (±16.008), 9.39 (±14.205), and 7.31 (±13.875) T cells per 10 HPFs. Statistically, no correlation between the 3 groups and localization of the inflammatory infiltrates in the lower esophagus was found (P = .281). In the upper and middle esophagus, we were able to show statistically significant differences between the different study groups (P = .015 and P = .047, respectively). By using post hoc testing with Mann-Whitney U test, we stratified data of the upper esophagus and found a significant difference between groups 2 and

Table 2 Morphologic findings

	Study group	Basal cell layer thickness	*P*	Epithelium thickness	*P*	Papilla length	*P*
Upper esophagus	Group 1	25.83	.649	444.00	.769	183.63	.130
	Group 2	30.33		422.00		156.00	
	Group 3	29.13		489.75		227.75	
Middle esophagus	Group 1	29.60	.293	450.27	.814	203.73	.794
	Group 2	23.30		435.33		176.17	
	Group 3	21.55		389.75		178.75	
Lower esophagus	Group 1	30.85	.795	440.78	.650	195.91	.710
	Group 2	27.79		446.50		137.61	
	Group 3	22.93		565.33		276.67	

NOTE. All data in micrometers. Group 1, without surgery; group 2, with surgery; group 3, end-stage disease.

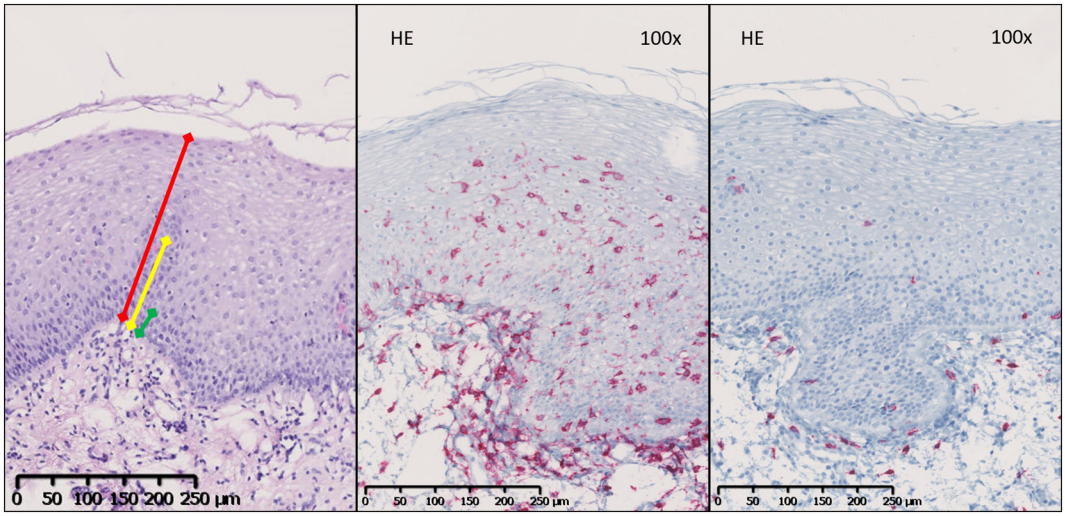

Fig. 2 Immunohistochemical measurement (example of specimens in HE, CD4 and CD8 staining, original magnification ×100).

Table 3 Immunohistochemical findings in the upper esophagus

Group	CD4	*P*	CD8	*P*	CD20	*P*	CD25	*P*	FoxP3	*P*
1	274.75		134.13		7.43		2.00		23.29	
2	235.50	.878	139.50	.277	.50	.314	3.40	.015 *	21.40	.288
3	506.00		215.50		1.50		30.00		40.50	

NOTE. Average cells count per 10 HPFs. Group 1, without surgery; group 2, with surgery; group 3, end-stage disease.
* Statistically significant.

Table 4 Immunohistochemical findings in the middle esophagus

Group	CD4	*P*	CD8	*P*	CD20	*P*	CD25	*P*	FoxP3	*P*
1	218.18		107.00		4.00		7.22		10.22	
2	261.67	.010 *	210.00	.011 *	10.00	.421	3.00	.047 *	56.50	.086
3	918.25		446.25		.00		22.25		71.00	

NOTE. Average cells count per 10 HPFs. Group 1, without surgery; group 2, with surgery; group 3, end-stage disease.
* Statistically significant.

3 (P = .014). Stratification of inflammatory cells in the middle esophagus showed significant differences between groups 2 and 3 (P = .027) and between groups 1 and 3 (P = .010).

We found an average cell count of 27.00 (±29.219), 35.41 (±51.212), and 32.43 (±33.233) FoxP3-positive lymphocytes per 10 HPFs in the upper, middle, and lower esophagus. No statistically significant differences could be found between the study groups in the upper, middle, and lower esophageal thirds (P = .288, P = .086, and P = .963, respectively).

There were 3.27% of all CD4-positive lymphocytes that were also CD25 positive, and there were 9.34% of all CD4 lymphocytes that were also FoxP3 positive in the upper esophagus. In the middle esophagus, 2.45% of all CD4-positive lymphocytes were also CD25 positive, and 9.25% showed an expression of FoxP3. There were 3.44% of all CD4-positive lymphocytes that were also CD25 positive in the lower esophagus, and there were 2.35% that expressed FoxP3, too.

On average, measurement of CD8-positive lymphocytes in the upper, middle, and lower thirds of the esophagus revealed 154.00 (±92.721), 201.05 (±179.930), and 125.31 (±119.733) T cells per 10 HPFs. Statistically, no correlation among the 3 groups and localization and distribution of the inflammatory infiltrates in the upper and lower esophagus was found (P = .277 and P = .423, respectively). In the middle esophagus, we were able to show statistically significant differences between the different study groups (P = .011). By using post hoc testing with Mann-Whitney U test, we stratified data and could find a significant difference between groups 1 and 3 (P = .004). No significant difference could be shown between groups 1 and 2 (P = .097) and groups 2 and 3 (P = .087).

In summary, we were able to show mild inflammation of the squamous epithelium with a predominance of CD4-positive cells, regardless of the site of biopsy (see Fig. 2). The average CD4/CD8 quotients were 2.08:1 (upper esophagus), 1.83:1 (middle esophagus), and 1.51:1 (lower esophagus). There were no significant differences between the 3 esophageal levels (P = .483).

We found an average cell count of 3.59 (±9.281), 5.00 (±10.756), and 5.52 (±11.567) CD20-positive lymphocytes per 10 HPFs in the upper, middle, and lower esophagus. No statistically significant differences could be found among the study groups in the upper, middle, and lower esophageal thirds (P = 3.140, P = .421 and P = .760, respectively).

For better comparability, immunohistochemical results stratified by the different study groups and expression profiles are shown in Table 3 for upper esophagus; Table 4 shows immunohistochemical findings in the middle esophagus; immunohistochemical findings in the lower esophagus are shown in Table 5.

Cells of the plexus myentericus were only present in group 3, but not in the superficial mucosal biopsies of groups 1 and 2. So, immunohistochemical investigation of the inflammation within the nerve plexus was only possible in group 3. We found 249.17 (±102.636) CD4-positive lymphocytes, no CD25-positive lymphocytes, 0.08 (±0.020) FoxP3-positive lymphocytes, 178.33 (±63.46) CD8-positive lymphocytes, and 1.75 (±4.050) CD20-positive lymphocytes per 10 HPFs. The CD4/CD8 ratio was 1.40:1.

4. Discussion

Achalasia is a long-recognized but rare and poorly understood disease with a variety of clinical symptoms [22]. Other diseases may present with similar symptoms (eg, eosinophilic esophagitis [11,12] or scleroderma [23]), and therefore, the diagnosis is challenging and often delayed [24]. Inflammation in achalasia is believed to be driven by CD8-positive cytotoxic T cells that were linked to a loss of ganglion cells in the Auerbach plexus [16,17]. The aim of our study was to investigate whether achalasia can be diagnosed in histologic specimens of the esophageal epithelium alone by evaluating the distribution and grading of inflammatory cells and morphologic changes.

We found a CD4-predominant T-cell inflammatory infiltrate in all stages of the disease and all esophageal levels. Only a few CD20-positive B cells could be detected indicating a negligible role of B cells in the pathology of achalasia. The main T-cell subpopulation was composed of T-helper cells; some of them representing CD4-CD25-FoxP3–positive regulatory T cells. Overall, statistical analysis of the inflammatory cell distribution among the study groups revealed mainly nonsignificant differences. The statistically significant increase of CD25-positive regulatory T cells in upper and middle esophagus as well as the increase of CD8-positive cytotoxic T cells in the middle esophagus in advanced disease may more likely be due to other causes than a true expression of an inflammatory infiltrate dominated by cytotoxic T cells, as assumed in the muscular nerve plexus in active disease. For example, this finding could instead represent an unspecific infiltrate of lymphocytes due to prolonged contact of food with the esophageal epithelium in hypomotile esophagus. We were not able to detect a shift in the CD4/CD8 ratio towards CD8-positive lymphocytes in any esophageal level at any time of disease

Table 5 Immunohistochemical findings in the lower esophagus

Group	CD4	P	CD8	P	CD20	P	CD25	P	FoxP3	P
1	202.74		113.30		6.05		7.29		30.00	
2	265.71	.236	185.71	.423	5.00	.760	7.17	.281	47.20	.963
3	65.00		52.00		1.50		8.00		21.00	

NOTE. Average cells count per 10 HPFs. Group 1, without surgery; group 2, with surgery; group 3, end-stage disease.

progression. In addition, the increase of CD25-positive regulatory T cells over time may be induced by bacterial colonization promoted by the motility disorder itself as a secondary sequel; it is known that an impaired esophageal motility leads to a decrease of esophageal self-cleaning function and favors bacterial growth. Furthermore, infection may cause a transient upregulation of FoxP3 in CD25-negative T-helper cells. This may be the reason why we found slightly more FoxP3-positive lymphocytes in the squamous epithelium than CD25-positive cells, the latter representing true CD25-positive and FoxP3-positive regulatory T cells.

We were not able to show hyperplasia of the squamous epithelium in our material throughout the esophagus. In addition, neither basal cell layer hyperplasia nor elongation of papillary length could be detected. Therefore, we conclude that elongated papillae or basal cell hyperplasia are not findings that are linked to achalasia or changes that may occur as a consequence of a slow esophageal transit. Interestingly, Lind et al [25] were not able to show a shift of the CD4/CD8 ratio in the inflammation appearing in reflux disease, either. Consequently, morphologic changes may not be useful in the diagnosis of achalasia but could be important in the differential diagnosis between achalasia and other esophageal diseases, such as gastrointestinal reflux disease [26].

We found neither neurons of the Auerbach plexus nor a CD8-dominant inflammation within the musculature of the muscularis propria in group 3 patients, which had undergone complete esophagectomy. The loss of neurons in advanced disease is part of its definition and was described before [16,27-30]. Inflammation in active disease is believed to target neuronal structures. The loss of ganglion cells may explain the absence of CD8-positive lymphocytes in our specimens, as no target protein remains.

The main result of our study is that the inflammation in our esophageal biopsy specimens is of reactive and unspecific nature—independent of disease stage or esophagus level—and does not reflect the composition of the pathognomic CD8-dominant inflammatory infiltrate within the muscular esophageal wall in active disease [16,17]. In conclusion, we assume that it is not possible to diagnose or confirm achalasia by means of esophageal biopsies alone. Additional tests should be performed if achalasia is suspected, and further studies will be necessary to identify better diagnostic histologic markers. Because of the retrospective nature of the study and the anonymization of personal data, no data regarding the type of achalasia according to the Chicago classification were available and further stratification of data regarding manometric findings was not possible.

Ethics approval and consent to participate

Ethical approval was given by the Research Ethics Committee of Friedrich-Alexander University Erlangen-Nuremberg, Faculty of Medicine, on August 29, 2016, under the reference number 237_16 Bc.

Availability of data and material

The data sets used and/or analyzed during the current study are available from Dr Lothar Veits (Institute of Pathology, Klinikum Bayreuth, Bayreuth, Germany) on reasonable request.

Author contributions

M.D. performed data curation, formal analysis, project administration, visualization, writing of the original draft. The work was performed in fulfillment of the requirements of Friedrich-Alexander-Universität Erlangen-Nürnberg for obtaining the degree “Dr med.”.

K.L. performed statistical analysis and visualization.

I.G., S.N., R.T, L.L. provided resources (data and material) and performed review & editing of the draft.

J.S. and J.B. performed statistical analysis and performed review & editing of the draft.

R.R. and A.H. provided resources and validated the draft.

M.V. and L.V. performed conceptualization, provided the methodology, resources and software, supervised the research process, acquired funds and performed review & editing of the manuscript.

Funding

The authors acknowledge funding from Wissenschaftsfoerderung der Klinikum Bayreuth GmbH, project number 50002, funding sum 10.000 €.

Acknowledgments

The authors thank Mrs Ingrid Hegenbarth and Mrs Nina Höhn for excellent technical support and Dr Liane Marciano and Mr Jay Marciano, USA, for their proofreading.

References

[1] Patel DA, Kim HP, Zifodya JS, Vaezi MF. Idiopathic (primary) achalasia: a review. Orphanet J Rare Dis 2015;10:103. https://doi.org/10.1186/s13023-015-0302-1.

[2] Boeckxstaens GE, Zaninotto G, Richter JE. Achalasia. Lancet 2014;383:83-93. https://doi.org/10.1016/S0140-6736(13)60651-0.

[3] Ramirez M, Patti MG. Changes in the diagnosis and treatment of achalasia. Clin Transl Gastroenterol 2015;6:e87. https://doi.org/10.1038/ctg.2015.8.

[4] Pandolfino JE, Kwiatek MA, Nealis T, Bulsiewicz W, Post J, Kahrilas PJ. Achalasia: a new clinically relevant classification by high-resolution manometry. Gastroenterology 2008;135:1526-33. https://doi.org/10.1053/j.gastro.2008.07.022.

[5] Herbella FA, Armijo PR, Patti MG. A pictorial presentation of 3.0 Chicago classification for esophageal motility disorders. Einstein 2016;14:439-42. https://doi.org/10.1590/S1679-45082016MD3444.

[6] Torresan F, Ioannou A, Azzaroli F, Bazzoli F. Treatment of achalasia in the era of high-resolution manometry. Ann Gastroenterol 2015;28:301-8.
[7] Gunasingam N, Perczuk A, Talbot M, Kaffes A, Saxena P. Update on therapeutic interventions for the management of achalasia. J Gastroenterol Hepatol 2016;31:1422-8. https://doi.org/10.1111/jgh.13408.
[8] Davis-Yadley AH, Neill KG, Malafa MP, Pena LR. Advances in the endoscopic diagnosis of Barrett esophagus. Cancer Control 2016;23:67-77.
[9] Meves V, Behrens A, Pohl J. Diagnostics and early diagnosis of esophageal cancer. Viszeralmedizin 2015;31:315-8. https://doi.org/10.1159/000439473.
[10] Lee KJ, Choi SJ, Kim WS, Park SS, Moon JS, Ko JS. Esophageal stricture secondary to candidiasis in a child with glycogen storage disease 1b. Pediatr Gastroenterol Hepatol Nutr 2016;19:71. https://doi.org/10.5223/pghn.2016.19.1.71.
[11] Martín Martín L, Santander C, Lopez Martín MC, et al. Esophageal motor abnormalities in eosinophilic esophagitis identified by high-resolution manometry. Gastroenterology 2011:1447-50. https://doi.org/10.1111/j.1440-1746.2011.06770.x.
[12] Nurko S, Rosen R. Esophageal dysmotility in patients who have eosinophilic esophagitis. Gastrointest Endosc Clin N Am 2008;18:73-9. https://doi.org/10.1016/j.giec.2007.09.006.
[13] Ates F, Vaezi MF. The pathogenesis and management of achalasia: current status and future directions. Gut Liver 2015;9:449-63. https://doi.org/10.5009/gnl14446.
[14] Gockel I, Becker J, Wouters MM, et al. Common variants in the HLA-DQ region confer susceptibility to idiopathic achalasia. Nat Genet 2014;46:901-4. https://doi.org/10.1038/ng.3029.
[15] Becker J, Niebisch S, Ricchiuto A, et al. Comprehensive epidemiological and genotype-phenotype analyses in a large European sample with idiopathic achalasia. Eur J Gastroenterol Hepatol 2016;28:689-95. https://doi.org/10.1097/MEG.0000000000000602.
[16] Clark SB, Rice TW, Tubbs RR, Richter JE, Goldblum JR. The nature of the myenteric infiltrate in achalasia: an immunohistochemical analysis. Am J Surg Pathol 2000;24:1153-8.
[17] Sodikoff JB, Lo AA, Shetuni BB, Kahrilas PJ, Yang GY, Pandolfino JE. Histopathologic patterns among achalasia subtypes. Neurogastroenterol Motil 2016;28:139-45. https://doi.org/10.1111/nmo.12711.
[18] Lehman MB, Clark SB, Ormsby AH, Rice TW, Richter JE, Goldblum JR. Squamous mucosal alterations in esophagectomy specimens from patients with end-stage achalasia. Am J Surg Pathol 2001;25:1413-8.
[19] Schneider NI, Plieschnegger W, Geppert M, et al. Validation study of the Esohisto consensus guidelines for the recognition of microscopic esophagitis (histoGERD trial). Hum Pathol 2014;45:994-1002. https://doi.org/10.1016/j.humpath.2013.12.013.
[20] Fiocca R, Mastracci L, Riddell R, et al. Development of consensus guidelines for the histologic recognition of microscopic esophagitis in patients with gastroesophageal reflux disease: the Esohisto project. Hum Pathol 2010;41:223-31. https://doi.org/10.1016/j.humpath.2009.07.016.
[21] Purdy JK, Appelman HD, Golembeski CP, McKenna BJ. Lymphocytic esophagitis: a chronic or recurring pattern of esophagitis resembling allergic contact dermatitis. Am J Clin Pathol 2008;130:508-13. https://doi.org/10.1309/D3PCF6D6YYMQRX9A.
[22] Galey KM, Wilshire CL, Niebisch S, et al. Atypical variants of classic achalasia are common and currently under-recognized: a study of prevalence and clinical features. J Am Coll Surg 2011;213:155-63. https://doi.org/10.1016/j.jamcollsurg.2011.02.008.
[23] Mainie I, Tutuian R, Patel A, Castell DO. Regional esophageal dysfunction in scleroderma and achalasia using multichannel intraluminal impedance and manometry. Dig Dis Sci 2008;53:210-6. https://doi.org/10.1007/s10620-007-9845-x.
[24] Gockel I, Müller M, Schumacher J. Achalasia—a disease of unknown cause that is often diagnosed too late. Dtsch Arztebl Int 2012;109:209-14. https://doi.org/10.3238/arztebl.2012.0209.
[25] Lind A, Koenderman L, Kusters JG, Siersema PD. Squamous tissue lymphocytes in the esophagus of controls and patients with reflux esophagitis and Barrett's esophagus are characterized by a non-inflammatory phenotype. PLoS One 2014;9:e106261. https://doi.org/10.1371/journal.pone.0106261.
[26] Vieth M, Mastracci L, Vakil N, et al. Epithelial thickness is a marker of gastroesophageal reflux disease. Clin Gastroenterol Hepatol 2016;14:1544-51. https://doi.org/10.1016/j.cgh.2016.06.018.
[27] Adams CW, Brain RHF, Trounce JR. Ganglion cells in achalasia of the cardia. Virchows Arch 1976:75-9.
[28] Goldblum JR, Rice TW, Richter JE. Histopathologic features in esophagomyotomy specimens from patients with achalasia. Gastroenterology 1996:648-54.
[29] Goldblum JR, Whyte RI, Orringer MB, Appelman HD. Achalasia: a morphologic study of 42 resected specimens. Am J Surg Pathol 1994;18:327-37.
[30] Misiewicz JJ, Waller SL, Anthony PP, Gummer JW. Achalasia of the cardia: pharmacology and histopathology of isolated cardiac sphincteric muscle from patients with and without achalasia. Q J Med 1969:17-30.