LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

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ENTERIC NERVOUS SYSTEM REMODELLING, GENETIC, NUTRITIONAL, AND ENVIRONMENTAL FACTORS IN COLONIC DIVERTICULOSIS

Doctoral Dissertation
Medical and Health Sciences,
Medicine (M 001)

Kaunas, 2022

The dissertation was prepared in the Medical Academy of Lithuanian University of Health Sciences during the period of 2018–2022.

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Dissertation will be defended at the open session of the Medical Research Council of Lithuanian University of Health Sciences on the 20th of June, 2022 at 2 p.m. in the Great Auditorium of the Hospital of Lithuanian University of Health Sciences Kauno klinikos.
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LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS

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STOROSIOS ŽARNOS DIVERTIKULIOZĖ: ENTERINĖS NERVŲ SISTEMOS REMODELIACIJOS, GENETINIŲ, MITYBOS IR APLINKOS VEIKSNIŲ POVEIKIO ANALIZĖ

Daktaro disertacija
Medicinos ir sveikatos mokslai,
medicina (M 001)

Kaunas, 2022

Disertacija rengta Lietuvos sveikatos mokslų universitete Medicinos akademijoje 2018–2022 metais.

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Disertacija ginama viešame Lietuvos sveikatos mokslų universiteto medicinos mokslo krypties tarybos posėdyje 2022 m. birželio 20 d. 14 val. Lietuvos sveikatos mokslų universiteto ligoninės Kauno klinikų Didžiojoje auditorijoje.
Adresas: Eivenių g. 2, LT-50161 Kaunas, Lietuva.

CONTENTS

ABBREVIATIONS

ADD – asymptomatic diverticular disease
ARHGAP15 – Rho GTPase–activating protein 15
BMI – body mass index
CD – colonic diverticulosis
CGRP – calcitonin gene–related peptide
CI – confidence interval
CM – circular muscles
COL1A1 – collagen type I alpha 1 chain
COL3A1 – collagen type III alpha 1 chain
COLQ – collagen–like tail subunit of asymmetric acetylcholinesterase
CRLR – calcitonin receptor–like receptor
DD – diverticular disease
DICA – diverticular inflammation and complication assessment
DNR – deoxyribonucleic acid
ENS – enteric nervous system
EU – European Union
FAM155A – family with sequence similarity 155A
FI – fluorescence intensity
GI – gastrointestinal
HWE – Hardy–Weinberg equilibrium
ICC–IM – intramuscular interstitial cells of Cajal
ICC–MY – interstitial cells of Cajal around the myenteric plexus
ICC–SM – interstitial cells of Cajal around the submucosal plexus
ICC – interstitial cells of Cajal
IR – immunoreactivity
ISP – inner submucosal plexus
LM – longitudinal muscles
MP – myenteric plexus
NO – nitric oxide
NOS1 – neuronal nitric oxide synthase
NS – nervous system
NSAIDs – nonsteroidal anti–inflammatory drugs
OR – odds ratio
OSP – outer submucosal plexus
PBS – phosphate-buffered saline

PFA – paraformaldehyde
PGP 9.5 – protein gene–product 9.5
RAMP1 – receptor activity modifying protein 1
ROI – region of interest
RR – relative risk
RT–PCR – real–time polymerase chain reaction
SDD – symptomatic diverticular disease
SN – sodium nitroprusside
SNP – single nucleotide polymorphism
SUDD – symptomatic uncomplicated diverticular disease
TTX – tetrodotoxin
USA – United States of America
VIP – vasoactive intestinal peptide

INTRODUCTION

Colonic diverticulosis (CD) is a chronic progressive disorder of the large bowel. It is characterized by the presence of diverticula, i.e., sac–like protrusions of the mucosa and submucosa through the muscular layer of the large bowel wall [1, 2]. Diverticulosis is one of the most common gastrointestinal disorders diagnosed on routine colonoscopy, with its prevalence being closely related to age [2, 3]. More than half of all individuals older than 60 years are expected to acquire CD [4]. The vast majority of patients with colonic diverticula do not develop symptoms throughout their lifetime. However, in up to 25 % of individuals, any one of many diverse combinations of inflammatory symptoms, changes, and complications may occur [2]. Symptoms may variably result from simple physiologic changes in colonic motility related to altered neuromuscular activity in the colon wall or varying degrees of the inflammatory response, leading to complications such as acute diverticulitis, segmental colitis, or even diffuse peritonitis [5].

Colonic diverticulosis was first described in the mid–19th century. However, with improved life expectancy and the adoption of a "Western" diet, since the early 20th century, the prevalence of CD has been continuously growing, consequently increasing its burden on national health care systems worldwide [6–11]. Despite this load, the etiology of CD remains complex and poorly understood.

In today's perspective, diverticulosis is thought to stem from a complex interaction of multiple factors, including both intrinsic and extrinsic. The rapid and continuous rise in CD incidence following the industrialization process in capitalist countries speaks of the importance of diet and lifestyle in disease development. Epidemiological studies show that certain factors are associated with an increased risk of developing diverticulosis. Physical inactivity [12–15], a low–fiber diet followed by constipation [16–20], red meat consumption [17, 20], obesity [21–23], and other factors such as smoking [24–26], alcohol [24, 27, 28], or certain medications, i.e., nonsteroidal anti–inflammatory drugs (NSAIDs) [29, 30], have all been proposed to lead to the onset of CD. However, due to differences in diagnostic modalities in cohorts of patients that are not comparable for age, symptoms, or ethnicity, available data is not homogeneous and is often challenging to interpret. It is also safe to speculate that the risk factors for the development of CD are probably different from those for its complications. This means that the research on determinants of diverticulosis fails to show consistent results. A high–powered comprehensive analysis of different dietary and lifestyle factors linked with CD is still lacking. Therefore, this study aimed to explore the association be-

tween potential risk factors and the prevalence of CD and diverticulitis using data from a colonoscopy–based European population.

While the pathogenesis of CD has primarily been attributed to environmental risk factors such as diet, new epidemiologic evidence suggests that heredity may also play a role in the disease's development. This is partly supported by the observation of transethnic disparities in prevalence rates and the predominant location of diverticula between Western and Asian populations [31]. Adding to that is the evidence that CD is associated with various monogenic connective tissue disorders, such as polycystic kidney disease, Coffin–Lowry syndrome, Ehler–Danlos syndrome, and Williams–Beuren syndrome [32, 33]. In the last decade, with the introduction of new genomic technologies and decreased genotyping costs, two sizeable population–based twin studies in Scandinavia were published, indicating that genetic factors are a strong contributor to the development of diverticulosis [34, 35]. The odds ratio (OR) of acquiring CD if one's co–twin was affected was 7.15 in monozygotic twins, compared to 3.2 in dizygotic twins, according to a Swedish Twin Registry study [34]. The Danish twin study reported a relative risk (RR) of 2.92 for diverticulosis in twin siblings compared to the general population [35]. In both studies, heredity was accounted for 40 % of the risk of developing the disease.

Most recently, results from three large genome–wide association studies in Europe and the United States were published, providing the most compelling evidence for the role of genetic predisposition in CD [36–38]. Among newly identified candidate genes associated with CD, many have a role in intestinal neuromuscular function and connective tissue support. Thus far, only a couple of small case–control studies using the candidate gene approach attempted to identify specific genetic variants for the disease development but were underpowered to provide conclusive results [39, 40]. Because of this, genetic variants implicated in the etiology of this widespread disease remain mostly unknown, and more extensive case–control studies analyzing the role of single nucleotide polymorphisms (SNPs) within genes encoding for collagens of the connective tissue are still needed.

Finally, when it comes to classical etiological mechanisms hypothesized in the context of CD, colonic neuromuscular dysfunction has traditionally been considered one of paramount importance. A plethora of colonic motor dysfunctions have been associated with the distinct abnormalities of the enteric nervous system (ENS) [41, 42]. The ENS is an integrative network located along the gastrointestinal (GI) tract wall. More than 30 neurotransmitters have been identified in the ENS, which interact with intricate peristaltic movements, initiating local reflexes to regulate motility and secretion [43, 44]. In the context of CD, it has been well established that patients display

increased intraluminal pressure profiles in the sigmoid colon and changes to the ENS and colonic musculature [45–51]. A long–held pathogenetic concept, proposed by Painter and Burkitt [52, 53] over 45 years ago, implied that this increase in intracolonic pressure is caused by a low–fiber diet reducing the stool volume. However, newer data calls this concept into doubt. In the setting of CD, the smooth muscle becomes hypersensitive to cholinergic stimulation [54, 55] and loses its relaxation ability when exposed to sodium nitroprusside (SN), a nitric oxide (NO) donor [56]. This phenomenon suggests that a disruption in neuromuscular transmission may be responsible for the impaired gastrointestinal motility observed in CD patients. Apart from NO and vasoactive intestinal peptide (VIP), another potent smooth muscle relaxant playing a significant role in the non–adrenergic non–cholinergic regulation of the GI tract motility is neuronal calcitonin gene–related peptide (CGRP) [57]. CGRP fibers innervate various targets within the digestive system (epithelia, myocytes, neuronal elements) [58], giving a morphological basis for the peptide's wide range of biological functions. Acting through a heteromeric receptor composed of a G–protein coupled receptor called calcitonin receptor–like receptor (CRLR) and a receptor activity–modifying protein 1 (RAMP1) [59], CGRP has a multidirectional impact on the alimentary tract. It plays a pivotal role in sensory and pain conduction [57], vasodilation [60], immunological response [61], absorption, and secretory activity [62]. In animal models, CGRP was shown to be capable of inducing peristaltic reflexes [63], relaxing smooth muscle cells [64, 65], inducing phasic contractile activity [66–68], and exciting myenteric neurons [69]. Nevertheless, the importance of CGRP in mediating gastrointestinal motor activity has never before been considered in the context of motility impairment observed in CD patients.

The aim of the study

To evaluate the role of enteric nervous system remodeling, genetic, nutritional, and environmental factors for the development of colonic diverticulosis.

The objectives of the study

1. To determine the role of single nucleotide polymorphisms within genes encoding for collagens of the connective tissue – COL3A1, COL1A1, ARHGAP15, COLQ, and FAM155A – for the development of colonic diverticulosis.
2. To investigate the CGRP signaling pathway within the enteric plexuses of sigmoid colon samples in symptomatic and asymptomatic diverticular disease.

3. To identify the ultrastructural changes of the enteric nervous system of the human sigmoid colon in diverticulosis patients.
4. To investigate the role of socio–demographic factors, dietary and bowel habits in the development of colonic diverticulosis.

Novelty of the study

Understanding the etiological mechanisms of disease development is crucial to developing advanced and effective treatment strategies and preventing the emergence of new cases. Over the past decades, our knowledge about the etiology and pathophysiology of CD and its related complications has considerably expanded. CD is now known to be a multifactorial process that involves both environmental factors and genetic predisposition. Furthermore, its pathogenesis is accompanied by structural and functional alterations within the colon wall. Despite a wealth of data published in this field, the incidence of diverticulosis continues to rise. This may be due to the high complexity of etiological factors involved and the variable spectrum of manifestations of the disease itself, which makes standardized research particularly difficult.

Observed transethnic differences between Western and Eastern populations [31] and data from the epidemiological twin studies [34, 35] leave no doubt about the importance of heredity in disease development. In addition, advances in genotyping techniques have permitted the identification of novel candidate genes associated with CD and diverticulitis. Recent GWAS consistently reported three primary genetic susceptibility factors for both conditions but did not differentiate between diverticulitis and diverticulosis in particular due to the limitations of registry–based approaches [36–38]. The GWAS mentioned above also revealed several genetic loci and polymorphisms involved in connective tissue maintenance and collagen degradation that affect the risk of developing CD [36–38]. However, these findings still needed to be replicated in independent cohorts of patients from different populations to be validated. This study aimed to confirm the role of the identified variants for diverticulosis and diverticulitis, respectively. To our best knowledge, this study is the first large–scale study assessing the role of genetic variation within COL3A1 (rs3134646 and rs1800255), COL1A1 (rs1800012), ARHGAP15 (rs4662344), and FAM155A (rs67153654) within a well–phenotyped cohort of colonoscopy–proven diverticulosis patients.

The second part of the study was dedicated to evaluating the association between our considered socio–demographic factors and dietary and bowel habits in the development of colonic diverticulosis and diverticulitis. One of the major strengths of this study over previously published reports was the availability of comprehensive clinical and endoscopic data and covariates

that have been linked with the risk of CD. Moreover, this was the first study that analyzed the relationship between the participants' educational and occupational status and the risk of CD. Contrary to popular belief, our findings revealed that participants in the diverticulosis group had a lower educational status than controls and were less likely to work in sedentary or night shift occupations. To our knowledge, there is no other large–scale multicentered colonoscopy–based analysis addressing this causality to date.

Our research's target was to go further into the structural remodeling of the ENS in the context of diverticulosis, combining both immunohistochemical and also transmission electron microscopy imaging modalities. Although the idea of nerve tissue remodeling in diverticulosis has been raised previously [70, 71], to our knowledge, this is the first study to analyze morphological changes within intrinsic plexuses of diverticulosis patients on ultrastructural level. Therefore, results described for the first time in the present work add to the observations of previous authors.

Another one of the major findings of this study was the significant changes in the CGRP signaling pathway in CD patients, elucidating the role of the impaired smooth muscle relaxation mechanism in the disease development. Our results have demonstrated that in symptomatic diverticulosis patients, CGRP expression is downregulated within all three enteric plexuses of the human sigmoid colon, with the asymptomatic group having the intermediate values. What is more, the data revealed that the expression of the CGRP receptor – CRLR – was upregulated oppositely in diverticulosis patients, with the symptomatic group having significantly greater values than control patients. This is the first study demonstrating this gradual decrease of CGRP as the disease progresses. Moreover, this study showed that CGRP has all the necessary components for the direct activation of VIPergic and nitrergic neurons within the ENS's myenteric, inner, and outer submucosal plexuses. To our knowledge, no reports examining this disbalance in CGRP's expression that may be responsible for the disordered myorelaxation in CD have been published previously. Thus, the results of this study add valuable insights into the etiogenesis of colonic diverticulosis and its related complications.

1. REVIEW OF LITERATURE

1.1. Terminology

Colonic diverticulosis (CD) is among the most common benign conditions of the gastrointestinal tract. It is characterized by the development of saccular outpouchings of the mucosa and submucosa through the weak areas in the muscular layer of the colon wall, referred to as diverticula [1, 2] (Fig.1.1.1 shows a schematic representation of the pathophysiology of diverticulosis development, adapted from *Tursi et al.* [2]). These lesions can occur in any part of the large intestine, although the most common site for diverticula formation is the sigmoid colon. CD is a benign condition, ranging from the presence of a single diverticulum to the abundant number of diverticula that may be too numerous to count. CD is, for the most part, detected incidentally in patients undergoing endoscopy or radiological examinations as it is generally asymptomatic [2].

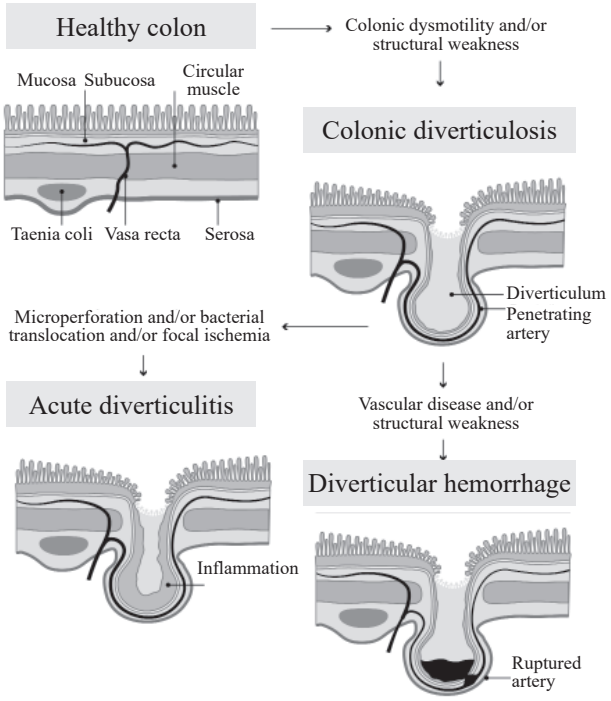


Fig. 1.1.1. *Pathophysiology of the development of diverticulosis from a healthy colon.*

Colonic diverticula are sac–like herniations of the mucosa and submucosa through the weak points of the large bowel wall; colonic dysmotility or structural weaknesses are possible contributors to this pathology

The terminology used in scholarly literature to describe CD is often replete and confusing – diverticular disease, symptomatic diverticulosis, and symptomatic uncomplicated diverticular disease, to name a few. According to currently accepted definitions, diverticulosis is merely the presence of colonic diverticula; these may or may not become symptomatic or complicated [2]. The term diverticular disease (DD) describes clinically significant and symptomatic diverticulosis estimated to develop in approximately 25 % of individuals with CD [2]. That is to say, the anatomical lesion (diverticulosis) has progressed to the point of an illness. Meanwhile, symptomatic uncomplicated diverticular disease (SUDD) is a subset of diverticular disease characterized by chronic abdominal symptoms attributable to diverticula, which typically involve bloating, abdominal pain, and changes in bowel habits in the absence of macroscopically overt colitis or diverticulitis. Diverticulitis is the macroscopic inflammation of diverticula with related acute or chronic complications such as diverticular hemorrhage, abscess formation, colonic perforation, or bowel obstruction [2, 5]. Within the scope of this study, the term asymptomatic diverticulosis will be used interchangeably with the asymptomatic diverticular disease (ADD) for simplicity purposes. The current terminology used to describe diverticulosis and its complications is shown in Fig. 1.1.2.

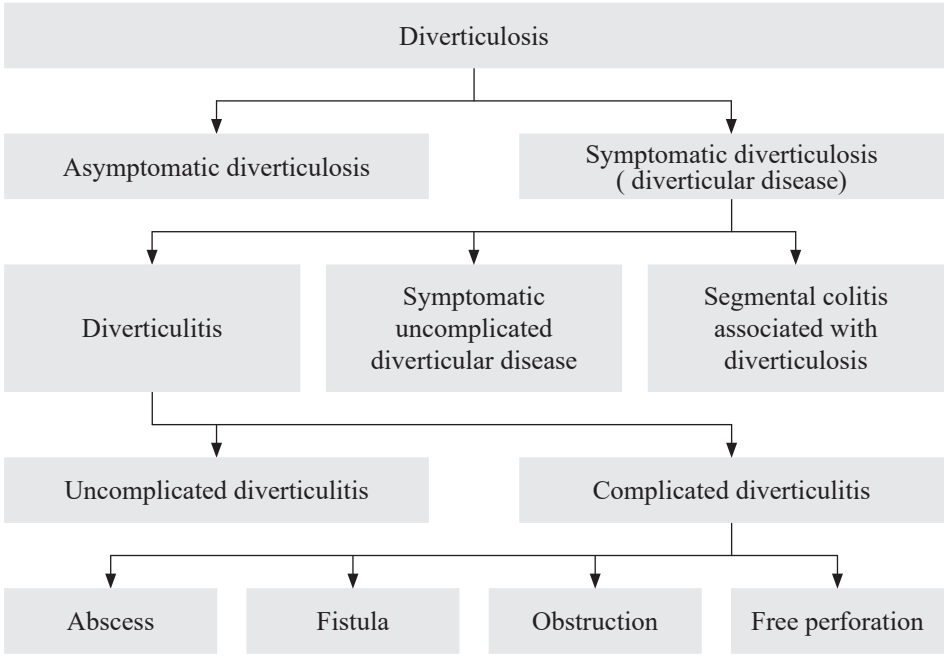


Fig.1.1.2. *Current classification of colonic diverticulosis, summarized from Tursi et al. [2]*

1.2. Epidemiology and prevalence

The incidence of colonic diverticulosis and diverticular disease is increasing worldwide [3, 6, 7, 52]. Nowadays, this condition represents the fifth most important gastrointestinal disorder in developed countries [8]. However, the actual prevalence of colonic diverticulosis is difficult to determine as most individuals with diverticula are asymptomatic. Available data are not homogenous and often difficult to analyze due to the difference in diagnostic modalities in cohorts of patients not comparable for age, symptoms, and ethnicity. Epidemiological studies report that the prevalence of diverticulosis in Europe and North America ranges from 20 to 42 % [72–74]. In Eastern countries, it is reported to be 13–25 %, reflecting not only different lifestyles and dietary habits but also genetic background [32]. In addition, several epidemiological studies report remarkable variations in the predominant location of diverticula depending on ethnicity. It is generally accepted that diverticula in Western countries are predominantly located in the left colon, whereas in Asian countries, they occur mainly in the right colon [75]. The difference is that diverticula, typically forming in the left colon, involve eversion of the mucosa and submucosa but not the muscular layer of the colon wall and, therefore, are called "false" or pseudodiverticula. Diverticulosis in the right colon is characterized by "true" diverticula, in which eversion can involve all colonic wall layers [1, 2].

The prevalence of the disease is also closely associated with age [2, 3]. European countries report a 5.3 % frequency of diverticulosis in patients aged 30–39, 8.7 % in those aged 40–49, 19.4 % between 50 and 59, and up to 29.6 % in subjects over the seventh decade, while maximal incidences were found in patients aged 70–79 and above 80 of 40.2 and 57.9 %, respectively [4]. Although DD is generally accepted to be more common among the elderly, data show that its prevalence among younger individuals (< 40 years of age) is currently increasing [4]. Overall, the incidence of diverticulosis appears to be slightly lower in Asia than in Europe and the United States; however, the tendency is that over the last few decades the prevalence of diverticulosis and DD, which includes both diverticulitis with its complications and SUDD, is continuously increasing on both sides of the globe [9, 10] consequently increasing its burden on national health care systems [3, 11].

Medicare expenditures for the diagnosis and treatment of DD and its complications have become a significant economic burden on societies all around the world. In 2003, *Delvaux et al.* reported an estimation of prevalence of DD in European Union (EU) which included at that time 15 countries with a total population of 376,481,775 inhabitants [3]. About 27.3 % of EU population, corresponding to more than one–hundred million people, had colonic

diverticula. According to the Authors' assumptions, the number of perforation cases/year was 60,237 and the annual rate of hospital admission for DD was nearly 800,000 [3]. In Europe DD accounts for about 13,000 deaths per year [3]. Finally, The Global Burden of Disease in 2019 assess DD as the leading cause of death in the group "other digestive diseases" which includes various codes of International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD–10) [76].

Despite its ever–growing economic burden on the national health care systems, high prevalence, and complicated clinical management, over the years DD has drawn relatively little research effort and has repeatedly been named the "neglected disease" in scientific literature [4, 77]. Up to now data on determinants of colonic diverticulosis and DD is sparse and the pathophysiology of the disease remains poorly understood. For numerous years, the Western lifestyle has been considered a key factor for the development of diverticulosis, owing to its comparatively high prevalence in developed countries. This long–standing pathogenetic concept, firstly introduced by Painter and Burkitt over 45 years ago [53], implied that a low–fiber diet reduces stool volume, increases intraluminal colonic pressure and consequently leads to mucosal herniation and creation of pseudodiverticula. However in recent years there has been a paradigm shift in our understanding of diverticulosis pathophysiology. Newer data suggest that diverticulosis develops as a complex interaction of genetics together with environmental factors and structural remodeling of the enteric nervous system [32, 78–80]. However, mechanisms for diverticula development are likely different from those for the manifestation of symptoms. For example, previously mentioned altered colonic motility and changes to colonic wall musculature might predispose individuals to the development of asymptomatic diverticulosis, whereas intestinal dysbiosis and a low–grade inflammation might have a bigger role in the development of SUDD [81–84].

1.3. The potential contributing factors to colonic diverticulosis

1.3.1. Genetic predisposition

Compiled data from recent decades suggest a genetic basis for the development of colonic diverticulosis and DD. Previously mentioned transethnic differences in prevalence rates and predominant location of diverticula between Western and Asian populations indicate a plausible underlying genetic background [31]. This hypothesis is further supported by the fact that in some hereditary disorders of the connective tissue including autosomal dominant polycystic kidney disease, Coffin–Lowry syndrome, Ehler–Danlos syndrome

type IV, and Williams–Beuren syndrome colonic diverticula are observed in increased frequency and at a very early age [32, 33].

First attempt to investigate the significance of genetics in the development of the disease was made in two large population–based familial aggregation studies in Scandinavia [34, 85]. *Granlund et al.* linked the Swedish Twin Registry to the Swedish Inpatient Registry [34]. With the use of mathematical models, the heritability was estimated to be 40 % in a cohort of twins with (2,296 people) or without (102,156 people) DD, and the nonshared environmental effects were calculated to account for 60 % of the trait variability [34]. A comparable result was evidenced by a Danish twin study. Using the Danish National Registry of Patients linked to the Danish Twin Registry, the heritability of DD was estimated to be 53 % in a twin cohort comprising twins with (923 people) and without (29,399 people) DD [85]. These findings imply an important role of genetic predisposition in the development of DD. However, possible genetic variants involved in the pathogenesis of this common disease remain widely unknown.

Thus far, only two small case–control studies using candidate gene approach attempted to identify certain SNPs in DD. One study found an association between rs7848647, a variant within TNFSF15 (encoding a cytokine of the TNF family) and diverticulitis requiring surgery [39]. Another study found that a variant in RPRM (encoding reprimo, a protein involved in cell cycle regulation and DNA repair), was linked to the presence of diverticulosis [40]. However, these studies were underpowered to provide conclusive results.

The most important data supporting the role of genetic predisposition in DD come from three very recent genome–wide association studies (GWAS). The first genome–wide association study in DD and diverticulitis was performed in 2017 in Iceland and the Netherlands [36]. The study showed that intronic variants rs4662344 and rs7609897, established within DNase hypersensitivity sites located in Rho–GTPase–activating protein 15 (ARHGAP15) and collagen–like tail subunit of asymmetric acetylcholinesterase (COLQ) genes, were linked with uncomplicated DD [36]. rs67153654 within an intron of FAM155A (family with sequence similarity 155A) was also significantly associated to diverticulitis occurrence [36]. These were the first loci shown to associate with diverticular disease in a genome–wide study.

Additionally, one of the two largest to date GWAS was conducted in 2018 in the United States [37]. *Maguire et al.* analyzed 27,444 cases and 382,284 controls from the UK Biobank and tested for replication in the Michigan Genomics Initiative (2,572 cases; 28,649 controls) [37]. Study identified 42 loci associated with DD (39 of them novel), genes that are significantly enriched for expression in the mesenchymal stem cells and multiple connective tissue

cell types and are co–expressed with genes that have a role in vascular and mesenchymal biology [37].

The largest GWAS study to date employed UK Biobank and imputed genotypes using 31,964 cases and 419,135 controls [38]. These associations were then replicated in a European sample of 3,893 cases and 2,829 diverticula–free controls and evaluated for risk contribution to diverticulitis and uncomplicated diverticulosis and identified 48 genetic risk loci. The most significant novel risk variant rs9960286 was located near CTAGE1 (cutaneous T–cell lymphoma–associated antigen 1), and the most significant novel replicated risk variant rs60869342 was located in NOV (nephroblastoma overexpressed) [38]. Based on 95 % Confidence Intervals (CIs), the authors found four loci having stronger effects for diverticulitis, namely variants at PHGR1 (proline, histidine and glycine rich 1), FAM155A–2, calcitonin–related polypeptide beta (CALCB), and the S100A10 locus [38].

The functional link between DD and many of the candidate genes identified by aforementioned GWAS is unknown. To confirm gene–variant associations, functional characterizations should yet be established. It is noteworthy, however, that among genes in newly determined risk loci associated with DD, many have a role in immunity, extracellular matrix biology, cell adhesion, membrane transport, and intestinal motility thus contributing to the pathophysiology of the disease [86].

Table 1.3.1.1. *Plausible functional characteristics of GWAS identified risk loci associated with diverticular disease*

Intestinal motility	Immunity	Membrane transport/ signaling	Cell adhesion
ANO1, CHRNB1, COLQ, PPP1R14A	ARHGAP15, FADD, HLA–DQA1, HLX, PLEKHA1	ANO1, CACNB2, CALCA, CALCB, CHRNB1, COLQ, CUTC, PPP1R16B, S100A10, SLC25A28, SLC35F3, SNX24, SPINT2, STARD13	BMPR1B, CLSTN2, COL6A1, CRISPLD2, EFEMP1, ELN, ENPP2, HAS2, ICSF10, LIMK1, LTBP1, LRRC17, NOV, PCSK5, S100A11, SHFM1, TCHH, TIMP2

Table content based upon the publication by *Maguire et al.* [37].

1.3.2. Impact of lifestyle

1.3.2.1. Nutrition

The importance of lifestyle, mediated by economic and cultural context, on the epidemiology of diverticular disease is well–known. Rapid and continuous increase in DD incidence following the Industrial Revolution in capitalist

countries is thought to be largely attributed to dietary and lifestyle changes. Among these changes, two major areas of modifiable factors can be distinguished, i.e. sedentary lifestyle [87] and nutrition [12, 88]. The latter might in turn be differentiated in two specific risk factors, namely the excessive consumption of meat [18, 87, 89] and the reduced intake of fiber, fruit and vegetables [18, 90].

The long–held hypothesis linked a low–fiber diet consumed in the West to an increase in intracolonic pressure that results in the formation of diverticula [52]. Despite limited research, this hypothesis has been widely accepted and for many years constipation from a low dietary fiber has been commonly cited as the main etiological factor for the development of CD [16, 52]. However, contradicting results from more recent studies put this theory into question. For example, *Peery et al.* [18] examined the relationship between bowel habits and dietary fiber intake in the development of asymptomatic diverticulosis and found that less frequent bowel movements and hard stools were associated with a reduced risk of diverticulosis. In addition, there was no association between dietary fiber intake and risk of diverticulosis [18, 20]. Although the association between fiber and asymptomatic diverticulosis is uncertain, several studies indicate that low dietary fiber is associated with risk of symptomatic diverticular disease [91] and its complications [90, 92]. In a large UK cohort, individuals who consumed more than 25 grams of fiber had a 40 % decreased risk of hospitalization for diverticulitis [90]. On the other hand, a diet rich in red meat consumption is positively associated with an increased incidence of diverticulitis [89]. The process by which red meat consumption causes symptomatic disease is unclear, nevertheless, observational studies show a 1.5 to 2 times increase in risk [18]. A cross–sectional colonoscopy based study of 2,104 patients reported a significantly increased risk of DD in diets that were high in total fat or red meat and low in fiber (RR: 2.35 *vs.* 3.32) [18]. Altogether, a prudent dietary pattern (high in fruits, vegetables and whole grains) seems to decrease the risk of diverticulitis, as opposed to an occidental pattern (high in red meat and refined grains) which increases the risk [17].

1.3.2.2. Physical activity and obesity

Physical activity, in terms of sedentary lifestyle, is also one of the main risk factors for DD [15, 87]. Studies that have analyzed the effect of exercise on the development of DD revealed that physical activity reduces the risk of developing symptomatic diverticular disease [14, 15, 18]. By contrast, obesity, and central obesity in particular, has been linked to an increased incidence of both diverticular bleeding and diverticulitis [14, 15, 21]. In a prospective

study by *Strate et al.*, men with a body mass index (BMI) > 30 kg/m^2 were shown to have a relative risk of 1.8 [21]. Surprisingly, there is conflicting data on the significance of physical activity in reducing the risk of diverticulosis. A study of 2,104 participants found no association between CD and physical activity [18]. Still, the pathophysiology of this risk factor is not clearly understood. Some authors suggest that obese individuals might have altered intestinal microbiota composition which can contribute to the development DD [93, 94]. More research into the shifts in gut microbiota in obese individuals is needed to establish if it can explain the increased risk of DD in this population.

1.3.2.3. Smoking and alcohol consumption

Over the years, several different studies, though mostly observational, have proposed excessive consumption of alcohol as a potential risk factor associated with DD. For example, a study in a Danish cohort showed that female drinkers had a three times higher risk of diverticulitis than the general population. In contrast, for male alcoholics, the risk was two times higher [95]. However, the results may be biased because of dietary and smoking habits associated with alcoholics. Another study by *Sharara et al.* [27] found that the adjusted odds ratio for diverticulosis in alcohol users was 1.91 (1.36 to 2.69), with higher alcohol use increasing the prevalence (p–value for trend = 0.001). There was a substantial link between national per–capita alcohol consumption rates and the prevalence of diverticulosis reported from 18 countries (Pearson correlation coefficient r = 0.68; p = 0.002) [27].

Another considerable risk factor for DD is smoking. According to one large case–control study, smokers are three times more likely than nonsmokers to have complications from the diverticular disease [96]. However, this same association was not found in a significant cohort study comprising over 46,000 men in the United States [24].

1.3.2.4. Medications and vitamin D

Diverticular disease has been associated with a number of different medications. Regular use of nonsteroidal anti–inflammatory drugs (NSAIDs) has a well–known association with a higher risk of gastrointestinal bleeding (i.e., ulcers and diverticular) but are also increasingly recognized as a risk factor for complicated diverticulitis, especially perforation [29, 97]. The likely mechanism of action is indirect, with cyclooxygenase inhibition and decreased prostaglandin synthesis in the intestines as a result. Prostaglandins are essential for maintaining mucosal blood flow and a healthy colonic mucosal bar-

rier. A direct mechanism exists as well, involving mucosal injury caused by NSAIDs, which leads to increased toxin and bacterium translocation [98, 99].

Strate et al. reported this association in a large prospective cohort study of 47,210 U.S. men in the Health Professionals Follow–Up Study [29]. The results of this study showed that regular use of NSAIDs or Aspirin (greater than or equal to two times per week) had a RR of 1.25 (95 % confidence interval [CI], 1.05 to 1.47) of diverticulitis and RR of 1.70 (95 % CI, 1.21 to 2.39) for diverticular bleeding in comparison to nonusers. Finally, the strongest evidence for this comes from a meta–analysis of 11 studies revealing the association between NSAIDs and diverticular perforation (OR, 3.4) and 12 studies significantly linking NSAIDs and diverticular bleeding (OR, 2.6) [30].

Opiate pain medications have also been demonstrated to increase intracolonic pressure and slow intestinal transit, both of which are risk factors for DD complications. Case studies have shown high percentages of patients with perforation taking opiate analgesics [98, 100].

Lately, the significance of vitamin D in DD has been investigated. *Maguire et al.* evaluated prediagnostic vitamin D (25–OH) levels between 9,116 patients with uncomplicated diverticulosis and 922 patients who developed diverticulitis requiring hospitalization in a retrospective cohort study [101]. In comparison to patients who required hospitalization for diverticulitis, those with uncomplicated diverticulosis had a statistically significant higher mean prediagnostic serum vitamin D level [101]. These findings were later corroborated by another major study, which found a link between low ultraviolet light exposure and diverticulitis [102]. The results of this study suggest that a lower serum vitamin D level may be associated with a higher risk of complicated diverticulitis, and that vitamin D deficiency may play a role in the pathogenesis of diverticulitis, though this (and any potential role of vitamin D treatment) is still a speculation. In order to confirm this hypothesis, larger cohort studies will be necessary.

1.3.3. Neuromuscular function abnormalities

1.3.3.1. Microscopic changes to enteric nervous system and colonic musculature

Historically, the pathogenesis of diverticulosis has been associated with a combination of an increased intracolonic pressure and a weakening of the colonic wall musculature, caused by structural alterations of the connective tissue [71, 103, 104]. It has been demonstrated by various studies that individuals with diverticulosis have altered colonic connective tissue composition and collagen metabolism. Both asymptomatic diverticulosis and complicated DD patients display an increased thickness of the circular and longitudinal

muscle layers, shortening of the taeniae and altered smooth muscle architecture, which includes an aberrant muscle bundle orientation and a decrease in smooth muscle myosin heavy chain gene and protein expression [103, 105, 106]. In addition, an abnormal deposition of two major extracellular matrix components, collagen and elastin, have been described in DD patients [103, 104]. Studies have shown that tissue–degrading matrix metalloproteinases or their inhibitors responsible for collagen metabolism are also altered in patients with DD [107, 108]. These changes in the colonic musculature could facilitate a decrease in tensile strength in the *tunica muscularis* thus leading to diverticula formation.

Derangement of enteric innervation [48] such as reduced number of glial cells, nerve cells [109] and intestinal pacemaker cells (also termed interstitial cells of Cajal or the ICCs) [110] as well as changes in the levels of neurotransmitters, neurotransmitter receptors and neurotrophic factors [49, 55] was also detected in individuals with diverticulosis. The ENS is a semiautonomous intrinsic neural network, composed of three distinct ganglionated plexuses: the myenteric (MP) – Auerbach's, the outer submucous (OSP) – Schabadasch's, and the inner submucous (ISP) – Meissner's [44, 111, 112]. MP and OSP primarily maintain intestinal motility, while OSP and ISP control epithelial functions [44, 111, 112]. The ENS contains primary afferent neurons, interneurons and motor neurons that act on different effector cells in both myenteric and muscular compartment, such as the ICCs and the smooth muscle cells [113, 114]. The ICCs generate spontaneous and rhythmic electrical activity as well as mediate signal transmission from enteric neurons to smooth muscle cells [44] and can be categorized into three main groups: submucosal ICCs (ICCs–SM) found in the submucosal layer of the colonic wall, myenteric ICCs (ICCs–MY) which form a cell network along the myenteric plexus between the longitudinal and circular layers of the tunica muscularis, and intramuscular ICCs (ICCs–IM) found throughout the longitudinal and circular muscle layers [44]. Another key component of the ENS is enteric glial cells (EGC) associated with submucosal and myenteric neurons and found in proximity to epithelial cells [44, 115, 116]. EGC terminal endfoot processes also connect to the epithelial basal membrane and blood vessels [117]. EGCs are vital in maintaining enteric neuron homeostasis, regulating colonic motility, and have recently been shown to protect the integrity of the intestinal epithelial barrier [116, 118, 119].

In terms of the ENS, all diverticulosis patients, including asymptomatic subjects with diverticula as well as symptomatic uncomplicated and complicated patients, have significant molecular and morphological changes in the enteric neuromuscular compartment, which may contribute to bowel dysfunctions such as motor disturbances and visceral hypersensitivity [120–

122]. Every individual with CD have an increased number of nerve fibers in the diverticular region; however patients with SUDD display an active nerve fiber outgrowth represented by an extensive axonal sprouting [123]. A loss of enteric neurons, hypoganglionosis, and an imbalance in myenteric (e.g. acetylcholine, substance P, nitric oxide, vasoactive intestinal polypeptide and serotonin, calcitonin gene–related peptide) and pain–mediating (e.g. galanin, neuropeptide K) neurotransmitters are also present in all DD patients [50, 109, 120, 122, 124]. For example, patients with diverticulitis have been shown to have a decrease in 5HT–4 serotonin receptors [50], while SUDD patients had an up–regulation of subtype 3 muscarinic receptors [55]. Furthermore, significant alterations in the density and morphology of both ICCs–MY and ICCs–SM in DD patients have been reported [120, 125]. In particular, c–Kit network was rarefied and disarranged as a consequence of morphological alterations of ICCs, which showed curtailed and blunted processes along with a decrease in ICC density in both circular and longitudinal layers [125]. These changes might explain the disrupted rhythmic activity of gut muscle cells observed in DD patients.

1.3.3.2. Altered colonic motility

Based on the observation of increased motility in both basal state and after meal ingestion, studies postulated that increased intraluminal pressures arising in the affected segments due to low intake of fiber might lead to the formation of diverticula [126, 127]. Pressure is directly proportional to wall tension and inversely proportional to bowel radius, according to Laplace's law. Because the sigmoid colon has the smallest intestinal diameter, it tends to create the highest luminal pressure. However, later studies attempting to corroborate this hypothesis have yielded (likely due to disparities in methods used to record intestinal motility in these patients) conflicting results. Indeed, some studies found considerable increases of motility both basally and after eating in DD patients, compared to controls [45, 128, 129], while others found no such differences [46]. The disparities between the studies could be due to the heterogeneous methods of recording colonic motility in these patients, the short recording periods and the fact that most investigations were conducted in the rectum or rectosigmoid junction, thus missing the diverticular area [130]. Following that, several subsequent colonic motility studies using 24–hour manometry (which measures the pressure and pressure waves in colon) were carried out, this time looking at more proximal colonic segments and positioning the recording catheters within the diverticular region by means of colonoscopy. *Bassotti et al.* found that patients with diverticulosis have increased intraluminal pressure, increased colonic response to eating

and increased number of high–amplitude contractions in segments harboring diverticula compared to controls [47, 110]. These findings suggest that motor abnormalities either contribute to the development of diverticulosis or are the result of diverticulosis. However, as indicated in a recent review, these studies may be insufficient to draw clear conclusions on the evidence of abnormal colonic motility in DD patients [131]. Indeed, preliminary data from recent high–resolution manometry recordings suggest that there may be no significant variations in colonic motility between diverticulosis patients and controls [132].

1.3.3.3. Visceral hypersensitivity

Visceral perception had also been a neglected topic in DD, with only a couple of studies devoted to the matter. One motility study by *Clemens et al.* investigated diverticular (sigmoid) and non–diverticular (rectal) segments, comparing patients with diverticulosis, SUDD, and healthy controls [133]. The perception of rectal distention was higher in SUDD patients compared to asymptomatic diverticulosis and controls; however, there were no significant variations in rectal compliance across the three groups. Compared to controls, but not asymptomatic diverticulosis, SUDD patients also had higher sigmoid perception before and after meals, whereas sigmoid colon compliance was equivalent in all three groups. These findings showed that colonic distention causes increased perception in both the affected (diverticular) and unaffected (rectal) segments in patients with SUDD, but not in those with asymptomatic diverticulosis. This increased perception is not associated with an altered colonic wall compliance, implying that colonic abnormalities may be related to motor/perceptive functions, and may be responsible for some of the patients' symptoms [133].

This hypothesis was later confirmed in a study by *Hellwig et al.* in which asymptomatic diverticulosis and SUDD patients were examined using sigmoidoscopy as well as biopsy sampling, followed by rectal barostat testing 5–10 days later [106]. SUDD patients had a lower initial pain threshold, a higher median overall pain score, and higher relative expression of neurokinin–1 and tumour necrosis factor alpha mRNA when compared to asymptomatic patients. Barostat pain scores and neurokinin–1 expression had a significant correlation. These findings suggested that patients with SUDD exhibit visceral hypersensitivity caused by low–grade inflammation and tachykinin overexpression [106].

Altogether, data shows that colonic diverticulosis remains one of the most prevalent disorders of the gastrointestinal tract in Western countries, with an increasing incidence in younger patients as well as in developing countries

that have begun to adopt Western diets. Despite its prevalence, the pathophysiology of the disease is yet unknown. The majority of our knowledge about diverticulosis is based on assumptions and hypotheses, often leading to contradictory conclusions. Diet, colonic motility abnormalities, genetic effects, and other bowel diseases are thought to interact in a complex manner. More basic science research on this topic is certainly needed to help us improve our understanding of risk factors and disease progression.

2. METHODS

2.1. Ethics

The study was approved by the local Research Ethics Committees of each study center: the Kaunas Regional Biomedical Research Ethics Committee (protocol No BE–10–2, issued on 8th of March, 2011), the Research Ethics Committee of the Saarland University (approval 63/11, issued on 10th of May, 2011) and the Research Ethics Committee of the University of Cologne (approval 16–397, issued on 12th of January, 2017). The study protocol conforms to the Code of Ethics of the World Medical Association (1975 Declaration of Helsinki). Every participant has signed informed consent before their inclusion in the study.

2.2. Overall study cohort

The study cohort was conducted between 2012 and 2016 at three tertiary referral centers in Germany and Lithuania: the Department of Gastroenterology at the Lithuanian University of Health Sciences, Kaunas, the Department of Medicine II, Saarland University Medical Center, Homburg, and the Clinic for Gastroenterology and Hepatology, University Hospital of Cologne, Cologne. Prior to their inclusion, all adult participants between the ages of 19 to 95 underwent a thorough clinical evaluation to exclude coexisting gastrointestinal or inherited connective tissue disorders, e.g., Ehlers–Danlos or Marfan syndrome. All study subjects were of self–reported Caucasian descent. A standardized questionnaire was used to assess epidemiological and baseline data. Participants were also interviewed for our considered risk factors for CD.

In all patients, the presence of diverticula was confirmed colonoscopically, which is the most widely accepted standard to detect diverticula. A senior gastroenterologist performed all colonoscopies using digital video endoscopes (high–resolution scopes Olympus CF 160, 180, or 190). Patients without a complete colonoscopy, including inspection of the cecum and at least moderate quality preparation, as assessed by the physician performing the examination, were excluded from the study. Patients in whom diverticula were absent were included in the control group.

Extension of diverticulosis was classified as follows: left–sided (including the sigmoid and descending colon), right–sided (transversal, ascending colon, and cecum), and pancolonic. The endoscopic severity of the CD was assessed using the Diverticular Inflammation and Complication Assessment (DICA) [134, 135]. The diagnosis of diverticulitis was made using the most

recent classifications for DD [136, 137]. It was established by either computed tomography (CT) or sonographic imaging, as well as clinical (pain in the lower left abdomen) and laboratory findings (increased serum inflammation markers). In all instances, suspected complicated diverticulitis was diagnosed with computed tomography. For the scope of this investigation, subjects were defined as patients with diverticulosis or diverticulitis, respectively.

2.3. Assessment of environmental and dietary risk factors

Before the colonoscopy, all participants completed a standardized questionnaire on the potential risk factors for developing CD with the assistance of a licensed physician. The following groups of risk factors were included in the study: socio–demographic factors, factors related to nutritional status and dietary habits, and factors associated with bowel habits. Socio–demographic factors covered age, gender, ethnicity, educational and occupational status, daily consumption of alcohol and tobacco, regular consumption of NSAIDs and laxatives. The number of meals per day, quantity of daily fluid intake, amount of fish and red meat servings per week, and whether participants followed a vegetarian or vegan diet were all used to establish nutritional status and dietary preferences. Body mass index (BMI) (kg/m^2) was calculated from self–reported height (cm) and weight (kg), and being overweight or obese was defined as a BMI ≥ 25 kg/m^2. In addition, self–reported bowel movement frequency, the average duration of defecation, if present – nightly excretion, as well as symptoms associated with bowel movements such as pain, feeling of incomplete bowel emptying, need for digital evacuation or enemas, false urge, and overall duration of constipation (years) were assessed. Finally, each patient's chart was reviewed to obtain missing data and double–check data gathered from the questionnaires.

Methods used for the assessment of variables and estimation of the odds ratio (OR) and 95 % confidence intervals (CI) are presented in detail in the publication by *Lukosiene et al.* [138].

2.4. Identification of single nucleotide polymorphisms of genes encoding for connective tissue

2.4.1. Study population for SNP analysis of COL3A1, COL1A1, ARHGAP15, COLQ, and FAM155A

From the overall study cohort, 707 individuals were investigated for genetic variations within genes encoding for collagens of the connective tissue in the colonic wall, i.e., COL3A1 (rs3134646, rs1800255) and COL1A1 (rs1800012) (a CONSORT diagram outlining inclusion and exclusion of pa-

tients is provided in Fig. 2.4.1.1). The study group consisted of patients with colonoscopically proven diverticulosis (n = 422), and the control group was composed of subjects without pathological changes in the colon on endoscopic examination (n = 285).

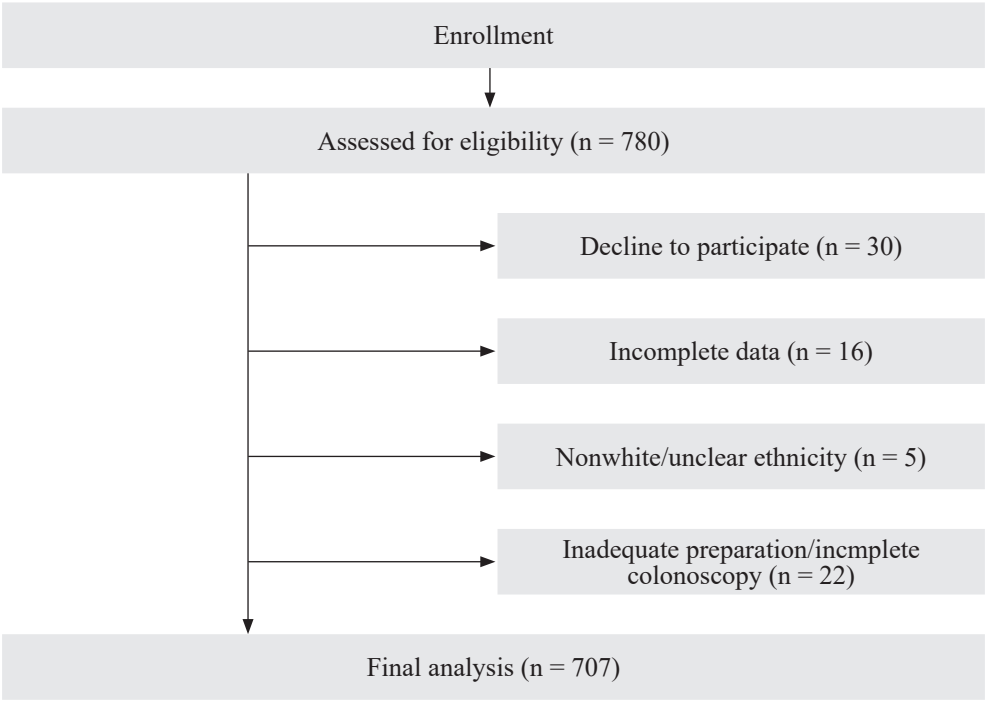


Fig. 2.4.1.1. *A CONSORT diagram outlining inclusion and exclusion of patients. Patient who had no diverticula on colonoscopy were used as controls.*

Following that, the association between three previously identified SNPs [36–38] associated with the risk of DD and diverticulitis was investigated. Genetic variants of ARHGAP15 (rs4662344), COLQ (rs7609897), and FAM155A (rs67153654) were determined in 1332 individuals of European descent. Subjects were divided into three groups: the first group consisted of diverticula–free controls (n = 479), the second group consisted of asymptomatic individuals in whom diverticula were present during endoscopy (n = 856), and the third group included patients with clinically and endoscopically proven diverticulitis (n = 198 out of 856).

2.4.2. DNA extraction

Genomic DNA was extracted from peripheral blood mononuclear cells using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, the Netherlands) follo-

wing the manufacturer's protocol. DNA concentrations were measured using a NanoDrop 2000 (Thermo Scientific) spectrophotometer. DNA samples were stored at –20 °C until analysis.

2.4.3. Genotyping

Genotyping of COL3A1 (rs3134646, rs1800255), COL1A1 (rs1800012), ARHGAP15 (rs4662344), COLQ (rs7609897), and FAM155A (rs67153654) genetic polymorphisms was performed using real–time polymerase chain reaction (RT–PCR). Genomic DNA isolated from peripheral blood mononuclear cells (1 μL, 10–50 μg) and factory–validated TaqMan® primer and probe sets (also known as TaqMan® Assays) were used for this study. The TaqMan® Assays consisted of two locus–specific primers amplifying a single nucleotide polymorphism region and two allele–specific TaqMan® probes. These probes were labeled with a fluorescent dye (FAMTM or VIC®) and glow inhibitor (TAMRATM). Genotyping was performed according to the manufacturer's recommendations and protocol by using the following conditions: 25 °C for 1 minute; 95 °C for 20 seconds (preheat); 95 °C for 5 seconds; 60 °C for 30 seconds (35 cycles). The Life Technologies 7500 Fast detection system (Life Technologies International, USA) was used for the RT–PCR assay.

For in–depth methodologic descriptions, see the following:

SNP genotyping for COL3A1 (rs3134646, rs1800255) and COL1A1 (rs1800012) is presented in detail in the publication by *Reichert et al.* [139];

Risk variant genotyping for ARHGAP15 (rs4662344), COLQ (rs7609897), and FAM155A (rs67153654) is thoroughly laid out in [140].

2.5. Investigation of the enteric nervous system

2.5.1. Study population for immunohistochemical and ultrastructural analysis

All colon specimens used for morphological examination of the ENS were obtained from the Department of Surgery at the Lithuanian University of Health Sciences Kaunas Clinics between December 2015 and May 2017. A total of 32 samples were collected, among which the following groups were distinguished: asymptomatic diverticular disease (ADD), symptomatic diverticular disease (SDD), and controls. Detailed patients characteristics used for immunohistochemical analysis are provided in Table 2.5.1.1.

Table 2.5.1.1. *Patient characteristics*

Group	n	Gender	Age, years (range)	BMI (range)
Control	11	5F/6M	64 (35–87)	25.18 (21.18–31.11)
ADD	10	6F/4M	62 (40–76)	27.59 (22.94–33.67)
SDD	10	7F/3M	62 (39–80)	26.11 (22.72–31.64)

ADD – asymptomatic diverticular disease; SDD – symptomatic diverticular disease; BMI – body mass index.

Patients undergoing surgery for non–obstructing colorectal carcinoma were used to obtain control (subjects who did not have macroscopic diverticula) and the ADD (patients in whom diverticula were present but were asymptomatic) samples. In addition, tissue specimens for the SDD group were obtained from patients undergoing elective surgery for recurrent attacks of diverticulitis. Patients with a clinical history of intestinal motility disorders or inflammatory bowel diseases were excluded from tissue sampling.

1–5 cm length circular colon segments were taken from the seemingly normal area adjacent to the diverticulum in diverticulitis patients or from macroscopically intact regions in cancer patients. Areas that displayed altered colonic morphology due to transmural outpouching of the diverticulum or had signs of inflammation or fibrosis were not sampled. All colon samples were collected in the operating theater and immediately placed at 4 °C pre–aerated (95 % O2, 5 % CO2) Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 11 mM glucose).

A fraction (25/32) of the collected tissue samples were also used for the ultrastructural investigation of both enteric plexuses with a transmission electron microscope: 9 controls, 7 ADD, and 9 SDD patients. The characteristics of the abstracted study group are shown separately in Table 2.5.1.2.

Table 2.5.1.2. *Patient characteristics*

Group	n	Age, years (range)	BMI (range)
Control	9	58.2	27.5
ADD	7	60.9	28.3
SDD	9	67.6	27.1

ADD – asymptomatic diverticular disease; SDD – symptomatic diverticular disease; BMI – body mass index.

2.5.2. Immunohistochemistry

Following resection, tissue samples were immersed in a 4 % PFA solution (Sigma–Aldrich) at room temperature for 150 minutes. After that, samples were rinsed in PBS (0.01 M) for 3 × 10 minutes and cut into 10 × 10 mm pieces. Segments were cryoprotected by immersion in 25 % sucrose (Sigma–Aldrich) and 0.05 % sodium azide (Carl–Roth) solution at 4 °C overnight. The following day, samples were embedded in OCT compound (ShandonTM CryomatrixTM, Thermo Fisher Scientific) and serially sectioned to obtain 16 μm full–thickness sections made along longitudinal and circular muscle axes in the cryostat (CryoStar NX70, Thermo Fisher Scientific, USA). Each subsequent section was deepened by > 1 mm to avoid recurrence of the same ganglia in adjacent sections. Sections were mounted on microscope slides, air–dried, and stored at 20 °C until use.

Before immunohistochemical staining, sections were rehydrated in PBS and permeabilized in a 0.5 % Triton X–100 (Carl Roth) and 10 % DMSO (Carl Roth) solution for 1 hour at room temperature. To prevent non–specific binding, samples were rinsed and incubated with 5 % NDS (Jackson ImmunoResearch Laboratories) for 1 hour. Next, double immunohistochemical staining was carried out by incubating samples in primary antisera (Table 2.5.2.1) overnight at 4 °C. The next day, samples were rinsed in PBS and incubated for 4 hours at room temperature with a suitable secondary antibody combination (Table 2.5.2.1). After the secondary antibodies' incubation, specimens were again rinsed in PBS for 3 x 10 minutes and cover–slipped using Vectashield® mounting medium (Vector Laboratories, USA).

The precise protocol used for immunohistochemical staining in this study is described in detail in the publication by *Pauza et al.* [141].

Table 2.5.2.1. *Primary and secondary antibodies used in the study*

Antigen	Host	Dilution	Source	Cat. #
Primary				
CGRP	Mouse	1:1000	Abcam[1]	Ab 10987
RAMP1	Rabbit	1:1000	Bioss[2]	BS–1567R
CRLR	Rabbit	1:1000	Bioss[2]	BS–1860R
PGP 9.5	Mouse	1:1000	Abcam[1]	Ab 72911
PGP 9.5	Rabbit	1:1000	Bio–Rad[3]	7863–0504
NOS1	Rabbit	1:500	Abcam[1]	EP 1855Y
NOS1	Mouse	1:1000	Santa Cruz[4]	SC–5302
VIP	Rabbit	1:1000	Chemicon[5]	AB982

Table 2.5.2.1 continuation

Antigen	Host	Dilution	Source	Cat. #
Secondary				
Rabbit Cy3	Donkey	1:500	Milipore[6]	AP182C
Rabbit AF488	Donkey	1:500	Invitrogen[7]	A21206
Mouse Cy3	Donkey	1:500	Milipore[6]	AP192C
Mouse AF488	Donkey	1:500	Invitrogen[7]	A21202

[1] Abcam, Cambridge, UK;
[2] Bioss Antibodies Inc., Woburn, Massachusetts, USA.
[3] Bio–Rad (Formerly AbD Serotec), Kidlington, UK.
[4] Santa Cruz biotechonology, Dallas, Texas, USA.
[5] Chemicon International, Temecula, California, USA.
[6] Millipore Corp., Temecula, California, USA.
[7] Invitrogen, Ltd., Paisley, UK

The amount of CGRP–IR nerve fibers and CRLR/RAMP1–IR neuronal somata and nerve fibers in the enteric ganglia was assessed using the quantitative fluorescence microscopy method following Waters methodology [142]. Samples were stored at 4 °C in the dark. To avoid fluorophore bleaching, images were taken no later than one week after the sample preparation.

Fluorescent images of intrinsic neural structures were acquired using Zeiss AxioImager Z1 wide–field microscope (Carl Zeiss, Germany) equipped with AxioCam MRm Rev.3 digital camera. The objective used in the study was 40×/0.9 EC Plan NeoFluar, and images were captured using AxioVision Rel.4.8.2 software (Carl Zeiss, Germany). The fluorescent light source was HXP 120 V illuminator using 38HE (EX 470/40, EM 525/50) and 43HE (EX 550/25, EM 605/70) filter set.

Images of enteric ganglia of both myenteric and submucosal plexuses were obtained in a Z–projection composed of 10 focal planes (2–D images) taken at 1 μm increment. A varying number of shots were made that would capture 10–20 autonomic ganglia of each enteric plexus (Table 2.5.2.2). For all images used for quantification, the channel of interest was photographed at a set exposure time (800 ms). All images used for quantification were obtained with a set exposure time (800 ms) for the channel of interest to avoid miscalculation of fluorescence intensity.

Table 2.5.2.2. *Number of enteric ganglia analyzed in the immunohistochemical study (n)*

	CGRP			CRLR			RAMP1		
	MP	**ISP**	**OSP**	**MP**	**ISP**	**OSP**	**MP**	**ISP**	**OSP**
Control (n)	372	243	288	205	153	177	142	84	93
ADD (n)	248	152	183	245	198	216	211	112	158
SDD (n)	292	197	212	221	188	167	191	94	118

ADD – asymptomatic diverticular disease; SDD – symptomatic diverticular disease; MP – myenteric plexus; OSP outer submucosal plexus; ISP – inner submucosal plexus.

Image analysis was performed using ImageJ/Fiji software. 2–D images were loaded into Fiji as a stack and Z–projected using average intensity projection. Fluorescence intensity was determined by selecting the region of interest (ROI) in PGP 9.5 signal view (selecting boundaries of the enteric ganglion) and measuring the densitometric fluorescence intensity (IntDen) within the ROI in the channel of interest (containing CGRP/RAMP1/CRLR signal). The obtained value was background corrected by subtracting the size of ROI multiplied by the average background intensity value of the image. Ganglia containing CGRP–IR neuronal bodies were excluded from the analysis.

Before imaging the colonic sections, a fluorescence standard Rose Bengal (Sigma–Aldrich) (0.25 g mL^{-1}) was used to adjust for potential inconsistencies of the fluorescent lighting and optical shading effects, as described by Model and Burkhardt [143]. The final normalized fluorescence intensity value (expressed in arbitrary units) was obtained by dividing the original (background corrected) intensity value by the average reference value of the fluorescent standard.

2.5.3. Contractility experiments *in vitro*

For physiological experiments *in vitro*, excised tissue was immediately placed in cold–aerated Krebs–Henseleit solution (4 °C) to separate the musculature from the mucosa. Colonic musculature was then cut into 2 × 10 mm strips orientated along longitudinal or circular muscle axes. Next, intestinal muscle strips were immersed in a heated (37 °C) aerated Krebs–Henseleit solution in individual 25 mL wells (Radnoti organ bath, AD instruments Pty, AU). The muscular strips were pre–stretched to a passive tension of 35–40 mN and equilibrated for at least 1 hour. Krebs–Henseleit solution replaced every 15 minutes until a stable baseline was established.

To obtain a maximal reference contraction, bethanechol (10^{-4} M) was added, and a contact period of up to 5 minutes was allowed. Following that,

human CGRP (10^{-7} M) was introduced, and a 15–minute contact period was allowed until the relaxation response plateaued. Then, sodium nitroprusside (SN) (10^{-3} M) was added to induce a maximal reference relaxation response. In a subset of experiments, tetrodotoxin (10^{-7} M) (TTX) was added 15 minutes before the administration of bethanechol.

For each recording, the relaxation response to CGRP was calculated within the range of maximal reference contraction and maximal reference relaxation. The magnitude of the response is expressed as a percentage of the maximal reference relaxation to SN. Untreated strips were run in parallel with strips subjected to experimental compounds. Each strip was only subjected to a single set of drugs used in experimental protocol, and all strips at one experimental session were taken from the same individual.

2.5.4. Transmission electron microscopy

Ultrastructural analysis was carried out as detailed in the publication by *Alaburda et al.* [144]. Briefly, tissue specimens cut for electron microscopy in cold Krebs–Henseleit (4 °C) solution were immersed for 2 hours in a 2.5 % glutaraldehyde 0.1 M PB solution (pH 7.4) at 4 °C. Then, samples were dissected into three layers: the mucosa separated along the submucosa and the remaining piece cut along annular muscles. Using a Stemi 2000CS stereomicroscope (Zeiss, Gottingen, Germany), these three layers were further dissected into 1 × 1 × 2 mm sized tissue samples and fixed in the same 2.5 % glutaraldehyde 0.1 M PB (pH 7.4) solution at 4 °C overnight. After that, samples were postfixed for 2 hours in 0.1 M PB (pH 7.4) with 1 % osmium tetroxide, dehydrated using a series of graded ethanol solutions, and embedded into a mixture of Epon 812 and Araldite resins (Sigma–Aldrich, Steinheim, Germany). Semi–thin sections (1 μm) were stained with methylene blue according to Ridgway [145] and were analyzed with a Zeiss AxioMat light microscope (Carl Zeiss, Jena, Germany). Following semi–thin sections, ultra–thin sections (50–70 nm) were cut using a Leica EMUC7 ultramicrotome (Leica Mikrosysteme Handelsges.m.b.H., Vienna, Austria) and mounted on 600–mesh thin bar support nickel grids (Agar Scientific, Essex, UK). Samples were stained with uranyl acetate and lead citrate for 7 minutes each.

Ultra–thin sections were analyzed using a Tecnai BioTwin Spirit G2 transmission electron microscope (FEI, Eindhoven, the Netherlands) at 100 kV. Images were taken at 4800x, 6800x, and 9300x magnifications using a bottom–mounted 16 MP Eagle 4K TEM CCD camera with TIA software (FEI, Eindhoven, the Netherlands).

Electron micrographs were analyzed morphometrically with Fiji's software package [146, 147]. Areas of neurite profiles were measured by ma-

nually marking the cross–sections of all completely visible neurites. Based on the shape and electronic density, the swelling of neurites was assessed. Unmyelinated neurites with a diameter of less than 0.2 μm were assigned to a separate category of very fine neurites. ICCs and mast cells were counted, and their distance to the nearest enteric nerve was determined.

2.6. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, version 20.0, IBM, Munich, Germany; version 24.0, SPSS Inc., Chicago, IL, USA), Prism (Version 5, Graphpad Software, La Jolla, USA) and R 3.5.1 (R Core Team, 2015). Data distribution was assessed by histograms and Q–Q plots, and the Shapiro–Wilk test was used to establish data normality. Differences in the data were evaluated using an unpaired Student's t–test with Welch's correction or Mann–Whitney U test where appropriate. Exact tests were performed to check the consistency of genotyping results with Hardy–Weinberg equilibrium (HWE). Power calculations were performed to determine sample size. Genotype frequencies were compared in contingency tables listing cases and controls. Dietary data, alcohol use, tobacco use, NSAID and laxative use, and bowel habits were converted into categorical indicator variables for analyses. Data are presented as means ± standard error, medians, ranges, frequency, and percentages where appropriate. CGRP, CRLR, and RAMP1 levels are expressed as ± percentage of the mean control group value. Univariate and multivariate logistic regression models were used to identify risk factors associated with DD. A p–value < 0.05 was considered statistically significant.

3. RESULTS

3.1. SNP studies of genes encoding for connective tissue

3.1.1. SNP genotyping for COL3A1 (rs3134646, rs1800255) and COL1A1 (rs1800012)

3.1.1.1. Patients characteristics

COL3A1 (rs3134646, rs1800255) and COL1A1 (rs1800012) SNPs were investigated in 707 individuals (349 men, 358 women). Subjects were divided into two groups: the study group, consisting of patients with colonoscopically proven diverticulosis (n = 422), and the control group, composed of subjects without pathological changes in the colon on endoscopic examination (n = 285). All patients were of European descent (328 German and 379 Lithuanian patients). The median age was 64 years (range, 19–95). The frequency of diverticulosis in the total cohort was similar to previous data [20]. Data on the reasons for colonoscopy were available in 606 of 707 (85.7 %) patients. Colorectal cancer screening was the most common reason for a colonoscopy in 324 cases (53.5 %), followed by abdominal pain in 178 cases (29.3 %) and rectal hemorrhage in 104 cases (17.2 %).

Clinical data showed that CD was characteristic of elderly patients ($p < 0.0001$), most of them being older than 60 years. Age distribution of patients with CD is provided in Fig. 3.1.1.1.1.

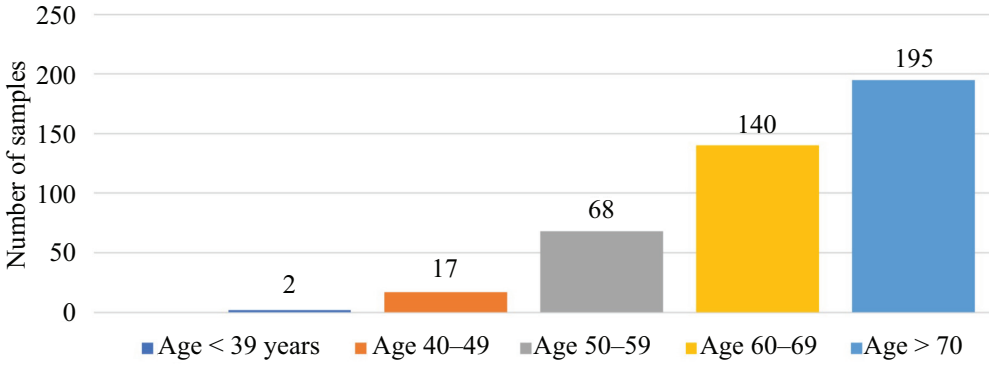


Fig. 3.1.1.1.1. *The distribution of age in diverticulosis patients.*

BMI was significantly higher ($p < 0.0001$) in patients with diverticulosis (median, 28 kg/m^2; range, 17–54) compared to controls (median, 26 kg/m^2; range, 17–48). No differences could be detected for sex ($p = 0.92$), ethnicity ($p = 1.00$), laxative use ($p = 0.09$), smoking ever ($p = 0.20$), or current daily

alcohol intake (p = 0.61). The clinical characteristics of study population is presented in Table 3.1.1.1.1.

Table 3.1.1.1.1. *Clinical characteristics of study population*

Parameter	Diverticulosis (n = 422)	Controls (n = 285)	Total (n = 707)	p value
Age, years	68 (32–95)	57 (19–83)	64 (19–95)	**<0.0001**
BMI, kg/m^2	28 (17–54)	26 (17–48)	27 (17–54)	**<0.0001**
Gender, female/male	209 (49.5 %)/ 213 (50.5 %)	140 (49.1 %)/ 145 (50.9 %)	349 (49.4 %)/ 358 (50.6 %)	0.92
Ethnicity, German/Lithuanian	207 (49.1 %)/ 215 (50.9 %)	121 (42.5 %)/ 164 (57.5 %)	328 (46.4 %)/ 379 (53.6 %)	1.00
Laxatives, yes/no	100 (24.3 %)/ 312 (75.7 %)	31 (17.8 %)/ 143 (82.2 %)	131 (22.4 %)/ 455 (77.6 %)	0.09
Smoking ever, yes/no	157 (38.3 %)/ 253 (61.7)	118 (43.2 %)/ 155 (56.8 %)	275 (40.3 %)/ 408 (59.7 %)	0.20
Alcohol (daily),yes/no	25 (6.1 %)/ 384 (93.9 %)	17 (7.1 %) /221 (92.9 %)	42 (6.5 %)/ 605 (93.5 %)	0.61

Values are given as median (range), or frequencies and percentages, n (%). Significant p values are highlighted in bold. BMI – body mass index.

3.1.1.2. Associations of SNPs and diverticulosis

The genotype distributions did not deviate from the HWE (overall $p < 0.05$). Examining the frequency of COL3A1 (rs1800255) genotypes in the control group, we found that 149 out of 279 patients had the GG genotype (53.4 %), 107 had the GA genotype (38.4 %), and 23 had the AA genotype (8.2 %). Assessing the frequency of COL3A1 (rs1800255) genotypes in the diverticulosis group, we found that 256 of 417 patients had the GG genotype (61.4 %), 142 patients had the GA genotype (34.1 %), and 19 patients had the AA genotype (4.6 %). Frequency studies of COL3A1 (rs3134646) genotypes revealed that in the control group, 69 of 278 had the GG genotype (24.8 %), 144 had the GA genotype (51.8 %), and 65 had the AA genotype (23.4 %). In the group of patients with CD, 100 out of 418 patients had the GG genotype (23.9 %), 189 patients had the GA genotype (45.2 %), and 129 patients had the AA genotype (30.9 %). Frequency studies of COL1A1 (rs1800012) genotypes showed that in the control group, 200 of 278 had the GG genotype (71.9 %), 73 had the GT genotype (26.3 %), and 5 had the TT genotype (1.8 %). In the group of patients with CD, 289 out of 416 patients had the CC genotype (61.4 %), 106 patients had the CA genotype (25.4 %), and 21 patients had the AA genotype (5.0 %). The genotype frequencies of selected variants in COL1A1 and COL3A1 of patients with diverticulosis and controls are shown in Table 3.1.1.2.1.

Table 3.1.1.2.1. *The genotype distribution of selected variants in COL1A1 and COL3A1 of patients with diverticulosis and controls*

Gene	Genotype/alleles	Controls		Diverticulosis	
		n	%	n	%
COL3A1					
rs1800255[a]	GG	149	53.4 %	256	61.4 %
	GA	107	38.4 %	142	34.1 %
	AA	23	8.2 %	19	4.6 %
rs3134646[b]	GG	69	24.8 %	100	23.9 %
	GA	144	51.8 %	189	45.2 %
	AA	65	23.4 %	129	30.9 %
COL1A1					
rs1800012[c]	GG	200	71.9 %	289	69.5 %
	GT	73	26.3 %	106	25.4 %
	TT	5	1.8 %	21	5.0 %

Values are given as frequencies and percentages, n (%).
[a]Eleven samples failed to be genotyped for rs180025.
[b]Eleven samples failed to be genotyped for rs3134646.
[c]Sixteen samples failed to be genotyped for rs1800012.

Further analysis of the genotype distribution of COL3A1 (rs3134646, rs1800255) and COL1A1 (rs1800012) revealed an association between specific genotype variants and clinical data. The results are shown in Table 3.1.1.2.2.

Table 3.1.1.2.2. *rs1800255, rs3134646, rs1800012 genotype variants association with clinical data*

Parameter	rs1800255 (AA genotype)	rs3134646 (AA genotype)	rs1800012 (TT genotype)
Gender, female/male	23 (54.8 %)/ 19 (45.2 %)	86 (46.7 %)/ 98 (53.3 %)	11 (42.3 %)/ 15 (57.7 %)
Age, years	60 (22–78)	64.5 (21–91)	68 (46–91)
BMI, kg/m^2	28.1 (18.4–40.6)	26.9 (16.6–52.4)	26.6 (20.1–37.8)
Ethnicity, German/Lithuanian	23 (54.8 %)/ 19 (45.2 %)	80 (43.5 %)/ 104 (56.5 %)	11 (42.3 %)/ 15 (57.7 %)
Laxatives, yes/no	3 (10.3 %)/ 26 (89.7 %)	12 (7.5 %)/ 148 (92.5 %)	3 (12.0 %)/ 22 (88.0 %)
Smoking ever, yes/no	22 (56.4 %)/ 17(43.6 %)	121 (65.8 %)/ 63 (34.2 %)	7 (26.9 %)/ 19 (73.1 %)
Alcohol (daily), yes/no	0 (0.0 %)/ 29 (100.0 %)	14 (8.7 %)/ 147 (91.3 %)	1 (4.0 %)/ 24 (96.0 %)

Values are given as median (range), or frequencies and percentages, n (%). BMI – body mass index.

Potential risk factors for CD were identified using a univariate logistic regression model. Older age ($p < 0.0001$), increased BMI ($p < 0.0001$), COL3A1 (rs1800255) AA genotype ($p = 0.01$), COL3A1 (rs3134646) AA genotype ($p = 0.033$), and COL1A1 (rs1800012) TT genotype ($p = 0.019$) were significantly associated with the risk of developing CD. However, after adjusting for confounding factors, only two, i.e., older age ($p < 0.0001$) and increased BMI ($p = 0.004$), remained significant in multivariate logistic regression analysis. When selectively analyzing the SNPs in sex–specific multivariate logistic regression analysis, we found that genotype AA of rs3134646 increases the risk of developing CD in men ($p = 0.037$). The logistic regression analysis results of the risk factors for the development of CD are presented in Table 3.1.1.2.3.

Table 3.1.1.2.3. *Logistic regression analysis of risk factors for colonic diverticulosis*

Parameter	OR	95 % CI	p value
Univariate analysis			
Age, years	1.09	1.07–1.10	**<0.0001**
BMI, kg/m^2	1.06	1.03–1.10	**<0.0001**
Gender, female/male	0.98	0.73–1.33	ns
Ethnicity, German/Lithuanian	0.77	0.57–1.04	ns
Laxatives, yes/no	1.33	0.62–2.89	ns
Smoking ever, yes/no	0.81	0.59–1.10	ns
Alcohol (daily), yes/no	0.56	0.30–1.06	ns
COL3A1 (rs1800255) AA genotype	0.44	0.24–0.82	**0.01**
COL3A1 (rs3134646) AA genotype	1.37	1.31–1.43	**0.033**
COL1A1 (rs1800012) TT genotype	2.9	1.08–7.80	**0.019**
Multivariate analysis with presence of diverticulosis as dependent variable			
Age, years	1.08	1.07–1.10	**<0.0001**
BMI, kg/m^2	1.05	1.02–1.09	**0.004**
COL3A1 (rs1800255) AA genotype	0.55	0.27–1.14	0.11
COL3A1 (rs3134646) AA genotype	1.34	0.89–2.00	0.16
COL1A1 (rs1800012) TT genotype	2.53	0.85–7.49	0.095
Multivariate analysis with presence of diverticulosis as dependent variable in men			
Age, years	1.07	1.05–1.09	**<0.0001**
BMI, kg/m^2	1.03	0.98–1.08	0.27
COL3A1 (rs1800255) AA genotype	0.71	0.28–1.81	0.21
COL3A1 (rs3134646) AA genotype	1.77	1.04–3.20	**0.04**
COL1A1 (rs1800012) TT genotype	1.74	0.40–7.56	0.40
Multivariate analysis with presence of diverticulosis as dependent variable in women			
Age, years	1.1	1.07–1.12	**<0.0001**
BMI, kg/m^2	1.06	1.01–1.11	**0.01**

Table 3.1.1.2.3 continuation

Parameter	OR	95 % CI	p value
COL3A1 (rs1800255) AA genotype	0.41	0.13–1.30	0.13
COL3A1 (rs3134646) AA genotype	1.04	0.58–1.84	0.90
COL1A1 (rs1800012) TT genotype	3.42	0.68–17.2	0.14

Significant p values are highlighted in bold. BMI – body mass index; CI – confidence interval; OR – odds ratio; ns – statistically non–significant.

3.1.2. SNP genotyping for ARHGAP15 (rs4662344), COLQ (rs7609897), and FAM155A (rs67153654)

3.1.2.1. Patients characteristics

Altogether, 1,332 individuals (635 men and 699 women) of European descent were analyzed. As in the previous study, the majority of patients with diverticulosis were significantly older ($p < 0.001$) and had a higher BMI ($p < 0.001$) than controls. Diverticulitis patients were significantly younger ($p < 0.001$), more often smokers ($p = 0.006$), and more frequently current alcohol drinkers ($p = 0.001$) compared to asymptomatic diverticulosis patients. No association was found for BMI ($p = 0.20$) and gender ($p = 0.26$). The clinical characteristics of this study cohort are summarized in Table 3.1.2.1.1.

Table 3.1.2.1.1. *Clinical characteristics of the study population*

Parameter	Diverticulosis (n = 856)	Controls (n = 479)	Diverticulitis (n = 198)	Total (n = 1332)	p value*	p value**
Age, years	67 (59–74)	57 (46–66)	61 (53–71)	64 (55–72)	**<0.001**	**<0.001**
BMI, kg/m^2	27.9 (25.2–31.6)	26.7 (23.7–30.3)	27.1 (24.8–30.5)	27.5 (24.8–31.2)	**<0.001**	0.20
Gender, female/ male	423 (49.4 %)/ 433 (50.6 %)	210 (44.3 %)/ 264 (55.7 %)	105 (53.0 %)/ 93 (47.0 %)	636 (47.6 %)/ 699 (52.4 %)	0.08	0.26
Smoking ever, yes/no	300 (35.6 %)/ 556 (64.4 %)	170 (36.7 %)/ 309 (63.3 %)	86 (43.9 %)/ 112 (56.1 %)	469 (36.0 %)/ 865 (64.0 %)	0.72	**0.006**
Alcohol (daily), yes/no	50 (6.0 %)/ 806 (94.0 %)	22 (4.6 %)/ 457 (95.4 %)	19 (9.9 %)/ 179 (90.1 %)	91 (6.8 %)/ 1243 (93.2 %)	0.31	**0.01**

Values are given as median (range), or frequencies and percentages, n (%). BMI – body mass index. Significant p values are highlighted in bold. *Diverticulosis patients compared to healthy controls; **Diverticulitis patients compared to asymptomatic diverticulosis.

3.1.2.2. Associations of SNPs and diverticulosis

The genotypes of ARHGAP15 (rs4662344) and FAM155A (rs67153654) were distributed according to the HWE model, so ARHGAP15 and FAM155A SNPs were investigated in further data analysis. The HWE for the variant in COLQ deviated in controls (both in diverticulosis and diverticulitis analyses $p < 0.001$) and was not included in further analysis.

The frequency of the ARHGAP15 (rs4662344) minor allele was increased in diverticulosis patients compared to controls and was consistent with the results of the previous GWAS [36–38]. The frequencies of the FAM155A (rs67153654) alleles did not differ between the study groups (OR 1.01; 95 % CI: 0.81–1.27 and OR 0.91; 95 % CI: 0.61–1.72), and association with diverticulosis was not detected. The ARHGAP15 (rs4662344) T allele was significantly associated with the risk of diverticulosis (OR 1.28; 95 % CI: 1.00–1.63, $p = 0.05$). The results are shown in Table 3.1.2.2.1.

Table 3.1.2.2.1. *Genotypic and allelic frequencies in ARHGAP15 and FAM155A in diverticulosis and controls*

Gene	A^{min} / A^{maj}	MAF (%)	f CT (%)	f TT (%)	OR	P_{trend}	OR (95 % CI)	$P_{allelic}$	OR 95 % CI)	$P_{genotypic}$
ARHGAP15 (rs4662344)										
Diverti-culosis	T/C	19.5	29.7	4.7	1.30	**0.02**	1.28 (1.00–1.63)	**0.05**	1.85 (0.97–3.50)	0.06
Controls	T/C	15.9	26.3	2.7						
FAM155A (rs67153654)										
			f TA (%)	**f TA (%)**						
Diverti-culosis	A/T	22.8	35.4	5.1	0.90	**0.01**	1.01 (0.81–1.27)	0.91	1.02 (0.61–1.72)	0.93
Controls	A/T	22.6	35.2	5.1						

Genotypic and allelic frequencies of the variants. Values are given as count and percentage. Significant p values are highlighted in bold. A^{maj} – major allele; A^{min} – minor allele; CI – confidence interval; MAF – minor allele frequency; OR – odds ratio.

However, this association was not maintained after adjusting for corresponding environmental cofactors in multivariate logistic regression (OR 1.22; 95 % CI: 0.93–1.61. Table 3.1.2.2.2).

Table 3.1.2.2.2. *Multivariate analysis of factors associated with diverticulosis compared to controls*

	OR* (95 % CI)	p value
ARHGAP15 rs4662344:T (CC+TC vs TT)	1.22 (0.93–1.6)	0.15
ARHGAP15 rs4662344:TC (CT vs TT+CC)	1.14 (0.86–1.52)	0.35
ARHGAP15 rs4662344:TT (TT vs TC+CC)	1.89 (0.84–4.29)	0.13
FAM155A rs67153654:T (TA+AA vs TT)	1.09 (0.84–1.00)	0.53
FAM155A rs67153654:AT (TA vs TT+AA)	1.02 (0.78–1.30)	0.89
FAM155A rs67153654:TT (TT vs AT+AA)	1.19 (0.67–2.10)	0.56

CI – confidence interval; OR – odds ratio. * adjusted for age, BMI, alcohol consumption, and smoking.

3.1.2.3. Associations of SNPs and diverticulitis

The ARHGAP15 (rs4662344) minor allele frequency was higher in diverticulitis cases than in the asymptomatic diverticulosis. This result coincided with the findings of previous GWAS [36–38]. On the other hand, the frequency of the FAM155A (rs67153654) gene A allele (OR 0.66; 95 % CI: 0.47–0.92) was significantly reduced in patients with prior diverticulitis compared to controls (Table 3.1.2.3.1) as also previously stated in [36–38].

Table 3.1.2.3.1. *Genotypic and allelic frequencies in ARHGAP15 and FAM155A in diverticulitis and asymptomatic diverticulosis*

Gene	A^{min} / A^{maj}	MAF (%)	f CT (%)	f TT (%)	OR	P_{trend}	OR (95 % CI)	$P_{allelic}$	OR 95 % CI)	$P_{genotypic}$
ARHGAP15 (rs4662344)										
Diverticulosis	T/C	19.5	32.8	3.1	1.20	0.16	1.27 (0.91–1.76)	0.16	1.38 (0.67–2.84)	0.38
Diverticulitis	T/C	18.8	28.8	4.4						
FAM155A (rs67153654)										
			f TA (%)	f TA (%)						
Diverticulosis	A/T	14.1	21.9	3.1	0.68	**0.01**	0.66 (0.47–0.92)	**0.01**	1.02 (0.19–1.09)	0.07
Diverticulitis	A/T	24.3	37.1	5.8						

Genotypic and allelic frequencies of the variants. Values are given as count and percentage. Significant p values are highlighted in bold. A^{maj} – major allele; A^{min} – minor allele; CI – confidence interval; MAF – minor allele frequency; OR – odds ratio.

These associations remained statistically significant after a multivariate logistic regression analysis, during which the results were adjusted for environmental cofactors. In this analysis, the variant rs4662344 in ARHGAP15 was borderline significantly (OR 1.43; 95 % CI 1.00–2.03; p = 0.05) associated with diverticulitis after adjusting for the corresponding cofactors. (Table 3.1.2.3.2).

Table 3.1.2.3.2. *Multivariate analysis of factors associated with diverticulitis*

	OR* (95 % PI)	**p value**
ARHGAP15 rs4662344:T (CC+TC vs TT)	1.43 (1.00–2.03)	**0.05**
ARHGAP15 rs4662344:TC (CT vs TT+CC)	1.35 (0.44–1.94)	0.11
ARHGAP15 rs4662344:TT (TT vs TC+CC)	1.40 (0.61–3.21)	0.43
FAM155A rs67153654:T (TA+AA vs TT)	0.70 (0.49–0.99)	**0.04**
FAM155A rs67153654:AT (TA vs TT+AA)	0.73 (0.51–1.06)	0.10
FAM155A rs67153654:TT (TT vs AT+AA)	0.68 (0.27–1.69)	0.41

CI – confidence interval; OR – odds ratio. * adjusted for age, BMI, alcohol consumption, and smoking.

3.2. Association of environmental and dietary risk factors and colonic diverticulosis

3.2.1. Patients characteristics

In total, 1,333 individuals were analyzed: 635 (47.6 %) males and 698 (52.4 %) females. The average age of male subjects was 61.89 years, and the average age of females was 62.95 years. Colonic diverticula were found in 858 (64.4 %) of the study subjects, with the remaining 475 (35.6 %) in whom diverticula were absent included in the control group. All patients were of European descent (61.1 % were Lithuanians and the remaining 38.8 % were Germans) (Table 3.2.1.1). Most CD patients (85.8 %) had descending or sigmoid colon diverticula. The rest had right–sided or pancolonic diverticula (14.1 %). Among diverticulosis patients, 198 (23.1 %) individuals had a diverticulitis episode in their medical history.

After completing the standardized questionnaire, comprehensive data on dietary and bowel habits and educational and occupational status was available for 844 study participants (523 for the diverticulosis group, 321 for the control group). Participants with diverticulosis were significantly older ($p < 0.001$) and had a higher BMI ($p < 0.001$) compared to controls (Table 3.2.1.1). No differences were detected for sex ($p = 0.08$), ethnicity ($p = 0.022$), NSAID use ($p = 0.683$), smoking ($p = 0.883$), or regular alcohol consumption ($p = 0.273$) (Table 3.2.1.1).

Table 3.2.1.1. *Characteristics of the study population*

	Diverticulosis (n = 858)	**Controls (n = 475)**	**p value**
	Total (n = 1333)		
	Mean ± standard deviation or n (%)		
Age, years	66.39 ± 10.4 (29–95)	55.24 ± 14.6 (19–92)	**<0.001**
Gender, male/female	424 (49.4 %)/ 434 (50.6 %)	211 (44.4 %)/ 264 (55.6 %)	0.08
Ethnicity			0.022
German	353 (41.1 %)	165 (34.7 %)	
Lithuanian	505 (58.9 %)	310 (65.3 %)	
BMI, kg/m^2	28.75 ± 5.4	27.27 ± 5.1	**<0.001**
Everyday cigarette smoker	300 (35.0 %)	168 (35.4 %)	0.883
Everyday alcohol user	50 (5.8 %)	21 (4.4 %)	0.273
NSAID use, ≥15 day/month	91 (10.6 %)	47 (9.9 %)	0.683

Values are given as count and percentage. Significant p values are highlighted in bold. BMI – body mass index; NSAID – non steroidal anti–inflammatory drugs.

3.2.2. Risk factors for colonic diverticulosis

Univariate analysis revealed that individuals with CD were significantly older (66.4 ± 10.4 years *vs.* 55.2 ± 14.7 years, $p < 0.001$) and also more overweight (28.75 ± 5.4 kg/m^2 *vs.* 27.27 ± 5.1 kg/m^2, $p < 0.001$) compared to control subjects (Table 3.2.1.1), which was in line with previous studies [148]. Data also suggested a significant association between CD and higher educational status ($p = 0.004$) as well as nighttime work shifts ($p < 0.0001$) (Table 3.2.2.1).

Regarding bowel habits, univariate analysis (Table 3.2.2.1) showed a significant correlation between CD and the sensation of incomplete bowel emptying after defecation ($p < 0.0001$) and sparse bowel movements (≤1 time/week; $p = 0.003$). However, painful defecation was reported by similar proportions of cases and controls (25.9 % *vs.* 25.3 %, $p = 0.846$), and there was no significant association between abdominal cramps unrelated to defecation and CD (23.1 % *vs.* 20.9 %, $p = 0.444$). Similarly, there was no difference in defecation duration (5.7 % *vs.* 7.1 %, $p = 0.413$) or overall duration of constipation (self–reported) (10.6 % *vs.* 12.3 %, $p = 0.467$) between cases and control subjects. Diverticulosis was also not associated with nighttime bowel movements (3.8 % *vs.* 3.4 %, $p = 0.819$).

Table 3.2.2.1. *Association of dietary and bowel habits with diverticulosis*

	Controls (n = 321)	**Diverticulosis n = 523)**	**p value**
	Total (n = 844)		
Vegetarianism	6 (1.9 %)	3 (0.6 %)	0.076
Number of meals per day, ≥3 servings/day	230 (71.7 %)	417 (79.9 %)	**0.006**
Red meat, ≥3 servings/week	32 (10.0 %)	41 (7.9 %)	0.289
Fish, ≥3 servings/week	279 (86.9 %)	431 (82.9 %)	0.117
Fluids, <1 liter/day	42 (13.1 %)	90 (17.2 %)	0.107
Bowel movements, ≤1 times/week	13 (4.0 %)	5 (1.0 %)	**0.003**
Pain with bowel movements, ≥25 % time	83 (25.9 %)	132 (25.3 %)	0.846
Feeling of incomplete bowel emptying, ≥25 % time	95 (29.7 %)	247 (47.3 %)	**<0.0001**
Abdominal pain, ≥25 % time	74 (23.1 %)	109 (20.9 %)	0.444
Prolonged duration of defecation, >10 min	18 (5.7 %)	37 (7.1 %)	0.413
Nightly defecation	12 (3.8 %)	18 (3.4 %)	0.819
Duration of constipation, ≥10 years	34 (10.6 %)	64 (12.3 %)	0.467
Laxative use, ≥1 day/week	41 (12.8 %)	57 (11.0 %)	0.417
Higher education	150 (46.9 %)	192 (36.9 %)	**0.004**
Night shifts	36 (11.2 %)	18 (3.4 %)	**<0.0001**

Values are given as count and percentage. Significant p values are highlighted in bold.

In terms of dietary habits (Table 3.2.2.1), CD subjects reported eating ≥3 meals per day substantially more often (79.9 % *vs.* 71.7 %, p = 0.006) when compared to controls; however, this was not associated with increased odds of diverticulosis. Consumption of red meat and fish, as well as a low fluid intake and vegetarian diet, were also not associated with CD. Similarly, no correlation was found between regular use of laxatives (12.8 % *vs.* 11.0 %, p = 0.417) or NSAIDs (10.6 % *vs.* 9.9 %, p = 0.683).

Variables associated with CD in the univariate analysis were reassessed using multivariate logistic regression analysis while adjusting for age and gender. Multivariate analysis confirmed that the risk of developing CD increases with age (OR 1.079, 95 % CI 1.06–1.1, p < 0.05) (Table 3.2.2.2). Higher BMI (≥25 kg/m^2) also increased odds for CD (OR 1.05, 95 % CI 1.02–1.09, p = 0.004) compared with normal BMI subjects (Table 3.2.2.2). In addition, patients who experienced a feeling of incomplete bowel emptying ≥25 % of the time had a higher risk of diverticulosis (OR 2.05; 95 % CI 1.47–2.87) than controls (Table 3.2.2.2). Sparse bowel movements were not associated with an increased prevalence of diverticulosis. On the contrary, data showed that individuals who had fewer bowel movements (≤1 time/week) also had a lower probability of developing diverticulosis compared with individuals with more than one bowel movement per week (OR 0.1; 95 % CI 0.03–0.33)

(Table 3.2.2.2). Higher educational status and nocturnal work shifts were not significant after adjusting for confounding factors.

Table 3.2.2.2. *Assessment of Risk Factors for Diverticulosis (Multivariate)*

	OR	95 % CI	p value
Age, years	1.079	1.06–1.1	**<0.001**
BMI, ≥25 kg/m^2	1.05	1.02–1.09	**0.004**
Feeling of incomplete bowel emptying, ≥25 % time	2.05	1.47–2.87	**<0.001**
Bowel movements, ≤1 times/week	0.1	0.03–0.33	**<0.001**

CI – confidence interval; OR – odds ratio. Significant p values are highlighted in bold.

3.2.3. Risk factors for diverticulitis

Potential risk factors for developing diverticulitis were also assessed. Older participants had a lower risk (OR 0.921, 95 % CI 0.89–0.95, $p < 0.05$) of diverticulitis compared to younger individuals (Table 3.2.3.2). Conforming to previous results, the feeling of incomplete bowel emptying after defecation also increased the odds of diverticulitis (OR 2.769, 95 % CI 1.35–5.7, $p < 0.006$) (Table 3.2.3.2). Additionally, individuals of higher educational status had increased odds (OR 2.453, 95 % CI 1.31–4.59, $p = 0.005$) for diverticulitis compared to the less educated group (Table 3.2.3.2). No other associations were found between the analyzed risk factors and the diverticulitis study group (Table 3.2.3.1).

Table 3.2.3.1. *Assessment of Risk Factors for Diverticulitis (Univariate)*

	Prior diverticulitis (n = 198)	No diverticulitis (n = 660)	p value
	Total (n = 858)		
	Mean ± standard deviation or n (%)		
Age, years	61.27 ± 12.34	67.93 ± 9.21	**<0.001**
Gender, male/female	105 (53.0 %)/ 93 (47.0 %)	319 (48.3 %)/ 341 (51.7 %)	0.246
BMI, kg/m^2	28.3 ± 6.0	28.88 ± 5.2	0.096
Everyday cigarette smoker	86 (43.4.0 %)	214 (32.4 %)	**0.004**
Everyday alcohol user	19 (9.6 %)	31 (4.7 %)	0.01
NSAID use, ≥15 day/month	19 (9.6 %)	72 (10.9 %)	0.599
Vegetarianism	0 (0.0 %)	3 (0.6 %)	0.543
Number of meals per day, ≥3 servings/day	44 (77.2 %)	373 (80.2 %)	0.591
Red meat, ≥3 servings/week	50 (87.7 %)	431 (92.7 %)	0.188
Fish, ≥3 servings/week	45 (78.9 %)	386 (83.4 %)	0.403
Fluids, < 1 liter/day	8 (14.0 %)	82 (17.6 %)	0.497

Table 3.2.3.1 continuation

	Prior diverticulitis (n = 198)	No diverticulitis (n = 660)	p value
	Total (n = 858)		
	Mean ± standard deviation or n (%)		
Bowel movements, ≤1 times/week	1 (1.8 %)	4 (0.9 %)	0.514
Pain with bowel movements, ≥25 % time	23 (40.4 %)	109 (23.5 %)	**0.006**
Feeling of incomplete bowel emptying, ≥25 % time	40 (70.2 %)	207 (44.5 %)	**<0.0001**
Abdominal pain, ≥25 % time	22 (38.6 %)	87 (18.7 %)	**<0.0001**
Prolonged duration of defecation, > 10 min	6 (10.5 %)	31 (6.7 %)	0.286
Nightly defecation	3 (5.3 %)	15 (3.2 %)	0.426
Duration of constipation, ≥10 years	4 (7.0 %)	60 (12.9 %)	0.199
Laxative use, ≥1 day/week	8 (14.0 %)	49 (10.6 %)	0.431
Higher education	31 (54.4 %)	161 (34.7 %)	**0.004**
Night shifts	3 (5.3 %)	15 (3.2 %)	0.426

Values are given as count and percentage. Significant p values are highlighted in bold.

Table 3.2.3.2. *Assessment of Risk Factors for Diverticulitis (Multivariate)*

	OR	95 % CI	p value
Age, years	0.921	0.89–0.95	**<0.0001**
Everyday cigarette smoker	0.516	0.25–1.08	0.078
Pain with bowel movements, ≥25 % time	1.655	0.79–3.46	0.181
Feeling of incomplete bowel emptying, ≥25 % time	2.769	1.35–5.7	**0.006**
Abdominal pain, ≥25 % time	1.608	0.82–3.17	0.17
Higher education	2.453	1.31–4.59	**0.005**

CI – confidence interval; OR – odds ratio. Significant p values are highlighted in bold.

3.3. Study of the enteric nervous system

3.3.1. Immunohistochemical and physiological examinations

3.3.1.1. CGRP expression

CGRP–IR nerves and nerve fibers were extended through all layers of the large intestine, concentrating mainly within the intrinsic plexuses. The majority of CGRP–IR fibers were surrounding the enteric neurons of the myenteric plexus (MP) but were also found in both the external (OSP) and internal (ISP) submucosal plexuses. CGRP–IR fibers encircled the intestinal glands and richly innervated the mucosa, whereas only sparse, minute CGRP–IR nerve fibers were located in the muscular layers. In some enteric ganglia, not only CGRP–IR fibers but also neurons were found (Fig. 3.3.1.1.1).

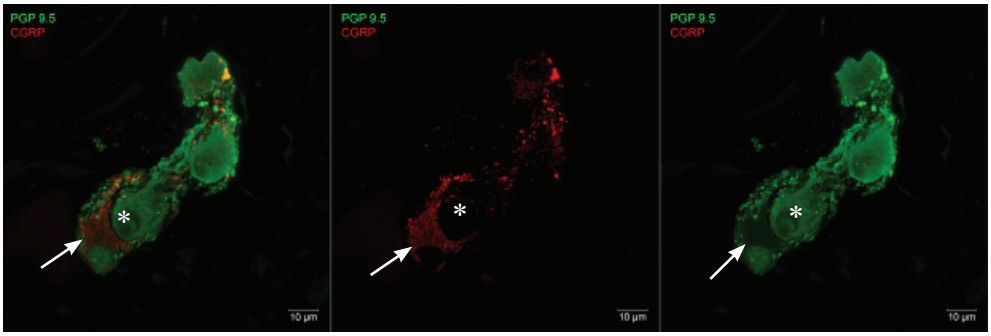


Fig. 3.3.1.1.1. *Confocal micrographs showing a CGRP–IR neuron (arrow) in the enteric ganglion. Next to it is a CGRP–negative neuron (asterisk).*

CGRP–IR nerve fibers were six times more abundant in the ganglia of MP than in the ganglia of both submucosal plexuses in the control samples (Table 3.3.1.1.1). The ISP had the lowest fluorescence intensity levels of CGRP–IR nerve fibers. Within the enteric ganglia of the OSP, the levels of GCRP fluorescence intensity were similar. In all experimental groups, the ratio of CGRP–IR nerve fibers in the MP, ISP, and OSP ganglia remained constant (Table 3.3.1.1.1).

Table 3.3.1.1.1. *Changes in the levels of CGRP, CRLR, and RAMP1 in the enteric ganglia between study groups*

		MP			OSP			ISP		
		SDD	ADD	Control	SDD	ADD	Control	SDD	ADD	Control
CGRP	N	292	248	372	197	152	243	212	183	288
	Median	61.62	117.0	127.6	14.25	16.00	19.74	7.297	13.72	15.33
	Δ (%)	–51.7	–8.31	–	–27.8	–19.0	–	–52.4	–10.5	–
	p value	<.0001	0.326	–	0.039	0.407	–	<.0001	0.804	–
CRLR	N	221	245	205	188	198	153	167	216	177
	Median	21.90	18.85	16.94	9.555	7.951	6.760	6.526	4.452	5.319
	Δ (%)	+29.3	+11.3	–	+41.3	+17.6	–	+22.7	–16.3	–
	p value	0.008	0.078	–	<.0001	0.415	–	0.022	0.199	–
RAMP1	N	191	211	142	94	112	84	118	158	93
	Median	8.406	10.86	9.858	4.879	5.183	3.988	2.814	3.363	2.811
	Δ (%)	–14.7	+10.2	–	+22.3	+30.0	–	+0.11	+19.6	–
	p value	0.677	0.057	–	0.043	0.057	–	0.807	0.243	–

ADD – asymptomatic diverticular disease; SDD – symptomatic diverticular disease; MP – myenteric plexus; OSP – outer submucosal plexus; ISP – inner submucosal plexus.

Quantitative fluorescence intensity analysis showed a decrease in CGRP–IR nerve fibers within the enteric ganglia of DD patients' sigmoid (Fig. 3.3.1.1.2). This difference was particularly evident in the MP, where SDD patients had half the quantity of CGRP–IR nerve fibers as the control group, i.e., a 51.7 % decrease ($p < 0.0001$, Mann–Whitney U test) (Fig. 3.3.1.1.2A–D). In parallel, CGRP–IR nerve fibers in the OSP and ISP decreased by 27.8 % ($p = 0.04$) and 52.4 % ($p < 0.0001$), respectively (Table 3.3.1.1.1). In the asymptomatic group, we found mean intensity values of 8.31 % ($p = 0.326$) for MP; 19 % ($p = 0.407$) for OSP and 10.5 % ($p = 0.804$) for ISP, which were not statistically different from those of the control group (Fig. 3.3.1.1.2E–F, Table 3.3.1.1.1).

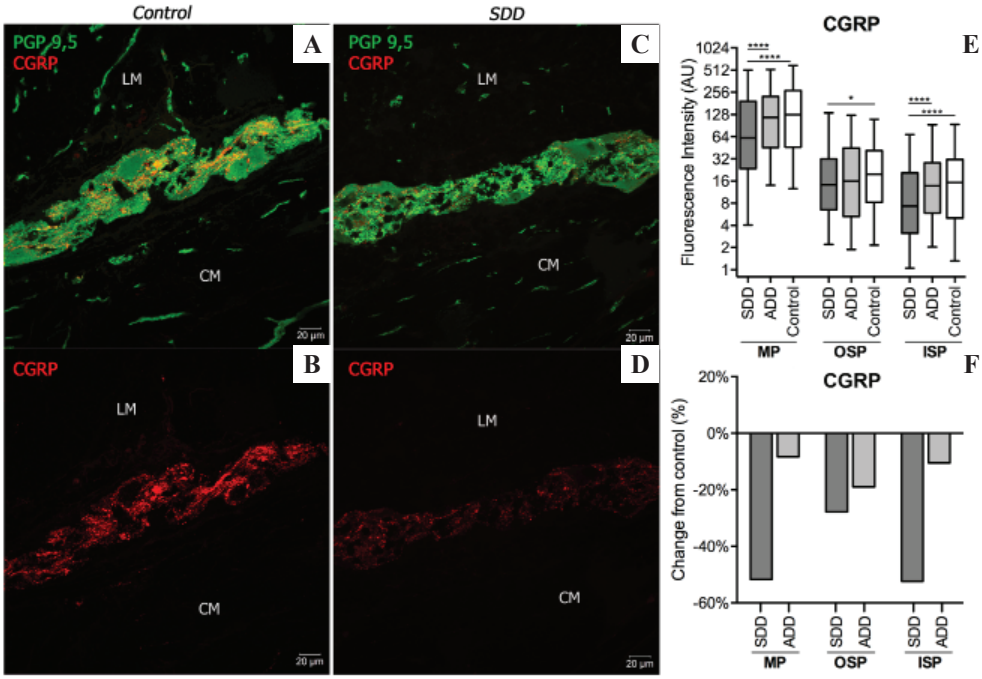


Fig. 3.3.1.1.2. *CGRP–IR nerve fibers in a myenteric ganglion of DD patients.*

(A–D) Confocal micrographs demonstrating differential abundance of CGRP–IR fibers within myenteric ganglion of control (A–B) and SDD (C–D) patients. (E) Quantification of FI within the ENS of DD patients. Whiskers – 5–95th percentile. *–$p < 0.05$, ****–$p < 0.001$ (Mann–Whitney U test). Log2 scale. (F) Percentage change from median control value. LM – longitudinal muscles, CM – circular muscles.

3.3.1.2. CRLR and RAMP1

The CRLR–IR structures were visible as granules, most of them concentrated within the neuronal bodies (Fig. 3.3.1.2A–B). In control tissues, CRLR–IR neuronal structures were 2.5 times more abundant in the MP compared to the submucosal plexuses, with a similar quantity in the OSP and ISP. The proportions of CRLR–IR distribution were consistent with the amount of CGRP–IR nerve fibers (Table 3.3.1.1.1).

In the ADD and SDD groups, the CRLR–IR assay showed the opposite trend compared to CGRP–IR nerve fibers. In SDD patients, the amount of CRLR–IR structures in the intestinal nerve plexuses was increased (Fig. 3.3.1.2C–D). The highest increase was in OSP at 41.3 % ($p < 0.0001$), whereas in MP and ISP CRLR–IR neural structures increased by 29.3 % ($p = 0.008$) and 22.7 % ($p = 0.022$), respectively (Table 3.3.1.1.1).

In the group of asymptomatic patients, the change in the amount of CRLR–IR structures was: MP: Δ+11.3 %, $p = 0.078$; OSP: Δ+17.6 %, $p = 0.415$; ISP: Δ–16.3 %, $p = 0.243$, and again the values were intermediate across the experimental groups (Table 3.3.1.1.1).

Signal localization of RAMP1–IR structures was consistent with that of the CRLR (Fig. 3.3.1.2E–F). The expression of RAMP1–IR structures found in the MP and ISP plexuses of SDD and control patients was not statistically different (MP: −14.7 %, $p = 0.67$; ISP: Δ+0.11 %, $p = 0.81$), while the OSP showed a 22.3 % increase ($p = 0.04$) (Fig. 3.3.1.2G–H, Table 3.3.1.1.1).

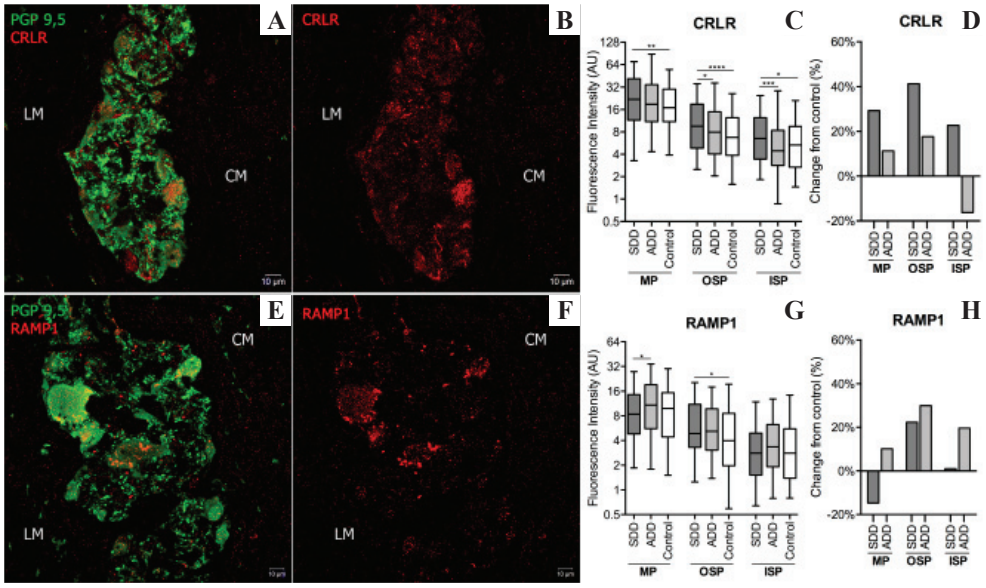


Fig. 3.3.1.2. *CRLR–IR and RAMP1–IR nerve structures within the enteric ganglia of DD patients.*

(A–B, E–F) Confocal micrographs showing CRLR and RAMP1 within the myenteric ganglia. Note the highest accumulations of CRLR–IR and RAMP1–IR fluorescence signal in the neuronal somata and in lesser amount dispersed in the neuropil. (C–D) Quantification of CRLR–IR structures FI within the MP, OSP and ISP ganglia of DD patients. (G–H) Quantification of RAMP1–IR structures abundance within the ENS of DD patients. Whiskers – 5–95 percentile. *–$p < 0.05$, **–$p < 0.01$, ***–$p < 0.005$, ****–$p < 0.001$ (Mann–Whitney U test). Log2 scale. (D, H) Percentage change from median control value. LM – longitudinal muscles, CM – circular muscles.

3.3.1.3. CGRP and smooth muscle relaxation response

Exogenous administration of CGRP induced a tonic relaxation response in both circular and longitudinal smooth muscle strips of the human sigmoid colon (Fig. 3.3.1.3.1A–B). The phasic contractile activity was not observed after the addition of CGRP to the medium. The application of tetrodotoxin did not affect the CGRP–induced response. The longitudinal muscle relaxation response to exogenous CGRP was increased in DD patients. In control samples, exogenous CGRP induced 69.5 % of the maximal relaxation value in the longitudinal muscles and 73 % in the circular muscles (Fig. 3.3.1.3.1B). In the asymptomatic and symptomatic patient groups, relaxation in longitudinal muscles increased by 4.54 % ($p = 0.677$) (unpaired Welch's test) and 10.5 % ($p = 0.033$), respectively, whereas in circular muscles it decreased (SDD:

–2.44 %, p = 0.536; ADD:–6.99 %, p = 0.149), but was not statistically different between the groups (Fig. 3.3.1.3.1B).

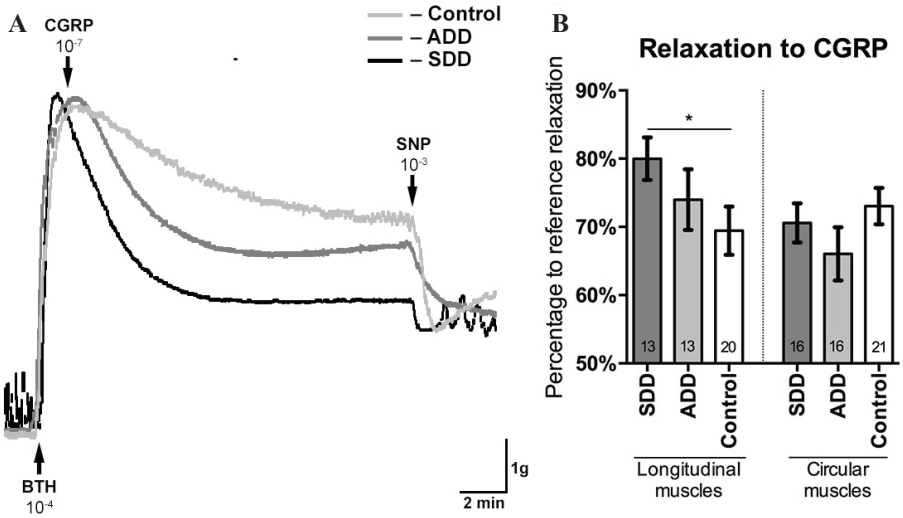


Fig. 3.3.1.3.1. *Smooth muscle relaxation to CGRP.*

(A) Representative curves demonstrating CGRP induced tonic relaxation of longitudinal smooth muscle. (B) Quantification of relaxation response to CGRP. *–p = 0.033 (unpaired Welch's t–test). Mean ± SEM. Numbers indicate repeats. n (SDD) = 6, n (ADD) = 6, n (Control) = 10.

3.3.1.4. Association between CGRP and NOS1/VIP

Double immunohistochemical staining with CGRP, CRLR, or RAMP1 and either NOS1 or VIP was used to highlight the involvement of CGRP in smooth muscle relaxation. Within the enteric ganglia, CGRP–IR nerve fibers were closely associated with NOS1–IR and VIP–IR neurons (Fig. 3.3.1.4.1A–B; Fig. 3.3.1.4.2A–B). Both VIP–IR and nitrergic neurons in the human enteric nervous system were immunoreactive for CRLR and RAMP1 (Fig. 3.3.1.4.1C–D; Fig. 3.3.1.4.2C–D), indicating CGRP activation. VIP–IR neurons were primarily detected in the inner and outer submucosal plexuses, whereas NOS1–IR neurons were predominantly in the myenteric plexus.

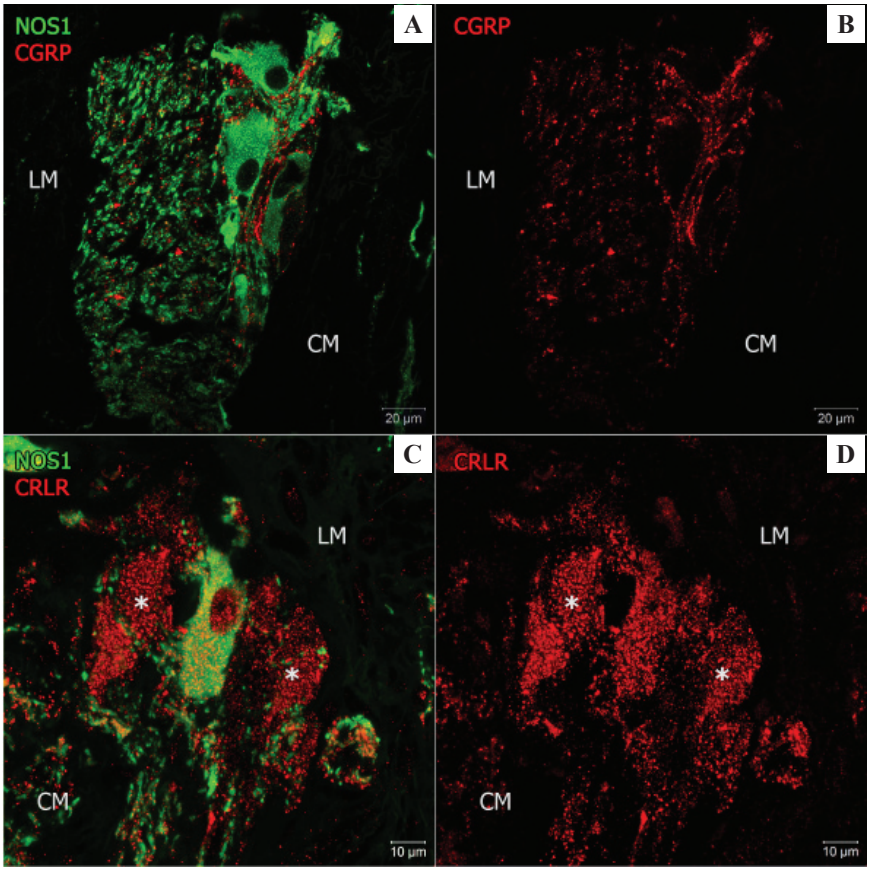


Fig. 3.3.1.4.1. *CGRP–IR nerve fibers innervating nitrergic components within ENS.*

(A–B) CGRP–IR fibers entangling nitrergic neurons within myenteric ganglion. (C–D) CLRL–IR structures in the myenteric ganglion are mainly accumulated in the neuronal somata and in lesser extent in the ganglion neuropil. Note that some neuronal somata (*) are strongly positive for CRLR, but are not labelled by NOS1. LM – longitudinal muscles, CM – circular muscles.

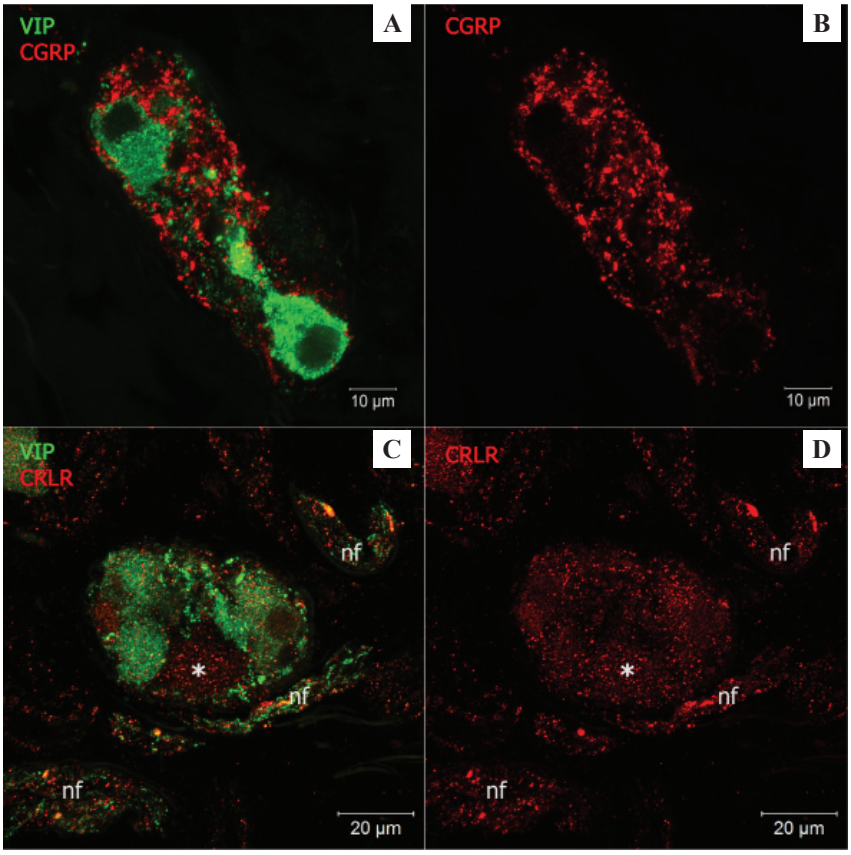


Fig. 3.3.1.4.2. *CGRP–IR nerve fibers innervating VIP–IR components within ENS.*

(A–B) CGRP–IR fibers in close association with VIP–IR neurons residing in the submucosal plexus. (C–D) CRLR–IR structures in the submucosal ganglion are mainly accumulated in the neuronal somata and in lesser extent in the ganglion neuropil. Note that some neuronal somata (*) are positive for CRLR, but are not labelled by VIP. LM – longitudinal muscles, CM – circular muscles, nf – nerve fibers.

3.3.2. Ultrastructural research

3.3.2.1. Ultrastructural changes of enteric ganglia

The enteric neurons in the control group had similar characteristics to those described by other authors [149] when assessed qualitatively. Abundant ribosomes were freely scattered in the cytosol or attached to the cisternae of the endoplasmic reticulum (Fig. 3.3.2.1.1). The mitochondria were round and arranged in clusters, but some were swollen. The Golgi apparatus was visible and was often scattered throughout the cytosol. Neurofilaments and microtu-

bules were visible in the neuronal somata and were particularly pronounced in neurites. One or more lipofuscin inclusions were observed in the cell bodies. Neurons were localized in the periphery of the ganglia. The somata was in direct contact with the basal membrane or partially covered by glial processes. Varicosities and axon terminals with synaptic vesicles were observed inside the ganglia, near the neurons. The neuropil was rich in axodendritic and axosomatic synaptic contacts. The nucleus was usually round, with shallow indentations, and the nucleoplasm largely contained euchromatin which was more concentrated at the nuclear lamina.

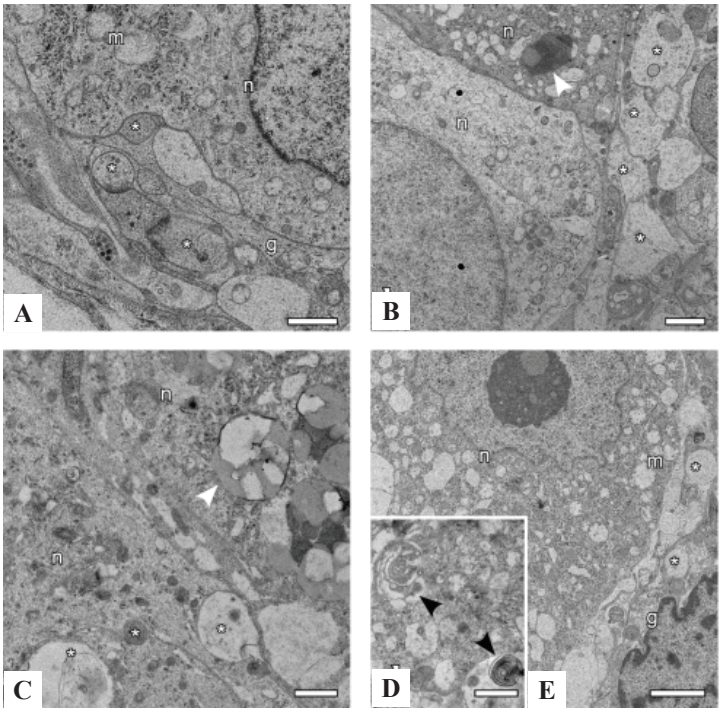


Fig. 3.3.2.1.1. *MP neurons in control (A), asymptomatic diverticulosis (B) and SDD (C–E).*

In the control group, the neuronal body (n) is intact, with adjacent glial processes (g) and axons (*). The surrounding neuropil is tightly packed and without gaps, with axodendritic synapses visible. The neuron is intact on the lower left in the asymptomatic diverticulosis group. In contrast, the neuron's cytoplasm is dense on the upper left, with swollen membranous organelles and visible lipofuscin inclusions (white arrowhead). The neurons in the symptomatic diverticulosis group are the most affected. They have the largest lipofuscin inclusions in the cytoplasm, swollen membrane organelles, mitochondria (m), and laminar bodies (black arrowheads (D)). The nucleus's surface is uneven, with deep and pronounced indentations (E), which were not present in the control or diverticulosis samples.
MP – myenteric plexus; SDD – symptomatic diverticular disease.
Scale bars a, b, c and d: 1 μm, e: 2 μm.

Most neurons were intact in the asymptomatic diverticulosis group, with only some neurons showing thickened cytoplasm, more prominent lipofuscin inclusions, and noticeable swelling of the organelles (Fig. 3.3.2.1.1B). The location of the neurons and their relationship to other structures in the ganglion did not differ from the control group.

In contrast, neurons in symptomatic diverticulosis samples were altered (Fig. 3.3.2.1.1C–E). Large lipofuscin inclusions, lamellar bodies, and swollen, vacuolated mitochondria were observed in most neurons. The rough endoplasmic reticulum was also swollen, and the Golgi apparatus was not discernible in the cytosol. The nucleus was often irregularly shaped, with multiple shallow indentations of various degrees, and also appeared enlarged and more condensed (Fig. 3.3.2.1.1E).

3.3.2.2. Ultrastructural changes of neurites

The enteric ganglia neuropil of control samples was rich in densely arranged neurite profiles (Fig. 3.3.2.2.1A). Although some axons appeared swollen, most of them showed well–contrasting microtubules. Synapses were visible in the myenteric and outer submucosal plexuses, and some ganglia had peg–and–socket type junctions. Mitochondria and neurotransmitter vesicles were visible in the varicosities, and microtubules were absent. The vesicles were primarily small and granular, with an electron–dense core, or small and agranular. In the enteric ganglia of asymptomatic diverticulosis samples, the neuropil was identical to that of controls, but areas of swollen axons were more frequent (Fig. 3.3.2.1.1B).

In contrast, the neuropil of the enteric ganglia of SDD showed apparent lesions. The affected neurites were swollen, free of microtubules and vesicles, the cytoplasm was translucent, and the neurofilaments were not contrasting (Fig. 3.3.2.1.1B). In addition, the plasma membrane was disintegrated and not continuous. As a result, some axons were fused to adjacent axons or glia. In the enteric ganglia of SDD, the axons were separated by fibers of glia cells and collagen fibers. The neuropil was also almost free of varicosities with neurotransmitter vesicles, and no synaptic contacts were found.

Damaged neurites were more abundant in every ganglionated plexus in SDD, but not in asymptomatic diverticulosis (Fig. 3.3.2.2.2). In the MP, 14.2 % of neurites in control samples were damaged compared with 13.9 % in asymptomatic diverticulosis (OR = 0.972, $p = 0.856$), whereas 24.6 % of axons were damaged in SDD (OR = 1.968, $p < 0.0001$). A similar trend was seen in the outer submucosal plexus (control 8.48 %, diverticulosis 8.51 %; SDD 15.82 % *vs.* control OR = 2.027, $p = 0.017$), and in the inner submuco-

sal plexus (control 15.9 %, diverticulosis 13.6 %; SDD 25.0 %, *vs.* control OR = 1.756, p < 0.001).

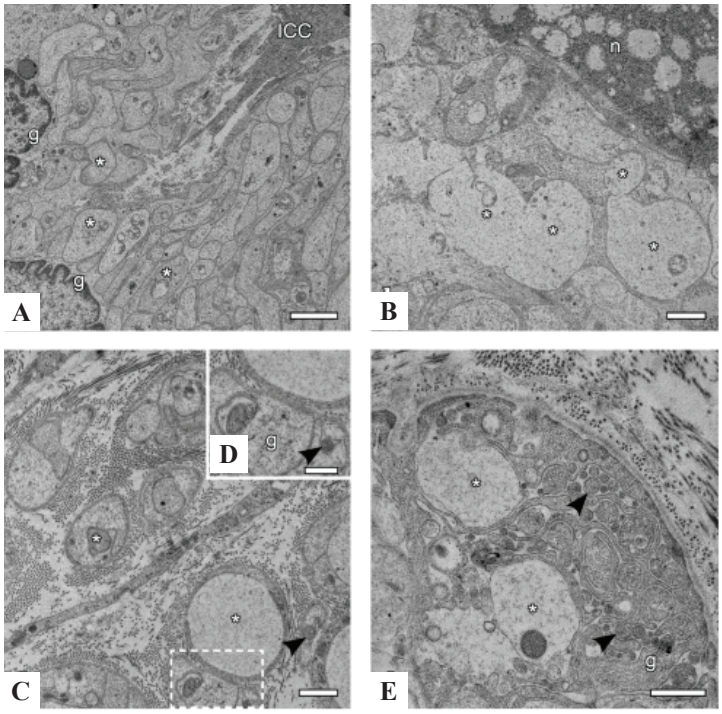


Fig. 3.3.2.2.1. *Changes in neurites of MP in SDD.*

In the control group (A), the neuropil of the myenteric ganglion is dense and rich in axons of different diameters (*), where neurofilaments and microtubules are prominent and visible. Some axons are intact in the MP of the SDD (B–E), but most have translucent cytoplasm, no microtubules, and damage to the continuous plasma membrane (B). In MP nerves (C), the axons are single, isolated by glial cells (g), and collagen fibers fill the large gaps. Some axons are very small in diameter (arrowhead) and are found either next to swollen axons (D) or as a separate group of small axons (E). n – neuron body; MP – myenteric plexus; SDD – symptomatic diverticular disease; ICC – interstitial cells of Cajal. Scale bar in panel a: 2 µm, b and c: 1 µm, d and e: 0.5 µm.

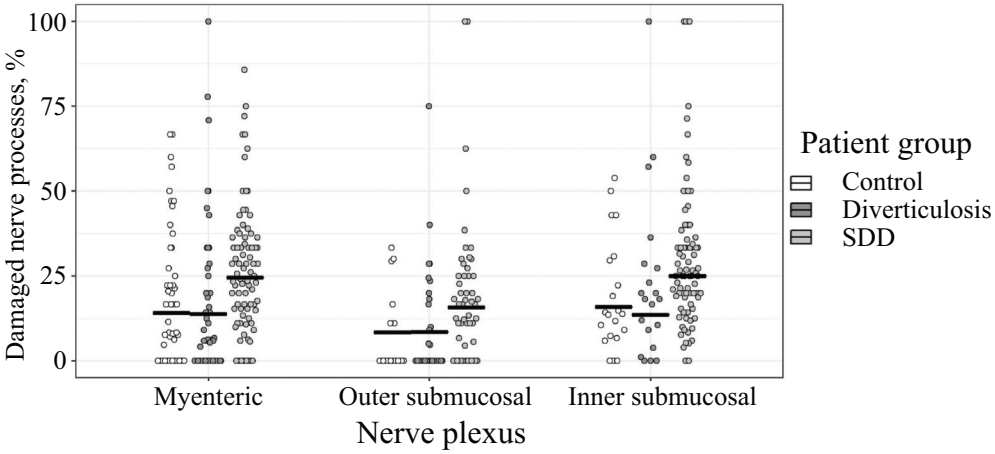


Fig. 3.3.2.2.2. *Percentage of damaged neurites in the myenteric, inner, and outer submucosal plexuses in all study groups.*

The dots show the percentage of damaged neurites found in each electrogram; the black line shows the mean percentage. The highest number of damaged neurites was found in the SDD group. SDD – symptomatic diverticular disease.

SDD samples contained not only damaged neurites, but also fine neurites were more frequent than in controls (Fig. 3.3.2.2.1C–E). These neurites were either adjacent to swollen axons, surrounded by glia outgrowths, or clustered in groups. They contained dense axoplasm and microfilaments. Such neurites were mostly found in the MP and ISP. In the MP, 2.79 % (control) and 2.02 % (asymptomatic diverticulosis) of such fine neurites were found, while in the SDD 5.02 % (OR = 1.845, p = 0.02) of such fine neurites were found. In the OSP, these neurites were more frequent in asymptomatic diverticulosis group (control 2.42 %, diverticulosis 6.96 %, OR = 3.012, p = 0.039, and SDD group 5.63 %, OR = 2.401, p = 0.099). In the ISP, the highest number of fine neurites was found in SDD (11.13 %, OR = 1.40, p = 0.102), while in the control and uncomplicated diverticulosis groups the rates were 8.23 % and 8.67 % respectively (OR = 1.06, p = 0.829).

Therefore, the change in the enteric plexus is twofold – an increase in the number of swollen, damaged neurites and the number of fine neurites. Therefore, the cross–sectional neurite area in SDD is more dispersed than in the other groups (Fig. 3.3.2.2.2, Fig. 3.3.2.2.3), but these tendencies were not statistically significant.

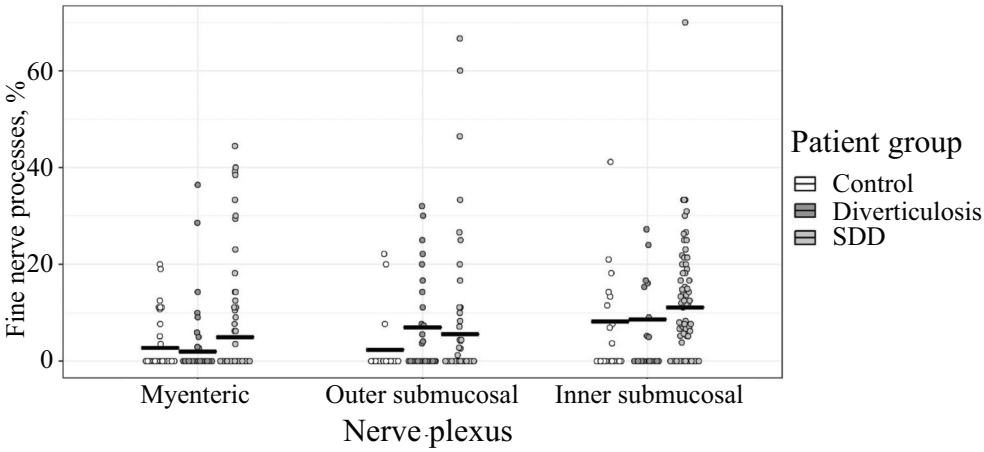


Fig. 3.3.2.2.3. *Percentage of fine neurites in the MP, OSP, and ISP in all study groups.*

The dots show the percentage of fine neurites found in each electrogram; the black line shows the mean percentage. The highest percentage of fine neurites was found in the ISP and MP in the SDD group compared to the other groups. MP – myenteric plexus; OSP – outer submucosal plexus; ISP – inner submucosal plexus; SDD – symptomatic diverticular disease.

3.3.2.3. Changes of mast cells

Most of the mast cells were found in the ISP. A few were also found in the OSP and the MP, but because they were only sporadic, they were not included in further analysis. In the control and asymptomatic diverticulosis samples, an average of 1 and 1,5 mast cells were found in each sample, respectively. In contrast, SDD samples contained an average of 2 cells each, were closer to the enteric nerves than in the other groups (Fig. 3.3.2.3.1A, B), and rarely came into contact with ICCs (Fig. 3.3.2.3.1C). The mean distance between mast cell and nerve in SDD samples was 0.83 µm (0.26 to 7.05 µm), whereas, in the control group, it was 2.25 µm (1.57 to 7.98 µm; $F = 6.44$, $p = 0.004$). No mast cells were found close to the nerve in the asymptomatic diverticulosis group. Enteric nerves rarely had a perineurium in the ISP, so the neurons were likely in direct contact with mast cells.

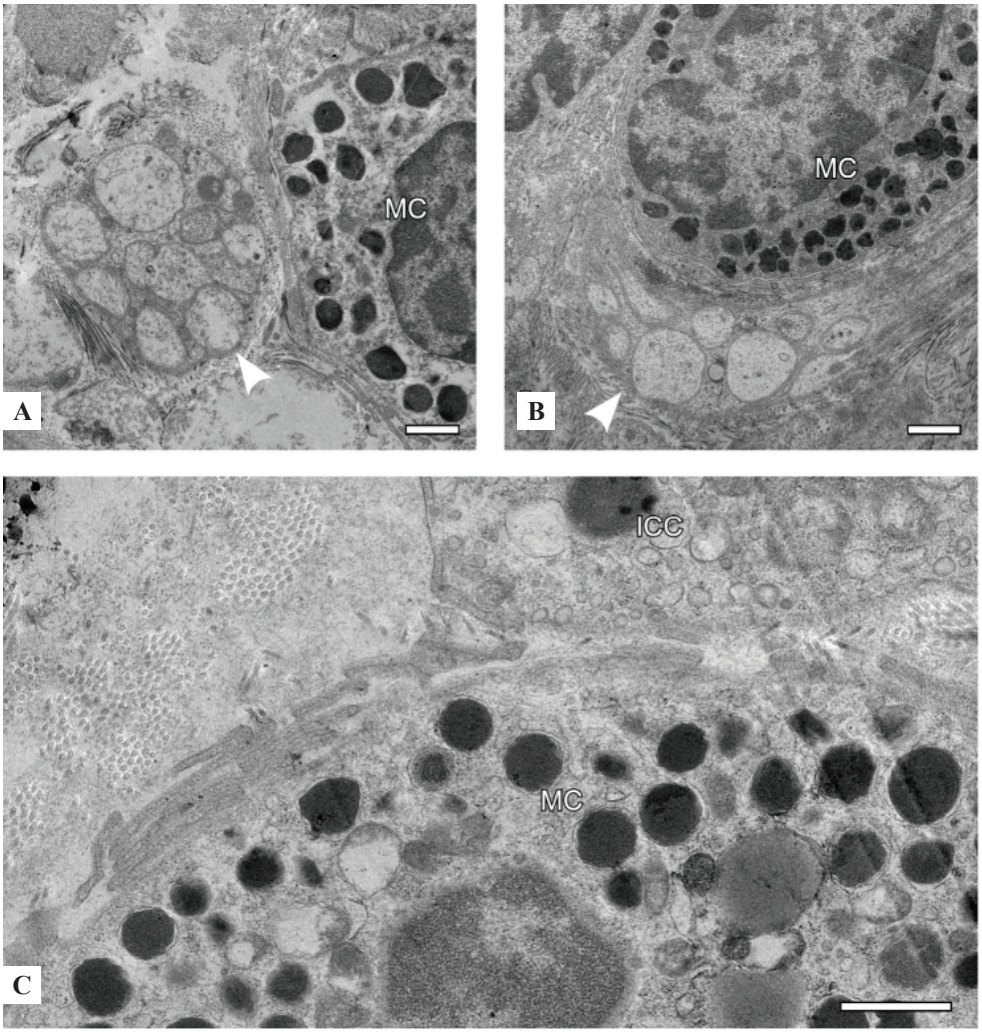


Fig. 3.3.2.3.1. *Mast cells in the ISP in SDD sample.*

The mast cell is located adjacent to the enteric nerve (arrowhead, A–B), with a distance of 250 nm between the structures. The mast cells are also in contact with ICC (C). MC – mast cell; ISP – inner submucosal plexus; SDD – symptomatic diverticular disease; ICC – interstitial cells of Cajal. Scale bar (A–C) is 1 μm.

3.3.2.4. Alterations of interstitial cells of Cajal

The ICCs around the myenteric plexus (ICC–MY) of the control samples appeared healthy, as previously described by other authors [150]. The defining features of these cells were caveolae and intermediate filaments (Fig. 3.3.2.4.1A). They lacked microtubules and lysosomes. In most cases, the rough endoplasmic reticulum and Golgi apparatus were poorly developed and were localized adjacent to the nucleus. Mitochondria were sometimes visible in the cell processes, except for intermediate filaments and caveolae.

ICC–MY in asymptomatic diverticulosis samples looked similar, except for lamellar bodies and sparsely distributed caveolae in some cells (Fig. 3.3.2.4.1B). Cell processes were closely associated with adjacent smooth muscle cells and enteric nerves or ganglia in both groups. ICCs located around the submucosal plexus (ICC–SP) fulfilled the same morphological criteria, except for more distant contacts with surrounding cells and more pronounced processes.

In SDD samples, ICC–MY exhibited structural changes. The cell shape remained the same, but depletion of cytoplasm was observed. The proliferation of ribosomes and rough endoplasmic reticulum in cell processes was observed (Fig. 3.3.2.4.1C, D). In contrast to control and asymptomatic diverticulosis samples, ICC–MY processes rarely contacted smooth muscle cells or enteric nerves but did contact other ICC–MY. The same changes were also observed in ICC–SP samples of SDD. Some of the processes were the same as the control samples, but others had no intermediate filaments. Longitudinal sections of ICC–SP processes showed the degradation of the rough endoplasmic reticulum. The processes were in contact with each other and with the enteric nerves of the submucosal plexus. No other signs of injury were found.

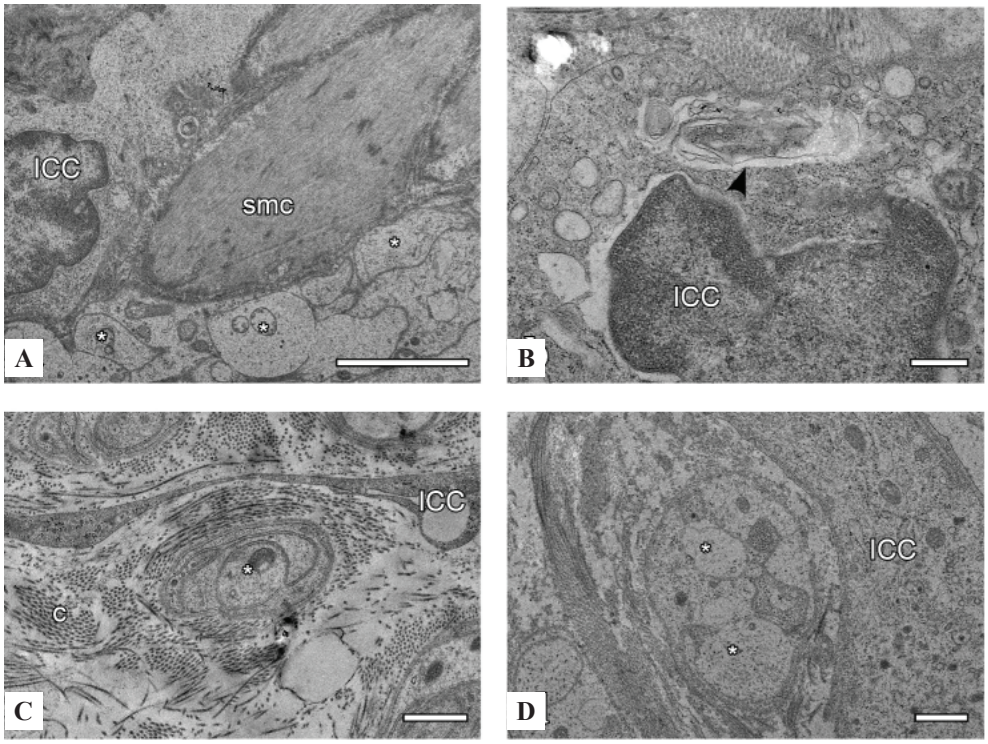


Fig. 3.3.2.4.1. *ICCs in the MP in control (A), asymptomatic diverticulosis (B), and SDD (C–D) groups.*

In control samples, ICCs were surrounded by SMCs and MP nerve fibers. ICCs were in close contact with both SMCs and nerve fibers. In the asymptomatic diverticulosis, most ICCs were similar to controls. However, there were signs of damage, such as lamellar bodies (arrowheads) and membrane ruptures, but close contact with the surrounding cells remained. In the SDD, collagen fibers (C) were interposed between ICC processes and nerve fibers (*). The ICC processes showed ribosomes and fragments of the rough endoplasmic reticulum. In the larger ICC processes (D), a proliferation of the rough endoplasmic reticulum and abundant small lysosomes were observed.
* – axons. ICC – interstitial cells of Cajal; SMC – smooth muscle cells; MP – myenteric plexus; SDD – symptomatic diverticular disease.
Scale bar a: 5 μm, b–d: 1 μm.

4. DISCUSSION

4.1. The role of SNPs within genes encoding for collagens of the connective tissue for the development of colonic diverticulosis

The development of colonic diverticula is attributable to various genetic, environmental, and epidemiological factors. Diverticulosis is generally accepted to be a disease of the elderly. In the age group under 40 years, diverticula are found in only up to 9 % of individuals, whereas in the age group over 75 years, they are already present in 40–60 % of individuals [4]. It is, therefore, not surprising that with the aging population, the incidence of CD and its complications is steadily increasing in societies globally [3, 6, 7, 52]. In recent years, epidemiological twin studies drew attention to the role of genetic factors in the development of CD, demonstrating that heredity accounts for 40 % of the risk of developing the disease [34, 35]. Environmental factors account for the remaining 60 % [34, 35]. Although the importance of genetics is evident, the specific genes and their variants that affect the development of the disease have not been thoroughly investigated. It is currently thought that mutations in genes encoding connective tissue or changes in gene expression may be responsible. It has been shown that patients with specific genetic syndromes (autosomal dominant polycystic kidney disease, Coffin–Lowry syndrome, Ehler–Danlos syndrome type IV, and Williams–Beuren syndrome) characterized by connective tissue abnormalities are more likely to develop colonic diverticula at a much younger age [32, 33].

This study aimed to investigate the role genetic variants of ARHGAP15 (rs4662344), COLQ (rs7609897), FAM155A (rs67153654), COL3A1 (rs3134646, rs1800255), and COL1A1 (rs1800012) for the development of CD. The distribution of genotype frequency of ARHGAP15 (rs4662344), FAM155A (rs67153654), COL3A1 (rs3134646, rs1800255), and COL1A1 (rs1800012) was found to be consistent with the conditions of Hardy–Weinberg equilibrium model. The genetic variants in these genes were analyzed in further data analysis. The HWE for the variant in COLQ deviated in controls, and therefore COLQ (rs7609897) was removed from the study. Logistic regression analysis revealed that the COL3A1 (rs3134646) AA genotype increases the risk of developing CD in men of European descent.

The role of SNPs of COL3A1 and COL1A1 in the development of colonic diverticulosis has not been investigated so far, so the results cannot be compared to other authors. A recent comprehensive population–based study in Denmark and Iceland showed that genetic variants in ARHGAP15 may be associated with the risk of diverticulosis and FAM155A with the risk of diverticulitis. The results of our study were similar, i.e., the A allele of

FAM155A (rs67153654) was associated with the risk of developing diverticulitis in patients with diverticulosis, but this genetic variant did not affect the development of diverticulosis. The risk variant of ARHGAP15 rs4662344 was associated with the risk of developing both diverticulitis and diverticulosis in our study cohort. Several other small case–control studies are known to have investigated the role of SNPs in the development of CD, i.e., one study found an association between rs7848647, a variant within TNFSF15, and diverticulitis requiring surgery [39], and another linked Reprimo 824 G > C variant to the presence of diverticulosis [40]. However, these studies were underpowered to provide conclusive results.

4.2. Enteric nervous system remodeling in colonic diverticulosis

In addition to the previously discussed genetic factors, structural and functional changes within the large bowel wall play an equally important role in developing diverticulosis. The pathogenesis of the disease has been significantly associated with a combination of impaired intestinal motility [45–47], weakening of the colonic wall musculature [103–106], and derangement of enteric innervation [53–55]. The ENS is an intrinsic neural network composed of three ganglionated plexuses: the myenteric (MP), the outer submucous (OSP), and the inner submucous (ISP). MP and OSP primarily regulate intestinal motility, while OSP and ISP control epithelial functions [44, 111, 112]. This arrangement of the ENS into three distinct enteric plexuses is found in all large mammals. Many colonic motor dysfunctions have been associated with the specific abnormalities of ENS [41, 42]. Therefore, it is not surprising that an increased intestinal smooth muscle sensitivity to acetylcholine [54, 55] and a loss of relaxation response to sodium nitroprusside [56] were also detected in diverticulosis patients.

CGRP is involved in a wide range of physiological processes throughout the gastrointestinal tract via a heteromeric receptor composed of CRLR and RAMP1 [59]. The effects of CGRP are manifold. It plays a significant role in absorption and secretion [57, 152, 153], nociception and immune response [57, 61], and, importantly, intestinal motility [66–68]. The main targets of CGRPergic innervation are the intrinsic plexuses, where they encircle and form connections with the neurons of enteric ganglia [58, 154, 155]. Therefore, it cannot be excluded that declining levels of CGRP can induce a whole range of negative downstream effects within the gastrointestinal tract.

In this study, we aimed to determine the effect of CGRP on intestinal smooth muscle relaxation in the context of CD. To this end, we quantified the abundance of CGRP–IR nerves and nerve fibers in all three plexuses of the ENS, i.e., MP, ISP, and OSP. The semi–quantitative approach used in this stu-

dy, using IHC staining and quantitative fluorescence microscopy, allowed the simultaneous examination of different ENS plexuses, identifying the precise anatomical localization of the examined proteins and the quantitative changes in their expression.

Most CGRP–IR nerve fibers within the intrinsic plexuses were located in the vicinity of enteric neurons. CGRP–IR neurons were scarce in the human colonic specimens studied. Moreover, we did not find any ganglia composed exclusively of this type of neuron, unlike those described in the human small intestine [156]. In contrast, in the descending colon of the swine model, CGRP–IR neurons accounted for 25 % of neurons in the MP and 40 % of neurons in the ISP [157]. Furthermore, CGRP–IR nerve fibers innervated colonic tissue similarly to canines [58]. This suggests that the precise role of CGRP is not just region–dependent but also exhibits some interspecific variation. In contrast, the CRLR signal was concentrated in the neuronal cell bodies of all enteric ganglia, as previously described by *Cottrell et al.* [154].

Our methodology allowed us to quantitatively demonstrate for the first time that the primary target of CGRP innervation is the MP, whereas the submucosal plexuses were innervated 6–10 times less. Furthermore, our results showed that CGRP expression was decreased within the enteric ganglia of the sigmoid colon of DD patients. Since the CGRP levels in asymptomatic group samples were intermediate, a gradual decrease of CGRP in DD can be assumed. Therefore, knowing that DD patients display impaired colonic motility, based on the obtained data, it is reasonable to speculate that the decline of the CGRP–IR nerve fibers in the MP ganglia of DD patients adds to the etiological causes of the disease or its symptoms.

Moreover, declined levels of CGRP–IR nerve fibers could account for the increased intracolonic pressure observed in DD patients [126, 158]. Given that CGRP is a potent smooth muscle relaxant, altered expression of CGRP could be associated with impaired muscle relaxation [56]. Smooth muscle contractility studies have confirmed the results of other researchers showing that the smooth muscle relaxation ability in DD is impaired [56]. Interestingly, CGRP–induced relaxation of longitudinal smooth muscle was higher in ADD samples by almost 5 % and in SDD by 10 % compared to the control group. This could mean that DD increases the sensitivity to CGRP and thus the importance of CGRP for smooth muscle relaxation. The increase in the CGRP receptor CRLR found in our study may have evolved as a compensatory mechanism for the gradual decrease of CGRP levels and explain the increased sensitivity of muscle to CGRP. In 2003, *Golder et al.* [55] demonstrated analogous changes with cholinergic innervation in DD patients.

RAMP1 is a second protein required to form a functional CGRP receptor [152]. However, our study did not find a clear pattern of changes in RAMP1

expression in the MP and ISP plexuses of DD patients. Furthermore, the levels of both components of the CGRP receptor, RAMP1, and CRLR, were reliably increased only in OSP ganglia. The fact that RAMP1 levels in DD patient samples show a non–uniform and unreliable variation, whereas CRLR levels increase in association with an increased response to CGRP raises the question of the heterogeneous interaction of these proteins in the ENS.

Both VIP and NO are the main gastrointestinal smooth muscle relaxant agents [159] and inhibitors of motor neuron activation [43]. In the vasculature, activation of CGRP receptors triggers the release of NO [57]. By double staining against NOS1–CGRP, NOS1–CRLR, and NOS1–RAMP1, we have demonstrated CGRP–IR nerve fibers to be closely associated with VIPergic and nitrergic neuronal structures in the enteric ganglia studied. Therefore, the relaxant effect of CGRP may be mediated through the activation of VIP–IR and NOS–IR neurons. Thus far, several studies have demonstrated that in DD, the NO–mediated response is altered and that colonic smooth muscle relaxes loses its ability to relax to sodium nitroprusside [51]. The reduced levels of CGRP in DD may likely reflect a general tendency toward intestinal denervation leading to an imbalance in neuromuscular transmission as a significant etiological factor in DD.

4.3. Ultrastructural changes in colonic diverticulosis

The idea of nerve tissue remodeling in diverticulosis has been raised previously. For example, a decrease in the amount and size of enteric ganglia [160], loss of enteric neurons [70], and changes in the intestinal muscle innervation [55, 71] have been reported. However, this is the first study to analyze further ultrastructural changes within intrinsic plexuses of diverticulosis patients using transmission electron microscopy. Therefore, we believe that the results described for the first time in the present work add to the observations of previous authors.

Although no significant changes were identified within the enteric plexuses of asymptomatic diverticulosis patients, in SDD samples, we observed marked alterations in both neuronal bodies and neurites. The study design did not allow us to determine whether these changes were the cause of DD or secondary to acute diverticulitis attacks. However, the identified loss of typical nerve fiber structure, swelling of neurites, and increase in glia and collagen deposits may explain nerve thickening previously reported by *Simpson et al.* [160]. In SDD, the observed swelling of axons and increased abundance of very fine neurites may be explained by the ongoing nerve regeneration and subsequent hyperinnervation, leading to the visceral hypersensitivity previously described in intestinal inflammation [149]. Given that significant ultras-

tructural changes were only found within the ENS of SDD patients, there is no reason to believe that they were the cause of DD but rather a consequence of acute diverticulitis attacks.

The principal function of the interstitial cells of Cajal (ICCs) is to generate spontaneous and rhythmic electrical activity as well as mediate signal transmission from enteric neurons to smooth muscle cells [44]. Therefore, it has been hypothesized that loss of normal ICC function may disturb regular intestinal motility [35]. Previous physiological studies have reported abnormal colonic motor patterns in DD patients [110], which could have resulted from rearranged ICC networks [120]. The ultrastructural changes observed in the ICCs in this study may contribute to the motility abnormalities observed in SDD.

Additionally, the ENS is known to integrate paracrine signals via mast cells. It has been demonstrated that the interaction between mast cells and the ENS not only influences the onset of symptoms of irritable bowel syndrome [161] but also correlates with symptom severity [162]. Furthermore, mast cell degranulation is associated with an increased visceral hypersensitivity [163]. Therefore, the increased number of mast cells in the vicinity of the ISP nerves found in this study suggests their possible role in the etiogenesis of DD. This is supported by the fact that the incidence rate of irritable bowel syndrome following an acute diverticulitis episode is higher [164] and that histamine receptor expression in the enteric nerves is increased in DD [165]. Therefore, further studies of mast cells in DD are warranted to better understand their role in the etiogenesis of diverticular disease.

4.4. Lifestyle, nutrition, and colonic diverticulosis

In this part of the study, we evaluated the association between our considered socio–demographic factors, dietary and bowel habits, and the risk of colonic diverticulosis and diverticulitis. One of the significant strengths of this study was that it used participants from a large multicentered colonoscopy–based cohort, thus providing access to highly detailed clinical and endoscopic data and covariates. According to our findings, older age, higher BMI, frequency of bowel movements, and a sensation of incomplete bowel emptying were all linked to an increased risk of CD. We also discovered that higher educational status, older age, and a sensation of incomplete bowel emptying were all associated with a higher incidence of diverticulitis in CD patients in our cohort.

Our findings were consistent with previous research [78, 148], demonstrating that diverticulosis prevalence rises with age. This corresponds to our earlier hypothesis that diverticula formation results from a weakening of con-

nective tissue within the colon wall and degenerative changes in the enteric neural structures presumably occurring with aging. In addition, above–mentioned GWAS [36–38] have identified diverticulosis–related genetic variants associated with relevance to intestinal neuromuscular function and connective tissue support, further adding to this link.

Similarly, participants with an overweight or obese BMI had a higher risk of diverticulosis than those with a healthy weight in our cohort. However, data on the association between obesity and CD has been inconsistent up to this point. While some authors state that a higher BMI increases the likelihood of developing CD [20, 21, 23], others have found no such association [166] or believe that having an increased waist circumference due to visceral and subcutaneous fat accumulation is a better predictor [22].

Several studies have initially suggested that males had a higher disease prevalence than females [167]. However, a more recent study by *Peery et al.* [168] has shown that women aged 40 to 49 have a reduced risk of any diverticulosis than men of the same age, although the strength of this link weakens with age. This could mean that sex hormones throughout the premenopausal period protect against disease development [168]. The average age of the enrolled participants in our cohort was over 50 years, and we found no gender–related variations in CD incidence.

Likewise, there was no significant correlation between daily cigarette smoking and CD. However, smoking was nearly twice as common among subjects with past diverticulitis episodes compared to the asymptomatic group. Although this factor did not reach statistical significance for increasing the risk of diverticulitis in our cohort, it could be argued that smoking is more likely to be related to symptomatic diverticular disease than asymptomatic diverticulosis.

One of the intriguing aspects of this study was the attempt to investigate the association between participants' educational and occupational status and the risk of CD. To our knowledge, there has been no other large–scale colonoscopy–based analysis addressing this causality to date. Contrary to popular opinion, our data demonstrated that diverticulosis patients had a lower educational status than controls (192 (36.9 %) *vs.* 150 (46.9 %)) and were less likely to work in sedentary or night shift occupations. It's uncertain whether the results have been influenced by the 10–year age disparity between research groups or whether CD is more prevalent among individuals from lower socioeconomic backgrounds. Although this tendency did not reach statistical significance in our analysis for increasing the risk of CD, we believe it is important to address. A higher educational degree, presumably indicating a more sedentary lifestyle, did, however, increase the odds of diverticulitis.

This supports the argument that the risk factors for CD development likely differ from those for its complications.

In the context of eating habits, we found no evidence that dietary choices impact CD development. None of the dietary components examined in our study were linked to the condition. Other studies have previously demonstrated that a Western dietary pattern (rich in red meat and refined grains) increases the odds of diverticulitis when compared to more prudent nutritional habits (high in fruits, vegetables, and whole grains) [89, 169]. Also, red meat consumption has been previously shown to increase the risk of diverticulitis (relative risk of 1.2 for each serving of red meat), although not diverticulosis [89, 169]. However, these results have not been corroborated in our analysis.

We have also found that less frequent bowel movements decrease the risk of CD. For more than 40 years, the concept that constipation caused by a "low residual diet" was to blame for the formation of colonic diverticula has been widely accepted [52, 53]. This hypothesis relied mainly on observational data showing that specific changes in colonic neurotransmission are present in both idiopathic constipation and diverticular disease [170]. However, several recent studies have disputed this hypothesis by demonstrating that increasing fiber intake raised diverticulosis risk in a dose–dependent manner [20]. After adjusting for compounding factors, individuals in the highest quartile of total fiber intake had a higher diverticulosis prevalence ratio (1.30; 95 % CI 1.13–1.50) than those in the lowest quartile [20]. Additionally, frequent bowel movements were associated with diverticulosis [20], which is consistent with our findings.

Moreover, in our study, the sensation of incomplete bowel emptying after defecation, which is a characteristic symptom of constipation, was strongly associated with an increased risk of diverticulosis. This can be attributed to an increased smooth muscle sensitivity to cholinergic stimulation observed in CD patients, previously described by other researchers [54, 55]. This change to colonic neuromuscular transmission possibly leads to a reduced ability of the smooth muscle to relax and, therefore, induces symptoms. Our contractility experiments discussed above have also demonstrated decreased smooth muscle relaxation response in CD. Furthermore, the feeling of incomplete bowel emptying after defecation was associated with not only asymptomatic but also symptomatic complicated diverticular disease, as it increased the risk of diverticulitis in our study. We believe this could indicate a gradual decline in intestinal neurotransmission as the disease progresses.

4.5. Outlook

Despite the extensive research, the prevalence of diverticulosis and DD, which includes both diverticulitis with its complications and SUDD, continues to rise, as does the socio-economic burden. The public health challenges this chronic-degenerative disease poses can no longer be postponed. However, due to the intricacy of this multifactorial condition, guidelines that explain causal pathways and management routes have yet to be developed. For this reason, it seems essential to deepen and implement the most recent scientific findings to guide the management of this condition.

We believe that the results of this study provide valuable insights into the etiogenesis and associated complications of colonic diverticulosis. The integration of research methodologies we have chosen to use in this study can be a helpful approach for combating the pathologies with multifactorial etiopathogenesis such as this one. Our study findings significantly support the accumulating evidence that the origins of diverticulosis are not purely mechanical but also involve a neurological component and genetic background. This marks a paradigm shift regarding the etiology of DD where the causal-consequential relationship between the two factors, i.e., neurological alterations of the ENS and the mechanical alterations of the colonic wall, remains the subject of future study. Identifying modifiable risk factors enables the development of evidence-based primary prevention measures in the future. Knowing defined prognostic factors is also crucial for predicting its main complication, acute diverticulitis while allowing for more personalized treatment strategies and preventing disease recurrence.

The work, however, continues as factors that promote the progression of diverticulosis to symptomatic disease need to be further elucidated. Although some pathophysiological mechanisms that trigger the occurrence of symptoms have been previously identified, understanding the role of altered CGRP signaling is relatively new and of pivotal importance. Although current results on CGRP in diverticular disease do not yet allow us to draw definitive conclusions about the precise changes in intestinal neuromuscular transmission associated with the disease, they are certainly worthy of consideration when deciding on possible treatment strategies. CGRP-directed therapies might, therefore, represent a rational approach to diverticular disease in the future.

In summary, our findings open the door for further research into the etiology of colonic diverticulosis. Additional experimental research will be needed to identify reliable prognostic factors that could be easily translated into clinical practice.

CONCLUSIONS

The following conclusions have been drawn from the results of the study:

1. Variants of COL3A1 (rs1800255), COL1A1 (rs1800012), and COLQ (rs7609897) are not associated with the risk of developing colonic diverticulosis. Multivariate logistic regression analysis revealed that a variant of COL3A1 rs3134646 increases the risk of diverticulosis in white men. FAM155A (rs67153654) A allele is associated with the risk of diverticulitis in patients with diverticulosis, but this genetic variation does not affect the development of diverticulosis. The risk variant of ARHGAP15 rs4662344 is associated with the risk of developing both diverticulitis and diverticulosis in our study cohort.
2. CGRP signaling pathway in diverticulosis patients is altered. These changes are most prominent in SDD, where CGRP expression is downregulated within all three enteric plexuses of the sigmoid colon, with the asymptomatic group having the intermediate values. The expression of the CGRP receptor – CRLR – is upregulated oppositely. CGRP also has all the necessary components for the direct activation of VIPergic and nitrergic neurons. This remodeling of neural structures is associated with a decreased relaxation response of intestinal smooth muscle observed in DD. The intermediate values of the results observed in the asymptomatic group suggest a gradual decrease of CGRP as the disease progresses. Study results demonstrate that an imbalance in the neuromuscular transmission is a major etiological of CD.
3. Ultrastructural changes occur only in SDD. All parts of the ENS are altered – intestinal ganglia, neuronal bodies, nerves, and nerve fibers – with a reduction in the number of neurotransmitter vesicles and synaptic contacts. Ultrastructural changes in the ICCs and an increased number of mast cells in direct contact with the nerves of the ISP and the ICCs are also observed.
4. Analysis of nutritional and environmental factors revealed that the risk of CD is associated with older age, higher BMI, frequency of bowel movements, and sensation of incomplete bowel emptying. Older age, higher BMI, and the sensation of incomplete bowel emptying increase the risk of CD, while infrequent defecation is associated with lower disease risk. In addition, the risk of diverticulitis in patients with diverticulosis is associated with older age, feeling of incomplete bowel emptying, and higher educational status in our study cohort.

SUMMARY IN LITHUANIAN

1. ĮVADAS

Storosios gaubtinės žarnos divertikuliozė yra lėtinė progresuojanti virškinamojo trakto patologija, kuriai būdingas divertikulų – gleivinės ir pogleivio maišelio pavidalo išgaubų per silpnąsias raumeninio sluoksnio vietas – formavimasis [1, 2]. Divertikuliozė yra viena dažniausių gastroenterologinių patologijų, kurios paplitimas, visuomenei senėjant, dažnėja [2, 3]. Vertinama, jog bendrojoje populiacijoje liga išsivysto daugiau nei 50 proc. vyresnių kaip 60 amžiaus asmenų [4]. Didesnei daliai pacientų ligos simptomai nepasireiškia (vadinama asimptominė divertikuliozė), bet likusiems 25 proc. yra būdinga simptominė ligos eiga [2]. Ligos sunkumas varijuoja nuo nekomplikuotos divertikulinės ligos (DL), kuriai būdingi į dirglios žarnos sindromą panašūs simptomai, iki gyvybei grėsmingų komplikacijų, tokių kaip ūminis divertikulitas, virškinamojo trakto kraujavimas ar žarnos perforacija [5].

Ligos paplitimas tarp išsivysčiusių ir besivystančių šalių ženkliai skiriasi. Nuo XX a. pradžios, ilgėjant vidutinei gyvenimo trukmei ir vis sparčiau įsigalint „vakarietiškam“ gyvenimo būdui, ligos paplitimas visame pasaulyje taip pat dažnėja, kartu didindamas ir ekonominę naštą šalių sveikatos sistemoms [6–11]. Nepaisant to, ligos etiologiją įtakojantys veiksniai iki šiol nėra suprasti.

Šiuolaikinė divertikuliozės patogenezės koncepcija teigia, jog ligos pasireiškimą lemia sudėtinga įvairių aplinkos ir vidinių faktorių tarpusavio sąveika. Nustatyta, kad aplinkos faktoriai sudaro apie 60 proc. ligos išsivystymo rizikos, o likusius 40 proc. – genetiniai veiksniai [35].

Industrinę revoliuciją sekęs sergamumo augimas yra siejamas su pasikeitusiais mitybos ir gyvenimo būdo įpročiais kapitalistinėse šalyse. Epidemiologiniai tyrimai atskleidė, jog tam tikri veiksniai, tokie kaip sumažėjęs fizinis aktyvumas [12–15], sumažėjęs skaidulų kiekis mitybos racione [16–20], gausus raudonos mėsos vartojimas [17, 20], antsvoris [21–23], rūkymas [24–26] ar reguliarus alkoholio vartojimas [24, 27] gali būti susiję su didesne ligos išsivystymo rizika. Vis dėlto, atliktų tyrimų duomenys dažnai yra nevienareikšmiai ir jų vertinimas sudėtingas. Tai lemia skirtumai tarp tiriamųjų populiacijų (skirtingas tiriamųjų amžius, etninė priklausomybė), naudotų diagnostikos metodų ir itin plataus ligos apraiškų spektro. Todėl išsamus galimų mitybos ir aplinkos veiksnių poveikio tyrimas, kuriame vertinami kolonoskopiškai atrinktos Europos populiacijos duomenys, yra itin aktualus.

Nepaisant to, jog storosios žarnos divertikuliozės etiologija tradiciškai siejama su aplinkos veiksnių įtaka, nauji epidemiologiniai duomenys rodo, kad paveldimumas taip pat yra reikšmingos ligos vystymuisi. Šį ryšį iš dalies

patvirtina pastebėti skirtumai tarp Vakarų ir Azijos populiacijų [31] bei ligos sąsaja su kitomis paveldimomis jungiamojo audinio ligomis, tokiomis kaip inkstų policistozė, Coffin–Lowry, Ehler–Danlos ar Williams–Beuren sindromai [32, 33]. Per pastarąjį dešimtmetį Skandinavijoje buvo atlikti du didelės apimties populiacijos dvynių tyrimai, įrodantys genetinių veiksnių reikšmę divertikuliozės išsivystymui [34, 35]. Visgi svariausi įrodymai šioje srityje paskelbti 2017–2019 m. trijuose viso genomo asociacijų (GWA) tyrimuose, atliktuose Europoje ir Jungtinėse Amerikos Valstijose [36–38]. Žinoma, kad daugelis naujai nustatytų su DL susijusių genų atlieka funkcijas virškinamojo trakto (VT) nerviniame–raumeniniame signalų perdavime ir jungiamojo audinio palaikyme. Nors atlikti tyrimai neleidžia abejoti genetinių veiksnių svarba DL išsivystymui, konkretūs genai ir jų sekų variacijos iki šiol nėra ištirti. Šiame darbe siekta atskleisti jungiamąjį audinį koduojančių genų vieno nukleotido polimorfizmų (VNP) įtaką DL išsivystymui.

Trečioji svarbi darbo kryptis – enterinės nervų sistemos (ENS) pokyčių analizė, sergant DL. Pakitusios nervinės reguliacijos hipotezė yra viena iš pagrindinių DL tyrinėjimo krypčių. Žinoma, kad ENS turi visą eilę veiklos programų ir reguliuoja VT sistemų aktyvumą [43, 44]. Nustatyta, kad sergančiųjų DL pacientų žarnyno lygieji raumenys yra jautresni cholinerginei stimuliacijai [54, 55] ir praranda gebėjimą atsipalaiduoti, juos paveikus natrio nitroprusidu, azoto oksido donoru [56]. Šis reiškinys leidžia manyti, kad VT motorikos sutrikimas, būdingas sergantiems DL, yra sąlygotas būtent nervinio–raumeninio perdavimo sutrikimo. Su kalcitonino genu susijęs peptidas (CGRP) yra stiprus žarnyno lygiųjų raumenų relaksantas [57]. Veikdamas per heteromerinį receptorių, sudarytą iš dviejų komponentų – į kalcitonino receptorių panašaus receptoriaus (CRLR) ir receptoriaus aktyvumą modifikuojančio baltymo 1 (RAMP1) [59] – CGRP atlieka reikšmingą rolę daugybėje VT funkcijų, tarp kurių ir VT motorikos reguliavimas [60–69]. Nepaisant to, CGRP signalo perdavimo kelio pakitimai, sergant DL, iki šiol nebuvo tirti.

2. TIKSLAS IR UŽDAVINIAI

2.1. Tikslas

Nustatyti enterinės nervų sistemos remodeliacijos, genetinių, mitybos ir aplinkos veiksnių įtaką storosios žarnos divertikuliozės išsivystymui.

2.2. Uždaviniai

1. Nustatyti su kolageno apykaita siejamų ir jungiamąjį audinį koduojančių genų – COL3A1, COL1A1, ARHGAP15, COLQ ir FAM155A – vieno nukleotido polimorfizmų įtaką divertikuliozės išsivystymui.

2. Ištirti CGRP nervinių skaidulų ir ląstelių kūnų pasiskirstymą storosios žarnos mienteriniame ir submukoziniame rezginiuose, sergant divertikulioze.
3. Įvertinti storosios žarnos enterinės nervų sistemos ultrastruktūrinius pakitimus, sergant divertikulioze.
4. Įvertinti sociodemografinių, mitybos ir tuštinimosi įpročių reikšmę storosios žarnos divertikuliozės išsivystymui.

2.3. Darbo naujumas

Norint sukurti pažangias ir veiksmingas gydymo strategijas bei užkirsti kelią naujų atvejų atsiradimui, labai svarbu perprasti ligos išsivystymo mechanizmus. Per pastaruosius dešimtmečius žinios apie DL etiopatogenezę reikšmingai išsiplėtė. Šiandieniniu požiūriu, DL išsivystymui įtakos turi ne tik aplinkos, bet ir genetiniai veiksniai [35]. Be pastarųjų, ne mažiau reikšmingais laikomi struktūriniai pokyčiai storosios žarnos sienoje [71, 109]. Nepaisant daugybės šioje srityje atliekamų mokslinių tyrimų, sergamumas divertikulioze visame pasaulyje ir toliau sparčiai didėja, o jos etiologiją įtakojantys veiksniai nėra iki galo suprasti.

Naujos genominės technologijos bei sumažėję genotipavimo kaštai leido identifikuoti genus, susijusius su DL ir dažnos jos komplikacijos – divertikulito – išsivystymu. Neseniai paskelbtuose viso genomo asociacijų tyrimų rezultatuose buvo įvardinti trys svarbiausi genų variantai, susiję su abejomis būklėmis [36–38], tačiau dėl taikytų metodų, šie VNP nebuvo diferencijuoti tarp atskirų divertikuliozės ir divertikulito tiriamųjų grupių. Šiuo tyrimu siekta įvertinti GWA nustatytų genų polimorfizmų reikšmę atskirai divertikuliozės ir divertikulito išsivystymo rizikai. Šis tyrimas yra pirmasis tokios apimties tyrimas, vertinantis DL ir COL3A1 (rs3134646 ir rs1800255), COL1A1 (rs1800012), ARHGAP15 (rs4662344) ir FAM155A (rs67153654) genų VNP ryšį gerai fenotipizuotoje kolonoskopiškai patvirtintos divertikuliozės pacientų grupėje.

Antroji tyrimo dalis buvo skirta įvertinti mūsų pasirinktų aplinkos rizikos veiksnių sąsajas su DL ir divertikulito išsivystymu. Vienas iš pagrindinių šio tyrimo privalumų buvo galimybė naudoti itin išsamius duomenis apie tiriamųjų mitybos ir tuštinimosi įpročius bei kitus kovariacinius rodiklius. Tai yra pirmasis tyrimas, kuriame analizuotas ryšys tarp tiriamųjų išsilavinimo, darbo pobūdžio bei DL rizikos. Priešingai paplitusiai nuomonei, tyrimo rezultatai atskleidė, kad divertikuliozė yra labiau paplitusi tarp žemesnio išsilavinimo asmenų, rečiau dirbančių sėdimą ar naktinį darbą. Mūsų žiniomis, iki šiol šis priežastinis ryšys nagrinėtas nebuvo.

Kitas svarbus tyrimo uždavinys – įvertinti divertikulioze sergančių pacientų ENS struktūrinus pokyčius. Nors pakitusios nervinės reguliacijos hipotezė

DL kontekste tyrinėta ir ankstesnių autorių darbuose [70, 71], tai pirmasis tyrimas, kuriame morfologiniai enterinių rezginių pokyčiai analizuojami abejais – imunohistocheminiu ir elektronomikroskopiniu – tyrimų metodais. Vienas iš svarbiausių šio darbo rezultatų buvo nustatyti reikšmingi CGRP signalo perdavimo kelio pokyčiai, galimai paaiškinantys sutrikusio lygiųjų raumenų atsipalaidavimo mechanizmą, sergant DL. Tyrimo metu gauti rezultatai atskleidė, kad sergant divertikulioze CGRP raiška visuose trijuose riestinės žarnos enteriniuose rezginiuose sumažėja. Šie pokyčiai buvo labiausiai išreikšti simptominės ligos atveju, lyginant su sveikais asmenimis, tuo tarpu besimptomėje grupėje stebėti tarpiniai rodikliai. Tai pirmasis tyrimas, leidžiantis įtarti laipsnišką CGRP mažėjimą ligai progresuojant. Mūsų žiniomis, iki šiol nėra paskelbtų tyrimų, nagrinėjančių CGRP raiškos pakitimus, sergant DL. Tyrimo metu gauti rezultatai yra reikšmingi, identifikuojant galimas storosios žarnos divertikuliozės etiologijos priežastis bei prisideda prie ankstyvos ligos diagnostikos ir personalizuotų prognozavimo bei profilaktikos strategijų kūrimo.

3. METODAI

Tyrimui suteikti Kauno regioninio biomedicininių tyrimų etikos komiteto (protokolo Nr. BE–10–2), Sarlando universiteto tyrimų etikos komiteto (protokolo Nr. 63/11) ir Kelno universiteto tyrimų etikos komiteto (protokolo Nr. 16–397) leidimai. Tyrimo protokolas parengtas vadovaujantis Pasaulio medicinos asociacijos etikos kodeksu (Helsinkio deklaracija). Visi tiriamieji pasirašė informuoto asmens sutikimo formą dalyvauti tyrime.

3.1. Tiriamoji populiacija

Tyrimas atliktas 2012–2016 m. trijuose tretinio lygio referenciniuose centruose Vokietijoje ir Lietuvoje: Lietuvos sveikatos mokslų universiteto Gastroenterologijos klinikoje, Kaune, Sarlando universiteto Medicinos centro II medicinos skyriuje, Hamburge, ir Kelno universitetinės ligoninės Gastroenterologijos ir hepatologijos klinikoje, Kelne. Į tyrimą įtraukti tik pilnamečiai 19–95 m. amžiaus europidų kilmės asmenys. Prieš įtraukiant į tyrimą, visiems dalyviams atliktas išsamus klinikinis ištyrimas, siekiant ekskliuduoti gretutines virškinamojo trakto ar paveldimas jungiamojo audinio ligas. Epidemiologiniai, gyvensenos, mitybos ir tuštinimosi įpročių duomenys surinkti, pasitelkiant standartizuotą klausimyną.

Visiems tiriamiesiems storosios žarnos divertikuliozė patvirtinta kolonoskopiškai. Endoskopinį ištyrimą atliko tik patyrę gydytojai gastroenterologai, naudodamiesi skaitmeniniais vaizdo endoskopais (Olympus CF 160, 180 arba 190). Į tyrimą neįtraukti pacientai, kurių žarnyno paruošimas neatitiko

esamo kokybės standarto ir kuriems kolonoskopijos metu apžiūrėta ne visa storoji žarna. Pacientai, kuriems endoskopinio tyrimo metu divertikulų nenustatyta, įtraukti į kontrolinę grupę. Visais atvejais, komplikuoto divertikulito diagnozė patvirtinta kompiuterine tomografija.

3.2. Tyrimo eiga ir metodai

3.2.1. Mitybos ir aplinkos veiksnių poveikio įvertinimui naudoti metodai

Prieš kolonoskopiją visi tiriamieji užpildė išsamų standartizuotą klausimyną apie vertinamus divertikuliozės išsivystymo rizikos veiksnius. Vertinimui pasirinkti sociodemografiniai veiksniai, taip pat veiksniai susiję su tiriamųjų mitybos ir tuštinimosi įpročiais. Sociodemografiniai veiksniai apėmė amžių, lytį, etninę kilmę, išsilavinimą ir profesinį statusą, alkoholio ir tabako vartojimą, dažną nesteroidinių vaistų nuo uždegimo (NVNU) ir vidurius laisvinančių preparatų vartojimą. Mitybos būklei ir įpročiams nustatyti buvo vertinamas valgymų skaičius per parą, suvartojamų skysčių kiekis per parą, žuvies ir raudonos mėsos porcijų kiekis per savaitę, taip pat tai, ar dalyviai laikosi vegetariškos ar veganiškos dietos. Kūno masės indeksas (KMI) (kg/m^2) buvo apskaičiuotas pagal pačių pateiktą ūgį (cm) ir svorį (kg), o antsvoris arba nutukimas buvo apibrėžtas kaip KMI $\geq$ 25 kg/m^2. Taip pat buvo vertintas respondento nurodytas tuštinimosi dažnis, vidutinė tuštinimosi trukmė, jei buvo – naktinis tuštinimasis, bei su tuštinimusi susiję simptomai, tokie kaip skausmas, nepilno išsituštinimo pojūtis ir klaidingas noras tuštintis; vertinta vidurių užkietėjimo trukmė (išreikšta metais) ir manualinės pagalbos ar klizmos poreikis tuštinantis.

3.2.2. Jungiamąjį audinį koduojančių genų VNP nustatymui naudoti metodai

DNR gryninimas iš periferinio kraujo mononuklearinių ląstelių buvo atliekamas naudojant komercinį rinkinį „DNeasy Blood & Tissue Kit“ (Qiagen, Venlo, Netherlands), remiantis gamintojų protokolu bei rekomendacijomis. DNR koncentracija bei kokybė buvo įvertinta matuojant mėginio 260/280 nm bangos ilgio sugertį, naudojantis „NanoDrop 2000“ (Thermo Scientific) spektrofotometru. Išskirti DNR mėginiai saugoti –20 °C temperatūroje iki panaudojimo genotipavimo tyrimams.

COL3A1 (rs3134646, rs1800255), COL1A1 (rs1800012), ARHGAP15 (rs4662344), COLQ (rs7609897) ir FAM155A (rs67153654) VNP nustatyti, taikant TL–PGR genotipavimo metodiką (angl. *real–time polymerase chain reaction*, tikralaikė polimerazinė grandininė reakcija). Šiam tyrimui atlik-

ti naudota iš periferinio kraujo mononuklearinių ląstelių išskirta genominė DNR (1 µl, 10–50 µg) bei gamykliškai validuoti „TaqMan®“ pradmenų ir zondų rinkiniai. „TaqMan®“ genotipavimo rinkinius sudarė du lokusui specifiniai pradmenys, amplifikuojantys vieno nukleotido polimorfizmo regioną, bei du aleliams specifiniai „TaqMan®“ zondai. Šie zondai buvo žymėti fluorescenciniais dažais (FAM™ arba VIC®) ir švytėjimo slopikiu (TAMRA™). Genotipavimas vykdytas vadovaujantis gamintojo rekomendacijomis ir protokolu (reakcijos sąlygos: 1 min. 25 °C; 20 s. 95 °C; 5 s. 5 °C; 30 s. 60 °C; 35 ciklai). TL–PGR tyrimui naudota „Life Technologies 7500 Fast“ detekcijos sistema (Life Technologies International, JAV).

3.2.3. Enterinės nervų sistemos tyrimui naudoti metodai

3.2.3.1. Imunohistocheminis ir elektronomikroskopinis tyrimas

Visi storosios žarnos mėginiai, naudoti ENS morfologiniam tyrimui, buvo surinkti LSMUL Kauno Klinikų Chirurgijos klinikoje. Iš viso tyrime naudoti 32 storosios žarnos mėginiai, tarp kurių išskirtos šios grupės: asimptomė divertikulinė liga (ADD) (n = 10, amžius: 57–76), simptominė divertikulinė liga (SDD) (n = 10, amžius: 39–80) ir kontrolė (n = 11, amžius: 50–75). Dalis šių mėginių (n = 25) taip pat naudoti elektronomikroskopiniam enterinių rezginių tyrimui.

Kontrolinės grupės audiniai buvo gauti iš pacientų, operuotų planine tvarka dėl neobstrukcinės kolorektalinės karcinomos. Jei šiems pacientams operacijos metu buvo randami divertikulai, tokie audiniai priskirti ADD grupei. SDD grupės audinių mėginiai buvo gauti iš pacientų, operuotų planine tvarka dėl besikartojančio divertikulito.

Kontrolės tyrimui 1–5 cm ilgio cirkuliarūs storosios žarnos segmentai buvo atkerpami iš makroskopiškai nepažeistos žarnos srities. ADD ir SDD grupių audinių mėginiai taip pat buvo imamai iš vizualiai sveikos vietos, esančios greta divertikulo, vengiant paimti patį divertikulą. Operacinėje mėginiai buvo talpinami į 4 °C aeruotą (95 proc. O_2, 5 proc. CO_2) Krebso–Henseleito tirpalą (118 mM NaCl, 4,7 mM KCl, 1,2 mM $MgSO_4$, 1,2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2,5 mM $CaCl_2$, 11 mM gliukozės).

Prieš imunohistochemines reakcijas, viso storio gaubtinės žarnos mėginiai buvo apdorojami pagal standartizuotą protokolą. Atliktos dvigubo imunohistocheminio dažymo reakcijos su CGRP, CRLR arba RAMP1, bei NOS1 arba VIP. Vėliau mėginiai buvo analizuojami kiekybinės fluorescencinės mikroskopijos metodu [142]. Baltymų raiška buvo nustatoma matuojant pasirinktų peptidų signalo tankį mienterinio, išorinio ir vidinio poodinio rezginio mazguose. Nervinės struktūros buvo fotografuojamos plataus lauko mikroskopu AxioImager Z1 (Carl Zeiss, Vokietija), su integruota skaitmenine

kamera AxioCam MRm Rev.3 ir apotomu. Gauti skaitmeniniai vaizdai buvo analizuoti AxioVision Rel.4.8.2 (Carl Zeiss, Vokietija) programa.

Elektronomikroskopinam tyrimui naudoti mėginiai buvo prefiksuojami 2,5 proc. glutaraldehido tirpale fosfatiniame buferyje. Vėliau, pagal standartizuotą protokolą paruošti ultraploni pjūviai (50–70 nm), bei dažomi uranilo acetate ir švino citrate. Mėginiai buvo analizuoti transmisiniu elektroniniu mikroskopu FEI® BioTwin G2 Spirit (Eindhoven, Olandija). Skaitmeninės elektronogramų nuotraukos buvo analizuojamos programa Fiji. Kokybiškai įvertinta enterinių mazgų ir nervų, mastocitų, bei intersticinių Kajalio ląstelių struktūra ir jų tarpusavio ryšys. Kiekybiškai išanalizuotas nervinių ataugų išsidėstymas ir dydis.

3.2.3.2. Raumenų susitraukimo eksperimentai *in vitro*

Fiziologiniams eksperimentams *in vitro* operacijos metu iškirpti audiniai buvo iš karto patalpinami į 4 °C aeruotą Krebso–Henseleito tirpalą ir jame raumeninė dalis atskiriama nuo gleivinės. Žarnos žiediniai ir išilginiai raumenys buvo sukarpomi į 2 × 10 mm juosteles, ir pakabinami į atskiras 25 ml voneles (Radnoti organ bath, AD instruments Pty, AU) su pašildytu (37 °C) aeruotu Krebso–Henseleito tirpalu. Raumenų juostelės buvo įtempiamos iki pasyvios 35–40 mN tempimo jėgos ir paliekamos kaboti apie valandą, keičiant kas 15 min. Krebso–Henseleito tirpalą, kol nusistovi stabili pradinė įtempimo jėga.

Po betanecholio (10^{-4} M) įdėjimo buvo laukiama apie 5 min. kol bus pasiekiama maksimali susitraukimo vertė, vėliau įdedama žmogaus CGRP (10^{-7} M) ir laukiama apie 15 min. kol nusistovės atsipalaidavimo atsakas. Tuomet įdedama natrio nitroprusido (10^{-3} M) kad žarna maksimaliai atsipalaiduotų. Kai kuriuose eksperimentuose, 15 min. iki betanecholio įdėjimo, buvo dedamas tetradotoksinas (10^{-7} M).

Tarp maksimalios susitraukimo vertės ir maksimalios atsipalaidavimo vertės buvo nustatomas atsipalaidavimo atsakas į CGRP. Atsako dydis išreiškiamas procentine išraiška nuo maksimalios atsipalaidavimo reikšmės įdėjus natrio nitroprusido. Kontrolinių raumenų juostelių susitraukimai buvo įrašinėjami paraleliai su eksperimentinėmis.

3.2.4. Statistinė duomenų analizė

Gautų duomenų statistinė analizė atlikta naudojant Statistical Package for the Social Sciences (SPSS, versija 24.0, SPSS Inc., Čikaga, IL, JAV; versija 20.0, IBM, Munich, Germany) ir „Prism“ (versija 5.0, Graphpad Software, La Jolla, USA). Duomenų išsibarstymas buvo tikrinamas naudojant histogramas, parametriniai duomenys buvo patvirtinti Shapiro–Wilk‘o testu. Statis-

tinis duomenų patikimumas buvo nustatomas Student'o t–testu su Welch'o korekcija arba Mann–Whitney U testu. VNP genotipų pasiskirstymo dažnis vertintas panaudojant Hardy–Weinbergo pusiausvyros modelį. Apskaičiuotas imties dydis ir tyrimo galia. Duomenys pateikiami kaip vidurkis ± standartinės paklaidos reikšmė, mediana, didžiausios ir mažiausios reikšmės skirtumas, dažnis ir procentai. CGRP, CRLR ir RAMP1 kiekis yra išreikštas ± procentine reikšme nuo vidutinės kontrolinės grupės reikšmės. Veiksnių, didinančius riziką susirgti DL, nustatymui taikyti vienanarės ir daugianarės logistinės regresijos modeliai. Duomenys buvo laikomi statistiškai patikimais, kai $p < 0,05$.

4. REZULTATAI

4.1. Mitybos ir aplinkos veiksnių poveikio analizė

Per penkerių metų laikotarpį į tyrimą įtraukti 1333 asmenys: 635 (47,6 proc.) vyrai ir 698 (52,4 proc.) moterys. Tiriamųjų grupių duomenys pateikiami 4.1.1 lentelėje.

Iš tyrime dalyvavusių pacientų, į standartinio klausimyno klausimus apie mitybos ir tuštinimosi įpročius, išsilavinimą bei profesinį statusą išsamiai atsakė 844 dalyviai (523 divertikuliozės grupėje, 321 kontrolinėje grupėje) (4.1.2 lentelė).

4.1.1 lentelė. *Tiriamųjų charakteristika*

	Sergantys divertikulioze (n = 858)	**Kontrolė (n = 475)**	**p reikšmė**
	Viso tiriamųjų (n = 1333)		
	Vidurkis ± standartinė paklaida arba n (%)		
Amžius, metais	66,39 ± 10,4 (29–95)	55,24 ± 14,6 (19–92)	**<0,001**
Lytis, vyrai/moterys	424 (49,4 %)/434 (50,6 %)	211 (44,4 %)/264 (55,6 %)	0,08
Tautybė			0,022
Vokiečių	353 (41,1 %)	165 (34,7 %)	
Lietuvių	505 (58,9 %)	310 (65,3 %)	
KMI, kg/m^2	28,75 ± 5,4	27,27 ± 5,1	**<0,001**
Rūkymo įpročiai (ar asmuo kada nors rūkė), taip/ne	300 (35,0 %)	168 (35,4 %)	0,883
Alkoholio vartojimas (ar vartojama kasdien), taip/ne	50 (5,8 %)	21 (4,4 %)	0,273
NVNU vartojimas (ar vartoja ≥ 15 d./mėn.), taip/ne	91 (10,6 %)	47 (9,9 %)	0,683

Vertės yra pateiktos kaip dažniai ir procentai. P reikšmė nurodo statistinį patikimumą (statistiškai patikimi duomenys pažymėti ryškiau). KMI – kūno masės indeksas; NVNU – nesteroidiniai vaistai nuo uždegimo.

Divertikulioze sergančių tiriamųjų amžiaus vidurkis (66,4 ± 10,4 metų prieš 55,2 ± 14,7 metų, p < 0,001) ir KMI (28,75 ± 5,4 kg/m^2 prieš 27,27 ± 5,1 kg/m^2, p < 0,001) buvo statistiškai reikšmingai didesni, palyginti su kontroline grupe (4.1.2 lentelė). Statistiškai reikšmingų skirtumų tarp lyties (p = 0,08), tautybės (p = 0,022), NVNU vartojimo (p = 0,683), rūkymo (p = 0,883) ar reguliaraus alkoholio vartojimo (p = 0,273) nenustatyta (4.1.2 lentelė).

Pritaikius vienanarės logistinės regresijos modelį, nustatytas statistiškai reikšmingas ryšys tarp storosios žarnos divertikuliozės išsivystymo ir aukštesnio išsilavinimo (p = 0,004) bei darbo naktinėse pamainose (p < 0,0001). Išanalizavus pasirinktus su tuštinimosi įpročiais susijusius veiksnius, nustatyta reikšminga asociacija tarp divertikuliozės ir tuštinimosi dažnio (p = 0,003) bei nepilno išsituštinimo pojūčio (p < 0,0001). Vienoda dalis respondentų nurodė jaučiantys skausmą tuštinimosi metu (25,9 proc. prieš 25,3 proc., p = 0,846), su tuštinimusi nesusijusį pilvo skausmą (23,1 proc. prieš 20,9 proc., p = 0,444) ir poreikį tuštintis naktį (3,8 proc. prieš 3,4 proc., p = 0,819). Tyrime taip pat nenustatytas ryšys tarp vidutinės tuštinimosi trukmės (5,7 proc. prieš 7,1 proc., p = 0,413) ar ilgalaikio vidurių užkietėjimo (10,6 proc., prieš 12,3 proc., p = 0,467).

Įvertinus pasirinktus su mitybos įpročiais susijusius veiksnius, divertikulioze sergantys tiriamieji dažniau nurodė valgantys ≥ 3 kartus per dieną (79,9 proc. prieš 71,7 proc., p = 0,006), tačiau šis veiksnys reikšmingai nedidino divertikuliozės išsivystymo rizikos daugianarėje analizėje. Dažnas raudonos mėsos ar žuvies vartojimas, per parą suvartojamų skysčių kiekis, vegetariška mityba nebuvo susiję su divertikuliozės išsivystymo rizika. Vienanarės analizės rezultatai yra pateikti 4.1.2 lentelėje.

4.1.2 lentelė. *Mitybos ir tuštinimosi įpročių ryšys su divertikulioze*

	Kontrolė (n = 321)	**Sergantys divertikulioze (n = 523)**	**p reikšmė**
	Viso tiriamųjų (n = 844)		
Vegetarizmas	6 (1,9 %)	3 (0,6 %)	0,076
Valgymų skaičius per dieną, ≥ 3 porcijos per dieną	230 (71,7 %)	417 (79,9 %)	**0,006**
Raudona mėsa, ≥ 3 porcijos per savaitę	32 (10,0 %)	41 (7,9 %)	0,289
Žuvis, ≥ 3 porcijos per savaitę	279 (86,9 %)	431 (82,9 %)	0,117
Skysčiai, < 1 litras per dieną	42 (13,1 %)	90 (17,2 %)	0,107
Tuštinimosi dažnis, ≤ 1 kartas per savaitę	13 (4,0 %)	5 (1,0 %)	**0,003**
Skausmingas tuštinimasis, ≥ 25 % laiko	83 (25,9 %)	132 (25,3 %)	0,846
Nepilno išsituštinimo pojūtis, ≥ 25 % laiko	95 (29,7 %)	247 (47,3 %)	**<0,0001**

4.1.2 lentelės tęsinys

	Kontrolė (n = 321)	Sergantys divertikulioze (n = 523)	p reikšmė
	Viso tiriamųjų (n = 844)		
Pilvo skausmas, ≥ 25 % laiko	74 (23,1 %)	109 (20,9 %)	0,444
Ilga tuštinimosi trukmė, > 10 min	18 (5,7 %)	37 (7,1 %)	0,413
Naktinis tuštinimasis	12 (3,8 %)	18 (3,4 %)	0,819
Vidurių užkietėjimas, ≥ 10 metų	34 (10,6 %)	64 (12,3 %)	0,467
Vidurius laisvinančių vaistų vartojimas, ≥ 1 dieną per savaitę	41 (12,8 %)	57 (11,0 %)	0,417
Aukštasis išsilavinimas	150 (46,9 %)	192 (36,9 %)	**0,004**
Naktinis darbas	36 (11,2 %)	18 (3,4 %)	**<0,0001**

Vertės yra pateiktos kaip dažniai ir procentai. P reikšmė nurodo statistinį patikimumą (statistiškai patikimi duomenys pažymėti ryškiau).

Veiksniai, susiję su divertikuliozės išsivystymo rizika vienanarėje analizėje, pakartotinai įvertinti daugianarės logistinės regresijos modelyje, koreguojant pagal amžių ir lytį. Patvirtinta, jog divertikuliozės rizika didėja su amžiumi (ŠS 1,079, 95 proc. PI 1,06–1,1, p < 0,05). Turintiems antsvorio ar nutukusiems asmenims ligos išsivystymo rizika taip pat yra didesnė (ŠS 1,05, 95 proc. PI 1,02–1,09, p = 0,004), kaip ir pacientams, dažniau patiriantiems nepilno išsituštinimo pojūtį (ŠS 2,05; 95 proc. PI 1,47–2,87). Retas tuštinimasis nebuvo susijęs su padidėjusia ligos rizika. Priešingai, duomenys atskleidė, kad retai besituštinančių tiriamųjų rizika susirgti divertikulioze yra mažesnė, lyginant su asmenimis, kurie tuštinasi reguliariai (ŠS 0,1; 95 proc. PI 0,03–0,33). Daugianarės logistinės regresijos rezultatai pateikiami 4.1.3 lentelėje.

4.1.3 lentelė. *Divertikuliozės išsivystymą galinčių sąlygoti rizikos veiksnių logistinės regresijos analizės rezultatai*

	ŠS	95 % PI	p reikšmė
Amžius, metais	1,079	1,06–1,1	**<0,001**
KMI, ≥ 25 kg/m^2	1,05	1,02–1,09	**0,004**
Nepilno išsituštinimo pojūtis, ≥ 25 % laiko	2,05	1,47–2,87	**<0,001**
Tuštinimosi dažnis, ≤ 1 kartas per savaitę	0,1	0,03–0,33	**<0,001**

ŠS – šansų santykis; PI – pasikliautinasis intervalas; KMI – kūno masės indeksas. P reikšmė nurodo statistinį patikimumą (statistiškai patikimi duomenys pažymėti ryškiau).

Vykdant tolimesnę analizę, ištirtas ryšys tarp anksčiau aptartų rizikos veiksnių ir divertikulito išsivystymo. Vyresnio amžiaus tiriamųjų divertikulito rizika buvo mažesnė (ŠS 0,921, 95 proc. PI 0,89–0,95, p < 0,05), palyginus su jaunesniais asmenimis. Kaip ir ankstesnėje analizėje, nepilno išsituš-

tinimo pojūtis (ŠS 2,769, 95 proc. PI 1,35–5,7, p < 0,006) buvo reikšmingai susijęs ir su didesne divertikulito išsivystymo rizika. Aukštesnį išsilavinimą turintys asmenys taip pat turėjo didesnę divertikulito išsivystymo riziką, lyginant su žemesnio išsilavinimo asmenimis (ŠS 2,453, 95 proc. PI 1,31–4,59, p = 0,005). Sąsajų tarp kitų analizuotų veiksnių ir divertikulito išsivystymo rizikos nenustatyta. Veiksniai, statistiškai reikšmingai susiję su didesne divertikulito rizika, pateikti 4.1.4 lentelėje.

4.1.4 lentelė. *Divertikulito išsivystymą galinčių sąlygoti rizikos veiksnių logistinės regresijos analizės rezultatai*

	ŠS	**95 % PI**	**p reikšmė**
Amžius, metais	0,921	0,89–0,95	**<0,0001**
Nepilno išsituštinimo pojūtis, ≥ 25 % laiko	2,769	1,35–5,7	**0,006**
Aukštasis išsilavinimas	2,453	1,31–4,59	**0,005**

ŠS – šansų santykis; PI – pasikliautinasis intervalas; KMI – kūno masės indeksas. P reikšmė nurodo statistinį patikimumą (statistiškai patikimi duomenys pažymėti ryškiau).

4.2. Jungiamąjį audinį koduojančių genų VNP tyrimai

4.2.1. COL3A1 (rs3134646, rs1800255) ir COL1A1 (rs1800012) VNP

COL3A1 (rs3134646, rs1800255) ir COL1A1 (rs1800012) VNP ištirti 707 asmenims (349 vyrams, 358 moterims). Atlikus klinikinių duomenų analizę nustatyta, kad divertikuliozė būdinga vyresnio amžiaus pacientams – didžioji dalis sirgusių pacientų buvo vyresni nei 60 metų amžiaus. Sirgusių asmenų kūno masės indeksas buvo statistiškai reikšmingai didesnis, lyginant su kontroline grupe. Kitų reikšmingų skirtumų tarp tiriamųjų grupių nenustatyta.

COL3A1 (rs3134646, rs1800255) ir COL1A1 (rs1800012) genotipų pasiskirstymo dažnis tenkino Hardy–Weinbergo pusiausvyros modelio sąlygas. Tirtų VNP genotipų pasiskirstymas kontrolinėje ir divertikulioze sergančių pacientų grupėse nurodytas 4.2.1.1 lentelėje.

4.2.1.1 lentelė. *COL1A1 ir COL3A1 genotipų variacijų pasiskirstymas tarp divertikuliozе sergančių ir kontrolinės grupės asmenų*

Geno polimorfizmas	Genotipas	Kontrolinė asmenų grupė		Divertikulioze sergančių pacientų grupė	
		n	%	n	%
COL3A1					
rs1800255[a]	GG	149	53,4 %	256	61,4 %
	GA	107	38,4 %	142	34,1 %
	AA	23	8,2 %	19	4,6 %
rs3134646[b]	GG	69	24,8 %	100	23,9 %
	GA	144	51,8 %	189	45,2 %
	AA	65	23,4 %	129	30,9 %
COL1A1					
rs1800012[c]	GG	200	71,9 %	289	69,5 %
	GT	73	26,3 %	106	25,4 %
	TT	5	1,8 %	21	5,0 %

Vertės yra pateiktos kaip dažniai ir procentai. [a] – vienuolikai asmenų nepavyko nustatyti rs180025 genotipą; [b] – vienuolikai asmenų nepavyko nustatyti rs3134646 genotipą; [c] – šešiolikai asmenų nepavyko nustatyti rs180012 genotipą.

Vykdant tolimesnę COL3A1 (rs3134646, rs1800255) ir COL1A1 (rs1800012) genotipų pasiskirstymo analizę nustatytas tirtų genų tam tikrų genotipų variacijų ryšys su klinikiniais duomenimis. Rezultatai yra pateikti 4.2.1.2 lentelėje.

4.2.1.2 lentelė. *rs1800255, rs3134646, ir rs1800012 genotipų variacijų ryšys su klinikiniais duomenimis*

Kriterijus	rs1800255 (AA genotipas)	rs3134646 (AA genotipas)	rs1800012 (TT genotipas)
Lytis, moterys/vyrai	23(54,8 %)/ 19 (45,2 %)	86(46,7 %) /98 (53,3 %)	11 (42,3 %)/ 15(57,7 %)
Amžius, metais	60 (22–78)	64,5 (21–91)	68 (46–91)
KMI, kg/m^2	28,1 (18,4–40,6)	26,9 (16,6–52,4)	26,6 (20,1–37,8)
Tautybė, vokiečių/ lietuvių	23(54,8 %)/ 19 (45,2 %)	80(43,5 %)/ 104(56,5 %)	11(42,3 %)/ 15(57,7 %)
Vidurius laisvinančių preparatų naudojimas taip/ne	3(10,3 %)/ 26 (89,7 %)	12(7,5 %)/ 148 (92,5 %)	3 (12,0 %)/ 22 (88,0 %)
Rūkymo įpročiai (ar kada nors asmuo rūkė) taip/ne	22(56,4 %)/ 17(43,6 %)	121(65,8 %)/ 63(34,2 %)	7(26,9 %)/ 19(73,1 %)
Alkoholio vartojimas (ar vartojama kasdien), taip/ne	0(0,0 %)/ 29(100,0 %)	14(8,7 %)/ 147(91,3 %)	1(4,0 %)/ 24(96,0 %)

Vertės yra pateiktos kaip medianos ir didžiausi/mažiausi reikšmių skirtumai arba kaip dažniai ir procentai. KMI – kūno masės indeksas.

Naudojant vienanarės logistinės regresijos modelį ištirti galimi divertikuliozės rizikos veiksniai. Nustatyta, kad vyresnis amžius ($p < 0,0001$), padidėjęs kūno masės indeksas ($p < 0,0001$), COL3A1 (rs1800255) AA genotipas ($p = 0,01$), COL3A1 (rs3134646) AA genotipas ($p = 0,033$) ir COL1A1 (rs1800012) TT genotipas ($p = 0,019$) yra statistiškai reikšmingai susiję su divertikuliozės išsivystymo rizika. Daugianarės logistinės regresijos analizė patvirtino tik du divertikuliozės išsivystymą lemiančius veiksnius, t.y. ligos atsiradimą gali sąlygoti vyresnis amžius ($p < 0,0001$) ir padidėjęs kūno masės indeksas ($p = 0,004$). Atlikus selektyvinę daugianarę logistinės regresijos analizę rasta, kad COL3A1 (rs3134646) VNP didina divertikuliozės išsivystymo riziką europidų kilmės vyrams mūsų tirtoje ligonių grupėje ($p = 0,037$). Šios ligos išsivystymą galinčių sąlygoti rizikos veiksnių logistinės regresijos analizės rezultatai pateikti 4.2.1.3 lentelėje.

4.2.1.3 lentelė. *Divertikuliozės išsivystymą galinčių sąlygoti rizikos veiksnių logistinės regresijos analizės rezultatai*

Kriterijus	**ŠS**	**95 % PI**	**p reikšmė**
A. Vienanarė analizė			
Amžius, metais	1,09	1,07–1,10	**<0,0001**
KMI, kg/m^2	1,06	1,03–1,10	**<0,0001**
Lytis, moterys/vyrai	0,98	0,73–1,33	ns
Tautybė, vokiečių / lietuvių	0,77	0,57–1,04	ns
Vidurius laisvinančių prieparatų naudojimas Taip/ne	1,33	0,62–2,89	ns
Rūkymas (ar kada nors rūkėte) taip / ne	0,81	0,59–1,10	ns
Alkoholis vartojimas (ar vartojama kasdien), taip / ne	0,56	0,30–1,06	ns
COL3A1 (rs1800255) AA genotipas	0,44	0,24–0,82	**0,01**
COL3A1 (rs3134646) AA genotipas	1,37	1,31–1,43	**0,033**
COL1A1 (rs1800012) TT genotipas	2,9	1,08–7,80	**0,019**
B. Daugianarė analizė kai divertikuliozė pasirinkta, kaip priklausomas kintamasis			
Amžius, metais	1,08	1,07–1,10	**<0,0001**
KMI, kg/m^2	1,05	1,02–1,09	**0,004**
COL3A1 (rs1800255) AA genotipas	0,55	0,27–1,14	0,11
COL3A1 (rs3134646) AA genotipas	1,34	0,89–2,00	0,16
COL1A1 (rs1800012) TT genotipas	2,53	0,85–7,49	0,095
C. Daugianarė analizė kai divertikulioze sergantys vyrai pasirinkti, kaip priklausomas kintamasis			
Amžius, metais	1,07	1,05–1,09	**<0,0001**
KMI, kg/m^2	1,03	0,98–1,08	0,27
COL3A1 (rs1800255) AA genotipas	0,71	0,28–1,81	0,21

4.2.1.3 lentelės tęsinys

Kriterijus	ŠS	95 % PI	p reikšmė
COL3A1 (rs3134646) AA genotipas	1,77	1,04–3,20	**0,04**
COL1A1 (rs1800012) TT genotipas	1,74	0,40–7,56	0,40
D. Daugianarė analizė kai divertikulioze sergančios moterys pasirinktos, kaip priklausomas kintamasis			
Amžius, metais	1,1	1,07–1,12	**<0,0001**
KMI, kg/m^2	1,06	1,01–1,11	**0,01**
COL3A1 (rs1800255)AA genotipas	0,41	0,13–1,30	0,13
COL3A1 (rs3134646) AA genotipas	1,04	0,58–1,84	0,90
COL1A1 (rs1800012) TT genotipas	3,42	0,68–17,2	0,14

KMI – kūno masės indeksas; PI – pasikliautinasis intervalas; ŠS – šansų santykis; p reikšmė – nurodo statistinį patikimumą (statistiškai patikimi duomenys pažymėti ryškiau), ns – statistiškai nepatikima.

4.2.2. ARHGAP15 (rs4662344), COLQ (rs7609897) ir FAM155A (rs67153654) VNP

ARHGAP15 (rs4662344), COLQ (rs7609897) ir FAM155A (rs67153654) VNP ištirti 1332 europidų kilmės asmenims (635 vyrams ir 699 moterims). Kaip ir ankstesnio tyrimo metu didesnė dalis pacientų, sergančių divertikulioze, buvo vyresnio amžiaus ir turėjo didesnį kūno masės indeksą nei kontrolinės grupės asmenys. Tuo tarpu pacientai, sergantys divertikulitu, buvo žymiai jaunesni ($p < 0,001$), jų tarpe nustatyta daugiau rūkančių ($p = 0,006$) ir vartojančių alkoholį asmenų ($p = 0,001$).

ARHGAP15 (rs4662344) ir FAM155A (rs67153654) genotipai pasiskirstė pagal Hardy–Weinbergo pusiausvyros modelį, todėl ARHGAP15 ir FAM155A VNP tirti vykdant tolimesnę duomenų analizę. COLQ (rs7609897) genotipų dažnis netenkino pusiausvyros modelio sąlygų. Šio geno variacijos į duomenų analizę neįtrauktos.

ARHGAP15 (Rs4662344) retojo alelio dažnis divertikulioze sergančiųjų pacientų tarpe buvo didesnis nei kontrolinėje tiriamųjų grupėje ir atitiko anksčiau aprašytus GWAS tyrimo rezultatus. FAM155A (rs67153654) alelių dažniai neišsiskyrė tarp tiriamųjų grupių (ŠS 1,01; 95 proc. PI: 0,81–1,27 ir ŠS 0,91; 95 proc. PI: 0,61–1,72). ARHGAP15 (rs4662344) T alelis buvo reikšmingai susijęs su divertikuliozės atsiradimo rizika (ŠS 1,28; 95 proc. PI: 1,00–1,63, $p = 0,05$). Rezultatai yra pateikti 4.2.2.1 lentelėje.

Atlikus daugianarės logistinės regresijos analizę, kurios metu gauti rezultatai koreguoti atsižvelgiant į aplinkos veiksnius, ARHGAP15 geno variacijos sąsajos su divertikuliozės atsiradimo rizika neišliko (ŠS 1,22; 95 proc. PI: 0,93–1,61).

ARHGAP15 (rs4662344) retojo alelio dažnis buvo didesnis divertikulitu sergančių pacientų tarpe nei tiriamojoje grupėje, kurią sudarė tik divertikulioze sergantys pacientai. FAM155A (rs67153654) geno A alelio dažnis (ŠS 0,66; 95 proc. PI: 0,47–0,92) sumažėjo pacientams, sergantiems divertikulitu, palyginti su asmenimis sergančiais divertikulioze. Asociacija išliko statistiškai reikšminga atlikus daugianarę logistinę regresijos analizę, kurios metu gauti rezultatai koreguoti atsižvelgiant į aplinkos veiksnius. Šios analizės metu, ARHGAP15 VNP susietas ir su divertikulito rizika divertikulioze sergantiems pacientams (4.2.2.2 lentelė).

4.2.2.1 lentelė. *ARHGAP15 ir FAM155A genotipų ir alelių pasiskirstymo dažnis kontrolinėje ir divertikulioze sergančių pacientų grupėse*

Genas	**A^{min} / A^{maj}**	**MAF (%)**	**f CT (%)**	**f TT (%)**	**ŠS**	**$P_{tendencija}$**	**ŠS (95 % PI)**	**P_{alelis}**	**ŠS (95 % PI)**	**$P_{genotipas}$**
ARHGAP15 (rs4662344)										
DD sergančių pacientų grupė	T/C	19,5	29,7	4,7	1,30	**0,02**	1,28 (1,00–1,63)	**0,05**	1,85 (0,97–3,50)	0,06
Kontrolinė grupė	T/C	15,9	26,3	2,7						
FAM155A (rs67153654)										
			f TA (%)	**f TA (%)**						
DD sergančių pacientų grupė	A/T	22,8	35,4	5,1	0,90	**0,01**	1,01 (0,81–1,27)	0,91	1,02 (0,61–1,72)	0,93
Kontrolinė grupė	A/T	22,6	35,2	5,1						

Genotipų ir alelinių variantų dažniai. Vertės pateikiamos kaip dažnis ar procentinė dalis. Reikšmingos P reikšmės yra pažymėtos ryškiau. A^{maj} – pagrindinis alelis; A^{min} – retas alelis; PI – pasikliautinas intervalas; MAF – retojo alelio dažnis (angl. *minor allele frequency*), ŠS – šansų santykis.

4.2.2.2 lentelė. *Daugianarė logistinės regresijos analizė, lyginami pacientai, sergantys nekomplikuota divertikulioze su pacientais, sergančiais divertikulitu*

	ŠS[*] (95 % PI)	**p reikšmė**
ARHGAP15 rs4662344:T (CC+TC vs TT)	1,43(1,00–2,03)	**0,05**
ARHGAP15 rs4662344:TC (CT vs TT+CC)	1,35(0,44–1,94)	0,11
ARHGAP15 rs4662344:TT (TT vs TC+CC)	1,40(0,61–3,21)	0,43
FAM155A rs67153654:T (TA+AA vs TT)	0,70(0,49–0,99)	**0,04**
FAM155A rs67153654:AT (TA vs TT+AA)	0,73(0,51–1,06)	0,10
FAM155A rs67153654:TT (TT vs AT+AA)	0,68(0,27–1,69)	0,41

PI – pasikliautinasis intervalas; ŠS – šansų santykis. [*] analizė atlikta atsižvelgiant į amžių, KMI, alkoholio vartojimą ir rūkymą.

4.3. Enterinės nervų sistemos tyrimas

4.3.1. Imunohistocheminis ir fiziologinis tyrimas

CGRP–IR nervai ir nervinės skaidulos driekėsi per visus storosios žarnos sluoksnius, koncentruodamiesi nerviniuose rezginiuose. Dauguma CGRP nervinių skaidulų supdavo mienterinio rezginio (MP) neuronus, taip pat buvo randami abiejuose – išoriniame (OSP) bei vidiniame (ISP) pogleivio rezginiuose. CGRP–IR skaidulų pluoštai buvo apipynę žarnyno liaukas, jomis taip pat gausiai inervuota gleivinė; tuo tarpu raumeniniuose sluoksniuose CGRP pozityvūs nerviniai pluoštai buvo reti. Kai kuriuose nerviniuose mazguose buvo aptinkamos ne tik CGRP pozityvios skaidulos, bet ir neuronai.

Kiekybinis fluorescencijos intensyvumo tyrimas parodė, kad CGRP–IR nervinių skaidulų tankumas riestinės žarnos enteriniuose mazguose DL atveju yra sumažėjęs. Šis pokytis buvo ypač akivaizdus MP, kur CGRP–IR nervų skaidulų kiekis SDD pacientams buvo perpus mažesnis nei kontrolinės grupės, t.y. sumažėjo 51,7 proc. ($p < 0,0001$). Tuo tarpu OSP ir ISP, CGRP–IR nervų pluoštai sumažėjo atitinkamai 27,8 proc. ($p = 0,04$) ir 52,4 proc. ($p < 0,0001$). Asimptominių pacientų grupėje nustatėme vidutines intensyvumo reikšmes: MP sumažėjo 8,31 proc., $p = 0,326$; OSP – 19 proc., $p = 0,407$; ISP – 10,5 proc., $p = 0,804$, kurios statistiškai nesiskyrė nuo kontrolinės grupės. CGRP kiekio pokytis enteriniuose mazguose tarp skirtingų tiriamųjų grupių pateiktas 4.3.1.1 lentelėje.

CRLR–IR struktūros buvo matomos granulių pavidalo, dauguma jų koncentravosi neuronų kūnuose. Kontroliniuose audiniuose CRLR–IR nervinės struktūros buvo 2,5 karto gausesnės MP lyginant su pogleivio rezginiais, o OSP ir ISP jų kiekis buvo panašus. CRLR–IR pasiskirstymo proporcijos atitiko CGRP–IR nervinių skaidulų kiekį (4.3.1.1 lentelė).

4.3.1.1 lentelė. *CGRP, CRLR ir RAMP1 kiekio pokytis enteriniuose mazguose skirtingose tiriamųjų grupėse*

		MP			OSP			ISP		
		SDD	ADD	Kontrolė	SDD	ADD	Kontrolė	SDD	ADD	Kontrolė
CGRP	N	292	248	372	197	152	243	212	183	288
	Mediana	61,62	117,0	127,6	14,25	16,00	19,74	7,297	13,72	15,33
	Δ (%)	–51,7	–8,31	–	–27,8	–19,0	–	–52,4	–10,5	–
	p reikšmė	<,0001	0,326	–	0,039	0,407	–	<,0001	0,804	–
CRLR	N	221	245	205	188	198	153	167	216	177
	Mediana	21,90	18,85	16,94	9,555	7,951	6,760	6,526	4,452	5,319
	Δ (%)	+29,3	+11,3	–	+41,3	+17,6	–	+22,7	–16,3	–
	p reikšmė	0,008	0,078	–	<,0001	0,415	–	0,022	0,199	–
RAMP1	N	191	211	142	94	112	84	118	158	93
	Mediana	8.406	10,86	9,858	4,879	5,183	3,988	2,814	3,363	2,811
	Δ (%)	–14,7	+10,2	–	+22,3	+30,0	–	+0,11	+19,6	–
	p reikšmė	0,677	0,057	–	0,043	0,057	–	0,807	0,243	–

ADD – asimptominė divertikulinė liga; SDD – simptominė divertikulinė liga; MP – mienterinis rezginys; OSP išorinis pogleivio rezginys; ISP – vidinis pogleivio rezginys.

ADD ir SDD grupėse CRLR–IR tyrimas parodė priešingą tendenciją, palyginti su CGRP–IR nervinėmis skaidulomis. SDD pacientams CRLR–IR struktūrų kiekis žarnyno nerviniame rezginyje padidėja. Didžiausias padidėjimas buvo OSP 41,3 proc. ($p < 0,0001$), o MP ir ISP CRLR–IR nervinių struktūrų padidėjo atitinkamai 29,3 proc. ($p = 0,008$) ir 22,7 proc. ($p = 0,022$). Asimptominių pacientų grupėje CRLR–IR struktūrų kiekio pokytis buvo: MP: +11,3 proc., $p = 0,078$; OSP: +17,6 proc., $p = 0,415$; ISP: –16,3 proc., $p = 0,243$, ir vėlgi reikšmės buvo tarpinės tarp kontrolinės ir SDD grupių (4.3.1 lentelė).

RAMP1–IR struktūrų signalo lokalizacija buvo atitinkama kaip ir CRLR. Rasta RAMP1–IR struktūrų ekspresija SDD ir kontrolinių pacientų MP ir ISP rezginiuose statistiškai nesiskyrė (MP: –14,7 proc., $p = 0,67$; ISP: +0,11 proc., $p = 0,81$), tuo tarpu ISP padidėjo 22,3 proc. ($p = 0,04$) (4.3.1.1 lentelė).

CGRP sukėlė atsipalaidavimo atsaką tiek žiediniuose, tiek išilginiuose riestinės žarnos raumenų mėginiuose. Divertikulioze sergančių pacientų išilginių raumenų atsakas į egzogeninį CGRP buvo padidėjęs. Kontroliniuose žmogaus riestinės žarnos mėginiuose, egzogeninis CGRP sukėlė 69,5 proc. maksimalios atsipalaidavimo vertės išilginiuose raumenyse ir 73 proc. žiediniuose raumenyse. Asimptominių ir simptominių pacientų grupių išilgi-

nių raumenų atsipalaidavimas padidėjo atitinkamai 4,54 proc. (p = 0,677) ir 10,5 proc. (p = 0,033), tuo tarpu žiediniuose raumenyse sumažėjo (SDD: −2,44 proc., p = 0,536; ADD: −6,99 proc., p = 0,149), tačiau statistiškai nesiskyrė tarp grupių.

Tam, kad patikrintume CGRP vaidmenį raumenų atpalaidavime, atlikome dvigubas imunohistochemines reakcijas su CGRP, CRLR arba RAMP1 bei NOS1 arba VIP. CGRP–IR nervinės skaidulos buvo glaudžiai susijusios su NOS1 ir VIP imunoreaktyviais enterinių mazgų neuronais. Tiek VIP–IR, tiek nitrerginiai neuronai žmogaus enterinėje nervų sistemoje buvo imunoreaktyvūs CRLR ir RAMP1, kas rodo CGRP aktyvaciją. NOS1–IR neuronai dažniausiai buvo sutinkami mienteriniame rezginyje, tuo tarpu VIP–IR neuronai dominavo vidinio bei išorinio pogleivio rezginių mazguose.

4.3.2. Elektronomikroskopinis tyrimas

4.3.2.1. Enteriniai mazgai

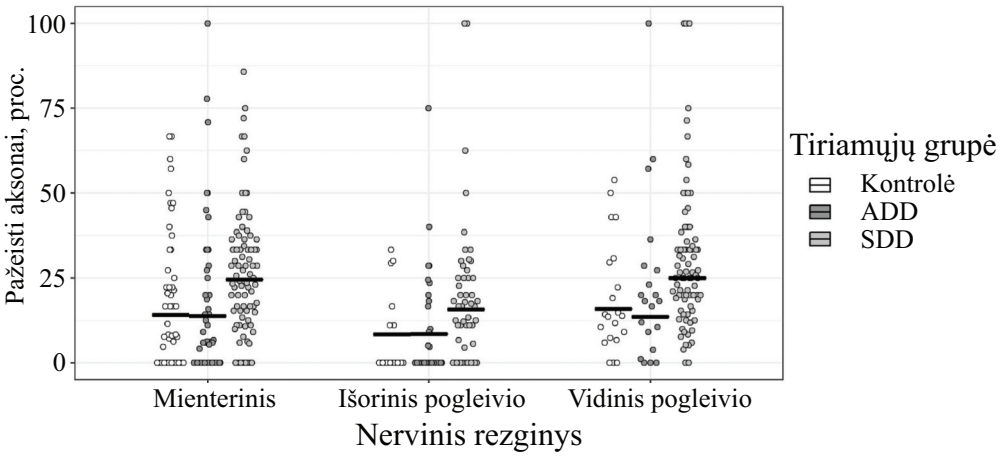
Besimptomės divertikuliozės grupės mėginiuose, dauguma neuronų buvo nepažeisti, tik kai kuriuose neuronuose buvo matoma sutankėjusi citoplazma, didesni lipofuscino intarpai, ir akivaizdus organelių pabrinkimas. Neuronų išsidėstymas ir jų santykis su kitomis mazgo struktūromis nesiskyrė nuo kontrolinės grupės mėginių.

Tuo tarpu simptominės divertikuliozės mėginiuose neuronai buvo akivaizdžiai pakitę. Daugumoje neuronų stebėti dideli lipofuscino intarpai, lamininiai kūneliai ir išbrinkusios, vakuolizuotos mitochondrijos. Grūdėtasis endoplazminis tinklas taip pat buvo išbrinkęs ir Goldžio aparatas buvo sunkiai įžiūrimas citozolyje. Branduolys dažnai būdavo netaisyklingos formos, su daugybe gilesnių invaginacijų, taip pat atrodė padidėjęs ir labiau kondensuotas.

4.3.2.2. Aksonai

Asimptominės divertikuliozės mėginių enteriniuose mazguose neuropilis buvo toks pats kaip kontrolinių, tačiau dažniau pasitaikydavo išbrinkusių aksonų plotai. Tuo tarpu SDD audinių enterinių mazgų neuropolyje akivaizdūs pažeidimai buvo pastebimi daug dažniau, lyginant su ADD grupe. Pažeisti aksonai buvo išbrinkę, be mikrotubulių ir pūslelių, citolazma buvo permatoma, nekontrastingi neurofilamentai. Plazminė membrana buvo su pertrūkiais, neištisinė. Dėl to, kai kurie aksonai buvo susilieję su greta esančiais aksonais ar glijos lastelėmis. SDD enteriniuose mazguose aksonai buvo atskirti glijos ląstelių ir kolageno skaidulų pluoštais. Taip pat neuropilyje beveik nebuvo varikozių su neuromediatoriaus pūslelėmis ir nebuvo rasta sinapsinių kontaktų. Gauti rezultatai yra pateikti 4.3.2.2.1 pav.

Be pažeistų aksonų, SDD pacientų mėginiuose daug dažniau buvo stebimi ir labai ploni aksonai. Jiems būdinga tanki aksoplazma. Šie aksonai buvo matomi arba šalia išbrinusių aksonų, apsupti glijos ataugų, arba susitelkę grupėmis. Dažniau tokio tipo aksonai buvo nustatomi MP ir ISP rezginiuose.



4.3.2.2.1 pav. *Tiriamųjų grupių MP, OSP ir ISP rezginių pažeistų aksonų procentinė dalis*

Grafoje taškeliai rodo kiekvienoje elektronogramoje rastų pažeistų aksonų procentinę dalį; juoda linija – procentų vidurkį.

4.3.2.3. Intersticinės Kajalio (ICC) ir putliosios ląstelės

Mienterinio rezginio intersticinės Kajalio ląstelės (ICC–MY), ADD mėginiuose atrodė panašiai kaip ir kontroliniuose mėginiuose, išskyrus kai kuriose ląstelėse pasitaikančius laminarinius kūnelius ir retai išsidėsčiusias kaveoles. ICC esančios pogleivio rezginyje (ICC–SP), atitiko tuos pačius morfologinius kriterijus, išskyrus tolimesnius kontaktus su aplinkinėmis ląstelėmis ir labiau išreikštas ataugas.

SDD mėginiuose ICC–MY ląstelių forma išliko nepakitusi, tačiau stebėtas citoplazmos išeikvojimas. Taip pat stebėtas ribosomų ir grūdėtojo endoplazminio tinklo išsiskaidymas ląstelių ataugose. Skirtingai nei kontroliniuose ir ADD mėginiuose, ICC–MY ataugos retai kontaktuodavo su lygiaisiais raumenimis ar enteriniais nervais, tačiau kontaktuodavo su kitomis ICC–MY. Tokie patys pokyčiai stebėti ir pogleivyje. Kai kurios ICC–SP ataugos nesiskyrė nuo kontrolės, bet kitose trūko tarpinių filamentų. Išilginiuose ICC–SP ataugų pjūviuose buvo matomas grūdėtojo endoplazminio tinklo skaidymasis. Ataugos kontaktuodavo viena su kita, taip pat su pogleivio rezginio enteriniais nervais. Kitų pažeidimo požymių neaptikta.

SDD grupėje vidutiniškai rasta du kartus daugiau putliųjų ląstelių nei kontroliniuose ir ADD mėginiuose, jos taip pat buvo arčiau enterinių nervų nei kitose tiriamųjų grupėse bei retais atvejais kontaktavo su intersticinėmis Kajalio ląstelėmis. Vidutinis atstumas tarp putliosios ląstelės ir nervo SDD mėginiuose buvo 0,83 μm (nuo 0,26 iki 7,05 μm), tuo tarpu kontrolinėje grupėje – 2,25 μm (nuo 1,57 iki 7,98 μm; $F = 6,44$, $p = 0,004$). ADD grupėje nebuvo rasta arti nervo esančių putliųjų ląstelių. ISP rezginyje enteriniai nervai retai turėjo perineuriumą, todėl tikėtina, kad neuronai tiesiogiai kontaktavo su putliosiomis ląstelėmis.

5. IŠVADOS

1. COL3A1 (rs1800255), COL1A1 (rs1800012), COLQ (rs7609897) VNP nėra susiję su divertikuliozės išsivystymo rizika. Daugianarė logistinės regresijos analizė atskleidė, kad COL3A1 (rs3134646) VNP yra susijęs su didesne divertikuliozės išsivystymo rizika europidų kilmės vyrams. FAM155A (rs67153654) A alelis yra susijęs su divertikulito rizika divertikulioze sergantiems pacientams, tačiau ši genetinė variacija neturi įtakos pačios divertikuliozės išsivystymui. ARHGAP15 (rs4662344) VNP sietinas tiek su divertikulito, tiek su divertikuliozės išsivystymo rizika.
2. Divertikulioze sergančių pacientų enterinės nervų sistemos CGRP reguliacija yra pakitusi. Šie pokyčiai ryškiausi tarp sergančių simptomine DL. Simptominės ligos atveju, CGRP–IR nervinių skaidulų enteriniuose nerviniuose mazguose sumažėja, o CGRP receptoriaus CRLR raiška išauga. Taip pat nustatytas glaudus CGRP ryšys su VIP–ergine nitrergine sistemomis. Šis nervinių struktūrų persitvarkymas yra susijęs su sumažėjusiu žarnos lygiųjų raumenų atsipalaidavimo atsaku. Tarpinės rezultatų vertės, stebimos asimptominėje tiriamųjų grupėje, leidžia įtarti laipsnišką CGRP mažėjimą ligai progresuojant. Nervinio–raumeninio perdavimo sutrikimai gali būti viena pagrindinių divertikuliozės etiologijos priežasčių.
3. Ultrastruktūriniai pakitimai pasireiškia tik simptominės DL atveju. Pažeidžiamos visos riestinės žarnos enterinės nervų sistemos dalys – nerviniai mazgai ir neuronų kūnai, nervai ir nervinės skaidulos, sumažėja varikozių su neuromediatoriaus pūslelėmis ir sinapsinių kontaktų. Taip pat stebimas padidėjęs skaičius putliųjų ląstelių, tiesiogiai kontaktuojančių su vidinio pogleivio rezginio nervais ir ICC, bei ultrastruktūriniai pakitimai pačiose ICC.

4. Išorinių veiksnių poveikio analizė atskleidė, jog storosios žarnos divertikuliozės rizika yra susijusi su vyresniu amžiumi, didesniu KMI, tuštinimosi dažnumu ir nepilno išsituštinimo pojūčiu. Vyresnis amžius, didesnis KMI ir nepilno išsituštinimo pojūtis didina storosios žarnos divertikuliozės riziką, o retas tuštinimasis, priešingai, yra susijęs su mažesne ligos rizika. Be to, rizika susirgti divertikulitu pacientams, sergantiems storosios žarnos divertikulioze, yra susijusi su vyresniu amžiumi, nepilno išsituštinimo pojūčiu ir aukštesniu išsilavinimu.

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LIST OF PUBLICATIONS

1. Reichert MC, Kupcinskas J, Krawczyk M, Jüngst C, Casper M, Grünhage F, Appenrodt B, Zimmer V, Weber SN, Tamelis A, **Lukosiene JI**, Pauziene N, Kiudelis G, Jonaitis L, Schramm C, Goeser T, Schulz A, Malinowski M, Glanemann M, Kupcinskas L, Lammert F. A Variant of COL3A1 (rs3134646) Is Associated With Risk of Developing Diverticulosis in White Men. Dis Colon Rectum. 2018 May;61(5):604–611.
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LIST OF SCIENTIFIC CONFERENCES

The results of the dissertation were presented in the scientific conferences:

1. The 3rd Meeting of the Federation of Neurogastroenterology and Motility, Amsterdam, The Netherlands, August 29 – September 1, 2018. Poster presentation "Nitrergic and vipergic neurons associated with decreased amount of calcitonin gene–related peptide in the enteric plexus of sigmoid colon in diverticular disease".
2. The 26th United European Gastroenterology Week, Vienna, Austria, October 20–24, 2018. Poster presentation "Changes in association between inhibitory motor neurons and calcitonin gene–related peptide in colonic diverticulosis".
3. The 1st International Conference on Xenobiotics and Endogens in Biological Matrices, Sassari, Italy, June 7, 2019. Poster presentation "Human endogenous calcitonin gene–related peptide expression is altered in patients with colonic diverticular disease".
4. The 27th United European Gastroenterology Week, Barcelona, Spain, October 19–23, 2019. Poster presentation "Evaluation of risk factors for colonic diverticulosis in Eastern and Central European population".
5. The 28th United European Gastroenterology Week, virtual, October 11–13, 2020. Poster presentation "Association between dietary, lifestyle factors, bowel habits and the risk of colonic diverticulitis".

ORIGINAL CONTRIBUTION

A Variant of *COL3A1* (*rs3134646*) Is Associated With Risk of Developing Diverticulosis in White Men

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BACKGROUND: Colonic diverticulosis is one of the most common gastroenterological disorders. Although diverticulosis is typically benign, many individuals develop diverticulitis or other aspects of diverticular disease. Diverticulosis is thought to stem from a complex interaction of environmental, dietary, and genetic factors; however, the contributing genetic factors remain unknown.

OBJECTIVE: The aim of our present study was to determine the role of genetic variants within genes encoding for collagens of the connective tissue in diverticulosis.

DESIGN: This was a transsectional genetic association study.

SETTINGS: This study was conducted at three tertiary referral centers in Germany and Lithuania.

PATIENTS: Single-nucleotide polymorphisms in *COL3A1* (*rs3134646*, *rs1800255*) and *COL1A1* (*rs1800012*) were genotyped in 422 patients with diverticulosis and 285 controls of white descent by using TaqMan assays.

MAIN OUTCOME MEASURES: The association of colonoscopy-proven diverticulosis with genetic polymorphisms with herniations was assessed in multivariate models.

RESULTS: The *rs3134646*, *rs1800255*, and *rs1800012* variants were significantly associated with the risk of developing diverticulosis in the univariate model; however, these associations were not significant in the multivariate logistic regression analysis including additional nongenetic variables. When selectively analyzing sexes, the genotype AA (AA) in *rs3134646* remained significantly associated with diverticulosis in men (OR, 1.82; 95% CI, 1.04–3.20; $p = 0.04$).

LIMITATIONS: Because a candidate approach was used, additional relevant variants could be missed. Within our cohort of patients with diverticulosis, only a small proportion had diverticular disease and thus, we could not examine the variants in these subgroups. Functional

Funding/Support: This study was supported by a grant from the Faculty of Medicine, Saarland University (HOMFOR grant T201000747) to Dr Reichert and a grant of the Research Council of Lithuania No. SEN-06/2015/PRM15-135.

Financial Disclosures: None reported.

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Dis Colon Rectum 2018; 61: 00–00
DOI: 10.1097/DCR.0000000000001001

studies, including the analysis of the involved collagens, are also warranted.

CONCLUSIONS: Our study shows that a variant of *COL3A1* (*rs3134646*) is associated with the risk of developing colonic diverticulosis in white men, whereas *rs1800255* (*COL3A1*) and *rs1800012* (*COL1A1*) were not associated with this condition after adjusting for confounding factors. Our data provide novel valuable insights in the genetic susceptibility to diverticulosis. See **Video Abstract** at http://links.lww.com/DCR/A504.

KEY WORDS: Colon; Diverticular disease; Diverticulosis; Gene polymorphism; Single-nucleotide polymorphism.

Colonic diverticulosis is one of the most common gastroenterological disorders, and its prevalence rises with age. Indeed, at the age of 60, up to 50% of the population might be affected by diverticulosis.[1] A significant amount of individuals develop diverticular disease (DD), in particular, inflammation of the diverticula (diverticulitis) or irritable bowel syndrome-like symptoms (symptomatic DD). Diverticulitis can lead to potentially life-threatening complications, including abscess formation, colonic perforation, or bowel obstruction. As a result, DD, and, in particular, diverticulitis, represent the fifth most costly GI disease in developed countries.[2] Consecutively, it has been reported that health care expenditures for the diagnosis and treatment of DD are constantly rising because of rapidly aging populations worldwide.[3,4]

To date, the pathogenesis of diverticulosis remains unknown. The long-standing pathogenetic concept introduced by Painter and Burkitt over 45 years ago,[5] implying that low-fiber diets reduce the stool volume, increase colonic pressure, and lead to the development of colonic diverticula, is questioned by newer data.[6,7] Diverticulosis is thought to develop as a complex interaction of environmental, including dietary and genetic, factors; however, the exact pathogenesis remains unknown.[6,7] Environmental factors supposedly associated with diverticulosis include age, constipation, obesity, smoking, and alcohol abuse.[8]

To date, little attention has been paid to the role of genetic factors in diverticulosis and diverticulitis. Recent epidemiological twin studies indicate a significant role of genetic factors for the development of diverticulosis: Granlund et al[9] linked the Swedish Twin Registry to the Swedish Inpatient Registry. With the use of mathematical models, the heritability was estimated to be 40%, and the nonshared environmental effects were calculated to account for 60% of the trait variability. In the Danish twin study using the Danish National Registry of Patients linked to the Danish Twin Registry,[10] the heritability was estimated to be 53%. It is also known that patients affected by diverticulosis in Western countries mostly develop diverticula in the left colon, whereas in Asia predominantly the right side is affected. These transethnic differences further strengthen the role of genetic factors for the development of diverticulosis.

The genetic variants involved in the pathogenesis of this common disease are widely unknown. To date, only 2 small case-control studies using the candidate gene approach and analyzing single-nucleotide polymorphisms (SNPs) in diverticulosis have been published; however, they do not provide conclusive results.[11,12] One hypothesis suggests that diverticulosis might develop because of weakness of the colonic wall,[13,14] caused by genetically altered structural proteins of the connective tissue. This hypothesis is supported by the fact that, in some genetic disorders of the connective tissue (such as autosomal dominant polycystic kidney disease, Coffin-Lowry syndrome, Ehler-Danlos syndrome, and Williams-Beuren syndrome), colonic diverticula are observed in increased frequency and already at a young age.[7,15]

In other diseases related to malfunctions of the connective tissue, genetic loci and polymorphisms involved in connective tissue maintenance and degradation of collagen could be identified: *rs2236479* located in the collagen XVIII (*COL18A1*) gene was identified as a candidate variant for pelvic organ prolapse in genome-wide association studies (GWAS).[16] Whole-genome linkage analysis revealed that *COL3A1* is linked with gastroesophageal reflux disease, and identified AA homozygotes in *rs3134646* as a risk factor for hiatal hernia in men.[17] In a Dutch study, the probability of pelvic organ prolapse was higher in women carrying the *COL3A1* 2209G>A allele.[18] A recent GWAS in patients with inguinal hernia identified 4 SNPs in genes involved in the regulation of connective tissue.[19] The evidence described above suggests an important role of genetic predisposition in weakness of the connective tissue, which also needs to be explored in diverticulosis, to identify variants for further validation in diverticular disease.

The aim of our study was to determine the role of genetic variation within genes encoding for collagens of the connective tissue in the colonic wall within our discovery cohort. Genetic polymorphism coding for collagens with proven associations with herniations of hollow luminal organs were selected: in *COL3A1* (*rs3134646* and *rs1800255*)[17,18,20] and in *COL1A1* (*rs1800012*).[21] To our best knowledge, this is the first large-scale study investigating the role of genetic polymorphisms predisposing to the development of colonoscopy-proven diverticulosis.

PATIENTS AND METHODS

Study Population

All patients taking part in the study were recruited at the Department of Medicine II, Saarland University Medical Center, Homburg, between 2012 and 2016, the Department

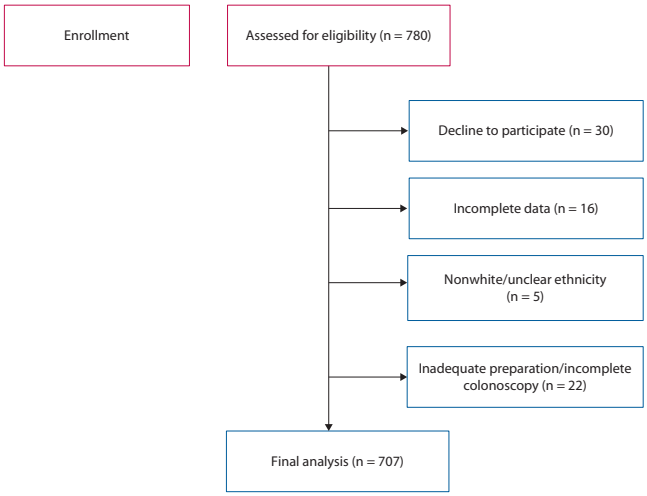

FIGURE 1. CONSORT diagram outlining inclusion and exclusion of patients. Patients with no diverticula in colonoscopy were used as controls.

of Gastroenterology and Hepatology, University Hospital Cologne and the Department of Gastroenterology at the Lithuanian University of Health Sciences, Kaunas in Lithuania, between 2012 and 2016, from patients referred for colonoscopy. A part of the samples from the Lithuanian cohorts came from our previous studies on colonic diseases.[22,23] All patients and controls underwent careful clinical examination and were of self-reported white ancestry including grandparents. Risk factors and epidemiological and baseline data were assessed by using a structured interview performed by a physician assisting the patients with the questionnaires. In addition, each patient's chart was reviewed to obtain missing data and double check data that were extracted from the questionnaires. The presence of diverticula was assessed by colonoscopy in all patients, which is the most widely accepted standard to detect diverticula. Only patients with complete colonoscopy including inspection of the cecum with at least adequate preparation, as assessed by the physician performing the colonoscopy, were included in the study. All colonoscopies were performed with the use of digital video endoscopes (high-resolution scopes, Olympus CF 160, 180, or 190) by a senior gastroenterologist. Extension of diverticulosis was graded as follows: few diverticula (1–15 diverticula), intermediate (16–100 diverticula), and extensive diverticulosis (>100 diverticula). Intestinal lavage for endoscopic examination was performed using 2 L of a solution containing polyethylene glycol. We excluded patients in whom complete colonoscopy, including inspection of the cecum, could not be performed or when preparation did not achieve at least moderate quality. Patients with inherited connective tissue disorders such as Ehlers-Danlos or Marfan syndrome, nonwhite ethnicity, or relatives of included patients were also excluded. The degree of chronic constipation was evaluated using the Cleveland Clinic constipation score.[24] In brief, this is a validated score that includes items for stool frequency, pain at evacuation, sensation of incomplete evacuation, abdominal pain, time spent on the toilet, requirement for digital evacuation, false urge, and duration of symptoms. Each item is added, and the sum of the item scores yields the constipation score. The study protocol was approved by the Research Ethics Committee of the Saarland University (approval 63/11) and the Regional Kaunas Ethics Committee protocol No BE-10–2. The study was performed according to the Declaration of Helsinki. All patients signed an informed consent form to participate in the study. A CONSORT diagram outlining inclusion and exclusion of patients is provided in Figure 1.

DNA Extraction and Genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells by using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). DNA concentrations were measured using a NanoDrop spectrophotometer. DNA samples were stored at –20°C until analysis. Genotyping of the 3 genetic polymorphisms (*rs1800255*, *rs3134646*, and *rs1800012*) was performed in 422 patients with diverticulosis and 285 controls of white de-

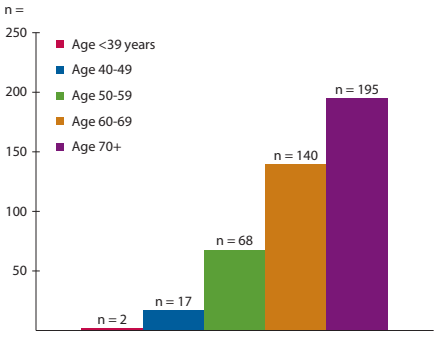

FIGURE 2. Distribution of age in patients with diverticulosis.

scent in our accredited laboratory (DIN EN ISO 15189) in Homburg by a technician blinded to the phenotype of the patients using TaqMan assays. The fluorescence data were analyzed with allelic discrimination 7500 Software v.2.0.6.

Supplemental Methods

One microliter of genomic DNA (10–50 µg) was mixed with 2.5 µL of GTXpress MasterMix (Thermoscientific, Waltham, MA, #4401892), 0.125 µL of Genotyping assay (C___7477926_10 for rs1800255, C___2326027_10 for rs3134646, and C___7477170_30 for rs1800012; all Thermoscientific), and 1.375 µL of water. Analyses were run on a TaqMan (7500 Fast Realtime PCR System, Applied Biosystems, Foster City, CA) by using the following conditions: 25°C for 1 minute; 95°C for 20 seconds (preheat); 95°C for 5 seconds; 60°C for 30 seconds (35 cycles).

Statistical Analysis

Statistical Package for the Social Sciences (SPSS, Version 20, IBM, Munich, Germany) and Prism (Version 5, Graphpad Software, La Jolla, CA) were used for statistical analysis. Quantitative data were expressed as medians and ranges. To determine sample size, power calculations were performed by using PS (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampeSize) to detect a significantly increased odds ratio of 2 with a power of 80%, based on the corresponding risk allele frequencies of *rs3134646*, *rs1800255*, and *rs1800012* and type I error rates of 0.05. Exact tests were performed to check the consistency of genotyping results with Hardy-Weinberg equilibrium (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Genotype frequencies were compared in contingency tables. Genotype association analysis between SNPs and diverticulosis was performed by using multiple logistic regression models. Because of the low number of homozygotes for the minor alleles of all SNPs, genetic analyses were performed assuming a dominant (by coding the genotypes as 0 and 1 for wild-type homozygotes and heterozygotes and as 2 for homozygous carriers if the variant allele, respectively)[25] and allelic model. *p* values of <0.05 were considered statistically significant.

RESULTS

Patients Characteristics

In total, 707 patients (349 men, 358 women) were included; all patients were whites (328 German and 379 Lithuanian patients). A breakdown by decades of ages is provided in Figure 2. Table 1 summarizes the baseline data of this study cohort. Data on the reasons for colonoscopy were available in 606 of 707 (85.7%) patients. The most common reason for a colonoscopy was colorectal cancer screening in 324 cases (53.5%), followed by abdominal pain in 178 (29.3%) and per rectal bleeding in 104 cases (17.2%). The median age was 64 years (range, 19–95). Frequency of diverticulosis in our total cohort was similar to prior data.[26] When comparing patients with diverticulosis with controls, patients with diverticulosis were significantly ($p < 0.0001$) older, with median age being 68 years (range, 32–95) versus 57 years (range, 19–83) in controls. BMI was significantly higher ($p < 0.0001$) in patients with diverticulosis (median, 28 kg/m^2; range, 17–54) than in controls (median, 26 kg/m^2; range, 17–48). No differences could

TABLE 1. Baseline data of study population

Parameter	*Diverticulosis (n = 422)*	*Controls (n=285)*	*Total (n=707)*	*p value*
Age, years	68 (32–95)	57 (19–83)	64 (19–95)	**<0.0001**
BMI, kg/m^2	28 (17–54)	26 (17–48)	27 (17–54)	**<0.0001**
Sex, men/women	209 (49.5)/213 (50.5)	140 (49.1)/145 (50.9%)	349 (49.4)/358 (50.6)	0.92
Ethnicity, German/Lithuanian	207 (49.1)/215 (50.9)	121 (42.5)/164 (57.5)	328 (46.4)/379 (53.6)	1.00
Laxatives, yes/no	100 (24.3)/312 (75.7)	31 (17.8)/143 (82.2)	131 (22.4)/455 (77.6)	0.09
CCCS	3 (0–18)	3 (0–13)	3 (0–18)	0.69
Smoking ever, yes/no	157 (38.3)/253 (61.7)	118 (43.2)/155 (56.8)	275 (40.3)/408 (59.7)	0.20
Alcohol (daily), yes/no	25 (6.1)/384 (93.9)	17 (7.1)/221 (92.9)	42 (6.5)/605 (93.5)	0.61

Values are given as median (range), or frequencies and percentages, n (%). Significant *p* values are highlighted in bold.
CCCS = Cleveland Clinic constipation score.

TABLE 2. Genotype distribution of selected variants in *COL1A1* and *COL3A1* of 707 patients with diverticulosis and controls

Gene	Genotype/alleles	Controls	Diverticulosis	OR (95% CI)	p value
COL3A1					
rs1800255[a]	GG	149 (53.4)	256 (61.4)		
	GA	107 (38.4)	142 (34.1)		
	AA	23 (8.2)	19 (4.6)		
	Allele G	405 (72.6)	654 (78.4)	0.73 (0.57–0.93)	0.012
	Allele A	153 (27.4)	180 (21.6)		
rs3134646[b]	GG	69 (24.8)	100 (23.9)		
	GA	144 (51.8)	189 (45.2)		
	AA	65 (23.4)	129 (30.9)		
	Allele G	282 (50.7)	389 (46.5)	1.18 (0.95–1.47)	0.13
	Allele A	274 (49.3)	447 (53.5)		
COL1A1					
rs1800012[c]					
	GG	200 (71.9)	289 (69.5)		
	GT	73 (26.3)	106 (25.4)		
	TT	5 (1.8)	21 (5.0)	1.23 (0.92–1.65)	0.16
	Allele G	473 (85.1)	684 (82.2)		
	Allele T	83 (14.9)	148 (17.8)		

Values are given as frequencies and percentages, n (%).
[a]Eleven samples failed to be genotyped for *rs180025*.
[b]Eleven samples failed to be genotyped for *rs3134646*.
[c]Sixteen samples failed to be genotyped for *rs1800012*.

be detected for sex ($p = 0.92$), constipation (Cleveland Clinic constipation score, $p = 0.69$), ethnicity ($p = 1.00$), laxative use ($p = 0.09$), smoking ever ($p = 0.20$), or current daily alcohol intake ($p = 0.61$) (Table 1).

Associations of SNPs and Diverticulosis

Table 2 presents the genotype frequencies. The genotype distributions did not deviate from the Hardy-Weinberg equilibrium (overall $p < 0.05$). In univariate regression analyses, older age ($P < 0.0001$), BMI ($p < 0.0001$), *rs1800012* ($p = 0.02$), *rs1800255* ($p = 0.01$), *rs3134646* ($p = 0.04$), and *rs1800012* ($p = 0.02$) were significantly associated with diverticulosis (Table 3). The presence of the homozygous variant (AA) in *rs1800255* was protective, whereas homozygous variants in *rs3134646* (AA) and *rs1800012* (TT) showed an increased risk for diverticulosis compared with controls (Tables 3 and 4). Subsequently, we included these variables in a multivariate logistic regression model. However, after adjusting for confounding factors, only age ($P < 0.0001$) and BMI ($p = 0.004$) remained significant in multivariate linear regression analysis (Table 3). When selectively analyzing the SNPs in sex-specific multivariate logistic regression analysis, in men, the genotype AA of *rs3134646* remained significantly associated with diverticulosis after the adjustment of all confounding cofactors ($p = 0.04$; OR, 1.82; 95% CI, 1.04–3.20). When we compared few + intermediate versus extensive diverticulosis and few versus intermediate + extensive diverticulosis, carriers of the genotype (*rs3134646*: AA) were less common in patients with extensive and intermediate diverticulosis (Table 4).

DISCUSSION

The aim of our present study was to determine the role of genetic variation within genes encoding for collagens and the risk of colonic diverticulosis. We show that a variant of *COL3A1 rs3134646* is associated with risk of developing colonic diverticulosis in white men, whereas *COL3A1 rs1800255* and *COL1A1 rs1800012* are not associated with this condition after adjusting for the confounding factors. To our best knowledge, this is the first large-scale colonoscopy-based study investigating the role of genetic polymorphisms predisposing to diverticulosis including 422 patients with diverticulosis and 285 controls of white descent. Germans and Lithuanians were found to have a similar genetic background.[27] Our study adds new valuable insights in the genetic predisposition of this common disorder.

Direct comparison of our study with previous results is not possible, because there are no other colonoscopy-based studies analyzing the association between *rs3134646*, *rs1800255*, or *rs1800012* and colonic diverticulosis. Overall, there are only 2 small previous studies addressing the role of genetic variations in DD. A small study from the United States containing 55 cases showed that *rs7848647* within the *TNFSF15* gene might be associated with surgically treated diverticulitis.[11] A second study including 90 healthy controls and 52 DD subjects suggested that the *Reprimo* SNP 824G>C might be linked with an increased risk for diverticulosis.[12] Therefore, our study represents the largest analysis in a colonoscopy-based cohort to date. All genotype distributions were consistent with Hardy-Weinberg equilibrium. Interestingly, the minor allele frequency of *rs1800255* was lower than in public databases (ExAC),[28]

TABLE 3. Logistic regression analysis of risk factors for diverticulosis

Parameter	*OR*	*95% CI*	*p value*
Univariate analysis			
Age	1.09	1.07–1.10	**<0.0001**
BMI	1.06	1.03–1.10	**<0.0001**
CCCS	0.99	0.95–1.06	NS
Sex, men/women	0.98	0.73–1.33	NS
Ethnicity, German/Lithuanian	0.77	0.57–1.04	NS
Laxatives, yes/no	1.33	0.62–2.89	NS
Smoking ever, yes/no	0.81	0.59–1.10	NS
Alcohol (daily), yes/no	0.56	0.30–1.06	NS
COL3A1 (*rs1800255*) AA genotype	0.44	0.24–0.82	**0.01**
COL3A1 (*rs3134646*) AA genotype	1.37	1.31–1.43	**0.04**
COL1A1 (*rs1800012*) TT genotype	2.90	1.08–7.80	**0.02**
Multivariate analysis with presence of diverticulosis as dependent variable[a]			
Age	1.08	1.07–1.10	**<0.0001**
BMI	1.05	1.02–1.09	**0.004**
COL3A1 (*rs1800255*) AA genotype	0.55	0.27–1.14	0.11
COL3A1 (*rs3134646*) AA genotype	1.34	0.89–2.00	0.16
COL1A1 (*rs1800012*) TT genotype	2.53	0.85–7.49	0.095
Multivariate analysis with presence of diverticulosis as dependent variable[a] in men			
Age	1.07	1.05–1.09	**<0.0001**
BMI	1.03	0.98–1.08	0.27
COL3A1 (*rs1800255*) AA genotype	0.71	0.28–1.81	0.21
COL3A1 (*rs3134646*) AA genotype	1.77	1.04–3.20	**0.04**
COL1A1 (*rs1800012*) TT genotype	1.74	0.40–7.56	0.40
Multivariate analysis with presence of diverticulosis as dependent variable[a] in women			
Age	1.1	1.07–1.12	**<0.0001**
BMI	1.06	1.01–1.11	**0.01**
COL3A1 (*rs1800255*) AA genotype	0.41	0.13–1.30	0.13
COL3A1 (*rs3134646*) AA genotype	1.04	0.58–1.84	0.90
COL1A1 (*rs1800012*) TT genotype	3.42	0.68–17.2	0.14

Significant *p* values are highlighted in bold.
CCCS = Cleveland Clinic constipation score; NS = not significant.
[a]Including all significant parameters from the univariate analysis.

but similar to other series from Lince et al[29] and Kluivers et al[18] with minor allele frequency between 0.03 and 0.07. The frequencies of genotypes and alleles of *rs3134646* and *rs1800012* fully corresponded to publically available data for white populations.[28] A recent register-based GWAS identified a variant in *FAM155A* that was associated with diverticulitis; the inability to replicate our results might be due to the different ethnicities of the patients included.[30]

TABLE 4. Extension of diverticulosis in men

Diverticulosis	*rs3134646 AA genotype (n = 66)*	*Controls (n = 193)*	*OR*	*95% CI*	*p value*
Few	29	48	0.42	0.36–0.81	**0.003**[a]
Intermediate	20	54			
Extensive	17	91	0.30	0.30–0.79	**0.002**[b]

Significant *p* values are highlighted in bold.
[a]Patients with few diverticula (1–15 diverticula) were compared versus patients with intermediate (16–100) + extensive diverticulosis (>100 diverticula).
[b]Patients with extensive diverticulosis were compared versus patients with few and intermediate diverticula.

One of the major strengths of our study is the availability of clinical and endoscopic data and covariates that have been linked with the risk of DD. Initial univariate analysis revealed that older age, BMI, and all 3 SNPs (*rs1800012*, *rs1800255*, and *rs3134646*) were significantly associated with diverticulosis. Our results are in line with previous studies supporting BMI and age as risk factors.[8,31] It is worth pointing out that the association of *rs1800012* and *rs1800255* did not remain significant in multivariate modeling after adjusting for age and BMI. Meanwhile, in men affected by diverticulosis, the *rs3164646* AA genotype remained significantly associated with diverticulosis even after adjusting for confounding factors. Interestingly, this corresponds to the findings of Åsling et al[17] for hiatal hernia in men. This supports the idea of a common etiology of hernias and diverticulosis mediated by a predisposing weakness of collagen in the abdominal connective tissue. As stated in the introduction, this hypothesis is further reinforced by the fact that, in some genetic disorders of the connective tissue, more colonic diverticula are observed already

at a younger age.[7,15] The reason for the sex-specific risk factors is currently unknown; even though a sex-specific genetic architecture is common in humans, and genotype-sex interactions contribute to the severity of the phenotype of many diseases,[32] a potential mechanism could be the hormonal regulation of the collagen metabolism. Interestingly, in patients with the genotype (*rs3164646* AA) extensive and intermediate diverticulosis is significantly less likely. It is possible that different mechanisms gain relevance in the development of mild versus extensive diverticulosis. Studies investigating this novel association, also in diverticular disease and, in particular, symptomatic diverticular disease, are warranted. Overall, within our study, for the first time, genetic variation contributing to the development of diverticula independent of known cofactors was identified in a large colonoscopy-based cohort. Interestingly, diverticula in patients with the risk variant were significantly smaller, which points to specific genetic backgrounds of few diverticula and extensive diverticulosis. To determine the impact on the clinical management of the disease, studies in patients with DD and diverticulitis are warranted.

Limitations of the Study

Our study has certain limitations that have to be acknowledged. First, we have used a candidate gene approach and could have missed other relevant variants that might be associated with the risk of developing diverticulosis. In addition, our results can not necessarily be transferred to nonwhites and have to be validated in other ethnicities as well. Within our cohort of patients with diverticulosis, only a small proportion had DD and previous diverticulitis, and, thus, we could not examine the effect of the analyzed SNPs in these subgroups of patients. The overall number of individuals within diverticulosis and control groups was not large enough for conclusive genotyping studies; however, we believe that our data provide novel valuable insights in the genetic susceptibility of diverticulosis. In addition, mechanistic studies including the analysis of the involved collagens are required, and replication of our results in other cohorts and ethnicities, preferable by GWAS, are warranted.

CONCLUSIONS

Our study shows that a variant of *COL3A1 rs3134646* is associated with the risk of colonic diverticulosis in white men, whereas *COL3A1 rs1800255* and *COL1A1 rs1800012* were not associated with this condition in multivariate regression analysis in our cohort.

ACKNOWLEDGMENTS

The authors thank Annika Bohner and Andrea Schmetz for their outstanding work in analyzing the blood samples, and Alexander Olbricht for excellent technical assistance.

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Common variation in *FAM155A* is associated with diverticulitis but not diverticulosis

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Colonic diverticulosis is a very common condition. Many patients develop diverticulitis or other complications of diverticular disease. Recent genome-wide association studies (GWAS) consistently identified three major genetic susceptibility factors for both conditions, but did not discriminate diverticulitis and diverticulosis in particular due the limitations of registry-based approaches. Here, we aimed to confirm the role of the identified variants for diverticulosis and diverticulitis, respectively, within a well-phenotyped cohort of patients who underwent colonoscopy. Risk variants rs4662344 in Rho GTPase-activating protein 15 (*ARHGAP15*), rs7609897 in collagen-like tail subunit of asymmetric acetylcholinesterase (*COLQ*) and rs67153654 in family with sequence similarity 155 A (*FAM155A*) were genotyped in 1,332 patients. Diverticulosis was assessed by colonoscopy, and diverticulitis by imaging, clinical symptoms and inflammatory markers. Risk of diverticulosis and diverticulitis was analyzed in regression models adjusted for cofactors. Overall, the variant in *FAM155A* was associated with diverticulitis, but not diverticulosis, when controlling for age, BMI, alcohol consumption, and smoking status ($OR_{adjusted}$ 0.49 [95% CI 0.27–0.89], p = 0.002). Our results contribute to the assessment specific genetic variants identified in GWAS in the predisposition to the development of diverticulitis in patients with diverticulosis.

Colonic diverticulosis is a widespread gastrointestinal condition described as formation of diverticula, which are sac-like protrusions of mucosa and submucosa through muscularis externa[1,2]. Although diverticulosis is predominantly asymptomatic, many patients develop symptoms[3,4]. These manifestations are classified as uncomplicated and complicated diverticular disease (DD), an inflammation of the tissue (diverticulitis) or diverticular bleeding. The prevalence of the disease and its complications is constantly increasing due to the aging populations[5,6]. Therefore, the identification of underlying pathobiological mechanisms of the disease is relevant for improving everyday clinical practice. It is hypothesized that environmental factors together with structural alteration of the colonic wall and remodeling of the enteric nervous system are all predisposing components in the development of colonic diverticula[5]. In recent years, epidemiological twin data have suggested that genetic factors are another major cofactor in the pathogenesis of DD[7,8]. Up to date, there has been relatively little research effort to determine these genes and their sequence variants that are associated with the development of DD[9]. Recently an Icelandic study group published a genome-wide association study (GWAS) searching for sequence variants that affect the

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risk of developing DD in the Icelandic population, containing a replication cohort of Danish individuals with DD[10]. The initial analysis included 15,220 Icelanders who were tested for associations with DD (5,426 cases) and its more severe form diverticulitis (2,764 cases). Subsequently, after applying weighted thresholds[11], 16 sequence variants identified in the GWAS were followed up in a DD sample from Denmark with 5,970 cases and 3,020 controls. In the combined analysis of these sample sets, three genetic loci that show genome wide-significance and may be associated with the risk of DD and/or diverticulitis were identified: intronic variants at the *ARHGAP15* (Rho GTPase-activating protein 15), *COLQ* (collagen-like tail subunit of asymmetric acetylcholinesterase) and *FAM155A* (family with sequence similarity 155 A) loci were significantly associated with DD. The second GWAS[12] included 27,444 patients from the European component of the UK Biobank resource and compared them with 382,284 controls. Overall, 154 associated variants were further tested in 31,221 patients from the Michigan Genomics Initiative, finally confirming 42 associated variants including the three previously identified variants[10]. Most recently, a third european GWAS[13] containing 451,099 patients in addition to identification of further loci, also confirmed these three major loci.

Notably, the dissection of the specific phenotypes diverticulosis and diverticulitis was incomplete in the GWAS, since the assessment of diverticulosis or diverticulitis is based on the ICD code. The studies applied the corresponding ICD codes in the ICD10 (K572-K579) and ICD9 (K562.10-13) systems, which also encompasses patients with diverticulitis (as well as diverticular bleeding), among the patients with diverticulosis. Even though ICD codes can identify patients with diverticulosis and DD, they do not discriminate between diverticulosis and diverticulitis[14]. A subanalysis of the patients from the Icelandic subcohort of the Icelandic/Danish GWAS included only patients with either surgically treated or complicated diverticulitis[10], whereas mild and often also outpatient-treated cases of diverticulitis were probably mostly missed. This information is not available in the Danish subcohort at all. Additionally, patients with asymptomatic diverticulosis were not analyzed in these GWAS studies. A part of our samples were used in the GWAS from Schafmayer *et al.*[13], and after adding further samples re-analyzed using additional clinical covariates from the database as outlined below and specifically focusing on patients with diverticulosis no prior diverticulitis as controls and (endpoint diverticulitis) and healthy with no diverticula (endpoint diverticulosis).

In this study, we therefore aimed to evaluate the associations between three major SNPs reported in the Icelandic GWAS applying weighted thresholds and confirmed in the North-American[12] and European[11,13] GWAS: *ARHGAP15* (rs4662344), *COLQ* (rs7609897) and *FAM155A* (rs67153654). The risk of developing diverticulosis and diverticulitis, respectively, was determined in a Caucasian (german/lithuanian) cohort phenotypically characterized for the specific phenotypes diverticulosis and diverticulitis. Due to the similar genetic background of Germans and Lithuanians[15], a combined analysis was performed.

Patients and Methods

Study population. All patients taking part in the study were recruited at the Department of Medicine II, Saarland University Medical Center, Homburg, the Clinic for Gastroenterology and Hepatology, University Hospital of Cologne, Cologne, Germany, and the Department of Gastroenterology at the Lithuanian University of Health Sciences, Kaunas in Lithuania between 2012 and 2016 from patients referred for colonoscopy. A part of the samples from the Lithuanian cohorts came from our previous studies on colonic diseases and diverticulosis[16–18] and were also used in a previous GWAS with less clinical information[13]. All patients and controls were of self-reported Caucasians ancestry (including grandparents). Risk factors, epidemiological and baseline data were assessed using a structured interview, performed by a physician assisting the patients with the questionnaires. The presence of diverticula was assessed by colonoscopy in all patients, which is the most widely accepted standard to detect diverticula. Only patients with complete colonoscopy including inspection of the cecum and at least adequate preparation, as assessed by the physician performing colonoscopy, were included in the study. All colonoscopies were performed using digital video endoscopes (high-resolution scopes Olympus CF 160, 180 or 190) by a senior gastroenterologist. Intestinal lavage for endoscopic examination was performed using 2 liters of a solution containing polyethylene glycol. Patients with inherited connective tissue disorders such as Ehlers-Danlos- or Marfan syndrome, non-Caucasian ethnicity or relatives of included patients were also excluded. The diagnosis of diverticulitis was established according to the current classifications for DD[19,20]. It was based on imaging by either computed tomography and/or ultrasound imaging as well corresponding clinical (pain in the lower left abdomen) and laboratory characteristics (increased serum inflammation markers). Suspected complicated diverticulitis was assessed with computed tomography in all cases. The study protocol was approved by the Research Ethics Committee of the Saarland University (approval 63/11), the Research Ethics Committee of the University of Cologne (approval 16–397) and the Regional Kaunas Ethics Committee (protocol No BE-10-2). The study was performed according to the Declaration of Helsinki. All patients have signed an informed consent form to participate in the study. For the purpose of this study, cases were defined as patients with diverticulosis or diverticulitis, respectively.

DNA extraction and genotyping. Genomic DNA was extracted from peripheral blood mononuclear cells using the DNeasy Blood& Tissue Kit (Qiagen, Hilden, Germany). DNA concentrations were measured using a NanoDrop spectrophotometer. DNA samples were stored at −20 °C until analysis. Genotyping of the three genetic polymorphisms (rs4662344, rs7609897 and rs67153654) with Taqman assays was performed in 856 patients with diverticulosis and 479 controls of Caucasian descent in our accredited laboratory (DIN EN ISO 15189) in Homburg by a technician blinded to the phenotype of the patients. The fluorescence data was analyzed with allelic discrimination 7500 software v.2.0.6.

Statistical analysis. Statistical Package for the Social Sciences (SPSS, Version 20, IBM, Munich, Germany) was used for statistical analysis. Power calculations were performed using PS (http://biostat.mc.vanderbilt.edu/

Parameter	Diverticulosis (n = 858)	Controls (n = 474)	Diverticulitis (n = 198)	Total (n = 1332)	P value*	P value**
Age, years	67 (59–74)	57 (46–66)	61 (53–71)	64 (55–72)	<**0.001**	<**0.001**
BMI, kg/m^2	27.9 (25.2–31.6)	26.7 (23.7–30.3)	27.1 (24.8–30.5)	27.5 (24.8–31.2)	<**0.001**	0.20
Gender, men/women	424 (49.4%)	210 (44.3%)	105 (53.0%)	634 (47.6%)	0.08	0.26
Smoking ever, yes/no	300 (35.6%)	168 (36.7%)	86 (43.9%)	468 (36.0%)	0.72	**0.006**
Alcohol (daily), yes/no	50 (6.0%)	21 (4.6%)	19 (9.9%)	71 (5.5%)	0.31	**0.01**

Table 1. Baseline data of study population. Values are given as median and interquartillic range (IQR), or frequencies and percentages. BMI = body mass index; Significant *P* values are highlighted in bold. *Diverticulosis versus healthy controls, **Diverticulitis versus Diverticulosis with no prior diverticulitis.

Gene	A_{min}/A_{maj}	MAF (%)	CT (%)	TT (%)	OR	P_{trend}	OR (95% CI)	$P_{allelic}$	OR (95% CI)	$P_{genotypic}$
ARHGAP15 (rs4662344)										
Diverticulitis*	T/C	19.5	32.8	3.1	1.20	0.16	1.27 (0.91–1.76)	0.16	1.38 (0.67–2.84)	0.38
Diverticulosis**	T/C	19.5	29.7	4.7	1.30	0.02	1.28 (1.00–1.63)	0.05	1.85 (0.97–3.50)	0.06
$Controls_{(no\ diverticulosis)}$	T/C	15.9	26.3	2.7						
$Controls_{(no\ prior\ diverticulitis)}$	T/C	18.8	28.8	4.4						
Iceland GWAS[10] $_{Diverticular\ disease***}$	T/C	20.9							1.23 (1.17–1.29)	<0.001
Iceland GWAS[10] $_{Controls***}$	T/C	17.7								
MGI GWAS[12] $_{****Diverticular\ disease}$	T/C	19.1/20.0							0.88/0.89	<0.001/0.07
MGI GWAS[12] $_{****Controls}$	T/C	17.3/18.0								
European GWAS[13] $_{Diverticular\ disease}$	T/C	17.9******								<0.001
European GWAS[14] $_{Controls}$	T/C	17.9******								
FAM155A (rs67153654)			TA (%) AA (%)							
Diverticulitis*	A/T	14.1	21.9	3.1	0.68	0.01	0.66 (0.47–0.92)	0.01	0.45 (0.19–1.09)	0.07
Diverticulosis**	A/T	22.8	35.4	5.1	1.01	0.90	1.01 (0.81–1.27)	0.91	1.02 (0.61–1.72)	0.93
Controls (no diverticulosis)	A/T	22.6	35.2	5.1						
Controls (no prior diverticulitis)	A/T	24.3	37.1	5.8						
Iceland GWAS[10] $_{Diverticular\ disease***}$	A/T	17.0							0.87 (0.83–0.91)	<0.001
Iceland GWAS[10] $_{Controls***}$	A/T	18.6								
MGI GWAS[12] $_{*****Diverticular\ disease}$	A/T	17.0/17.8							0.87/0.90	<0.001/0.024
MGI GWAS[12] $_{*****Controls}$	A/T	19.0/19.5								
European GWAS[13] $_{Diverticular\ disease}$	A/T	19.6******								
European GWAS[13] $_{Controls}$	A/T	19.6******								

Table 2. Genotypic and allelic frequencies in *ARHGAP15* and *FAM155A* of the combined German/Lithuanian cohort and published data from published GWAS. Genotypic and allelic frequencies of the variants. Values are given as count and percentage. A_{maj} = major allele; A_{min} = minor allele; CI = confidence interval; MAF minor allele frequency; MGI Michigan Genomics Initiative; OR = odds ratio. P Values calculated for *Diverticulitis versus Diverticulosis with no prior diverticulitis; **Diverticulosis versus healthy controls; ***refers to combined Icelandic/Danish sample; ****Data from variant rs6734367 which is in LD ($r^2 = 0.82$) with variant rs4662344 in caucansians[31], data from UK biobank/MGI; *****Data from the variant rs11619840 which is in LD ($r^2 = 0.93$) with variant rs67153654 in caucansians[31], data from UK biobank/MGI; ******Data only combined cases/controls available.

wiki/Main/PowerSampeSize) to detect a significantly increased OR of 2 with a power of 80%, based on the corresponding frequencies of the risk alleles in rs4662344, rs7609897 and rs67153654, and assuming type I error rates of 0.05. Quantitative data were expressed as medians and ranges. Comparisons of frequencies of genotypes at the three loci were performed in 3×2 contingency tables listing cases and controls. Genotypic and allelic association tests were performed using χ^2-square or Fisher's exact tests (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Due to the few homozygous mutants of the risk alleles we applied a dominant model. Genotype association analysis between SNPs and diverticulosis was performed using multiple logistic regression models adjusted for age, BMI, smoking status, and alcohol consumption, assuming log-additive effects. P-values < 0.05 were considered statistically significant. Results are expressed as odds ratios (OR) and 95% confidence intervals (CI). Pairwise linkage disequilibrium (r^2) was calculated utilizing LDpair[21] using a caucasian reference population (CEU) of the identified variants in all three GWAS[10,12,13].

Results

Patient characteristics. In total, 1,332 patients (634 men, 47.6%) were included. Table 1 summarizes the baseline data of this study cohort. Frequency of diverticulosis in our cohort was similar to prior data[20]. The median age was 64 years (IQR 55–72). Characteristics of the subgroup of patients with diverticulitis are presented

		Variant present with significant association in GWAS (Reference number)	*ARHGAP15*		*FAM155A*				
			rs4662344	rs6734367	rs9520344	rs11619840	rs67153654	rs9555371	rs9520339
ARHGAP15	rs4662344	[10,13]		**0.82**	0.001	0.019	0.014	0.009	0.001
	rs6734367	[12,13]			0.001	0.035	0.028	0.020	0.001
FAM155A	rs9520344	[12,13]				0.0021	0.0008	0.0043	**1.0**
	rs11619840	[12,13]					**0.93**	**0.83**	0.0021
	rs67153654	[10,13]						**0.90**	0.0008
	rs9555371	[13]							0.0043
	rs9520339	[13]							

Table 3. Pairwise linkage disequilibrium (r^2) calculated using LDpair (Machiela *et al.*[31]) with a caucasian reference population (CEU) of the identified variants in all three GWAS[10,12,13]. Bold values indicate where r^2 is >0.8 representing strong LD. Variants from the initial GWAS[10] are marked in underline.

Parameter	Adjusted OR* (95% CI)	P value
ARHGAP15 rs4662344:T (CC + TC vs TT)	1.22 (0.93–1.61)	0.15
ARHGAP15 rs4662344:TC (CT vs TT + CC)	1.14 (0.86–1.52)	0.35
ARHGAP15 rs4662344:TT (TT vs TC + CC)	1.89 (0.84–4.29)	0.13
FAM155A rs67153654:T (TA + AA vs TT)	1.09 (0.84–1.00)	0.53
FAM155A rs67153654:AT (TA vs TT + AA)	1.02 (0.78–1.30	0.89
FAM155A rs67153654:TT (TT vs AT + AA)	1.19 (0.67–2.10)	0.56

Table 4. Multivariate analysis of factors associated with diverticulosis versus controls. CI = confidence interval. OR = odds ratio. *Adjusted for age, BMI, alcohol and smoking status.

Parameter	Adjusted OR* (95% CI)	P value
ARHGAP15 rs4662344:T (CC + TC vs TT)	1.43 (1.00–2.03)	**0.05**
ARHGAP15 rs4662344:TC (CT vs TT + CC)	1.35 (0.44–1.94)	0.11
ARHGAP15 rs4662344:TT (TT vs TC + CC)	1.40 (0.61–3.21)	0.43
FAM155A rs67153654:T (TA + AA vs TT)	0.70 (0.49–0.99)	**0.04**
FAM155A rs67153654:AT (TA vs TT + AA)	0.73 (0.51–1.06)	0.10
FAM155A rs67153654:TT (TT vs AT + AA)	0.68 (0.27–1.69)	0.41

Table 5. Multivariate analysis of factors associated with diverticulitis in patients with diverticulosis. CI = confidence interval, OR = odds ratio. *Adjusted for age, BMI, alcohol and smoking status. Significant P values are highlighted in bold.

in Supplementary Table 1. The call-rate for the variations were >95% for all variants. The genotype frequencies (cut-off $P > 0.05$) were in Hardy-Weinberg equilibrium (HWE) in all controls for the variants in *ARHGAP15* and *FAM155A*. The HWE for the variant in *COLQ* deviated in controls (both in diverticulosis and diverticulitis-analyses $p < 0.001$), and was not included in further analysis. The minor allele frequencies (MAF) (Table 2) were similar to prior data[10,12,13]. In comparison of patients with diverticulosis and controls, patients with diverticulosis were significantly ($P < 0.001$) older and more obese ($P < 0.001$) than individuals with no diverticulosis. When comparing patients with diverticulitis, with diverticulosis and no prior diverticulitis, patients with diverticulitis where significantly younger ($P < 0.001$), more often smokers ($P = 0.006$), and more frequently current alcohol drinkers ($P = 0.001$). No association was detected for BMI ($P = 0.20$) and gender ($P = 0.26$). Table 3 presents the data on linkage disequilibrium (LD) of all variants identified in the GWAS[10,12,13].

Associations of variants and diverticulosis. Table 2 presents the allelic and genotypic frequencies comparing patients with diverticulosis to controls. MAF of the variant in *ARHGAP15* (rs4662344) was increased compared to controls, as described in the GWAS[10,12,13]. The major (T) allele of rs4662344 in *ARHGAP15* was significantly (OR 1.28; 95% CI 1.00–1.63) associated with diverticulosis. This association did not withstand after adjusting for corresponding environmental cofactors though (OR 1.22; 95% CI = 0.93–1.61) (Table 4). Neither was the MAF of the variant in rs67153654 in *FAM155A* different between cases and controls, nor could an association with diverticulosis be detected (Table 4).

Associations of SNPs and diverticulitis. The minor allele of the variant rs4662344 in *ARHGAP15* was more frequent in diverticulitis cases in comparison to controls with diverticulosis and no prior diverticulitis, as similarly described previously in the GWAS[10,12,13]. The MAF of the major (A) allele of rs67153654 in *FAM155A* was markedly (OR 0.66; 95% CI 0.47–0.92) reduced in patients with prior diverticulitis compared to controls (Table 2) as also previously described[10,12,13]. These associations remained significant after adjusting for

environmental cofactors (Table 5). The variant rs4662344 in *ARHGAP15* was borderline significantly (OR 1.43; 95% CI 1.00–2.03; P = 0.05) associated with diverticulitis after adjusting for the corresponding cofactors. Even though hampered by small sample size, similar results (n = 64) were obtained when analyzing patients with surgically treated diverticulitis (Supplementary Tables 2 and 3).

Discussion

The aim of our present study was to assess the role of genetic variations consistently identified in the three large recent GWAS[10,12,13] for the specific risks for diverticulosis and diverticulitis, respectively. Our results are in line with previous data concerning the association of diverticulosis with age and BMI as risk factors (diverticulosis)[22–24], as well as alcohol consumption[25,26] and smoking status[27–29] as risk factors for diverticulitis.

Our major finding is that the rs67153654 risk allele in *FAM155* is significantly associated with diverticulitis after adjusting for cofactors, but not with diverticulosis. The data on the risk variant rs4662344 in *ARHGAP15* was less consistent, it was borderline significantly associated with both diverticulosis and diverticulitis, but for confirmation additional larger studies are warranted. MAF of the analyzed SNPs was similar to previous data[10,12,13,30]. Even though the variants in *ARHGAP15* and *FAM155A* initially discovered in the Icelandic/Danish GWAS[10] are at partially different genetic positions on the same genes compared to the variants identified in the following GWAS[12,13], their LD indicates their common heritability. Therefore, an analysis using the SNPs initially identified in the Icelandic GWAS[10], which applied weighted thresholds, is justified.

All of the analyzed SNPs are located in introns, supporting a molecular mechanism at the level of RNA-expression in the surrounding gene or LD to another, yet unidentified causal variant.

One of the major strengths of our study is the availability of clinical and endoscopic data and covariates, allowing the exact separation of patients with uncomplicated diverticulosis from patients developing diverticulitis. Patients treated for diverticulitis as outpatients were also included in our analysis. Our study adds new insights into the susceptibility for diverticulitis, specifically attributing the association with the risk variant in *FAM155A* (protective effect) to diverticulitis, but not diverticulosis. Our study has certain limitations though, that have to be acknowledged. Due to the retrospective design of the study, we could not investigate the outcomes of diverticulosis and diverticulitis, including DD-associated mortality. Even though a similar genetic background is shared between Germans and Lithuanians[15] our results can not necessarily be transferred to non-Caucasians and have to be validated in other ethnicities ethnicity-specific analysis was not feasible due to sample size. Furthermore, several other variants associated to DD in the prior GWAS were not assessed in our analysis, and could also contribute significantly to the genetic risk to develop diverticulitis in patients with diverticulosis. Ultimately, the development of a genome-wide polygenic score[31] should be strived for in both clincal entities diverticulosis and diverticulitis. The role of the risk variants in complicated DD could also not be explored, as our sample size for these additional subgroups was too small. Additionally, to the best of our knowledge only one study from 2010 assessing the application of ICD codes for the discrimination of diverticulosis and diverticulitis is available. Therefore, confirmatory investigations are necessary. Further studies are also needed to understand which of the variants in *FAM155A* are the causal mutations. Furthermore, as the function of the involved genes is largely known, further elucidation of the molecular background of *FAM155A* deficiency in the pathogenesis of diverticulitis is warranted.

Conclusions

Our results indicate, that the variant in *FAM155A* is associated with diverticulitis, but not diverticulosis in Caucasians, whereas a risk variant in *ARHGAP15* might be associated with both diverticulosis and diverticulitis. Our results contribute to the assessment of these genetic variants identified in GWAS in the predisposition to the development of diverticulitis in patients with diverticulosis.

Data availability

The datasets generated during and/or analyzed during the current study are available on request as permitted by data protection laws and patients consent.

Received: 24 April 2019; Accepted: 14 January 2020;
Published online: 03 February 2020

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Acknowledgements
The authors would like to thank Annika Bohner, Irina Nowak and Friederike Reuner (all Homburg) for their outstanding work in analyzing the blood samples. This study was supported by a grant from the Faculty of Medicine, Saarland University (HOMFOR grant T201000747 to Matthias C. Reichert) and a grant of the Research Council of Lithuania No. SEN-06/2015/PRM15-135.

Author contributions
M.C.R., J.K. and F.L. designed the study; M.C.R., J.K., F.G., V.Z., B.A., A.T., J.I.L., M.K., M.C., C.J., A.S., M.M., M.G., C.S., G.K., L.J., N.P., S.N.W., T.G. and L.K. participated in acquisition and collection of the data, drafted the manuscript, and together with F.L. analyzed the data and finalized the manuscript, which was then revised by all authors. The final draft of the manuscript has been approved by all authors. The contents of this manuscript are our original work and have not been published, in whole or in part, prior to or simultaneous with our submission of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-58437-1.

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ORIGINAL PAPER

DOI: http://dx.doi.org/10.15403/jgld-3208

Environmental and Dietary Risk Factors for Colonic Diverticulosis and Diverticulitis

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Received: 08.11.2020
Accepted: 07.02.2021

ABSTRACT

Background & Aims: Colonic diverticulosis (CD) is among the most common conditions of the large bowel. Several factors have been associated with an increased risk of CD and its complications, including advanced age, obesity, physical inactivity, and a low-fiber diet. Available data is conflicting and a comprehensive analysis of different bowel, dietary and environmental habits linked with CD is lacking. We aimed to investigate the relationship between potential risk factors and CD prevalence using full data from a colonoscopy-based cross-sectional study in Europe.

Methods: The study was conducted at three tertiary referral centers in Germany and Lithuania. It included consecutive adult patients referred for routine colonoscopy who completed a detailed questionnaire on our considered multiple risk factors for diverticulosis and diverticulitis, including dietary and environmental factors, and bowel habits.

Results: The study included 1,333 patients, 696 women and 635 men. Colonic diverticulosis was diagnosed in 858 (64%) of patients. Multivariate analysis revealed that age (OR: 1.08, 95%CI: 1.06–1.10, p<0.001) and obesity (OR: 1.05, 95%CI: 1.02–1.09, p=0.004) were associated with CD. We also revealed new risk factors for CD: increased frequency of bowel movements (OR: 0.10, 95%CI: 0.03-0.33, p<0.001) and feeling of incomplete bowel emptying (OR: 2.05, 95%CI: 1.47–2.87, p<0.001). Older participants had reduced odds (OR: 0.921, 95 CI: 0.89–0.95, p<0.05) of diverticulitis compared to younger subjects. Feeling of incomplete bowel emptying after defecation was associated with increased odds (OR: 2.769, 95% CI 1.35–5.7, p<0.006) for diverticulitis. Moreover, participants with a higher educational status had increased odds (OR: 2.453, 95%CI: 1.31–4.59, p=0.005) for diverticulitis compared to the lower education group.

Conclusions. Study shows that older age, obesity, frequency of bowel movements, and feeling of incomplete bowel emptying are associated with the risk of CD. Furthermore, older age, feeling of incomplete bowel emptying, and higher education were associated with the risk of diverticulitis among CD patients.

Key words: ecolonic diverticulosis – diverticular disease – risk factors – pathophysiology.

Abbreviations: CD: colonic diverticulosis; CI: confidence interval; BMI: body mass index; DD: diverticular disease; DICA: Diverticular Inflammation and Complication Assessment; OR: odds ratio; NSAIDs: nonsteroidal anti-inflammatory drugs.

INTRODUCTION

Colonic diverticulosis (CD) is a chronic progressive disease of the large bowel characterized by the formation of diverticula, out-pouching of the mucosa and submucosa at weak points in the muscular layer of the colon wall. It is one of the most common gastroenterological disorders diagnosed on routine colonoscopy and its complications are an important cause of hospital admissions in Western populations [1]. The prevalence of the disease is closely associated with age [2, 3]. It is estimated that more than 50% of people older than age 60 will develop CD [4]. The majority of individuals with colonic diverticula will remain asymptomatic throughout their lifetime (termed CD) but up to 25% of them will develop symptomatic disease referred to as colonic diverticular disease [5]. The severity of the disorder can then vary from symptomatic uncomplicated diverticular disease (DD) characterized by irritable bowel syndrome-like symptoms, to complications such as diverticulitis, diverticular hemorrhage and perforation [3].

With improved life expectancy in Western societies, the prevalence of CD and DD is continuously growing, consequently increasing its burden on National Health Care systems [6]. Despite this load, data on determinants of CD is sparse and the pathophysiology of the disease remains poorly understood. To date, diverticulosis is thought to develop as a complex interaction of environmental and genetic factors [7-10]. There is also evidence showing that structural changes in the enteric nervous system and imbalances in neuromuscular transmission are other major etiological factors for the development of CD [11, 12]. However, rapid and continuous increase in DD incidence following the industrialization process in Western cultures undoubtedly points to the significance of environmental and dietary factors for the disease development.

During the last decade a number of different studies have proposed physical inactivity, low-fiber diet followed by constipation, red meat consumption, obesity, and some alternative factors such as use of nonsteroidal anti-inflammatory drugs (NSAIDs), smoking and alcohol abuse as environmental factors associated with symptomatic DD [13-20]. Nonetheless, risk factors for developing CD are likely to differ from those for CD complications. A high-powered comprehensive analysis on different dietary and environmental factors linked to CD development is thus lacking. Therefore, the aim of our study was to explore the association between potential risk factors and the prevalence of CD and diverticulitis using inclusive data from a colonoscopy-based cross-sectional study in eastern and central European population.

METHODS

Study Population

The study was conducted between 2012 and 2016 at three tertiary referral centers in Germany and Lithuania: Department of Medicine II, Saarland University Medical Center, Homburg, the Department of Gastroenterology and Hepatology, University Hospital Cologne and the Department of Gastroenterology, Lithuanian University of Health Sciences, Kaunas. Adult participants with ages 19 to 95, and without coexisting gastrointestinal disorders underwent routine screening colonoscopy. The presence of diverticula was determined by colonoscopy in all patients. Only patients with complete colonoscopy including inspection of the cecum with at least adequate preparation, as assessed by the physician performing the colonoscopy, were included in the study. All colonoscopies were performed with the use of digital video endoscopes by a senior gastroenterologist. Extension of diverticulosis was classified as follows: left-sided (including sigmoid and/or descending colon), right-sided (transversal, ascending colon and cecum) and pancolonic. Endoscopic severity of the CD was assessed using the Diverticular Inflammation and Complication Assessment (DICA) [21, 22]. Complete data for DICA score calculation was available for 523 out of 858 (60.9%) participants in the CD group of the cohort. Patients in whom complete colonoscopy, including inspection of the cecum, could not be performed or when preparation did not achieve at least moderate quality were excluded from the study. Patients in whom diverticula were absent were included in the control group.

Assessment of Variables

Prior to colonoscopy, all participants with the help of a qualified research assistant completed a standardized questionnaire on the risk factors for developing CD. Risk factors included in the study were categorized into the following groups: socio-demographic factors, factors related to nutritional status and dietary habits, and factors related to bowel habits. Among socio-demographic factors were included age, gender, ethnicity, educational and occupational status, regular consumption of alcohol and tobacco, regular consumption of prescription and over-the-counter medication (e.g. NSAIDs and laxatives). Factors related to nutritional status and dietary habits were defined by the number of meals per day, amount of daily fluid intake, amount of fish and red meat servings per week and whether participants followed a vegetarian or vegan diet. Body mass index (BMI) (kg/m^2) was calculated from self-reported height (cm) and weight (kg), and being overweight or obese was defined as a BMI ≥ 25 kg/m^2. Bowel habits were assessed as self-reported frequency of bowel movements, average duration of defecation, if present - nightly excretion, also symptoms associated with defecation such as pain, feeling of incomplete bowel emptying, requirement for digital evacuation or enemas, false urge and overall duration of constipation (years). Each patient's chart was reviewed to obtain missing data and double check data that were extracted from the questionnaires. Complete data on bowel habits and dietary factors were available for 844 study participants.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS version 24.0, SPSS Inc., Chicago, IL, USA). Means and standard deviations were reported for continuous variables. The Chi-squared test was used for the comparison of discrete variables and the Student's t-test for the comparison of continuous variables. Dietary data, alcohol use, tobacco use, NSAID and laxative use, and bowel habits were converted into categorical indictor variables for analyses. Variables found to be associated with CD in the univariate analysis were reassessed using logistic regression analysis to estimate odds ratios (OR) and 95% confidence intervals (CI) while adjusting for age and gender. A p-value ≤ 0.05 was considered as statistically significant.

Ethical Approval

The local Research Ethics committees of each study center approved the study: the Regional Kaunas Ethics Committee (protocol No BE-10-2, issued on 8th of March, 2011), the Research Ethics Committee of the Saarland University (approval 63/11, issued on 10th of May, 2011) and the Research Ethics Committee of the University of Cologne (approval 16–397, issued on 12th of January, 2017). All patients signed an informed consent form to participate in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

RESULTS

The study included 1,333 participants, 635 (47.6%) males with a mean age of 61.89 years and 698 (52.4%) females with a mean age of 62.95 years. Colonic diverticulosis was diagnosed in 858 (64.4%) of the patients while the remaining 475 (35.6%) in whom diverticula were absent were assigned to the control group. Most of the participants were Lithuanians (61.1%) while the remaining 38.8% were Germans: 31.7% from Homburg and 7.1% from Cologne, accordingly (Table I).

Most CD cases (85.8%) had descending or sigmoid colon diverticula. The rest had right sided or pancolonic diverticula (14.1%). During endoscopy the DICA score was evaluated for 523 (60%) patients within the CD group. The majority (93.9%) of cases were graded as DICA I, while the rest (6.1%) were DICA II and none belonged to DICA III class. From 858 participants in the CD group, 198 (23.1%) had previous diverticulitis episodes in their medical history.

Participants with diverticulosis were older and had a higher mean BMI compared to controls (Table I). Patients with diverticulosis were significantly older than patients without diverticulosis (66.4±10.4 years vs. 55.2±14.7 years, p<0.001). There was no significant difference between groups in term of gender; 49.4% of patients in the CD group and 44.4% in the control group were males (p=0.08) (Table I). Likewise, we did not find a significant association between CD and NSAID use. There was no difference between CD and controls with respect to regular alcohol and tobacco consumption (Table I). Multivariate analysis revealed that chances of developing CD increase with age (OR: 1.079, 95%CI: 1.06–1.1, p<0.05). Being overweight or obese (BMI ≥25 kg/m^2) also had increased odds of CD (OR: 1.05, 95%CI: 1.02–1.09, p=0.004) compared with subjects with normal BMI (<25 kg/m^2).

Comprehensive data for dietary and bowel habits together with information about education and employment status was available for 844 study participants. Among them, 523 were from the CD group and the remaining 321 subjects were grouped as controls. Univariate analysis (Table II) showed a significant association between CD and higher education (p=0.004) as well as nocturnal work shifts (p<0.0001). However, these results did not reach statistical significance in the multivariate logistic regression.

Table I. Characteristics of the study population

	Diverticulosis (n=858)	Controls (n=475)	p
Age, years	66.39 ± 10.4 (29-95)	55.24 ± 14.6 (19-92)	**<0.001**
Gender,male/female	424 (49.4%)/434 (50.6%)	211 (44.4%)/264 (55.6%)	0.08
Ethnicity			0.022
German	353 (41.1%)	165 (34.7%)	
Lithuanian	505 (58.9%)	310 (65.3%)	
BMI, kg/m^2	28.75 ± 5.4	27.27 ± 5.1	**<0.001**
Everyday cigarette smoker	300 (35.0%)	168 (35.4%)	0.883
Everyday alcohol user	50 (5.8%)	21 (4.4%)	0.273
NSAID use, ≥ 15 day/month	91 (10.6%)	47 (9.9%)	0.683

Data are expressed as mean ± standard deviation or n (%)

Table II. Association of dietary and bowel habits with diverticulosis

	Controls (n=321)	Diverticulosis (n=523)	p
Vegetarianism	6 (1.9%)	3 (0.6%)	0.076
Number of meals per day, ≥ 3 servings/day	230 (71.7%)	417 (79.9%)	**0.006**
Red meat, ≥ 3 servings/week	32 (10.0%)	41 (7.9%)	0.289
Fish, ≥ 3 servings/week	279 (86.9%)	431 (82.9%)	0.117
Fluids, < 1 liter/day	42 (13.1%)	90 (17.2%)	0.107
Bowel movements, ≤ 1 times/week	13 (4.0%)	5 (1.0%)	**0.003**
Pain with bowel movements, ≥ 25% time	83 (25.9%)	132 (25.3%)	0.846
Feeling of incomplete bowel emptying, ≥ 25% time	95 (29.7%)	247 (47.3%)	**<0.0001**
Abdominal pain, ≥ 25% time	74 (23.1%)	109 (20.9%)	0.444
Prolonged duration of defecation, > 10 min	18 (5.7%)	37 (7.1%)	0.413
Nightly defecation	12 (3.8%)	18 (3.4%)	0.819
Duration of constipation, ≥ 10 years	34 (10.6%)	64 (12.3%)	0.467
Laxative use, ≥ 1 day/week	41 (12.8%)	57 (11.0%)	0.417
Higher education	150 (46.9%)	192 (36.9%)	**0.004**
Night shifts	36 (11.2%)	18 (3.4%)	**<0.0001**

With respect to bowel habits, univariate analysis revealed a significant association between the feeling of incomplete bowel emptying after defecation (p<0.0001) and scarce bowel movements (≤ 1 time/week; p=0.003), and CD. Participants who reported feeling of incomplete bowel emptying ≥25% of the time had increased odds for diverticulosis (OR 2.05; 95%CI: 1.47–2.87) compared to the control group (Table III). Infrequent bowel movements were not associated with an increased prevalence of diverticulosis. Instead, those having less frequent bowel movements (≤1 times/week) had reduced odds compared with individuals with more than 1 bowel movement per week (OR: 0.1; 95%CI: 0.03–0.33). Colonic diverticulosis subjects reported to be having ≥3 meals per day significantly more often (79.9% vs. 71.7%, p=0.006) compared to controls, but it was not associated with increased odds of diverticulosis. The same proportions of cases and controls reported abdominal pain associated with bowel movements (25.9% vs. 25.3%, p=0.846) and there was no significant association between abdominal cramps unrelated to defecation and CD (23.1% vs. 20.9%, p=0.444). Likewise, we found no difference between cases and controls with respect to duration of defecation (5.7% vs. 7.1%, p=0.413) or overall duration of self-reported constipation (10.6% vs. 12.3%, p=0.467). There was no relationship between nocturnal bowel movements and diverticulosis (3.8% vs. 3.4%, p=0.819). There was no association between self-reported regular laxative use and CD (12.8% vs. 11.0%, p=0.417). Red meat and fish consumption, low fluid intake and vegetarian diet also had no association with CD.

Table III. Assessment of risk factors for diverticulosis (multivariate analysis)

	O.R.	95% C.I.	p-value
Age, years	1.079	1.06-1.1	**<0.001**
BMI, > 25 kg/m^2	1.05	1.02-1.09	**0.004**
Feeling of incomplete bowel emptying, ≥ 25% time	2.05	1.47-2.87	**<0.001**
Bowel movements, ≤ 1 times/week	0.1	0.03-0.33	**<0.001**

We also assessed risk factors for developing diverticulitis. In the univariate analysis the risk factors not associated with a higher risk of developing colonic diverticulitis are listed in Table IV. Older participants had reduced odds (OR: 0.921, 95 CI: 0.89–0.95, p<0.05) of diverticulitis compared to younger subjects (Table V). In accordance to prior findings, the feeling of incomplete bowel emptying after defecation was associated with increased odds (OR: 2.769, 95% CI 1.35–5.7, p<0.006) for diverticulitis (Table V). Moreover, participants with a higher educational status had increased odds for diverticulitis compared to the lower education group (Table V).

DISCUSSION

We explored the relationship between environmental and dietary factors, bowel habits and the risk of developing colonic diverticulosis and diverticulitis among participants enrolled in a large multicenter colonoscopy-based study. Our study shows that older age, obesity, frequency of bowel movements and feeling of incomplete bowel emptying were associated with a higher risk of CD. We also found that older age, feeling of incomplete bowel emptying and higher education status were associated with risk of diverticulitis among CD patients. Considering the importance of symptomatic diverticular disease for health care systems worldwide, we believe that our study adds valuable insights in understanding the risk factors for CD and diverticulitis.

The majority of patients in the CD group were diagnosed with left-sided diverticula, which coincides with the published data on individuals of European descent [1, 2]. Our analysis in a large patient cohort clearly showed that prevalence of diverticulosis increased with age. This is in line with previous studies explaining the underlying mechanisms of diverticula formation by a weakening of connective tissue in the colonic wall and/or degenerative changes in the enteric nerves subsequently leading to increased intraluminal pressure, which presumably occurs with aging [5]. The hypothesis of CD as a disorder of intestinal neuromuscular malfunction was further supported by a recent genome-wide association study. The authors demonstrated that certain genetic risk variants may lead to the weakening of the connective tissue in the colonic wall and potential cause the formation of diverticula [23].

Up to now, data on the association between obesity (BMI ≥ 30 kg/m^2) and CD is conflicting. Whereas some authors report that higher BMI increases the risk for developing CD [24-26], others find no such association [27] or propose an increased waist circumference due to visceral and subcutaneous fat collection to be a better predictive factor [28]. Our findings, using a colonoscopy-based cohort, are consistent with earlier studies demonstrating that obese participants (BMI≥30 kg/m2) have increased odds of diverticulosis compared with subjects a normal BMI [24-26]. The cited studies were limited by the lack of information on other important risk factors such as bowel habits and dietary patterns. Therefore, our study which shows a significant association between obesity and CD even after carefully adjusting for confounding factors, adds further emphasis to this link. To explain a mismatch with some published reports, we suggest that previous studies in which no such link was found were limited by either relying on a self-reported diagnosis, possibly leading to diagnostic bias [28] or were conducted in Asian populations in which the prevalence of obesity and diverticulosis is significantly lower [27]. The mechanism by which obesity is associated with colonic diverticula remains unknown. Although the association observed in our study does not imply causation, given the complex relationship between metabolism, the gut microbiota and the immune system [29], nutritional factors linked with BMI most likely have a role in mediating disease progression thus a further research into understanding this pathway is required.

In accordance to previous findings, we found no correlation between diverticulosis and gender. This contrasts with several published reports suggesting higher disease prevalence in males compared to females [30]. However, a recent study found that 40-49 years old women may have lower odds of all types of diverticulosis compared with men of the same age, but the strength of this association tends to decrease with aging [31]. This could indicate that sex hormones in premenopausal

Table IV. Assessment of risk factors for diverticulitis (univariate).

	Prior diverticulitis (n=198)	No diverticulitis (n=660)	p
Age, years	61.27 ± 12.34	67.93 ± 9.21	**0<.001**
Gender, male/female	105 (53.0%)/93 (47.0%)	319 (48.3%)/341 (51.7%)	0.246
BMI, kg/m^2	28.3 ± 6.0	28.88 ± 5.2	0.096
Everyday cigarette smoker	86 (43.4.0%)	214 (32.4%)	**0.004**
Everyday alcohol user	19 (9.6%)	31 (4.7%)	0.01
NSAID use, ≥ 15 day/month	19 (9.6%)	72 (10.9%)	0.599
	Total (n=522)		
	Prior diverticulitis (n=57)	No diverticulitis (n=465)	
Vegetarianism	0 (0.0%)	3 (0.6%)	0.543
Number of meals per day, ≥ 3 servings/day	44 (77.2%)	373 (80.2%)	0.591
Red meat, ≥ 3 servings/week	50 (87.7%)	431 (92.7%)	0.188
Fish, ≥ 3 servings/week	45 (78.9%)	386 (83.4%)	0.403
Fluids, < 1 liter/day	8 (14.0%)	82 (17.6%)	0.497
Bowel movements, ≤ 1 times/week	1 (1.8%)	4 (0.9%)	0.514
Pain with bowel movements, ≥ 25% time	23 (40.4%)	109 (23.5%)	**0.006**
Feeling of incomplete bowel emptying, ≥ 25% time	40 (70.2%)	207 (44.5%)	**<.0001**
Abdominal pain, ≥ 25% time	22 (38.6%)	87 (18.7%)	**<.0001**
Prolonged duration of defecation, > 10 min	6 (10.5%)	31 (6.7%)	0.286
Nightly defecation	3 (5.3%)	15 (3.2%)	0.426
Duration of constipation, ≥ 10 years	4 (7.0%)	60 (12.9%)	0.199
Laxative use, ≥ 1 day/week	8 (14.0%)	49 (10.6%)	0.431
Higher education	31 (54.4%)	161 (34.7%)	**0.004**
Night shifts	3 (5.3%)	15 (3.2%)	0.426

Data are expressed as mean ± standard deviation or n (%)

Table V. Assessment of risk factors for diverticulitis (multivariate)

	OR	95% CI	p
Age, years	0.921	0.89 - 0.95	**<0.0001**
Everyday cigarette smoker	0.516	0.25 - 1.08	0.078
Pain with bowel movements, ≥ 25% time	1.655	0.79 - 3.46	0.181
Feeling of incomplete bowel emptying, ≥ 25% time	2.769	1.35 - 5.7	**0.006**
Abdominal pain, ≥ 25% time	1.608	0.82 - 3.17	0.17
Higher education	2.453	1.31 - 4.59	**0.005**

period might play a protective role in the disease development [31]. Within our cohort the average age of enrolled subjects was over 50 years and no gender-related differences of CD incidence were evidenced.

We consider the analysis of the relationship between the participants' educational and occupational status and the prevalence of CD to be one of the novelties of this study. To our knowledge, up to this date, there is no other large-scale colonoscopy-based analysis addressing this causality to date. Our results showed that, contrary to popular belief, participants in CD group had a lower educational status compared to controls and were not as likely to be working in either sedentary jobs nor night shifts. It is debatable if the average 10-year age difference between study groups might have affected this result or that CD is more prevalent among individuals with a lower socioeconomic status. Although this tendency did not reach statistical significance for increasing the risk of CD in our analysis, we find its importance worth considering. On the other hand, higher educational status related to a more sedentary lifestyle did increase odds for diverticulitis (2.453; 95%CI: 1.31–4.59). Similar to diverticulosis, the etiopathogenesis of diverticulitis is poorly understood. Our finding supports the idea that risk factors for the development of CD are likely different from those related to its complications. Among many factors that may contribute to the development of diverticulitis, studies consistently point towards Western dietary pattern and physical inactivity [15-18]. A recent study has shown that men who adhered to five healthy lifestyle factors (BMI 18-25 kg/m^2, fiber intake > 23 g/day, red meat < 4 servings/week, two hours of exercise/week and no smoking) had a 75% reduced diverticulitis risk compared to men who did not adhere to any healthy lifestyle habits [20]. The hypothesis is that these factors might be associated with gut dysbiosis which in turn may cause mucosal barrier defects and local immune dysfunction resulting in the mucosal inflammation of diverticulitis [32]. Adding to that, genetic data from a genome wide association study demonstrate that risk of diverticulitis might be conferred by genes related to epithelial dysfunction

[23]. Although our data showed no direct association between dietary patterns and the increased risk of diverticulitis, our assumption is that a higher educational status could be viewed as an implication of Western style of living and, therefore, as a support to aforementioned studies.

Our results showed no significant association between daily cigarette smoking and CD. Nevertheless, among subjects with prior diverticulitis, smoking was almost two times more prevalent compared to the asymptomatic group. Although in our study this factor did not reach statistical significance for increasing the odds of diverticulitis, constant smoking is more likely to be associated with symptomatic diverticular disease than asymptomatic diverticulosis in contrast to other studies [14].

We discovered that a low frequency of defecation was associated with a decreased risk of CD. The hypothesis that constipation caused by a "low residual diet" was responsible for the development of colonic diverticula was proposed and widely accepted for over 40 years [33]. Although mostly based on observational data, the hypothesis was strongly supported by evidence of alterations in colonic neurotransmission (in particular vasoactive intestinal peptide) seen in both idiopathic constipation and diverticular disease [34]. However, several recent studies have questioned the association between constipation and diverticulosis stating that an increase in fiber consumption increased the risk of diverticulosis in a dose-dependent fashion [24]. After adjusting for other variables such as age, race, and body mass index, individuals in the highest quartile of total fiber intake had an increased diverticulosis prevalence ratio (1.30; 95%CI: 1.13–1.50) compared to the lowest quartile [24]. In addition, frequent bowel movements were positively associated with diverticulosis, which corresponds well with our findings [24]. Feeling of incomplete bowel emptying after defecation, which is a classical symptom of constipation, showed to be significantly associated with a higher risk for diverticulosis in our analysis. Although rectal tenesmus is primarily associated with constipation, we argue that the experience expressed by our subjects could be explained by a smooth muscle hypersensitization to cholinergic stimulation, previously described by other groups [11, 35, 36]. This alteration to colonic musculature means the reduction in smooth muscle relaxation ability and thus infliction of symptoms. Furthermore, the feeling of incomplete bowel emptying after defecation showed an association not only with asymptomatic but with symptomatic diverticular disease as well, as it further increased odds for diverticulitis. We argue that this could indicate a gradual change in enteric neurotransmission as the disease progresses [11].

In parallel with previously published studies, we found no proof that dietary choices could have an impact on the development of CD. None of dietary elements included in our study turned out to be associated with the disease. It has been demonstrated that red meat consumption is positively associated with the risk of diverticulitis (relative risk 1.2 for each serving of red meat) but not with diverticulosis [16, 37]. A Western dietary pattern (high in red meat and refined grains) was shown to increase the risk of diverticulitis, as opposed to a prudent pattern (high in fruits, vegetables and whole grains) [16, 37], although this proved not to be the case in our analysis.

CONCLUSIONS

Our study evidenced older age, obesity and the feeling of incomplete bowel emptying as risk factors associated with CD. We found that older age, feeling of incomplete bowel emptying and higher education level were associated with the risk of diverticulitis among CD patients.

Conflicts of interest: None to declare.

Authors' contribution: J.I.L. and J.K. conceived and designed the study. All authors contributed to the data acquisition. J.I.L. performed the statistical analysis, interpreted the results and drafted the manuscript. All authors critically revised the manuscript, approved the final version to be published, and agree to be accountable for all aspects of the work.

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Autonomic Neuroscience: Basic and Clinical 216 (2019) 63–71

Contents lists available at ScienceDirect

Autonomic Neuroscience: Basic and Clinical

journal homepage: www.elsevier.com/locate/autneu

Alterations in enteric calcitonin gene-related peptide in patients with colonic diverticular disease
CGRP in diverticular disease

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ARTICLE INFO

Keywords:
CGRP
Colon
CRLR
Diverticular disease
RAMP1

ABSTRACT

Diverticular disease (DD) is one of the most prevalent diseases of the large bowel. Lately, imbalance of neuromuscular transmission has been recognized as a major etiological factor for DD. Neuronal calcitonin gene-related peptide (CGRP) is a potent gastrointestinal smooth muscle relaxant shown to have a widespread effect within the alimentary tract. Nevertheless, CGRPergic innervation of the enteric ganglia has never been considered in the context of motility impairment observed in DD patients.

Changes in CGRP and calcitonin receptor-like receptor (CRLR) abundance within enteric ganglia were investigated in sigmoid samples from symptomatic and asymptomatic DD patients using quantitative fluorescence microscopy. CGRP effect on gastrointestinal smooth muscle was investigated using organ bath technique.

We found CGRP levels within the enteric ganglia to be declined by up to 52% in symptomatic DD patients. Conversely, CRLR within the enteric ganglia was upregulated by 41% in symptomatic DD. Longitudinal smooth muscle displayed an elevated (+10.5%) relaxant effect to the exogenous application of CGRP in colonic strips from symptomatic DD patients. Samples from asymptomatic DD patients consistently showed intermediate values across different experiments.

In conclusion, the present study demonstrates that CGRPergic signaling is subject to alteration in DD. Our results suggest that a hypersensitization mechanism to gradually decreasing levels of CGRP-IR nerve fibers takes place during DD progression. Alterations to CGRPergic signaling in DD disease may have implications for physiological abnormalities associated with colonic DD.

1. Introduction

Formation of a diverticulum, a sac-like protrusion of the mucosa and submucosa through the muscular layers, is regarded as the onset of colonic diverticular disease (DD). With high prevalence in the elderly population, DD is one of the most common diseases of the large bowel (Reichert and Lammert, 2015). Despite its ever-growing burden on the national health systems, high prevalence, and complicated clinical management, over the years DD has drawn relatively little research effort and has repeatedly been named the “neglected disease” in scholarly literature (Kruis et al., 2012; Tursi, 2016).

The etiology of colonic DD is known to be a multifactorial process which involves both environmental factors and genetic predisposition (Von Rahden and Germer, 2012). Furthermore, pathogenesis of DD is accompanied by structural remodeling and functional alterations of the colon. An eminent cause of diverticula formation appears to be altered colonic motility, as DD patients display increased intraluminal pressure profiles in the sigmoid (Arfwidsson et al., 1964; Painter, 1964; Parks and Connell, 1969), along with numerous changes to colonic musculature (Alvarez-Berdugo et al., 2015; Gallego et al., 2013; Hellwig et al.,

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https://doi.org/10.1016/j.autneu.2018.09.006
Received 2 July 2018; Received in revised form 4 August 2018; Accepted 16 September 2018

2014) and the enteric nervous system (ENS) (Bassotti et al., 2015; Böttner et al., 2013; Wedel et al., 2010).

In large mammals the ENS is composed of three distinct intrinsic ganglionated plexuses (the myenteric (MP) – Auerbach's, the outer submucous (OSP) – Schabadasch's, and the inner submucous (ISP) – Meissner's), where MP and OSP maintain intestinal motility, while OSP and ISP governs the epithelial functions (Brehmer et al., 2010; Hansen, 2003; Timmermans et al., 2001).

An array of colonic motor dysfunctions are linked with distinct abnormalities of the ENS (De Giorgio and Camilleri, 2004; Di Nardo et al., 2008). In case of DD, it is established that colonic smooth muscle becomes hypersensitive to cholinergic stimulation (Alvarez-Berdugo et al., 2015; Golder et al., 2003) and loses its capacity to relax to sodium nitroprusside (SNP), a NO donor (Golder et al., 2007). Thus, an imbalance in neuromuscular transmission is suspect to impair gastrointestinal motility observed in DD patients.

Calcitonin gene-related peptide (CGRP) is a potent smooth muscle relaxant involved in multiple physiological processes throughout the body (Russell et al., 2014). CGRP acts through a heteromeric receptor composed of a G-protein coupled receptor called calcitonin receptor-like receptor (CRLR) and a receptor activity-modifying protein 1 (RAMP1) (Hay and Pioszak, 2016). Within the digestive system, CGRP fibers innervate a multitude of targets (epithelia, muscle cells, neuronal elements) (Sternini et al., 1992), providing a morphological basis for the range of biological activities exerted by this peptide. In the alimentary tract CGRP exerts multidirectional action and has a prominent role in sensory and pain conduction (Russell et al., 2014), vasodilation (Nuki et al., 1993), immune response (Assas et al., 2014), absorption and secretory activity (Barada et al., 2000). Major targets of CGRPergic innervation are the intrinsic plexuses (Cottrell et al., 2012; Makowska and Gonkowski, 2018; Sternini et al., 1992), and in animal studies, CGRP was demonstrated to induce peristaltic reflexes (Grider, 1994, 2003; Grider et al., 2006), increase the peristaltic threshold (Holzer et al., 1989), relax intestinal smooth muscle cells (Katsoulis and Conlon, 1989; Takaki et al., 1989), induce phasic contractile activity (Holzer et al., 1989; Maggi et al., 1996, 1997) and excite myenteric neurons (Palmer et al., 1986). Regardless, the importance of CGRP in mediating gastrointestinal motor activity is mostly overlooked in recent reviews.

One downstream target of CRLR activation is neuronal NO synthase (NOS1), known to be subject to alteration in DD (Espin et al., 2014; Golder et al., 2007; Tomita et al., 2000). We hypothesized that CGRP signaling might be affected to counteract changes in NO production in a negative feedback loop. Information about CGRP in the human gastrointestinal tract is limited, and changes in CGRP innervation has never been considered in a context of motility impairment observed in DD. Thus, we set out to investigate CGRP innervation in sigmoid colon samples of DD patients using quantitative fluorescence microscopy and in vitro organ-bath technique.

2. Methods and materials

2.1. Patients and tissue samples

Control samples were obtained from patients undergoing surgery for non-obstructing colorectal carcinoma, who did not have symptoms of clinical motility disorders or previous episodes of symptomatic complicated or uncomplicated DD. This type of operation was a source of both control and asymptomatic diverticular disease (ADD) samples if diverticula were found to be present in these patients. Tissue specimens for the symptomatic diverticular disease (SDD) group were obtained from patients who underwent sigmoid resection or left hemicolectomy for symptomatic DD. Patients were operated after recurrent attacks of diverticulitis by elective surgery (Table 1).

Segments were taken from macroscopically normal regions of colon cancer patients, or in patients with diverticulitis, from the apparently normal area adjacent to the diverticulum. Diverticula containing areas that displayed altered colonic wall anatomy due to transmural mucosal/submucosal outpouchings or signs of inflammation and fibrotic scaring were excluded from tissue sampling. Colon segments were collected in the operating room, immediately placed at 4 °C pre-aerated (95% O_2, 5% CO_2) Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 11 mM glucose).

Table 1
Patient characteristics.

Group	n	Gender	Age, years (range)	BMI (range)
Control	11	5F/6M	64 (35–87)	25.18 (21.18–31.11)
ADD	10	6F/4M	62 (40–76)	27.59 (22.94–33.67)
SDD	10	7F/3M	62 (39–80)	26.11 (22.72–31.64)

ADD – asymptomatic diverticular disease; SDD – symptomatic diverticular disease; BMI – body mass index.

All experimental procedures with the human samples were approved by the Kaunas Regional Biomedical Research Ethics Committee, Kaunas, Lithuania (Permission number: BE-2-10) in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Immunohistochemistry

Following resection, tissue was submerged in 4% PFA solution (Sigma-Aldrich) for 150 min in RT. Later, samples were rinsed 3 × 10 min in PBS (0.01 M) and cut into 10 × 10 mm pieces. Segments were cryoprotected by immersion in 25% sucrose (Sigma-Aldrich) and 0.05% sodium azide (Carl-Roth) solution overnight in 4 °C. Next day, samples were embedded in OCT compound (Shandon™ Cryomatrix™, Thermo Fisher Scientific) and serially sectioned to obtain 16 µm full-thickness sections made along both longitudinal and circular muscle axes, changing the orientation of the mounted sample in the cryostat (CryoStar NX70, Thermo Fisher Scientific, USA). Sections were made no < 1 mm apart from one another to prevent measuring the same ganglia in adjacent sections. Sections were mounted onto microscope slides, air-dried and stored at −20 °C until use.

Sections were rehydrated by successive rinses in PBS and permeabilized in 0.5% Triton X-100 (Carl Roth) and 10% DMSO (Carl Roth) solution for 1 h in RT. Later, samples were rinsed and incubated in 5% NDS (Jackson ImmunoResearch Laboratories) for 1 h to block non-specific binding. Double immunohistochemical staining was carried out by incubating samples in primary antisera (Table 2) overnight at 4 °C. Next day, samples were rinsed in PBS and incubated in an appropriate combination of secondary antibodies (Table 2) for 4 h in RT. For fluorescence quantification experiments, pan-neuronal marker (PGP 9.5) was used in combination with antibodies against CGRP, CRLR or RAMP1. Finally, specimens were rinsed in PBS and cover-slipped using Vectashield® mounting medium (Vector Laboratories, USA).

Double staining with primary antibodies from the same host species was performed as detailed by Balen et al. (2008). Briefly, samples were incubated (in sequence) with first primary antibody (either *rb* anti-CRLR or *rb* anti-RAMP1, 1:500) overnight in 4 °C, rinsed in PBS and incubated with first secondary antibody (anti-*rb* Cy3, 1:500) for 4 h in RT. Later, samples were rinsed in PBS and incubated with the second primary antibody (*rb* anti-VIP, 1:1000) overnight at 4 °C. Next day, samples were rinsed in PBS and incubated for 20 min in second secondary antibody (anti-*rb* AF488, 1:50).

For all antibodies used in this study, negative controls were processed as outlined above except that either the primary or the secondary antibody was omitted. In all trials this eliminated detection of histofluorescence. Single positive controls were processed for all primary antibodies used in the study. In all trials, the signal of single positive controls was indistinguishable to that of experimental samples.

Table 2
Primary and secondary antibodies used in the study.

Antigen	Host	Type	Dilution	Source	Cat. #
Primary					
CGRP	Mouse	Monoclonal	1:1000	Abcam[a]	Ab 10987
RAMP1	Rabbit	Polyclonal	1:1000	Bioss[b]	BS-1567R
CRLR	Rabbit	Polyclonal	1:1000	Bioss[b]	BS-1860R
PGP 9.5	Mouse	Monoclonal	1:1000	Abcam[a]	Ab 72911
PGP 9.5	Rabbit	Polyclonal	1:1000	Bio-Rad[c]	7863-0504
NOS1	Rabbit	Monoclonal	1:1000	Abcam[a]	EP 1855Y
NOS1	Mouse	Monoclonal	1:500	Santa Cruz[d]	SC-5302
VIP	Rabbit	Polyclonal	1:1000	Chemicon[e]	AB982
Secondary					
Anti-Rabbit Cy3	Donkey	Polyclonal	1:500	Millipore[f]	AP182C
Rabbit AF488	Donkey	Polyclonal	1:500	Invitrogen[g]	A21206
Mouse Cy3	Donkey	Polyclonal	1:500	Millipore[f]	AP192C
Mouse AF488	Donkey	Polyclonal	1:300	Invitrogen[g]	A21202

[a] Abcam, Cambridge, UK.
[b] Bioss Antibodies Inc., Woburn, Massachusetts, USA.
[c] Bio-Rad (Formerly AbD Serotec), Kidlington, UK.
[d] Santa Cruz biotechonology, Dallas, Texas, USA.
[e] Chemicon International, Temecula, California, USA.
[f] Millipore Corp., Temecula, California, USA.
[g] Invitrogen, Ltd., Paisley, UK.

A full list of the chemicals and reagents used in the study can be found in the supplement material Table A.1.

2.3. Microscopy and image analysis

Amount of CGRP-IR nerve fibers and CRLR/RAMP1-IR neuronal somata and nerve fibers in the enteric ganglia was estimated using quantitative fluorescence microscopy method in accordance with Waters (2009). Samples were kept in the dark at 4 °C. Images were acquired no later than one week following the sample preparation to avoid fluorophore bleaching. Fluorescent images of intrinsic neural plexuses were acquired using Zeiss AxioImager Z1 wide-field microscope (Carl Zeiss, Germany) equipped with AxioCam MRm Rev.3 digital camera. The objective used in the study was 40×/0.9 EC Plan NeoFluar and images were captured using AxioVision Rel.4.8.2 software (Carl Zeiss, Germany). The fluorescent light source was HXP 120 V illuminator using 38HE (EX 470/40, EM 525/50) and 43HE (EX 550/25, EM 605/70) filter set.

Images of enteric ganglia of myenteric (Auerbach), outer submucosal (Schabadasch) and inner submucosal (Meissner) plexuses were captured in three dimensions throughout the section. Varying number of images were made that would capture 10–20 autonomic ganglia of each enteric plexus (this was done due to smaller size of the submucosal ganglia; since magnification (objective) was kept constant, some images contained several submucosal ganglia leading to a fewer images being made for that neural plexus). Each image consisted of a Z-projection composed of 10 focal planes (2-D images) made at 1 μm increment. Exposure interval that would not result in pixel saturation was selected during the pilot experiments. Images were taken at a fixed exposure time (800 ms) for the channel of interest for all images used for quantification.

It must be noted that CGRP-IR signal investigated in the present study represents primary afferent nerve fibers of either intrinsic or extrinsic origin, composed of numerous smooth and varicose axons, and intraganglionic laminar endings (IGLEs) (Furness et al., 2004). IGLEs are complex branching nerve endings that give rise to flat (laminar) expansions within the enteric ganglia (Zagorodnyuk et al., 2001).

Image analysis was performed in ImageJ/Fiji software. In brief, images were loaded into Fiji as a stack and Z-projected using average intensity projection. Fluorescence intensity was determined by selecting the region of interest (ROI) in PGP 9.5 signal view (selecting boundaries of the enteric ganglion) and measuring the densitometric fluorescence intensity (IntDen) within the ROI in the channel of interest (containing CGRP/RAMP1/CRLR signal). The obtained value was background corrected by subtracting the size of ROI multiplied by the average background intensity value of the image. Since CGRP-IR neurons occurred inconsistently and were absent in majority of the samples, ganglia containing CGRP-IR neuronal bodies were excluded from the analysis. This was done to prevent irregular sampling due to the large proportional area of CGRP-IR cell bodies that would positively shift FI measurements.

To correct for potential inconsistencies of the fluorescent lighting and optical shading effects, the resulting intensity values are reported as a fraction of a fluorescence standard as described by Model and Burkhardt (2001) Rose Bengal (Sigma-Aldrich) (0.25 g mL^{-1}) was used as a fluorescence standard and a series of images of this reference were made each time before imaging the colonic sections. The original (background corrected) intensity value was divided by the average reference value of the fluorescent standard to obtain the final normalized fluorescence intensity value in arbitrary units (AU).

Images for illustrations were generated using Zeiss LSM 700 laser-scanning confocal microscope equipped with a dual T-PMT sensor and using 40×/1.4 Plan Apochromat and 60×/1.46 αPlan Apochromat oil immersion objectives in ZEN Black SP1 2010 software (Carl Zeiss, Germany). Confocal images were processed into final figures by adjusting image size, brightness, and contrast using Photoshop CS6 (Adobe Systems, San Jose, USA).

2.4. In vitro contractility experiments

Following resection, tissue was placed in cold-aerated Krebs-Henseleit solution (4 °C) where the musculature was separated from the mucosa. Then, colonic musculature was cut into 2 × 10 mm strips orientated along longitudinal or circular muscle axes. Intestinal muscle strips were submerged in warm (37 °C) aerated Krebs-Henseleit solution in individual 25 mL wells (Radnoti organ bath, AD instruments Pty, AU) anchored by a metallic hook at the lower end and attached by silk suture to a force transducer for isometric recording of muscular activity (PowerLab®, AD Instruments Pty). The muscular strips were pre-stretched to a passive tension of 35–40 mN and allowed to equilibrate for at least 1 h, replacing Krebs-Henseleit solution every 15 min, until a stable baseline was established.

Bethanechol (10^{-4} M) was added and a contact period of up to 5 min was allowed to obtain a maximal reference contraction. Human CGRP (10^{-7} M) was then added and a contact period of 15 min was allowed until the relaxation response would plateau. Then SNP (10^{-3} M) was added to induce maximal reference relaxation response. In a subset of experiments, tetrodotoxin (10^{-7} M) (TTX) were administered 15 min before the addition of bethanechol.

Relaxation response to CGRP was calculated within the range of maximal reference contraction and maximal reference relaxation for every recording. The magnitude of the response is expressed as a percentage of the maximal reference relaxation to SNP. Untreated strips were run in parallel with strips subjected to investigational compounds. Each strip was only subjected to a single set of drugs used in experimental protocol and all strips at one experimental run were taken from the same individual.

2.5. Statistical analysis

The distribution of data was inspected using histograms and data normality was confirmed with the Shapiro-Wilk test. Differences in the data were assessed using unpaired Student's *t*-test with Welch's correction or Mann–Whitney *U* test where appropriate (GraphPad Prism 6, San Diego, USA). $p < 0.05$ was used as a threshold for statistical significance. Data are presented as mean ± SEM if not stated otherwise. Changes in CGRP, CRLR and RAMP1 abundance is expressed as ± %

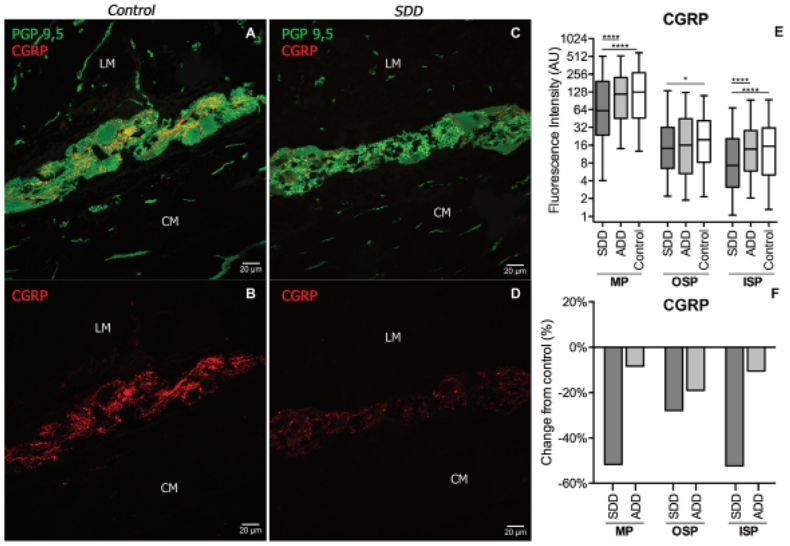

Fig. 1. CGRP-IR nerve fibers in a myenteric ganglion of DD patients. (A–D) Confocal micrographs demonstrating differential abundance of CGRP-IR fibers within myenteric ganglion of control (A–B) and SDD (C–D) patients. (E) Quantification of FI within the ENS of DD patients. Whiskers – 5–95th percentile. *–p < 0.05, ****–p < 0.001 (Mann-Whitney *U* test). Log2 scale. (F) Percentage change from median control value. LM – longitudinal muscles, CM – circular muscles.

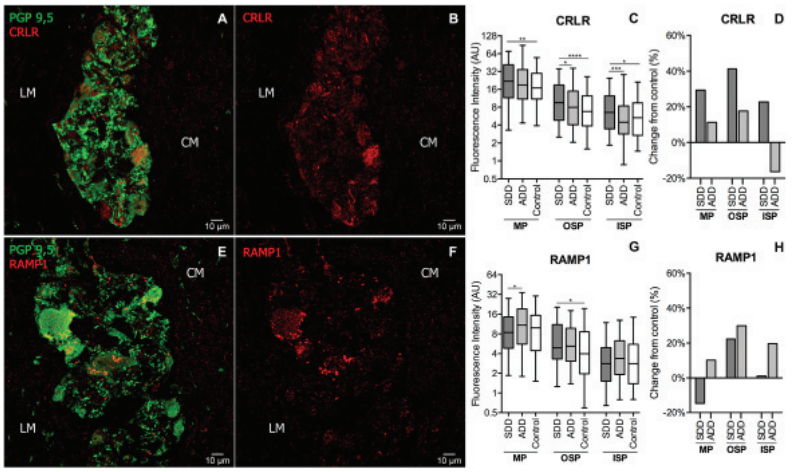

Fig. 2. CRLR-IR and RAMP1-IR nerve structures within the enteric ganglia of DD patients. (A–B, E–F) Confocal micrographs showing CRLR and RAMP1 within the myenteric ganglia. Note the highest accumulations of CRLR-IR and RAMP1-IR fluorescence signal in the neuronal somata and in lesser amount dispersed in the neuropil. (C–D) Quantification of CRLR-IR structures FI within the MP, OSP and ISP ganglia of DD patients. (G–H) Quantification of RAMP1-IR structures abundance within the ENS of DD patients. Whiskers – 5–95 percentile. *–p < 0.05, **–p < 0.01, ***–p < 0.005, ****–p < 0.001 (Mann-Whitney *U* test). Log2 scale. (D, H) Percentage change from median control value. LM – longitudinal muscles, CM – circular muscles.

from median control value.

3. Results

3.1. CGRP is decreased in SDD

CGRP-IR nerve fibers were situated throughout the colonic sections with the primary target of innervation being the intrinsic plexuses. The majority of CGRP-IR fibers were located in the vicinity of enteric neurons within the myenteric (MP) and both submucosal plexuses (OSP, ISP). CGRP-IR fibers entangled intestinal glands and abundantly innervated the mucosa, whereas only sparse, minute CGRP-IR nerve fibers were located in the muscular layers (Fig. 1B).

Seldom, enteric ganglia contained CGRP-IR neurons in which case that ganglion was excluded from further analysis (Figs. A.1–A.2).

In control samples, CGRP-IR nerve fibers were six-fold more abundant in the ganglia of MP compared to SP ganglia. The lowest fluorescence intensity levels of CGRP-IR nerve fibers were found in the ISP. The levels were similar within the enteric ganglia of the OSP. (Fig. 1E, Table A.2). The ratio of CGRP-IR nerve fibers between MP, OSP and ISP plexuses was constant between the experimental groups.

Quantification of fluorescence intensity revealed that abundance of CGRP-IR nerve fibers to be decreased within the enteric ganglia of sigmoid colon of DD patients (Fig. 1). This change was most evident within the MP where CGRP-IR nerve fibers amount in SDD patients was half that of the control group (Fig. 1A–D). When we compared relative change from median control value (Δ), we found that in MP ganglia CGRP-IR nerve fibers were decreased by 51.7% ($p < 0.0001$, Mann-Whitney *U* test) in SDD.

While that in OSP and ISP, CGRP-IR nerve fibers decreased by 27.8% ($p = 0.04$) and 52.4% ($p < 0.0001$), respectively. ADD patients had intermediate values throughout the enteric plexuses (MP: Δ-8.31%, $p = 0.326$; OSP: Δ-19%, $p = 0.407$; ISP: Δ-10.5%, $p = 0.804$) that did not differ from that of control (Fig. 1E–F, Table A.2).

3.2. CRLR-IR nerve structures are increased in SDD

CRLR-IR structures had a granular appearance, with the majority of the signal contained within neuronal cell bodies (Fig. 2A–B). In control samples, CRLR-IR nerve structures were was 2.5 times more abundant in the MP compared to SP and similar in quantity between OSP and ISP (Fig. 2C). In this regard, abundance of CRLR-IR nerve structures was similar to that of amount of CGRP-IR nerve fibers.

Amount of CRLR-IR structures was increased within the enteric ganglia of SDD patients (Fig. 2C–D, Table A.2). Compared to CGRP-IR nerve fibers, this reflected an opposite trend between the experimental groups. The greatest increase of 41.3% ($p < 0.0001$) was found in the OSP, whilst in MP and ISP amount of CRLR-IR nerve structures was increased by 29.3% ($p = 0.008$) and 22.7% ($p = 0.022$), respectively. Abundance of CRLR-IR structures in ADD (MP: Δ + 11.3%, $p = 0.078$; OSP: Δ + 17.6%, $p = 0.415$; ISP: Δ-16.3%, $p = 0.243$) again had intermediate values across the experimental groups.

Signal of RAMP1-IR structures mirrored CRLR-IR with regards to its localization. We found expression of RAMP1-IR structures not to differ statistically between SDD and control patients within MP and ISP ganglia (MP: −14.7%, $p = 0.67$; ISP: Δ + 0.11%, $p = 0.81$) (Fig. 2G–H, Table A.2), whereas that in OSP was increased by 22.3% ($p = 0.04$).

3.3. Relaxation response to CGRP is increased in longitudinal muscle of SDD patients

Exogenous application of CGRP induced a tonic relaxation response in both circular and longitudinal smooth muscle strips of the human sigmoid colon (Fig. 3A). We have observed no phasic contractile activity induced by CGRP application. Application of TTX had no effect on the CGRP induced response. Longitudinal muscles of DD patients displayed an increased relaxation response to exogenous CGRP stimulation (Fig. 3A–B, Table A.3). In control human sigmoid colon samples CGRP induced 69.5% of maximum reference relaxation in longitudinal muscle and 73% in circular muscle (Fig. 3B). CGRP induced relaxation was increased by 4.54% ($p = 0.677$) (unpaired Welch's *t*-test) and 10.5% ($p = 0.033$) in ADD and SDD longitudinal smooth muscle strips, respectively. CGRP induced relaxation was decreased in the circular muscle layer (SDD: −2.44%, $p = 0.536$; ADD: −6.99%, $p = 0.149$) but did not differ statistically between the experimental groups.

3.4. Association between CGRP and NOS1/VIP

To elucidate the role of CGRP in smooth muscle relaxation we performed double immunohistochemical staining for CGRP, CRLR or RAMP1 and either NOS1 or VIP (Figs. 4, 5). CGRP-IR fibers were found in close association with both NOS1-IR and VIP-IR neurons within the enteric ganglia of human sigmoid colon samples. Both VIP-inergic and nitrergic neurons within human ENS were found to express CRLR (Figs. 4C–D, 5C–D) and RAMP1 thus subject to CGRP activation. NOS1-IR neurons were predominantly located within the MP, whereas VIP-IR neurons were predominantly situated throughout OSP and ISP ganglia.

4. Discussion

The key findings of the present study are that CGRPergic signaling is subjected to alteration in DD where (1) the amount of CGRP-IR nerve fibers is decreased and (2) CRLR-IR structures are increased within the enteric ganglia of the sigmoid colon. (3) Moreover, the longitudinal smooth muscle of the sigmoid colon displays an elevated response to exogenous application of CGRP in DD patients.

CGRP is involved in an array of physiological processes throughout the alimentary tract. These include a role in nociception, immune response (Assas et al., 2014), secretion (Ahren and Pettersson, 1990; Esfandyari et al., 2000) and gastrointestinal motility (Grider, 1994; Maggi et al., 1997). Therefore, declining levels of CGRP may be suspected to consequence in a range of detrimental downstream effects within the GI tract. It must be noted that the present study was designed with an emphasis on CGRP involvement in smooth muscle relaxation, but effects on other associated functions cannot be out ruled.

To elucidate the scope of these effects, we investigated the abundance of our proteins of interest within different plexuses of the ENS, i.e., myenteric (MP), outer (OSP) and inner (ISP) submucosal plexuses. Apposed to experimental rodents, large mammals harbour two interconnected but morphologically and functionally distinct intrinsic submucous networks: one located close to the muscularis mucosae (ISP), the other near the circular layer of the external muscle (OSP) (Timmermans et al., 2001). Main distinction being the distribution pattern and neurochemical profile of the ganglionic cells, it is regarded that MP and OSP together maintain the intestinal motility, while ISP governs the absorption/secretory function (Brehmer et al., 2010). Some authors suggest that in the human intestine, even a third intermediate submucous nerve network can be discerned (Hoyle and Burnstock, 1989). It must be noted, neurochemical similarities between the intermediate plexus and either the inner or outer plexus in the human small and large intestine, appear to be region-dependent (Timmermans et al., 2001), second to being difficult to identify morphologically (Brehmer et al., 2010). For this reason, intermediate submucosal plexus was not investigated in the present study.

A semi-quantitative approach used in this study allowed estimation of protein levels with precise anatomical localization and enabled quantification of differential protein expression within different ENS plexuses in the same sample. This revealed the primary target of CGRP innervation to be the MP. The ratio of abundance of CGRP-IR nerve fibers between different plexuses was consistent between experimental groups. In our view, these results reflect (at least by quantitative means) CGRP involvement in distinct functional roles associated with MP and

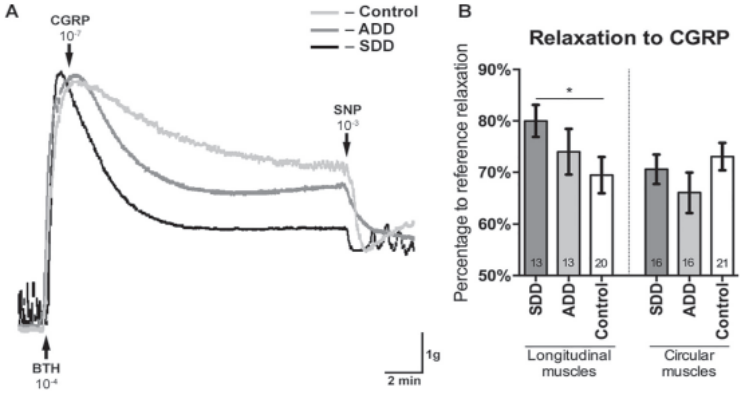

Fig. 3. Smooth muscle relaxation to CGRP. (A) Representative curves demonstrating CGRP induced tonic relaxation of longitudinal smooth muscle. (B) Quantification of relaxation response to CGRP. *–p = 0.033 (unpaired Welch's *t*-test). Mean ± SEM. Numbers indicate repeats. n(SDD) = 6, n(ADD) = 6, n (Control) = 10.

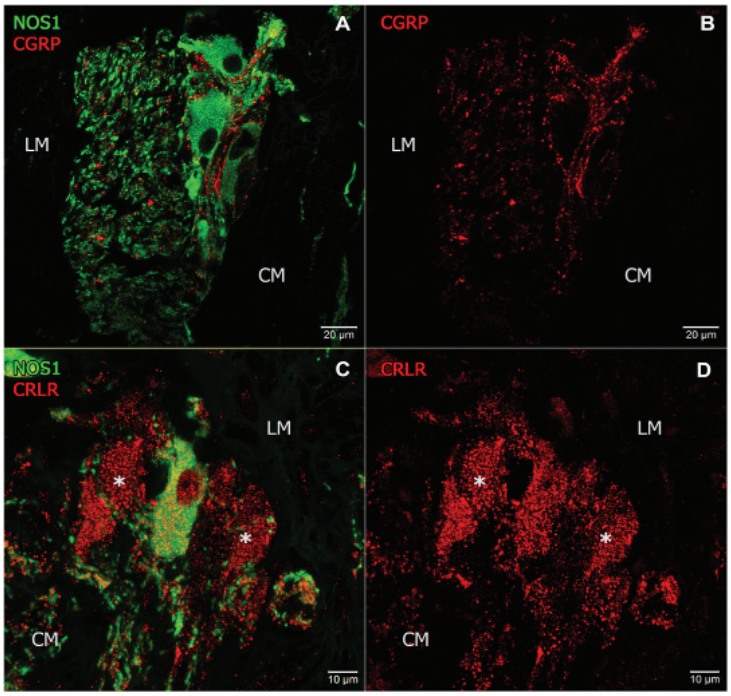

Fig. 4. CGRP-IR nerve fibers innervating nitrergic components within ENS. (A–B) CGRP-IR fibers entangling nitrergic neurons within myenteric ganglion. (C–D) CLRL-IR structures in the myenteric ganglion are mainly accumulated in the neuronal somata and in lesser extent in the ganglion neuropil. Note that some neuronal somata (*) are strongly positive for CRLR, but are not labelled by NOS1. LM – longitudinal muscles, CM – circular muscles.

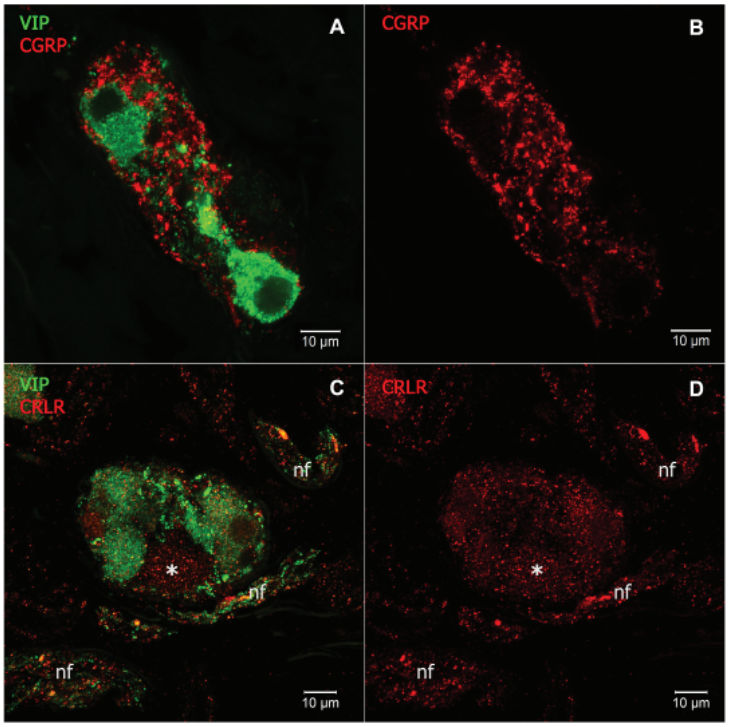

Fig. 5. CGRP-IR nerve fibers innervating VIP-IR components within ENS. (A–B) CGRP-IR fibers in close association with VIP-IR neurons residing in the submucosal plexus. (C–D) CRLR-IR structures in the submucosal ganglion are mainly accumulated in the neuronal somata and in lesser extent in the ganglion neuropil. Note that some neuronal somata (*) are positive for CRLR, but are not labelled by VIP. LM – longitudinal muscles, CM – circular muscles, nf – nerve fibers.

SP ganglia.

In the present study, CGRP-IR nerve fibers innervated the colonic tissue as previously described in canines (Sternini et al., 1992), but different than that detailed in the human small intestine (Timmermans et al., 1992) or swine descending colon (Makowska et al., 2017; Makowska and Gonkowski, 2018). In regards that we rarely detected CGRP-IR ganglionic cells and never identified a ganglion composed solely of CGRP-IR neurons as detailed by Timmermans et al. (1992). Similarly, descending colon of a swine model has been reported to contain numerous CGRP-IR neurons which made up to 25% of the total cell count in the MP, and up to 40% in the ISP (Makowska et al., 2017). This suggests that the exact function of CGRP may not only be region dependent, but also display a degree of interspecific differences. Whereas CRLR signal was contained within the neuronal cell bodies of all enteric ganglia as previously described by Cottrell (Cottrell et al., 2012).

CGRP is involved in the generation of GI motility patterns in a number of ways. Extrinsic and intrinsic sensory pathways which mediate peristaltic response to muscle stretch and mucosal stimulation were shown to utilize CGRP as a sensory neurotransmitter (Grider, 1994). Mechanism of this effect was elucidated showing that the peristaltic reflex is initiated by mucosal release of 5-HT and subsequent activation of 5-HT4 receptors on CGRP sensory neurons that in turn relay the signal via interneurons to VIP/NOS inhibitory and acetylcholine/tachykinin excitatory motor neurons (Grider, 2003; Grider and Piland, 2007). Potentially, declined levels of CGRP could interfere with this pathway leading to an imbalance of stimulatory and inhibitory input driving the peristaltic reflex in DD. Finding of high amount of CGRP-IR nerve fibers within the MP is consistent with functional studies showing CGRP role in gastrointestinal motility. Conversely, a profound decline of CGRP-IR nerve fibers in the MP ganglia of DD patients would be consistent with the altered motor function associated with DD. Moreover, Holzer reported that CGRP inhibited peristalsis by increasing the intraluminal pressure required to trigger peristaltic waves in the guinea pig (Holzer et al., 1989). Thus it could be hypothesized that reduction in CGRP expression could lead to lowering of the peristaltic threshold eliciting enhanced propagated high-amplitude contractions, intraluminal high-pressure zones, and excessive segmental contractions observed in the colonic musculature of DD patients (Bassotti et al., 2001, 2005; Painter, 1964; Parks and Connell, 1969). However, this hypothesis requires further functional assessment that could not be provided in the present study.

Given that CGRP is a potent smooth muscle relaxant (Katsoulis and Conlon, 1989; Maggi et al., 1996; Takaki et al., 1989) we hypothesized that declined levels of CGRP-IR nerve fibers observed in DD could have a direct effect on colonic smooth muscle relaxation. Smooth muscle contractility results revealed an elevated relaxant effect of CGRP in the longitudinal smooth muscle strips of sigmoid colon of SDD patients.

This could be explained by our subsequent finding that amount of CRLR-IR structures was upregulated in the enteric ganglia of SDD patients. Taken together these findings suggest that a local hypersensitization mechanism might take place to counteract gradually increasing smooth muscle tone. Moreover, ADD patients had transitional values across the experimental groups thus a gradual decrease of CGRP could be anticipated. This would imply that decreasing levels of CGRP-IR nerve fibers might play a role in the progression of DD and coincide with the emergence of DD symptoms. This trend was present across all enteric plexuses investigated in the study.

CGRP acts through a heteromeric receptor composed of a G-protein coupled receptor (CRLR) and a receptor activity-modifying protein 1 (RAMP1). RAMPs are required to chaperone CRLR to the cell surface, and heteromeric RAMP1-CRLR complex act as receptors for CGRP (Hay and Pioszak, 2016). We hypothesized that sensitization for CGRP could take place by upregulation of either CRLR or RAMP1. However, in the present study we did not find any clear trend in RAMP1 expression within MP and ISP ganglia of DD patients. However, RAMP1-IR structures were upregulated in the OSP which was in line with increased abundance of CRLR-IR structures indicating an increase of both components necessary for CGRP sensitization.

Both VIP and NO are the primary relaxant agents of the intestinal musculature (Van Geldre and Lefebvre, 2004). In the vasculature, CGRP receptor activation precedes NO release (Russell et al., 2014). Potentially, an analogous mechanism of relaxation is employed within the GI tract. Here, we immunohistochemically demonstrate CGRP-IR nerve fibers to be in close association with VIP-ergic and nitrergic components, suggesting that the relaxant effect of CGRP may act through downstream NOS1-IR and VIP-IR neuronal activation. Furthermore, differential localization of NOS1-IR and VIP-IR neurons within the ENS may suggest a different mechanism of relaxation/inhibition by which MP and OSP govern GI motility. Although, in the present study the CGRP induced relaxation response was TTX insensitive. Previous studies reported TTX to enhance the relaxant effect of CGRP by excitatory component inhibition (Maggi et al., 1996). These results suggest that smooth muscle relaxation reported in the present study might be due to secondary activation of ATP-sensitive potassium channels located on smooth muscle cells (Russell et al., 2014).

It is known that enteric neuropathies tend to preferentially affect neurons containing NOS1 (Rivera et al., 2011). It was demonstrated that enteric neuropathy is an underlying trait of DD (Wedel et al., 2010), and several other studies showed altered NOS1 expression and contractility responses to NO in SDD (Golder et al., 2007; Tomita et al., 2000). Furthermore, Espin reported increased NO-mediated responses together with upregulated NOS1 mRNA levels in ADD (Espin et al., 2014). These findings suggest that NOS1 overexpression at the early stages of DD may lead to excessive production of NO and commence neuropathic processes within the ENS. This explanation is coherent with a number of studies reporting other neurotransmitters to be declined in DD (Böttner et al., 2013; Golder et al., 2003, 2007; Tomita et al., 1993). However, the significance of any given ENS component for the intestinal motor disturbances observed in DD patients remains elusive as the detrimental effects of all associated factors are likely to take place simultaneously. It is feasible that decreased amount of CGRP-IR nerve fibers may reflect an overall trend of gut denervation in DD, further establishing an imbalance of neuro-muscular transmission as a major etiological factor of colonic DD.

It has been suggested that nerve tissue remodeling and neuropathic processes observed in DD are driven by inflammation of the enteric plexuses (Bassotti et al., 2015; Humes et al., 2012; Simpson et al., 2009; Wedel et al., 2010). In this study, SDD patients were operated on after recurrent episodes of diverticulitis thus it can be argued that the decrease in the abundance of CGRP-IR nerve fibers within the enteric ganglia is evoked by inflammatory processes of the diseased state. It is known that inflammation initially destroys nerves endings, and may lead to long term changes in gene expression and protein levels, which may represent changes observed in the present study. That being said, it must be noted that these considerations warrants additional experiments to reveal mechanistic basis of these findings.

Limitation of the present study was restricted functional assessment of the array of physiological responses mediated by CGRP. Here we were only able to investigate the relaxant effect elicited by CGRP and further studies elucidating alternative mechanisms are requisite.

5. Conclusions

Taken together, our results show that CGRP signaling is subject to alteration in symptomatic DD disease. These findings further demonstrate that an imbalance in neuro-muscular transmission is a major etiological factor of colonic DD. Lastly, our results suggest that CGRP is an important molecular mediator in DD and may have further implications for targeted disease prevention or treatment strategies which need to be evaluated in the future.

Acknowledgments

This study was generously supported by the Research Council of Lithuania (Grant SEN-06/2015/PRM15/135). Authors would like to thank Professor Aidas Alaburda, Josef Sundström and Justas Zilinskas for their support and contribution to the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.autneu.2018.09.006.

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Abbreviations

ADD: asymptomatic diverticular disease
CGRP: calcitonin gene-related peptide
CRLR: calcitonin receptor-like receptor
DD: diverticular disease
ENS: enteric nervous system
FI: fluorescence intensity
IR: immunoreactive
ISP: inner submucosal (Meissner's) plexus
MP: myenteric (Auerbach's) plexus
NO: nitric oxide
NOS1: neuronal nitric oxide synthase
OSP: outer submucosal (Schabadasch's) plexus
PGP 9.5: protein gene-product 9.5 (pan-neuronal marker)
RAMP1: receptor activity modifying protein 1
SDD: symptomatic diverticular disease (diverticulitis)
SNP: sodium nitroprusside
TTX: tetrodotoxin
VIP: vasoactive intestinal peptide

Histol Histopathol (2020) 35: 147-157

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Ultrastructural changes of the human enteric nervous system and interstitial cells of Cajal in diverticular disease

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Summary. Background. In spite of numerous advances in understanding diverticular disease, its pathogenesis remains one of the main problems to be solved. We aimed to investigate the ultrastructural changes of the enteric nervous system in unaffected individuals, in asymptomatic patients with diverticulosis and in patients with diverticular disease.

Methods. Transmission electron microscopy was used to analyse samples of the myenteric, outer submucosal and inner submucosal plexuses from patients without diverticula (n=9), asymptomatic patients with diverticulosis (n=7) and in patients with complicated diverticular disease (n=9). We described the structure of ganglia, interstitial cells of Cajal and enteric nerves, as well as their relationship with each other. The distribution and size of nerve processes were analysed quantitatively.

Results. In complicated diverticular disease, neurons exhibited larger lipofuscin-like inclusions, their membranous organelles had larger cisterns and the nucleus showed deeper indentations. Nerve remodeling occurred in every plexus, characterised by an increased percentage of swollen and fine neurites. Interstitial cells of Cajal had looser contacts with the surrounding cells and showed cytoplasmic depletion and proliferation of the rough endoplasmic reticulum. In asymptomatic patients with diverticulosis, alterations of enteric nerves and ICC were less pronounced.

Conclusions. In conclusion, the present findings suggest that most ultrastructural changes of the enteric nervous system occur in complicated diverticular disease. The changes are compatible with damage to the enteric nervous system and reactive remodeling of enteric ganglia, nerves and interstitial cells of Cajal. Disrupted architecture of enteric plexuses might explain clinical and pathophysiological changes associated with diverticular disease.

Key words: Diverticular disease, Colon, TEM, ENS, Interstitial cells of Cajal

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DOI: 10.14670/HH-18-136

Introduction

Colonic diverticula are herniations of the mucosa and submucosa through weak points in the muscular layer of the colon (Meyers et al., 1973). In most cases, the presence of diverticula (also known as diverticulosis) causes neither symptoms nor complications (Strate et al., 2012). However, diverticulosis becomes more frequent with age and 20% of affected individuals develop symptomatic uncomplicated diverticular disease (Everhart and Ruhl, 2009; Feuerstein and Falchuk, 2016). Moreover, the disease in some patients will advance to complicated diverticular disease (CDD),

Abbreviations. CDD, complicated diverticular disease; ICC, interstitial cells of Cajal; ICC-MY, interstitial cells of Cajal around the myenteric plexus; ICC-SM, interstitial cells of Cajal around the submucosal plexus.

causing acute or chronic diverticulitis and subsequently leading to lower quality of life, hospital admissions, surgical intervention or even death (Unit, 2003).

During the last decade a number of studies have identified that obesity, use of NSAIDs, smoking and genetic predisposition are definite risk factors for the disease (Schieffer et al., 2018). Additionally, a substantial amount of neuromuscular changes have been documented in diverticular disease. There is a decrease in the number and size of enteric ganglia (Simpson et al., 2009), a decrease in neuron numbers (Deduchovas et al., 2008) and changes in innervation patterns of the colonic musculature (Golder et al., 2003; Böttner and Wedel, 2012). Interstitial cells of Cajal (ICC), whose role is to receive regulatory inputs from the enteric nervous system and generate electrical activity and motor coordination (Sanders et al., 2014), decrease in number too (Bassotti et al., 2005a). Finally, the muscular layer of the colon shows disturbed ultrastructural architecture of smooth muscle cells (Hellwig et al., 2014), as well as increased collagen deposits in longitudinal muscle (Pantaroto et al., 2015). Based on these changes, diverticular disease is classified as a subtype of enteric myopathy characterised by muscular degeneration and smooth muscle cell myofilament deficiency (Knowles et al., 2010).

The pathogenesis of diverticular disease remains one of the main problems to be solved. A number of morphological studies have compared unaffected individuals to patients with complicated diverticular disease or diverticulitis. Regardless of numerous documented changes associated with diverticular disease, the causality of these changes remains mostly unaddressed. For this reason, multiple reviews conclude that the etiology of diverticular disease has not been understood completely (Tursi, 2016; Rezapour et al., 2018; Schieffer et al., 2018).

An ultrastructural study would offer further insight into the changes that occur on a larger level, such as enteric neuropathy (Wedel et al., 2010) or myenteric plexitis (Bassotti et al., 2015). Ultrastructural studies on Crohn's disease have shown that enteric nerves and ICC sustain damage (Dvorak and Silen, 1985; Wang et al., 2007). Furthermore, experimental studies have also found that morphological ICC changes during and after infection are only visible using electron microscopy (Wang et al., 2002). Considering the evidence that inflammatory bowel diseases and diverticular disease show overlap (Collins and Winter, 2015), the possibility of previously undocumented changes of the enteric nervous system and ICC is likely.

We aimed to investigate the ultrastructural changes of the enteric nervous system and ICC. To better understand the timing of these changes, we investigated the enteric nervous system in unaffected individuals, in patients with asymptomatic diverticulosis and in patients with CDD. We employed transmission electron microscopy to analyse the morphology of the three main ganglionated - myenteric, outer submucosal and inner submucosal - plexuses of the enteric nervous system and the ICC around them.

Materials and methods

Patients

Colon samples from each of 25 patients were received from the Department of Surgery at the Lithuanian University of Health Sciences Kaunas Clinics between December 2015 and May 2017. 16 patients were operated on for non-obstructing colon cancer: 9 patients did not have macroscopic diverticula (control), 7 patients had macroscopic diverticula (asymptomatic diverticulosis, later referred to as diverticulosis). 9 were electively operated on for recurrent episodes of diverticulitis (complicated diverticular disease).

Patients with previous or current irritable bowel syndrome or inflammatory bowel disease were excluded from the study. One patient in the diverticulosis group had received chemotherapy six months before the operation. Among patients with complicated diverticular disease, the median duration of symptoms was 16 months (range 2-72 months). Study group characteristics are described in Table 1.

The experimental procedures were approved by the Kaunas Regional Biomedical Research Ethics Committee, Kaunas, Lithuania (code BE-2-10). The study was performed in accordance with the ethical standards of the Declaration of Helsinki. All patients gave written informed consent prior to their inclusion in the study.

Tissue processing

1-5 cm length circular segments of the sigmoid colon were taken from the distal resection margin without any signs of macroscopic diverticula and immediately placed in a cold saline solution in the operation room. Within 30 minutes, 1x1x0.5 cm samples were cut from the main sample in cold saline and placed in a 2.5% glutaraldehyde 0.1 M PB solution (pH 7.4) at 4°C for 2 hours for immersion prefixation.

After the serosa was removed, the samples were then dissected in a Petri dish with the same fixative into three layers: the 1st layer containing the inner submucosal plexus was made by dissecting the mucosa from the rest of the sample by cutting along the submucosa, the 2nd layer containing the outer submucosal plexus was made by cutting along the circular muscle layer, and the 3rd layer containing the myenteric plexus between the outer part of the circular muscle layer and the longitudinal muscle layer. These three layers were further dissected into 1x1x2 mm sized tissue samples using a Stemi 2000CS stereomicroscope (Zeiss, Gottingen, Germany), fine scissors and tweezers. The samples were fixed in a 2.5% glutaraldehyde 0.1 M PB (pH 7.4) solution at 4°C overnight.

Afterwards, samples were postfixed for 2 h with 1%

osmium tetroxide in 0.1 M PB (pH 7.4), dehydrated using a series of graded ethanol solutions and embedded into a mixture of Epon 812 and Araldite resins (Sigma-Aldrich, Steinheim, Germany) using a LYNX II automatic tissue processor (EMS, Hatfield, PA, USA). Tissues were carefully oriented in flat embedding molds for transverse sectioning. Semi-thin sections (1 μm) were stained with methylene blue according to Ridgway (Ridgway, 1968) and were analysed with a Zeiss AxioMat light microscope (Carl Zeiss, Jena, Germany) to confirm the sections contained structures of the enteric nervous system. Ultrathin sections (50-70 nm) were cut using a Leica EMUC7 ultramicrotome (Leica Mikrosysteme Handelsges.m.b.H., Vienna, Austria) and mounted on 600-mesh thin bar support nickel grids (Agar Scientific, Essex, UK). Samples were stained with uranyl acetate and lead citrate for 7 minutes each.

Transmission electron microscopy

Ultrathin sections were analysed using a Tecnai BioTwin Spirit G2 transmission electron microscope (FEI, Eindhoven, the Netherlands) at 100 kV. Images were collected using a bottom-mounted 16 MP Eagle 4K TEM CCD camera, using TIA software (FEI, Eindhoven, the Netherlands) at 4800x, 6800x and 9300x magnification. Electron micrographs were analysed morphometrically with the software package Fiji (Schindelin et al., 2012; Rueden et al., 2017).

Neurons were identified based on descriptions by Gabella (1972) and compared between the three patient groups. Furthermore, the size of lipofuscin inclusions were measured when found in a neuron, and the density of lipofuscin inclusions was expressed as the ratio of lipofuscin area to neuron area, excluding the nucleus from the neuron area. Neurons with no lipofuscin inclusions were included when comparing results across the patient groups. The cross-sectional area of all completely visible neurites found in all three of the studied plexuses was measured and the resulting measurements were classified into either damaged or healthy neurites based on observations by Iijima-Ando and colleagues (Iijima-Ando et al., 2012). We based the classification on the translucency of the neurite, the presence of cytoskeleton, the shape of the neurite and the integrity of the membrane. We also created a category of fine neurites which are unmyelinated neurites with a diameter of less than 0.2 μm based on the widely recognized range of 0.2 to 1.5 μm of unmyelinated nerve fibres (Kiernan and Rajakumar, 2014). ICC were identified using the following morphological criteria: numerous mitochondria, abundant intermediate filaments, the presence of caveolae and close contact to adjacent smooth muscle cells and enteric nerves (Faussone-Pellegrini and Thuneberg, 1999). Occurrence of properties associated with abnormal ICC as described by Wang and colleagues (Wang et al., 2005) were described between the patient groups, counted and presented as proportions.

Statistical analysis

Statistical analysis was performed using R 3.5.1 (R Core Team, 2015). Data was imported with dplyr (Wickham et al., 2016) and readr (Wickham et al., 2017) packages and was visualised with ggplot2 (Wickham and Chang, 2016). In order to estimate the effect of diverticulosis and CDD on the percentage of fine neurites and damaged neurites, logistic regression was used, with type of neurite as the outcome variable and study group as the predictor variable. To estimate the overall change of variance of neurite profiles, linear regression was used, with the variance of measured cross-sectional areas per tissue sample as an outcome variable and layer of the colon and patient group as predictor variables. The control group was used as a reference group when fitting the models. Because the distribution of the area of neurite profiles was log-normal, logistic transformation was done before calculating the variance. For proportional data, the Chi-square test was used to estimate differences of morphological occurrences across different patient groups.

Results

Ultrastructural changes in ganglia

Using qualitative assessment, neurons in the control group showed similar characteristics to those described in other studies (Gabella, 1972). The neurons contained numerous ribosomes, either free in the cytosol or

Table 1. Patient characteristics.

Group	Number of patients	Age (mean)	Age (SD)	BMI (mean)	BMI (SD)
Control	9 (5 F/4 M)	58.2	13.3	27.5	8.25
Diverticulosis	7 (4 F/2 M)	60.9	10.4	28.3	4.18
CDD	9 (6 F/3 M)	67.6	11.4	27.1	2.93
Total	25	62.3	12.1	27.6	5.79

CDD, complicated diverticular disease; F, Female; M, Male; SD, standard deviation; BMI, body mass index.

attached to the endoplasmic reticulum (Fig. 1a). Mitochondria were usually round and distributed in groups, however, some of them were swollen. The Golgi apparatus was frequent, usually interspersed in the cytosol. Neurofilaments and microtubules were clearly visible in neuronal somata and were especially pronounced in dendrites. Cell bodies occasionally contained one or two small lipofuscin inclusions.

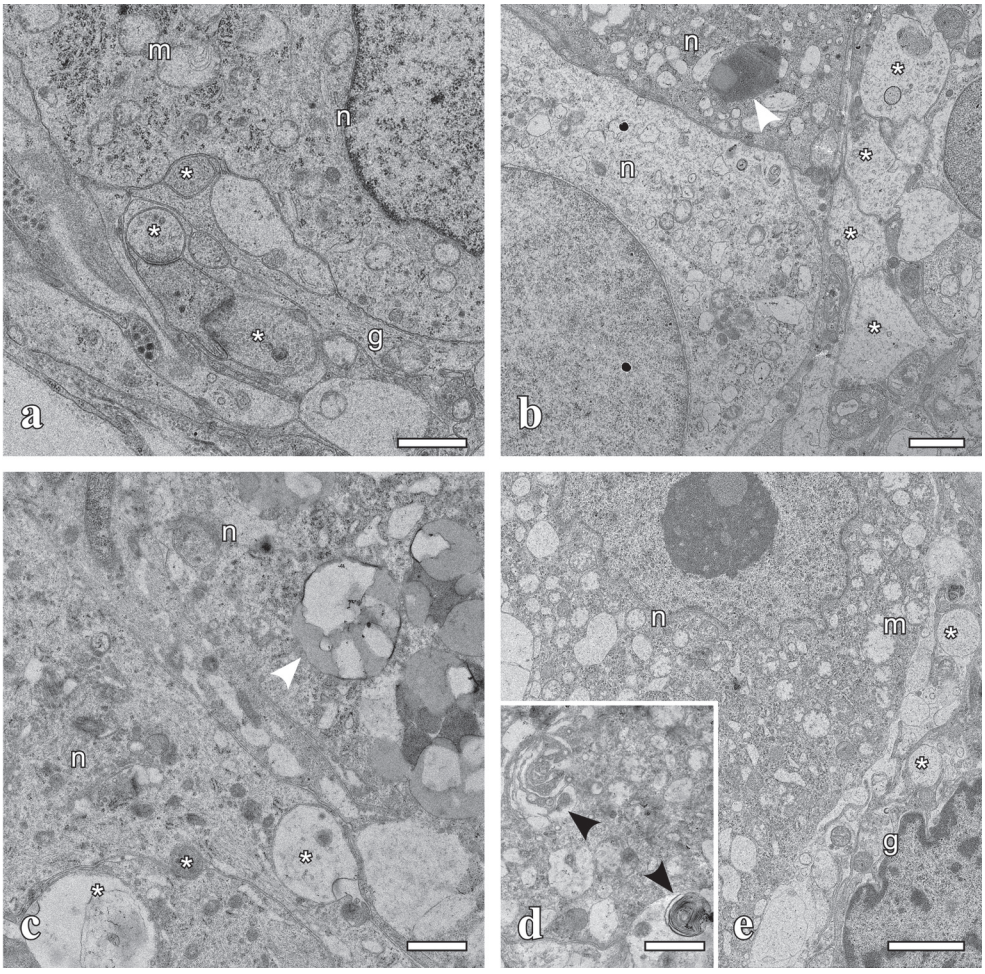

Fig. 1. Myenteric plexus neurons in the control **(a)**, diverticulosis **(b)** and CDD group **(c-e)**. In the control group, the neuronal soma (n) is intact, contacting adjacent glial processes (g) and axons (*), and the surrounding neuropil shows tight packing and axodendritic synapses. In diverticulosis, the neuron on the lower left side has a similar morphology to healthy neurons, while the neuron on the upper left side contains denser cytoplasm, swollen membranous organelles and a lipofuscin inclusion (white arrowhead). Neurons in the CDD group shows the largest degree of damage. Their cytoplasm contains much larger lipofuscin inclusions, swollen membranous organelles and mitochondria (m) and lamellar bodies (black arrowhead, **d**). The nucleus contains multiple prominent indentations **(e)** unseen in control or diverticulosis samples. Scale bars: a-d, 1 μm; e, 2 μm.

Neurons were usually located at the periphery of ganglia. Somata either contacted the basal lamina directly or were partially covered by glial processes. Inside ganglia, varicosities and axon terminals with synaptic vesicles were found near somata. Axodendritic and axosomatic synaptic contacts were common in the neuropil. The nucleus was usually round with few shallow indentations and the nucleoplasm largely contained euchromatin, with slight condensation near the nuclear lamina.

In diverticulosis samples, the majority of neurons were intact, except for cases where the neurons contained electrodense cytoplasm, larger lipofuscin inclusions and noticeable organelle swelling (Fig. 1b). The position of neurons and their relationship to other structures in ganglia did not differ compared to controls.

Meanwhile, neurons in CDD samples showed more pronounced changes (Fig. 1c-e). Most neurons contained larger lipofuscin inclusions, lamellar bodies and swollen mitochondria, with some of the neurons containing little to no lipofuscin inclusions. While the number of lipofuscin inclusions did not show large differences between all three patient groups, the inclusions were on average larger (Table 2, F=4.078, p=0.018) and occupied a larger area of the neuron body (F=4.501, p=0.013) in the diverticulosis and CDD groups compared to the control group. Mitochondrial vacuolisation was not only more frequent but also more pronounced than in the other groups. The rough endoplasmic reticulum was swollen and the Golgi apparatus was not discernible in the cytosol. The nucleus was usually irregular, with multiple shallow indentations of various degrees (Fig. 1e) that were uncommon in control and diverticulosis groups. The nucleolus appeared enlarged and more condensed.

Ultrastructural changes of neurites

The neuropil in control samples exhibited a variety of tightly packed neurite profiles (Fig. 2a). Though some of the neurites appeared swollen, most contained well contrasted microtubules. Synapses were visible in the myenteric and the outer submucosal plexuses, and some of the ganglia contained peg-and-socket junctions as well. Varicosities contained mitochondria and neurotransmitter vesicles, and lacked microtubules. The vesicles were usually small and granular with an electron-dense core or small and agranular. Neuropil in diverticulosis samples showed a similar pattern to healthy neuropil, however, patches of swollen axons were found (Fig. 1b).

Neurites in CDD samples displayed a degree of damage not seen in control and diverticulosis samples. In neuropil of the many enteric ganglia of CDD samples, tight packing of axons was disrupted. Nerve fibres individually enveloped in glial cell processes appeared more spread apart with collagen deposits in between them (Fig. 2b). Damaged neurites were swollen, lacked microtubules and vesicles, contained translucent cytoplasm, and neurofilaments were poorly visible (Fig. 2d). Due to this, some of them impinged on adjacent neurites and glial processes. The plasma membrane was disintegrated to various degrees. Furthermore, we failed to find clearly delineated synaptic contacts between neurites, suggesting decreased synaptic transmission.

Damaged neurites were more frequent in every ganglionated plexus in complicated diverticular disease but not in diverticulosis samples (Fig. 3). In the myenteric plexus, 14.2% of neurites in control cases were damaged compared with 13.9% in diverticulosis samples (OR=0.972, p=0.856) and 24.6% in complicated diverticular disease (OR=1.968, p<0.0001). A similar tendency followed in the outer submucosal (control 8.48%, diverticulosis 8.51%; CDD 15.82%, compared to control OR=2.027, p=0.017) and the inner submucosal (control 15.9%, diverticulosis 13.6%; CDD 25.0%, compared to control OR=1.756, p<0.001) plexuses.

In addition to damaged neurites, CDD samples contained a higher percentage of fine neurites (Fig. 4). Some of these neurites were closely enveloped by glial processes and were isolated from each other (Fig. 2b). These neurites contained dense axoplasm and microfilaments. Other fine neurites appeared in clusters enveloped by glial processes (Fig. 2e). A majority of these neurites were smaller than the usual lower diameter of unmyelinated axons, suggesting degeneration. The appearance of fine neurites was most pronounced in the myenteric and inner submucosal plexuses (Fig. 4). The myenteric plexus contained 2.79% and 2.02% fine neurites in control and diverticulosis samples, respectively, as opposed to 5.02% in CDD samples (OR=1.845, p=0.02). In the outer submucosal plexus, fine neurites were more frequent in diverticulosis samples (control 2.42%, diverticulosis 6.96%,

Table 2. Lipofuscin inclusion characteristics.

	Control	Diverticulosis	CDD
Size of lipofuscin granules (μm^2)*	2.25 μm^2 (0.02-14.67)	3.90 μm^2 (0.03-25.56)	4.84 μm^2 (0.01-47.71)
Relative lipofuscin area to neuron area (%)**	2.66% (0-22.20)	4.77% (0-20.26)	4.91% (0-31.80)

* F=4.078, p=0.018, control as reference; Control vs Diverticulosis, t=2.225, p=0.027; Control vs CDD, t=2.526, p=0.012; Diverticulosis vs CDD, t=0.284, p=0.777; ** F=4.501, p=0.013, control as reference; Control vs Diverticulosis, t=0.59, p=0.556; Control vs CDD, t=2.914, p=0.004; Diverticulosis vs CDD, t=1.76, p=0.080. Values are expressed as means (min-max). CDD, complicated diverticular disease.

OR=3.012, p=0.039) and in CDD samples (5.63%, OR=2.401, p=0.099). In the inner submucosal plexus, control samples and diverticulosis samples contained a similar amount of fine neurites (8.23% and 8.67%, respectively, OR=1.06, p=0.829), whereas CDD samples contained higher numbers of fine neurites (11.13%, OR=1.40, p=0.102).

Overall, the enteric plexus change is two-fold. Since

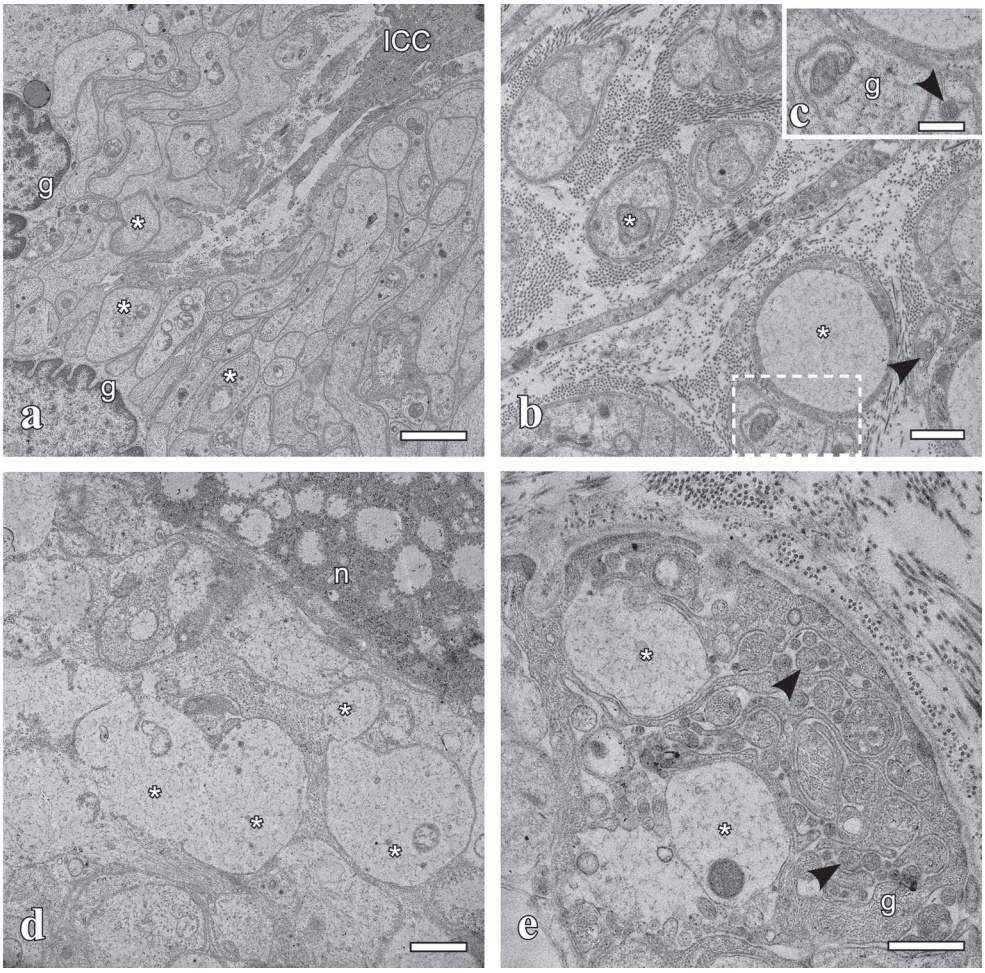

Fig. 2. Neurite changes in diverticular disease. In the control group **(a)**, neuropil in the myenteric ganglion is tightly packed, containing neurites (*) of various sizes, most of which contain neurofilaments and microtubules. In nerve bundles at the myenteric plexus **(b)**, glial processes (g) form sheaths around individual axons and nerve bundles are separated by collagen fibres. Furthermore, some of the axons have a much smaller diameter (arrowhead) and appear either near swollen axons **(c)** or as groups of fine neurites **(e)**. In the CDD group, some neurites contained translucent cytoplasm, lacked microtubules and had a disrupted plasma membrane **(d)**. n, neuronal soma; ICC, interstitial cell of Cajal. Scale bars: a, 2 μm; b, d, 1 μm; c, e, 0.5 μm.

damaged neurites were usually larger than other neurites, we found that CDD neurite distributions are more dispersed compared to control and diverticulosis groups, although the effect of these changes was only slightly pronounced and was not statistically significant. The mean variance of neurite cross sectional areas was slightly higher in the CDD group compared to the control group (β=0.165, SE 0.120, p=0.175) and the mean variance in the diverticulosis group was similar compared to the control group (β=0.069, SE 0.133, p=0.605).

Alterations of interstitial cells of Cajal in diverticular disease

ICC around the myenteric plexus (ICC-MY) of control samples exhibited features of healthy ICC that have been described in previous research (Yang et al., 2012). The defining properties of ICC were the presence of caveolae and intermediate filaments (Fig. 5a). Microtubules and lysosomes were not present. In most cases, the rough endoplasmic reticulum and the Golgi appratus were poorly developed and were located around

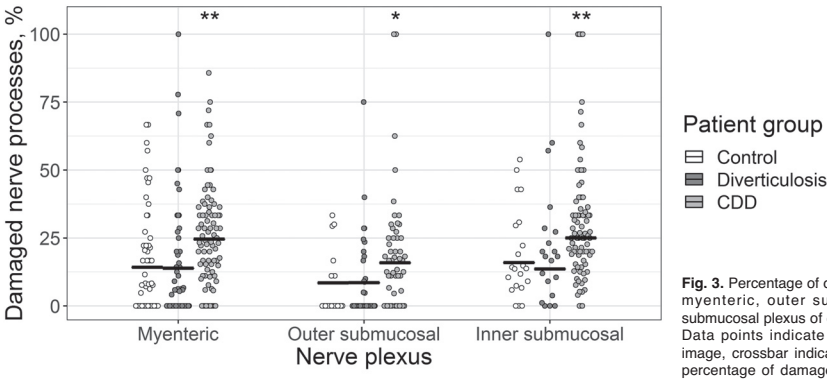

Fig. 3. Percentage of damaged neurites in the myenteric, outer submucosal and inner submucosal plexus of different patient groups. Data points indicate percentage within an image, crossbar indicates overall mean. The percentage of damaged neurites was higher in the CDD group compared to the control and diverticulosis groups in every major ganglionated plexus: the myenteric (OR=1.968, **p<0.0001, compared to control), the outer submucosal (OR=2.027, *p=0.017), and the inner submucosal plexus (OR=1.756, **p<0.001).

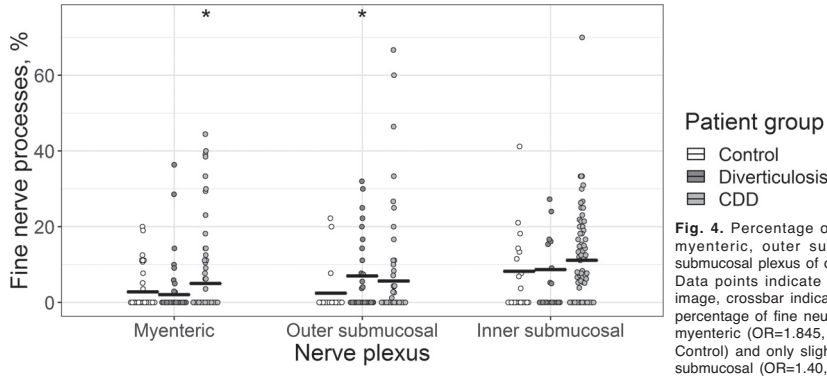

Fig. 4. Percentage of fine neurites in the myenteric, outer submucosal and inner submucosal plexus of different patient groups. Data points indicate percentage within an image, crossbar indicates overall mean. The percentage of fine neurites was higher in the myenteric (OR=1.845, *p=0.02, compared to Control) and only slightly higher in the inner submucosal (OR=1.40, p=0.102, compared to Control) plexus in the CDD group compared to controls and the diverticulosis group. In the outer submucosal plexus, fine neurites were more frequent in both the diverticulosis (OR=3.012, *p=0.039) and, to a lesser extent, the CDD group (OR=2.401, p=0.099) compared to the Control group.

the nucleus. The cell processes, apart from intermediate filaments and caveolae, sometimes contained mitochondria. ICC-MY in diverticulosis samples exhibited similar characteristics with the exception of lamellar bodies in some of the cells and sparsely distributed caveolae (Fig. 5b). In both samples, cell processes were closely associated with adjacent smooth muscle cells and enteric nerves or ganglia. ICC around the submucosal plexus (ICC-SM) showed similar ultrastructural characteristics with the exception of looser contact with surrounding cells and more pronounced processes.

In CDD samples, ICC-MY showed structural changes (Fig. 5c). The cells were still spindle shaped, but they showed cytoplasmic depletion. With a reduction of cytoplasm, contents of the perinuclear region resolved poorly. There was notable proliferation of ribosomes and rough endoplasmic reticulum in the cell processes (Fig. 5c,d), with most ICC-MY cells exhibiting changes (Control 2/7 cells, diverticulosis 6/13 cells, CDD 20/23 cells, $\chi^2=10$, $p=0.004$). Unlike in the control and diverticulosis samples, ICC-MY processes rarely contacted smooth muscle cells or enteric nerves but did contact other ICC-MY.

The morphology of ICC-SM in CDD samples showed changes similar to those found in affected ICC-MY, however, these changes were less consistent across the samples. Some of the processes were similar to those

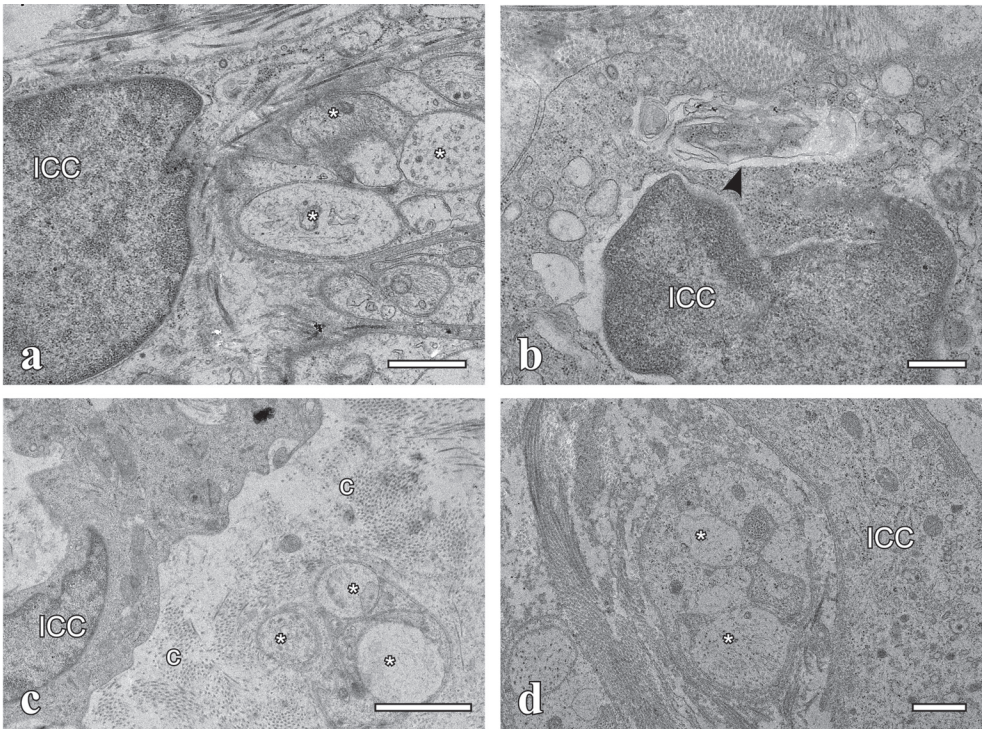

Fig. 5. Interstitial cells of Cajal (ICC) in the myenteric plexus in control **(a)**, diverticulosis **(b)** and CDD **(c-d)** groups. In control tissue, the ICC contained less dense cytoplasm that and retained close contact to the myenteric plexus (*). The ICC establishes close contacts with the myenteric plexus, usually with little to no gaps or collagen fibers in between **(a)**. In the diverticulosis group, most ICC were similar to those in the control group but there were signs of cell damage. The ICC contains a lamellar body (arrowhead), indicating membrane rupture, but maintains close contact with adjacent cells. In the CDD group, large deposits of collagen fibers **(c)** interrupt contact between the ICC and enteric nerve (*). The ICC processes contains ribosomes and patches of rough endoplasmic reticulum. In larger ICC processes **(d)**, proliferation of rough endoplasmic reticulum is visible alongside the appearance of small lysosomes. *, axons. Scale bars: a, b, d, 1 μm; c, 2 μm.

in control samples, but others lacked intermediate filaments and showed changes consistent with those in affected ICC-MY. In longitudinal sections of ICC-SM processes, proliferation of rough endoplasmic reticulum was notable and comparatively more frequent in CDD samples (Control 3/18, Diverticulosis 1/6, CDD 3/6, χ^2=3, p=0.2). The processes themselves were in close contact with each other and with enteric nerves of the submucosal plexuses. Other signs of injury were absent.

Discussion

We sought to investigate ultrastructural changes of the enteric nervous system in patients with asymptomatic diverticulosis and patients with complicated diverticular disease compared to unaffected patients. We found that the enteric nervous system in diverticulosis does not change dramatically. Marked changes appeared in complicated diverticular disease, including degenerative changes in neurons, a higher frequency of swollen and fine neurites, and injury to ICC. All of these changes are compatible with damage and remodeling of the enteric nervous system in complicated diverticular disease.

One shortcoming of our study is that we did not investigate the enteric nervous system in symptomatic uncomplicated diverticular disease. Patients were operated on for diverticular disease due to recurrent episodes of diverticulitis, after which low grade inflammation and extensive damage to the colonic wall could occur. Because of this, complicated diverticular disease could confound the changes seen in the enteric nervous system during an uncomplicated course of the disease. This is especially important considering that alterations described in this study might play a role in symptom development. In spite of this, it is also possible that these changes could be related to exacerbated symptoms after an episode of diverticulitis (Simpson et al., 2003). The present study cannot distinguish a direct connection between the morphological changes and the course of the disease. Therefore, without uncomplicated disease samples it is hard to judge whether the changes are due to complications of the disease or due to the disease itself.

Additionally, some of the morphological changes can be attributed to age-dependent degeneration as can be seen in quantitative changes in the diverticulosis and CDD groups. Specifically, the frequency of cytoplasmic lipofuscin deposits and changes to organelles (e.g. the nuclear envelope) become more pronounced with age and since both the diverticulosis and the CDD group are older than the control, degenerative changes may be attributed to age dependent changes.

Another drawback of the study was the use of human tissue received from surgical procedures. In control and diverticulosis samples, mitochondria were also swollen, and some of the neurites appeared damaged. Furthermore, as is evident in the distribution of damaged nerve processes in Fig. 3, some of the non-CDD samples contained damage equivalent to that seen in CDD samples, possibly due to ischemic damage during surgery and delayed fixation. Even though the overall degree of vacuolisation and neurite damage was higher in CDD samples, it is quite possible the ultrastructural changes seen in this study are not exclusively a correlate of the underlying disease but an artifact of the work up of the specimens.

This study is the first to describe ultrastructural changes that occur in diverticular disease. However, the idea of nerve tissue remodeling in diverticular disease has been raised previously - Simpson and colleagues showed that enteric nerves had increased in thickness and that small diameter nerves had increased in frequency in diverticular disease (Simpson et al., 2009). The authors suggested that these changes are secondary to acute diverticulitis and smooth muscle hypertrophy. Our results complement the previous findings by showing that neurites are damaged during diverticular disease. The neurites lacked microtubules, suggesting disrupted neurotransmission. The previously found increase in nerve thickness could be explained by loss of typical nerve fibre structure, swollen neurites, larger glial processes and higher collagen deposition. Usually axons are embedded in supporting glial processes (Baumgarten et al., 1970), however, in CDD, axons were found to be individually ensheathed in glial processes. Furthermore, we have shown an increase in fine neurites which coincides with new nerve sprouting but also shows regeneration of damaged nerves. Considering that ganglionic nerve cells and enteric nerves decrease in number during diverticular disease (Deduchovas et al., 2008; Wedel et al., 2010), it is most likely that fine neurites appear in response to damage to the enteric nervous system. There have been suggestions that nerve regeneration and subsequent hyperinnervation could be a cause of hypersensitivity (Stead, 1992), and the same could be the case in diverticular disease. Taking into account the absence of remarkable ultrastructural remodeling in asymptomatic patients, it is not unlikely that the changes developed as a result of acute diverticulitis.

Nonetheless, despite being less pronounced, we were able to detect changes in asymptomatic patients which were similar to changes in CDD. This calls into question whether morphological changes in complicated diverticular disease are caused exclusively by complications of the disease. Previous studies have suggested enteric neuropathy as a potential etiological factor (Wedel et al., 2015; Barrenschee et al., 2017) and the present study provides additional evidence for the hypothesis.

Morphological changes seen in ICC are similar to those found in previous studies on Crohn's disease (Wang et al., 2007). Wang and colleagues investigated ICC during and after inflammation. Their morphological description of ICC 60 days after infection would most likely correspond to the findings of the present study. The proliferation of the rough endoplasmic reticulum is

a sign of new protein synthesis and regeneration of cell processes. The presence could be explained by the fact that CDD patients for this study were not operated urgently and suffered episodes of diverticulitis in the past. However, ICC-MY failed to show close contact with smooth muscle cells and enteric nerves. In light of a previously found decreased density of ICC (Bassotti et al., 2005a), this shows that the ICC network remains impaired even after overt inflammation has subsided. However, it is not yet possible to discern whether these changes are primary or secondary to the disease, warranting further research.

Consistent injury to ICC-MY and ICC-SM only in CDD samples has multiple possible implications for understanding the disease. Since the ICC network is highly connected, it is believed that patchy injury to ICC should not impair normal peristalsis (Wei et al., 2017). Pathological changes of both ICC-MY and ICC-SM were rare in diverticulosis samples. If these changes represent actual damage to the ICC network, then it is possible they could play a role in the pathogenesis of the disease given sufficient injury. Such a hypothesis would require a driving force in the presence of diverticulosis, but a recent study failed to find mucosal inflammation in the presence of diverticulosis (Peery et al., 2017). However, if these changes are spurious, it could be assumed that asymptomatic individuals with diverticula do not have a damaged ICC network and, therefore, ICC might potentially be ruled out as a pathogenetic step of uncomplicated diverticular disease. This hypothesis is limited by the small number of morphological studies performed on ICC in diverticular disease which have yet to uncover whether there is a causal relationship between ICC and diverticular disease exists.

Additionally, previous physiological studies have found abnormal motor patterns in patients with diverticular disease (Bassotti et al., 2005b). Slow-wave activity of the colon is generated by ICC around the myenteric plexus (Koh et al., 1998) and it has been hypothesised that loss of normal ICC function may disturb normal motility (Strate et al., 2012). In our study, even though new terminal processes were present, they did not form the same connectivity as seen in healthy tissue. In fact, previous experimental studies have been able to show that abnormal motor patterns in the colon persist in spite of ICC plasticity (Wang et al., 2005). Considering such evidence, disturbed ICC might be a source of aggravated symptoms in patients who had experienced an episode of diverticulitis (Simpson et al., 2003; Lahat et al., 2019). Further mechanical and electrophysiological studies with a focus on ICC could help identify their exact role in diverticular disease.

In summary, the ultrastructural changes seen in the enteric nervous system are compatible with structural remodeling and injury to the ICC network in complicated diverticular disease that persist even after acute complications. Most changes appeared only during complicated diverticular disease and are absent in unaffected individuals and patients with diverticulosis. The current evidence suggests that damage occurs during a complicated course of the disease, provoking changes and adaptations that might be related to long-term effects on disease development. It is likely that diverticular disease has multiple forces that drive the generation of symptoms. Understanding how each of them influences the course of the disease individually may be crucial for future studies and treatment options.

Acknowledgements. The Research Council of Lithuania provided full financial support for this project (Grant SEN-06/2015/PRM15/135). We thank Aiste Masaityte and Jurgita Sventoraitiene for technical assistance throughout the study.

Conflict of interest. The authors declare that they have no conflict of interest.

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Accepted June 12, 2019

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