Microscopic colitis - factors that influence disease onset and disease course

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Microscopic colitis

Factors that influence disease onset and disease course

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The overarching aim of this thesis was to achieve a better understanding of the pathogenetic mechanisms, epidemiology and prognosis of microscopic colitis.

In study I, all new cases of MC between 1991-2013 were linked with a prospective population-based cohort from Malmö Diet and Cancer Study. The prediagnostic data acquired from the cohort concerning dietary habits and lifestyle confirmed the previously observed risk increase in smoking and female gender. High intake of alcohol increased the risk for MC. No association were found between intake of protein, carbohydrates, different types of fat, fibre or zinc and occurrence of MC. Lifestyle factors such as physical activity and education were not associated with MC.

In study II, blood samples from 66 CC patients were analyzed with ELISA to identify the expression of collagen-associated autoantibodies (matrix metalloproteinase-9, tissue inhibitors of metalloproteinase-1, collagen-III, collagen-IV, and tenascin-c). In comparation to controls, no increased presence was seen.

In study III, the incidence of cancer in patients with CC in Scotland and Sweden were calculated with age-standardized rate (ASR) and standardized incidence ratio (SIR), respectively. The risk for cutaneous squamous cell carcinoma (cuSCC) was elevated (SIR 3.27, 95% CI 2.42-4.32) whereas the risk for colon cancer was reduced (SIR 0.23, 95% CI 0.04-0.75).

In study IV, the incidence of MC in Skåne between 2010-2020 was investigated with focus on temporal and geographical variations. All new cases of MC during the time period were retrieved from the Department of Pathology and ASR per 100 000 person-years were calculated. In total, 1985 patients were identified whereof 71% women. The incidence for CC was stable (ASR 6.34 [95% CI 5.9-6.8], range per calendar year 4.6-8.1). The incidence of LC was significantly higher and increased markedly 2015-2020 (ASR 7.90 [95% CI 7.4-8.4], range per calendar year 1.7-15.2). The north-west part of Skåne demonstrated the highest incidence of LC, peaking in Båstad municipality with 79.6 per 100 000 person-years in 2019.

In conclusion, food habits are not associated with MC, whereas smoking and high alcohol consumption are risk factors. In CC patients, no autoantibodies associated to the collagen layer were detected. The risk of cuSCC is elevated and for colon cancer decreased in CC-patients. In view of the marked and rapid increase of LC in Skåne 2010-2020, causative agents such as water contamination or infectious agents could be contemplated.

Key words: microscopic colitis, collagenous colitis, lymphocytic colitis, risk factors, cancer, epidemiology, autoantibodies, incidence

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To my beloved children
Silja, Iben and Manfred

Table of Contents

Abstract

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In conclusion, food habits are not associated with MC, whereas smoking and high alcohol consumption are risk factors. In CC patients, no autoantibodies associated to the collagen layer were detected. The risk of cuSCC is elevated and for colon cancer decreased in CC-patients. In view of the marked and rapid increase of LC in Skåne 2010-2020, causative agents such as water contamination or infectious agents could be contemplated.

Populärvetenskaplig sammanfattning

Sjukdomen mikroskopisk kolit beskrevs första gången 1976 i Malmö av patologen Clas Lindström. Han undersökte ett vävnadsprov taget från tjocktarmens slemhinna hos en kvinna som led av långvarig, icke-blodtillblandad diarré, men där man inte sett några tecken på sjukdom med blotta ögat. Med mikroskop identifierade han ett kollagenband som var tjockare än vad som sågs hos friska personer. Han benämnde det nyupptäckta tillståndet för ”kollagen kolit” där kolit är latin och ordagrant betyder ”inflammation i tjocktarmen”. Under 80-talet beskrevs ytterligare karaktäristika över tjocktarmslemhinnans utseende hos denna patientgrupp: fler tecken på inflammation i slemhinnan, bland annat med en ökad närvaro av lymfocyter (en typ av vita blodkroppar). Dock sågs inte alltid Lindströms beskriva förtjockade kollagenband. Nu delas därför mikroskopisk kolit (MK)in i två subgrupper: Kollagen kolit (KK) och lymfocytär kolit (LK).

Sjukdomen betraktades till en början som mycket ovanlig, men allt eftersom kännedomen om tillståndet har ökat i forskarvärlden, bland läkarkåren och bland patienter, betraktas den nu som en relativt vanlig sjukdom. MK drabbar främst kvinnor i övre medelåldern och utbredningen har stadigt ökat sedan de första studierna publicerades. Under åttio- och nittiotalet bedömdes ca 10 personer per 100 000 insjukna per år. Studier från idag visar betydligt högre siffror, där man bland annat i Danmark kunde beskriva tre gånger så många insjuknade år 2011. I avhandlingens **fjärde studie** har jag undersökt utbredningen av MK i Skåne mellan åren 2010–2020.

De typiska symtomen för MK är långvarig diarré som inte är blodtillblandad. Därtill får en del buksmärta, viktnedgång, inkontinens och allmän trötthetskänsla. Enda metoden för att utreda MK är att göra en kameraundersökning av tjocktarmen (koloskopi) och i samband med det ta vävnadsprover som undersöks med mikroskop. Ses där de typiska tecknen för KK eller LK kan diagnosen ställas.

Orsaken till sjukdomen är i stor mån okänd. Den nuvarande hypotesen är att flera faktorer verkar samverka för sjukdomsuppkomst: immunförsvaret i tjocktarmen reagerar på något som passerar i tarmen hos vissa individer med extra benägenhet att drabbas. Den extra benägenheten att drabbas som åsyftas är ett genetiskt bärarskap av en speciell receptor som förekommer hos en del personer med MK. Receptorns funktion är att ”visa upp” ämnen för immunförsvaret som av kroppen ska betraktas som farliga. Receptorn kan vara olika benägen att bedöma ämnens

”farlighet” och är det en känslig variant kommer ibland ofarliga ämnen presenteras som farliga. Bärarskap av genen som bildar receptorn med hög känslighet är vanlig i Sverige och är kopplad till många autoimmuna tillstånd, tex glutenintolerans. Därför kan man också se ett samband mellan MK och glutenintolerans, dvs att samma person drabbas av båda sjukdomarna. Det som ”passerar” tjocktarmens slemhinna (i dagligt tal: bajs) verkar också påverka genom att på något vis trigga immunförsvaret. Det är dock oklart om det rör sig om komponenter från kosten, specifika bakterier eller giftiga ämnen. I slemhinnan aktiveras celler som reagerar på ”fienden” och en inflammation uppstår, vilken i sin tur orsakar diarré.

Det förtjockade kollagenlagret i KK uppstår på grund av en störning i kollagenomsättningen i tarmslemhinnan, både på grund av att ämnen som reglerar nedbrytningen minskar i aktivitet, och för att ämnen som bromsar tillväxten hämmas. I avhandlingens **andra studie** undersökte jag om någon av dessa strukturer har antikroppar riktade till sig som en förklaring till den ändrade aktiviteten.

Faktorer som ökar risken för att insjukna i MK är bland annat rökning samt vissa läkemedel såsom inflammationsdämpande läkemedel som tex Ipren och syrahämmande medel som tex Omeprazol. Innan det här avhandlingsarbetet påbörjades hade kostens betydelse för sjukdomen inte undersökts. I avhandlingens **första studie** studerade jag om kost, alkohol och livsstilsfaktorer såsom träning och kroppskonstitution skulle kunna påverka risken att insjukna i MK.

Den mest välstuderade o erkända behandlingen för MK är ett kortisonpreparat som minskar inflammationen vilket i sin tur leder till besvärsfrihet åtminstone under perioder. Om kortisonet inte har effekt kan andra läkemedel som påverkar immunförsvaret provas. Om inga läkemedel ger önskad effekt kan i sista hand operation så att tjocktarmen avlastas övervägas, men detta är mycket ovanligt

Majoriteten av patienterna har övergående besvär. Prognosen är generellt sett god och sjukdomen kopplas sällan till några allvarliga komplikationer. Innan den är avhandlingen påbörjades var det till stor del inte känt om risken för cancer är ökad hos dessa patienter. I avhandlingens **tredje studie** undersöktes om patienter med kollagen kolit får cancer i större utsträckning jämfört med befolkningen i stort.

Målet med denna avhandling var således att få mer kunskap om eventuella riskfaktorer, sjukdomens utbredning, risk för cancer och om några antikroppar riktade mot strukturerna i kollagenlagret kunde ses.

I **första studien** användes data insamlad från Malmö Kost Cancer-studien, en omfattande studie i Malmö som påbörjades under 90-talet där information om livstilsvanor och kostvanor samlades in hos cirka 28 000 malmöbor. Av de som deltog i den studien hade 135 insjuknat i MK fram till 2013. MK-patienterna jämfördes med de övriga deltagarna för att se om några vanor skiljde sig åt. Vi kunde se att MK-patienterna var, som förväntat, i större utsträckning rökande kvinnor i 60-årsåldern. Någon skillnad i intag av olika kostfaktorer såsom fleromättade fetter,

fibrer eller zink kunde inte ses mellan MK-patienterna och de andra deltagarna. Inte heller avseende träning, utbildningsnivå och vikt noterades någon skillnad. Däremot ökande risken för att insjukna ju mer alkohol man drack, vilket var ny information.

I avhandlingens **andra studie** undersöktes huruvida kroppens immunförsvar bildar antikroppar mot strukturer i det förtjockade kollagenlagret. Det skulle i så fall kunna förklara varför kollagenlagret är förtjockat, men också öka förståelsen för hur kollagen kolit uppstår. En antikropp skulle dessutom kunna användas som ett diagnostiskt verktyg: Patienter med misstänkt KK skulle kunna lämna ett blodprov i stället för att genomgå den betydligt mer obekväma och kostsamma kameraundersökningen av tjocktarmen. Antikropparna kunde emellertid inte ses i större utsträckning än hos friska blodgivare. Det är dock viktigt att understryka att alla potentiella strukturer inte undersöktes, varför fler studier med liknande frågeställning behövs.

I den **tredje studien** undersöktes sambandet mellan KK och cancer. Individer från Edinburgh, Malmö, Örebro och Linköping med KK följdes under cirka ett decennium. Cancerfall i gruppen registrerades och jämfördes sedan med resten av befolkningen i de nämnda städerna. Här sågs en trefaldigt ökad risk för skivepitelcancer i huden och en minskad risk för tjocktarmscancer. Bakomliggande orsaker till dessa samband är osäkra då vi inte hade någon information om patienternas livsstil. Möjligen drabbas de av hudcancer i större omfattning på grund av en störning i immunförsvaret. Att risken för tjocktarmscancer är minskad kan möjligen bero på att inflammationen i tjocktarmsslemhinnan verkar skyddande mot cancerceller, eller att diarrén i sig skyddar mot att cancerämnen hinner påverka tarmen pga. den ökade passagehastigheten.

I den **fjärde studien** undersöktes utbredningen av MK i Skåne under åren 2010 till 2020. Sammantaget under hela perioden drabbades 14,2 personer per 100 000 invånare och år, vilket är högre än tidigare uppmätt i Sverige men fortfarande lägre än tex Danmark. Insjuknandet av LK var vanligare än KK och ökade signifikant, speciellt under den senare halvan av uppföljningstiden. Här sågs även en ansamling av fall i nordvästra Skåne, där mer än dubbelt så många drabbades. I denna studie undersökte vi även om befolkningstäthet skulle kunna påverka risken, men inga skillnader sågs. Orsaken till den kraftiga ökningen av LK, (speciellt i nordväst) är oklar, men sannolikt beror det på en okänd miljöfaktor som dessa individer utsatts för. Detta är föremål för vidare studier.

Sammanfattningsvis har avhandlingsarbetet således visat att kost, träning, utbildningsnivå och vikt inte verkar vara en faktor som påverkar insjuknande av MK, men att rökning och hög alkoholkonsumtion gör det. Kollagenlagret verkar inte vara föremål för antikroppsbildning, men fler studier behövs för att klargöra detta. Risken för skivepitelcancer är förhöjd och tjocktarmscancer är minskad. MK, framför allt LK, fortsätter att bli alltmer vanligt i Skåne och sannolikt orsakas detta av en eller flera än så länge okända miljöfaktorer.

List of Papers

Paper I

The association between the intake of specific dietary components and lifestyle factors and microscopic colitis.

Larsson JK, Sonestedt E, Ohlsson B, Manjer J, Sjöberg K.

European Journal of Clinical Nutrition 2016 ; 70 :1309-1317

Paper II

Lack of autoantibodies against collagen and related proteins in collagenous colitis.

Larsson JK, Roth B, Ohlsson B, Sjöberg K.

BMC Immunology 2022; 23: 29

Paper III

Cancer Risk in Collagenous Colitis

Larsson JK, Dabos KJ, Höglund P, Bohr J, Münch A, Giannakou A, Nemeth A, Wurm-Johansson G, Toth E, Plevris JN, Fineron P, Koulaouzidis A, Sjöberg K.

Journal of Clinical Medicine 2019; 8: 1942

Paper IV

Regional variations in the incidence of microscopic colitis in the region Skåne 2010-2020

Larsson JK, Clarkson S, Sjöberg K.

Manuscript.

Abbreviations

aHR	adjusted Hazard ratio
ASCA	Anti-Saccharomyces cerevisiae antibodies
ASR	Age-standardized rate
BCC	Basal cell carcinoma
CC	Collagenous colitis
CD	Crohn's disease
CeD	Celiac Disease
CI	Confidence interval
CI	Confidence interval
Col III	Collagen type III
Col IV	Collagen type IV
CRC	Colorectal cancer
cuSCC	Cutaneous squamous cell carcinoma
DSA	Demographical Statistical Areas
E%	Energy percent
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
F:M ratio	Female:male ratio
FEIA	Fluorescent enzyme immunoassay
HLA	Human Leukocyte antigen
HR	Hazard ratio
HRT	Hormone replacement therapy
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocytes
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
IQR	Interquartile range

IR	Incidence ratio
LC	Lymphocytic colitis
MC	Microscopic colitis
MDC	Malmö Diet and Cancer
MMP	Matrix metalloproteinase
MUFA	Monounsaturated fatty acids
NMSC	Non-melanoma skin cancer
NR	Not reported
NSAID	Non-steroidal anti-inflammatory drugs
PPI	Proton pump inhibitor
PUFA	Polyunsaturated fatty acids
RNA	Ribonucleic acid
RR	Relative risk
RU	Relative units
SCB	Statistics Sweden
SD	Standard deviation
SE	Standard error
SFA	Saturated fatty acids
SIR	Standardized incidence ratio
SNOMED	Systematized Nomenclature of Medicine
SRR	Standardized rate ratio
Th	T-helper cell
TIMP	Tissue inhibitors of metalloproteinase
TNC	Tenascin-C
TNF	Tumour necrosis factor
TPO	Thyroid peroxidase

Preface

During medical school, my first impression of immune driven diseases was rather negative. Immunology is extremely complex and there are still many mechanisms that we do not yet understand. And to be honest- I did not even understand the things that were explained and well investigated. It was just too difficult. This resulted in my first and only missed exam during medical school.

My first experiences of research were also rather discouraging. This was also during medical school. Although the subject was interesting, I had problems to motivate myself, and got insufficient support from my supervisor. During the summer of 2012, I was still a medical student and worked as an intern (or in other words: "a summer doctor") at the in-patient ward of gastroenterology at Skåne University Hospital in Malmö. When the senior physician once asked me if I was interested in research in general and in immune-driven diseases in particular my answer couldn't be clearer: No! I said, and the conversation was over. However, that senior physician had planted a seed and one year later my first research study concerning microscopic colitis was published and I was registered as a PhD-student. And that senior physician? He had signed up to be my supervisor.

I experienced that there are few things that are so inspiring and exciting as to overcome big intellectual challenges. Together with my enthusiastic supervisor I found out that research is fun, stimulating and involves a feeling of doing something important. It is often frustrating to not understand. But patients with MC or other immune-driven inflammatory diseases will exist regardless of if we understand the mechanisms or not and we owe it to them to learn more about the diseases. By my research about MC, small steps for a better understanding about epidemiology and pathogenesis have been taken. And in the end, every step counts. This thesis is my contribution to help the affected patients with MC.

During the time of writing this thesis, I have gained some great experiences. I have been to international and national congresses, which in one I won a price for the best oral presentation at the congress. I have met people that burns for MC-research, and I have participated in interesting discussions. In different courses I have achieved new knowledge and reflections on research, and most importantly, met people from different clinics and shared experiences. I have met colleagues from other parts of Europe in collaborations. All over, I have met engaged people that burns for research. After this, I hope I still can contribute to the research field in

gastroenterology by co-supervising. I also look forward to exploring a new research field within a well-known clinical field, namely gynaecology and obstetrics. Even though I will change organ of primary interest, I am sure that the knowledge I have gained through this thesis will serve me and the research society well in the future.

Introduction

Background

In Malmö in 1976, the Swedish pathologist Clas Lindström described *collagenous colitis* (CC) for the first time. He noticed a remarkable, thick collagen band under the colonic epithelium in a woman who was suffering from chronic watery diarrhoea and abdominal pain (1). A few years later, in 1980, Read and colleagues reported the results of a group of patients with diarrhoea of unknown origin. A significant part of the group had a macroscopically normal mucosal appearance, but histologically (microscopic) mild inflammatory changes were described. The authors named these findings *microscopic colitis* (MC) (2). In 1989, Lazenby and his colleagues showed that an increased number of colonic intraepithelial lymphocytes (IELs) was the most characteristic feature of MC and suggested the term *lymphocytic colitis* (LC) (3).

Nowadays, microscopic colitis (MC) is an umbrella term used for the two diseases lymphocytic colitis (LC) and collagenous colitis (CC), characterized by chronic non-bloody diarrhoea with a macroscopic almost normal mucosa, but with histologically typical changes. The following years, the disease was considered to be rare and only some case reports and small patient series were published. Over the years, especially the last two decades, the scientific knowledge, as well as the clinical experience, have increased and MC is now a well-established and a commonly known cause of chronic diarrhoea.

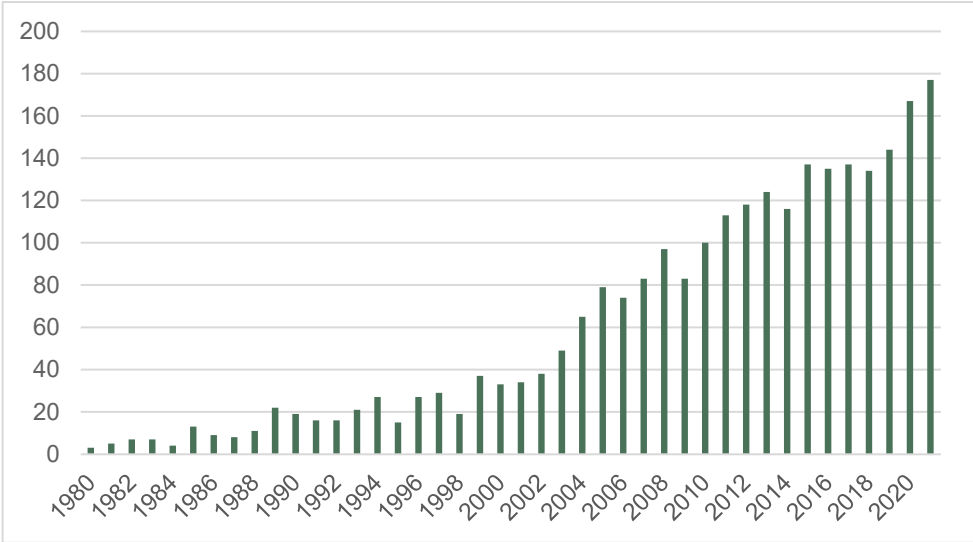


Figure 1, Number of publications per calendar year matched by the search term "Micrsoscopic colitis" on PubMed.

Epidemiology

MC mostly affects females and the mean age at diagnosis is 60-65 years (4). Rather small epidemiological studies from the 80s and 90s from Europe and the USA demonstrate incidence rates of MC less than 10 cases per 100 000 person-years (5-7). Thereafter, increasing numbers of both CC and LC have been reported. Although a worldwide increase, substantial variation between different regions has been observed. A recent nationwide study from Denmark reported the highest incidence numbers worldwide in 2011 (32.3 MC cases per 100 000 person-years) (8). In contrast, in Netherlands, the mean incidence per 100 000 person-years for MC 2000-2012 was 3.4 (9).

Table 1, Relevant incidence studies published on MC

				Collagenous colitis			Lymphocytic colitis			Microscopic colitis			
Region	**Study period**	**Author**	**Publ. year**	**IR**	**F:M ratio**	**Cases**	**IR**	**F:M ratio**	**Cases**	**IR**	**F:M ratio**	**Cases**	**CC:LC ratio**
Örebro, Sweden	1984–1993	Bohr *et al.* (10)	1995	1.8	9:1	30	NR	NR	NR	NR	NR	NR	NR
Örebro, Sweden	1993–1998	Olesen *et al.* (11)	2004	4.9	7.5:1	51	4.4	2.1:1	46	9.3	4.3:1	97	1.1:1
Örebro, Sweden	1999–2008	Wickbom *et al.* (12)	2013	5.2	3.6:1	96	5.0	4.6:1	90	10.2	4.0:1	186	1.1:1
Olmsted County, USA	1985–2001	Pardi *et al.* (7)	2007	3.1	4.4:1	46	5.5	1.2:1	84	8.6	1.8:1	130	0.6:1
Olmsted County, USA	2002–2010	Gentile *et al.* (13)	2014	9.1	NR	78	12.0	NR	104	21.0	3.2:1	182	0.8:1
Olmsted County, USA	2011-2019	Tome *et al.* (14)	2022	9.9	NR	103	15.8	NR	165	25.8	NR	268	0.6:1
Calgary, Canada	2002–2004	Williams *et al.* (15)	2008	4.6	3.4:1	75	5.4	6.3:1	89	10.0	4.4:1	164	0.9:1
Calgary, Canada	2004-2008	Steward *et al.* (16)	2011	NR	NR	347	NR	NR	759	16.9 - 26.2	NR	1106	NR
Terrassa, Spain	1993–1997	Fernández-Bañares *et al.* (6)	1999	2.3	4.75:1	23	3.7	2.7:1	37	6.0	3.3:1	60	0.6:1
Terrassa, Spain	2004–2008	Fernández-Bañares *et al.* (17)	2011	2.9	3.4:1	40	2.3	2.0:1	32	5.2	2.6:1	72	1.3:1
Tomelloso, Spain	2008–2010	Guagnozzi *et al.* (18)	2012	<1	NR	2	16	NR	30	18	1.1:1	32	0.1:1
Zealand, Denmark	1999–2010	Bjornbak *et al.* (19)	2011	10.8	NR	270	6.7	NR	168	17.5	NR	438	1.6:1
Zealand, Denmark	2010-2016	Davidson *et al.* (20)	2018	16.4	3.0:1	670	11.1	1.7:1	453	27.5	2.4:1	1123	1.5:1
Uppsala, Sweden	2005–2009	Thörn *et al.* (21)	2013	7.0	4.3:1	154	4.8	2.0:1	118	11.9	3.0:1	272	1.5:1
Southern Sweden	2001–2010	Vigren *et al.* (22)	2012	5.4	2.8:1	198	NR	NR	NR	NR	NR	NR	NR
Southern Sweden	2011-2015	Davidson *et al.* (20)	2018	5.9	2.8:1	379	2.7	1.7:1	170	8.6	2.4:1	549	2.2:1
Stockholm, Sweden	1980-2010	Mellander *et al.* (23)	2016	NR	3.7:1	344	NR	2.7:1	451	NR	3.1:1	795	0.8:1
Nationwide studies													
Iceland	1995–1999	Agnarsdottir *et al.* (5)	2002	5.2	7.9:1	71	4.0	5.0:1	54	9.2	NR	125	1.3:1
Denmark	2002-2011	Bonderup *et al.* (24)	2015	8.8	3.1:1	4749	5.6	1.8:1	3028	14.4	NR	7777	1.6:1
Netherlands	2000-2012	Verhaegh *et al.* (9)	2014	1.8	3.1:1	3741	1.3	2.3:1	2718	3.4	2.6:1	6459	1.4:1
Denmark	2001-2016	Weimers *et al.* (8)	2020	12.2	3.1:1	8437	8.5	1.8:1	5865	20.7	2.3:1	14302	1.4:1
Sweden	1995-2015	Bergman *et al. (25)*	2019	2.4	3.6:1	4606	4.8	2.4:1	9238	7.2	2.7:1	13844	0.5:1

IR, Incidence rate; F:M ratio, Female:male ratio; NR, Not reported. Red colour: estimates from data in the studies.

Clinical presentation and diagnosis

Patients with MC suffer from chronic, non-bloody, watery diarrhoea. These symptoms might be accompanied by abdominal pain, weight loss, faecal urgency, nocturnal diarrhoea, and fatigue (26). The onset of symptoms can be sudden but is often insidious (27). Laboratory tests and stool cultures seldom show abnormalities but are often carried out to rule out differential diagnoses (28). Hence, no relevant biomarkers exist to guide diagnosis. At endoscopic examination, the macroscopic picture is often normal, even though findings such as mucosal changes can be found (29). Histologic assessment of multiple colon biopsies is therefore mandatory to confirm the diagnosis of MC. The histological criteria for CC and LC are presented in Table 2 and the histology of CC is illustrated in Figure 2.

Table 2, Histological criteria for microscopic colitis (30)

	Collagenous colitis	Lymphocytic colitis
Mandatory	Thickened (> 10 µm) subepithelial collagenous layer	Intraepithelial lymphocytosis, at least 20 IEL per 100 surface epithelial cells
	Chronic inflammatory infiltrate in lamina propria	Chronic inflammatory infiltrate in lamina propria
Not mandatory	Epithelial damage such as flattening and deattachment	Epithelial damage such as flattening and deattachment
	Increased number of IEL	Slight thickening of the subepithelial collagen layer

IEL, intraepithelial lymphocytes.

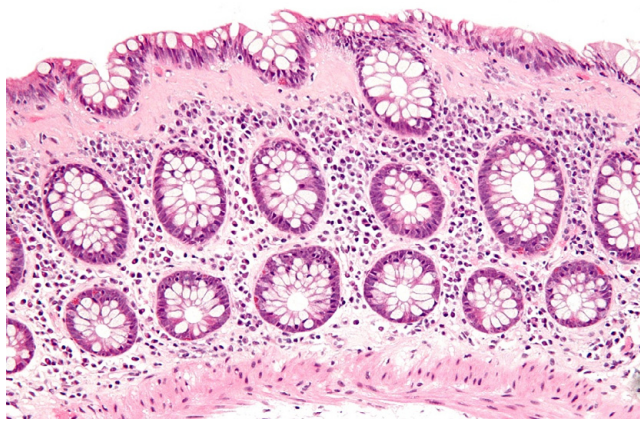


Fig. 2 Histological features of collagenous colitis

High magnification micrograph of collagenous colitis, H&E stain by Michael Bonert, MD, FRCPC. Copyright 2011 Michael Bonert (Nephron) (https://commons.wikimedia.org/wiki/File:Collagenous_colitis_-_high_mag.jpg), "Collagenous colitis - high mag", Licensed under CC BY-SA 3.0, https://creativecommons.org/licenses/by-sa/3.0/legalcode

Pathogenesis

The mechanisms behind MC are still largely unclear. Based on our current knowledge, it is regarded to be a multifactorial process, involving an uncontrolled mucosal immune response to various unknown luminal and mucosal agents in genetically predisposed individuals (31). Furthermore, microscopic colitis seems to be an immune-mediated disorder with prominent response from the cell-mediated part of the adaptive immune system and cytotoxic responses (32).

Genetics

The genetic predisposition is recognized for the development of MC but few studies have investigated this in detail. The human leukocyte antigen (HLA) class II DQ2 haplotype, frequently expressed in individuals from northern Europe (33) is known to be important in autoimmune diseases, such as coeliac disease (CeD), diabetes type 1 and thyroid disease (34, 35). Patients with MC often suffer from other autoimmune diseases, in particular CeD and thyroid disease (36). An association between MC, in particular CC, with certain HLA haplotypes has been observed, especially HLA DQ2, HLA DQ 1,3 (37, 38) and DQ2.5 (39). Westerlind *et al.* also described a clear overlap between CC and CeD in the HLA DQ2 haplotype (39). The relationship between MC and CeD and other autoimmune diseases, which all are associated to the HLA DQ2 haplotype, can indicate a common pathogenesis.

Luminal factors

It is suggested that among other factors that lead to disease development in MC, luminal factors/components such as microbiological agents and endogenous toxins can trigger the immune system. In 1995, Jarnerot *et al*. reported that eight out of eight therapy resistant patients with CC experienced clinical and histopathological remission with faecal stream diversion, indicating an unknown luminal factor is of pathogenetic importance (40). However, the role of gut microbiota has not yet been deeply studied. In ten patients with active MC, faecal samples showed decreased levels of *akkermansia mucinphilia* which is normally one of the most prevalent bacterial strains in the large intestine (41). If this is a consequence of smoking (which also decreases the concentrations of *akkermansia mucinphilia*) (42) or if it may be an actual causal relationship is not yet clear. Whether certain dietary factors may influence the pathogenetic development of MC *e.g.* as a triggering luminal agent, has not been investigated. However, since a decreased epithelial barrier function is suggested to exist in patients with MC (32) any influx of a luminal agent through the gut mucosa is probably facilitated.

Mucosal immune response

The mucosal immune response in MC is mainly dominated by cytokines activated by the Th1/Th17 pathway, resulting in overexpression of pro-inflammatory cytokines such as TNF- α, INF- γ, IL-15, and IL-6 (43-45). Günaltay *et al.* also reported a decreased expression of the IL-1 family cytokine IL-37, which exerts anti-inflammatory effect through attenuation of production o pro-inflammatory cytokines (46). FOXP3 positive cells, one of the more predominant regulatory type of T-cells, has also an increased expression in patients with MC (43, 44, 47, 48).

Collagen metabolism in CC

In CC, the collagen layer is thickened and is believed to reflect a local disturbance of the extracellular matrix (ECM) turnover resulting in the formation of a disrupted ECM. Previous studies have shown contradictory results with increased levels of both type I, III, IV and VI collagens in the pathognomic thickened collagen layer (49-52) as well as increased transcripts of procollagen I and IV (53). Certain matrix metolloproteinases (MMPs) that are involved in the degradation of collagen may contribute to the disrupted collagen layer. An allelic variation of MMP-9 has been suggested to be a risk factor for CC (54) and Liu *et al.* detected enhanced mRNA expression levels of the gene MMP-9 in CC-patients (44). MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) and previous studies have shown increased transcripts of TIMP-1 in patients with CC (44, 51). The extracellular matrix glucoprotein Tenascin-C (TNC) is increased in the collagen layer, something that is not the case in a healthy population (49, 51, 55, 56). TNC also interacts with MMPs (57).

Risk factors

Smoking, both former but in particular current smoking, is a risk factor for MC. Smokers develop MC ten years earlier than non-smokers (58). Smokers and former smokers are also less likely to obtain clinical remission in comparison to non-smokers (59). MC is also associated with drug exposure. Strongest associations are seen in non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors (PPI) and selective serotonin-reuptake inhibitors (SSRIs), (60-62) especially current users and if the medications are combined (63). Some studies have also described an association to statin use, but the evidence for this is weaker (4). As MC mostly is affecting females, hormonal factors should be taken o consideration. Hormone replacement therapy (HRT) has been reported to be associated with MC, but with contradictory outcomes. One study demonstrated a lesser exposure for HRT in MC patients compared with controls, and in a prospective cohort study, increased risk för MC was reported in current users of HRT (Hazard ratio [HR] 2.64, 95% CI 1.78-

3.90) (64, 65). In the prospective cohort study, ever-use of oral contraceptives also increased the risk. Reported data on alcohol consumption up until 2015 are not conclusive, but a possible association could exist (66). Whether other lifestyle factors may act as risk factors of MC has up until 2015 not been investigated.

Therapy

The goal for treatment is to achieve clinical remission defined from the Hjortswang criteria: <3 stools per day and a mean <1 water stool per day during one-week registration (67). Oral budesonide is the only established drug with effect to induce and maintain remission in patients with MC. At least four randomized controlled trials have been conducted on CC and at least three on LC. Budesonide is effective in both inducing and maintaining remission in CC, and to induce remission in LC. The proportions of patients in remission after 6-8 weeks of therapy range from 73-100% in CC and 71-91% in LC (26). At corticosteroid dependence, bile acid sequestrant therapy may be an alternative (68). Immunomodulators (tiopurines) and biological therapy (TNF-blockers and integrin-inhibitors) can be considered in selected patients who fail to respond to budesonide, which is less than 5% according to a recent Danish study (26, 69). Surgery such as ileostomy and colectomy can be considered as a last option if all medical therapies fail (40).

Prognosis

The disease course is self-limiting or transient in over 50% of the patients (69). Active disease often leads to impaired quality of life and patients in clinical remission can still suffer from abdominal pain and other extra-intestinal symptoms (70). However, the long-term prognosis is good and serious complications are rare (71). The risk for colorectal cancer (CRC) seems not to be increased, but data concerning this is from small studies (66). Lung cancer risk has been described to be increased, but adjustment for smoking was not done in that study (72). Up until 2015, it has not been investigated whether CC or LC patients have an increased risk for other extra-intestinal cancers. The risk for death seems to be increased in patients with MC in comparation to matched controls (adjusted HR [aHR] 1.17; 95% confidence interval [CI] 1.12-1.22). However, after adjustment for comorbidity burden, no difference was seen. Hence, the increased mortality in MC patients is probably caused by the comorbidity burden rather than the disease itself (73).

Rationale- a brief introduction to the papers in this thesis

In accordance with other gastrointestinal immune-mediated diseases such as inflammatory bowel disease (IBD), the incidence of MC is increasing globally (4). One explanation may be an increased awareness of the existence of the disease, but improved diagnostic procedures could also contribute (12). However, it is likely that an increased exposure to identified as well as unidentified risk factors, such as environmental factors, also is present. Whether food habits, lifestyle or socioeconomic factors contribute to the disease development of MC is not yet clear. In ***study I*****,** we wanted to investigate if such potential risk factors were associated to MC.

There is, this far, no evidence that CC is an autoimmune disorder. Nevertheless, the disease has many characteristics in common with other autoimmune diseases: (i) it affects predominantly a certain age and gender group (elderly females), something that is typical for autoimmune diseases in general, (ii) there is a genetic predisposition with certain HLA haplotypes that furthermore are frequent in patients with other autoimmune diseases such as CeD (37-39, 74) and (iv) the mucosal cytokine profile in CC is Th1-dominated (43, 45). However, according to Witebsky's postulate concerning autoimmune diseases, such a disease "requires the recognition of a particular autoantigen to which antibodies or cells are autoreactive" (75) which has not been described in CC. In ***study II***, we aimed to investigate the prevalence of autoantibodies targeted to structures in the thickened subepithelial collagen layer. If a specific autoantibody for CC would be found, this would contribute to a better understanding of the pathogenetic processes in CC but it could also serve as a diagnostic tool. We also aimed to study the presence of other putative antibodies that might play a role in the immune-driven process in CC.

Patients with CC suffer from a limited chronic inflammation in the colonic mucosa. Chronic inflammation is a risk factor for cancer, both in the affected organ/system but also in other sites of the body. For instance, the risk of lymphoma is increased in patients with rheumatoid arthritis (76), and the incidence of basal cell carcinoma (BCC) and CRC in increased in patients with CeD (77). The incidence of malignancies in patients with CC is not deeply studied. In an international multi-center setting, ***study III*** was designed to investigate the cancer incidence in patients diagnosed with CC.

Since microscopic colitis was described for the first time for more than forty years ago, it is now possible to obtain epidemiological information over longer time periods. Several studies have revealed that the incidence is increasing over the world but there are also geographical variations. This could indicate that environmental factors contribute to the pathogenesis. A mapping of the fluctuations in time and space could give information about possible contributing factors. The purpose of ***study IV*** was to investigate the overall incidence of MC in Skåne 2010-2020, with focus on both temporal and spatial variations.

Aims

The overarching aim of this thesis was to achieve a better understanding of the pathogenetic mechanisms, epidemiology and prognosis of microscopic colitis.

Specific aims

- In a prospective study setting, to investigate whether diet and other lifestyle factors such as education, obesity and physical activity are associated with the development of MC.
- To identify if specific autoantibodies targeted against the thickened subepithelial collagen layer and associated protein structures in the mucosa are present in patients with CC.
- To determine the incidence of cancer in patients with CC in Sweden and Scotland.
- To determine the incidence of LC and CC in Skåne during the period 2010-20, with focus on temporal and spatial variations.

Materials and methods

MC-patients

MC patients (or only CC patients when appropriate) were retrieved from the local Departments of Pathology of interest through the Systematized Nomenclature of Medicine (SNOMED) codes (M40600 for CC and M47170 for LC). The validity of MC cases in the Swedish Pathology registries has recently been evaluated with a positive predictive value of 95% (78).

In ***study I***, we matched all new cases of MC in Malmö between 1991 and 2013 with the Malmö Diet and Cancer-register (MDC) to identify all MC-patients in Malmö included in the MDC-study. Those who were diagnosed with MC prior the inclusion in MDC were excluded.

In ***study II***, a female cohort diagnosed with MC in Skåne between 2002 and 2010 was achieved from a previous investigation in the research group (79) where also blood samples and clinical information from questionnaires had been collected. For our study, only CC cases were selected. The patient's records were manually scrutinized and only those with at least two flare-ups or/and two positive histological specimens verifying CC were selected to guarantee that only cases with persistent disease were included. In 2017, patients with disease onset later than 2010 were identified and included in the study if they matched the inclusion criteria.

In ***study III***, stage one, new CC cases between the years 2000 to 2013 from Malmö and from the local pathology registry in Edinburgh were selected. In the second stage, all new CC patients from Skåne 2000-2015 were selected, as well as all new CC patients from local registries in Linköping (2000-2015) and Örebro (2008-2015).

In ***study IV***, all new MC cases diagnosed between 2010 and 2020 in Skåne were selected. To assure only new cases of MC were included, the data set was compared with data from 2000-2009, and duplicates were eliminated.

Study I

The Malmö Diet and Cancer study cohort

The extensive MDC study was initiated in the beginning of the nineties with the primary aim to investigate the association between dietary habits and subsequent risk of cancer. All men born between 1923 and 1945 and all women born between 1923 and 1950 living in Malmö during the screening period 1991-1996 were invited to participate. In the end of the period, 28 098 individuals had been recruited (mean age 58 years, 61% women), which corresponds to a participation rate of 40%. The cohort went through a wide and detailed examination concerning foods intake, socio-economic and lifestyle factors and anthropometry. A dietary assessment method was developed specifically for the MDC-study and data on dietary habits were achieved through this method. It included a dietary questionnaire, a seven-day food diary and an interview about dietary habits. The average daily intake of foods (grams per day) was calculated based on the frequency and portion size estimates from the food dairy, questionnaire and interview. Based on the questionnaires, socio-economic and lifestyle factors were collected. Data on anthropometry was measured.

MC-patients

See above.

Dietary factors

The average daily intake, adjusted for total energy intake, of following dietary factors were registered:

- Protein (energy percent [E%])
- Carbohydrates (E%)
- Sucrose (E%)
- Total fat (E%)
- Saturated fatty acids (SFA) (E%)
- Monounsaturated fatty acids (MUFA) (E%)
- Polyunsaturated fatty acids (PUFAs) (E%)
    - n-3 PUFA (omega-3)
    - n-6 PUFA (omega-6)
- Dietary fibre (g/MJ)
- Zinc (mg/MJ)

Other examined variables

- Smoking (never, former or active)
- Alcohol (five groups; zero consumers and gender-specific quartiles based on level of consumption)
- Body mass index (quartiles)
- Education level (five groups; elementary school, primary/secondary school, upper secondary school, further education without a degree and university degree)
- Physical activity (quartiles, based on level of daily activity)

Statistics

MC-patients were compared with non-cases within the total cohort. Associations were estimated through Cox proportional-hazards regression models with the underlying time variable from baseline until MC-diagnosis, death or end of follow-up. Adjustment for potential confounding factors were made. By using the examined factors as continuous variables (for example, 1-4 for dietary factors) we were able to test the p-trend, which analyses whether increasing amounts of exposure have a negative or positive association with risk of MC. Values were given in HR or in aHR with 95% CIs or in mean and range when appropriate. All dietary parameters were also tested in a multiple logistic regression model with the same non-significant outcome. P-values below 0.05 were considered statistically significant. SPSS Version 22 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses.

Study II

Study population

For CC cases, see above. Healthy blood donors served as controls for all immunological analyses.

Immunological analyses

For this study, in-house enzyme-linked immunosorbent assay (ELISA) methods were setup for analysis of anti-IgM and IgG antibodies against collagen III and IV and TIMP-1. See Figure 3 for ELISA diagram. For MMP-9 and TNC, an in-house ELISA method was conducted as previously already described and tested in another study (80). Antibody levels were presented as RU (relative unit; absorbance values after subtracted background) and the concentration in each doublet was interpolated

from the standard curve. The inter- and intra-assay coefficients of variation were calculated for each antibody. To determine the presence of antibodies, the cut-off value was defined as RU >97.5th percentile.

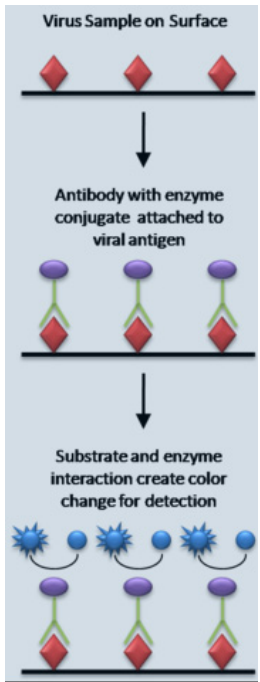


Figure 3, diagram of the direct ELISA principle

Direct ELISA diagram by "Cavitri" (https://commons.wikimedia.org/wiki/File:ELISA_diagram.png), https://creativecommons.org/licenses/by/3.0/legalcode

ASCA, total IgE and TPO

At the Department of Immunology at Skåne University Hospital, Malmö, anti-saccharomyces cerevisiae antibodies (ASCA) and total IgE were analysed. ASCA IgG were analysed by a fluorescent enzyme immunoassay method (FEIA) for which the reference value is set >10 U/mL in accordance with the manufacturer's recommendation (Orgentec Diagnostika, AlegriaH, Mainz, Germany). Out of 50 healthy blood donors tested at our laboratory, five (10%) were positive for ASCA IgG. Total IgE were also analysed by a FEIA method. To determine increased titers of total IgE, a cut-off value was set to >129 kU/L. In our laboratory, 14 of 100 healthy blood donors (14%) had increased titers. By a chemiluminescence enzyme immunological method, (Atellica IM, Siemens Healthcare GmbH, Erlangen, Germany) anti-thyroid peroxidase (TPO) antibodies were analysed at the Department of Clinical Chemistry at Skåne University Hospital, Malmö. If the cut-off level is set to 60 kIU/L, TPO antibodies are found in 10% of blood donors.

Statistical analyses

To calculate differences in antibody prevalence between controls and patients, Fisher's exact test was used. To calculate differences between mean values of continuous variables, Student's t-test was used. A p-value below 0.05 was considered statistically significant.

Study III

This international, multi-center cohort-study was carried out in two stages, compromising two sizeable cohorts of patients diagnosed with CC.

Study population

First stage, Scotland and Sweden (Edinburgh and Malmö)

For CC-cases, see above. Data on extra-colonic cancer were collected through manual search of the records of those with CC.

Second stage, Sweden (multi-center)

The investigation was remade with focus on the Swedish data since the distribution of cancer cases in the first stage of the study were unexpectedly skewed. Patients with CC diagnosis were in the second stage achieved from the Departments of Pathology in Malmö, Linköping and Örebro, for details, see above. The follow-up period began at the time of CC diagnosis and continued until end of the observation period or death. The CC cohort was linked up with the National Cancer Register in each region. To examine the incidence risk for all cancers during follow-up, none of the patients were excluded after their first diagnosis of cancer. Cancer diagnosis that occurred before the diagnosis of CC were excluded. For cancers that typically occur several times in one individual, the number of tumours was recorded instead of individual cases in both the CC-cohort and the control group.

Statistical Analysis

First stage

Person-years at risk were calculated by age-specific categories up to 85 years. Using Poisson approximation, the standard error (SE) was calculated. The age-standardized rate (ASR) was compared to public data that was available from UK's National Cancer Intelligence Network. The relative risk (RR) for each cancer was calculated by comparing ASRs for our cohort with the ASRs in Lothian region, Scotland.

Second stage

By gender and 5-year age groups, person-years at risk were calculated separately for the three geographical regions (Skåne, Linköping and Örebro). For each of the reported cancers, standardized incidence ratios (SIR) were calculated. The expected numbers of cancers were calculated by pooling patients and linking each area to existing regional cancer registries. By multiplying the number of person-years for each gender, age and area group by the corresponding specific cancer incidence rates in the respective regions, the expected numbers of cases of cancer and specific cancer types were calculated. Assuming a Poisson distribution of the observed number of cases, SIR and their 95% CI were calculated. Mid-P exact test was used and values below 0.05 were considered significant.

Study IV

Study population

See above.

Identification of population and population density

Population data by municipality, region and the whole country sorted by age and gender was achieved from Statistics Sweden (SCB) through their open data base. SCB also offers data on population concentration by organization the population in Demographical Statistical Areas (DSA). Based on population concentration, DSA is divided in three main categories: A (rural), B (mixed, *e.g.,* small towns) and C (urban areas). There are 789 DSAs in Skåne, where 15% constitute category A, 14% B and 71% C (81).

Statistics

By dividing the total number of cases by the total number of person-years of observation, multiplied by 100 000, crude and age-specific incidence rates for gender, age, calendar year, municipality of residence and DSA-category were calculated. Based on a Poisson distribution, two-sided exact CI were calculated. ASR were calculated by multiplying the age-specific rates with weights from the 2020 Swedish population to account for different demographics in the different municipalities. For ASR, SE and two-sided CI were calculated based on binominal approximation and Poisson approximation, resulting in the same numbers. Standardized rate ratio (SRR) was calculated by dividing ASR1 by ASR2 to estimate the relative risk of disease between two ASRs. Smith's formula (82) was used to calculate CIs, based on the SEs retrieved from the previous calculations on

ASR for each group. Student's t-test was used to calculate differences in age at diagnosis. To determine whether an increase in ASR was significant, linear regression analysis was used. To compare differences in ASR between two time periods, Kruskal-Wallis rank test was used. A p-value below 0.05 was considered statistically significant. Statistical calculations were performed with IBM SPSS Statistics editor version 25.

Ethics

Study I

The design of the original MDC study protocol, which complied the Declaration of Helsinki (LU-51-90) was approved by the Ethics Committee in Lund. For the present investigation, the study protocol was approved separately (LU 2013/650)

Study II

The ethics committees in Lund (2009/565 and 2011/209) and Stockholm (2016/271-31/1) approved the study. A written informed consent to participate in the study was provided from all participants.

Study III

The study was approved by the ethics committee in Lund (2013/650 and 2016/788) and by Lothian NHS, Scotland. The ethical board approved that written informed consent was not necessary prior to data collection since this study was a retrospective registry study.

Study IV

The Swedish Ethical Review Authority 2020-06631 and 2021-01554 approved the study. Since this was a registry-based study no approval from participants was required.

Results

A summary of the main findings of the four studies are presented in the following text. More comprehensive and complete reviews are found in respective papers presented later in this book.

Study I

During a follow-up period of 22 years, 135 patients with incident MC were identified in the MDC-cohort. Seventy-three of the MC-patients were diagnosed with CC and 62 with LC- In both subtypes, ten cases were men. For details, see table 3.

Table 3, Patients characteristics

	Non-cases	MC-cases	HR	95% CI
Total (n)	27 960	135		
Gender (% female)	60.5%	85.2%	3.57	2.22-5.74
Age (years)				
Minimum	44.5	44.9		
Maximum	73.6	72.8		
Mean	58.1	58.2		
Median	57.8	58.2		
25th percentile	51.3	51.3		
75th percentile	64.2	64.0		

MC, microscopic colitis; HR, Hazard Ratio; CI, Confidence Interval. HR was estimated through Cox proportional-hazards regression models with corresponding 95% CI.

Intakes of foods and food intake pattern

Intakes of protein, carbohydrates, sucrose, total fat, SFA, MUFA, Omega-3, Omega-6, dietary fiber and zinc were not associated with MC (Table 2). Subgroup analysis of CC and LC patients did not reveal any significant differences between cases and controls.

Table 4, Risk estimates for intake of different food components and microscopic colitis

	Quartiles	Quartile range	aHR	95% CI	*P* for trend
Protein					
	1	<= 14.01	1.00		
	2	14.02 – 15.53	1.22	(0.74-2.00)	
	3	15.54 – 17.20	1.23	(0.75-2.03)	
	4	17.21+	1.26	(0.69-1.88)	
					0.641
Carbohydrates					
	1	<= 41.11	1.00		
	2	41.12 – 44.90	0.87	(0.53-1.41)	
	3	44.91 – 48.66	0.89	(0.55-1.45)	
	4	48.67+	1.14	(0.72-1.81)	
					0.565
Sucrose					
	1	<= 6.08	1.00		
	2	6.09 – 8.04	0.81	(0.49-1.35)	
	3	8.05 – 10.34	1.03	(0.63-1.66)	
	4	10.35+	1.06	(0.65-1.71)	
					0.782
Fat					
	1	<= 33.56	1.00		
	2	33.57 – 37.54	0.75	(0.47-1.20)	
	3	37.55 – 41.57	0.80	(0.50-1.28)	
	4	41.58+	0.82	(0.51-1.30)	
					0.454
Saturated fatty acids					
	1	<= 13.59	1.00		
	2	13.60 – 15.73	0.82	(0.51-1.32)	
	3	15.74 – 18.32	0.83	(0.52-1.33)	
	4	18.33+	0.80	(0.50-1.29)	
					0.394
Monounsaturated fatty acids					
	1	<= 11.64	1.00		
	2	11.65 – 13.09	0.77	(0.48-1.23)	
	3	13.10 – 14.53	0.89	(0.56-1.40)	
	4	14.54+	0.75	(0.46-1.22)	
					0.346
Polyunsaturated fatty acids					
	1	<= 4.85	1.00		
	2	4.86 – 5.77	1.05	(0.66-1.66)	
	3	5.78 – 6.83	0.93	(0.57-1.50)	
	4	6.84+	1.00	(0.62-1.63)	
					0.899
Omega-3					
	1	<=0 .77	1.00		
	2	0.78 -0.92	0.70	(0.44-1.13)	
	3	0.93 – 1.12	0.71	(0.44-1.15)	
	4	1.13+	0.89	(0.57-1.41)	
					0.625

	Quartiles	Quartile range	aHR	95% CI	*P* for trend
Omega-6					
	1	<= 3.80	1.00		
	2	3.81 – 4.63	0.85	(0.61-1.51)	
	3	4.64 – 5.60	0.84	(0.52-1.34)	
	4	5.61+	0.84	(0.51-1.36)	
					0.383
Fibre					
	1	<= 1.78	1.00		
	2	1.79 – 2.15	1.32	(0.82-2.11)	
	3	2.16 – 2.59	0.74	(0.44-1.27)	
	4	2.60+	0.93	(0.57-1.54)	
					0.320
Zinc					
	1	<= 1.11	1.00		
	2	1.12 – 1.25	0.80	(0.48-1.34)	
	3	1.26 – 1.45	1.19	(0.75-1.90)	
	4	1.46+	1.01	(0.66-1.68)	
					0.493
Total zinc*					
	1	<= 9.18	1.00		
	2	9.19 – 11.37	1.30	(0.83-2.03)	
	3	11.38 – 14.70	0.98	(0.59-1.64)	
	4	14.71+	1.12	(0.68-1.82)	
					0.912

aHR, Adjusted hazard ratio; CI, Confidence Interval. aHR was estimated through Cox proportional-hazards regression models with corresponding 95% CI. HR (95% CI) are stated for quartiles of intake in energy percent (E%) of protein, carbohydrates, fat, fatty acids, omega-3 and omega-6, of fibre and zinc in mg/MJ and for total zinc in mg. Variables were adjusted for age, sex, smoking, season and method version of the dietary protocol in the Malmö Diet and Cancer study. To calculate P for trend, a general linear model was used. The quartiles were used as continuous variables. N cases: 135, N non-cases: 27960. *, including supplementary zinc intake

To determine whether a certain food pattern was associated with risk of MC, a diet quality index score on adherence to the recommended intakes of dietary factors was also applied. However, no statistically significant associations between adherence to recommendations and MC could be found.

Lifestyle factors

There was a strong association between smoking and MC risk, both for current smokers (aHR 2.53; 95% CI 1.66-3.84) as well as for former smokers (aHR 1.61; 95% CI 1.03-2.51). P for trend <0.001, adjusted for sex and age. Increased alcohol intake was associated with risk of developing MC (p for trend = 0.032), see figure 1 and Table 3. No associations between risk of MC and body mass index, physical activity level or education level were seen.

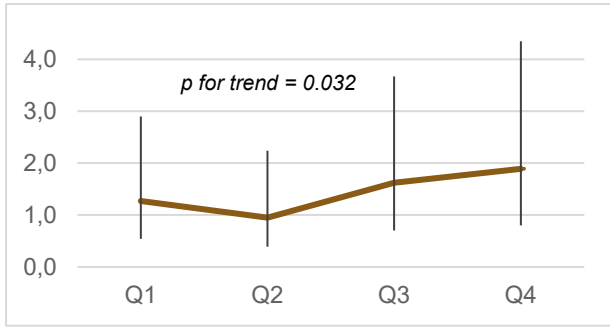


Figure 4, Alcohol consumption and risk of MC

Q, Quartiles. Y-axis: adjusted hazard ratio (aHR), with corresponding 95% confidence interval estimated through Cox proportional-hazards regression models. HR was adjusted for sex, age and smoking. X-axis: gender-specific quartiles based on level of consumption (grams/day) where Q4 is the highest. Zero consumers serve as reference.

Table 5, Definition of alcohol consumption for each group and conversion table

	gram/day	SD/week
ZC	0	0
Q1	<=2.55	< 1.5
Q2	2.6-7.9	~ 1.5-4.5
Q3	8.0-16.0	~ 4.5-9.0
Q4	16.0-194	> 9.0

ZC, Zero consumers; Q, Quartiles; SD, Standard drink (12 grams of pure alcohol). The threshold for low-risk consumption is approx. 8 SD per week (100 grams alcohol per week) (83).

Study II

Patient characteristics

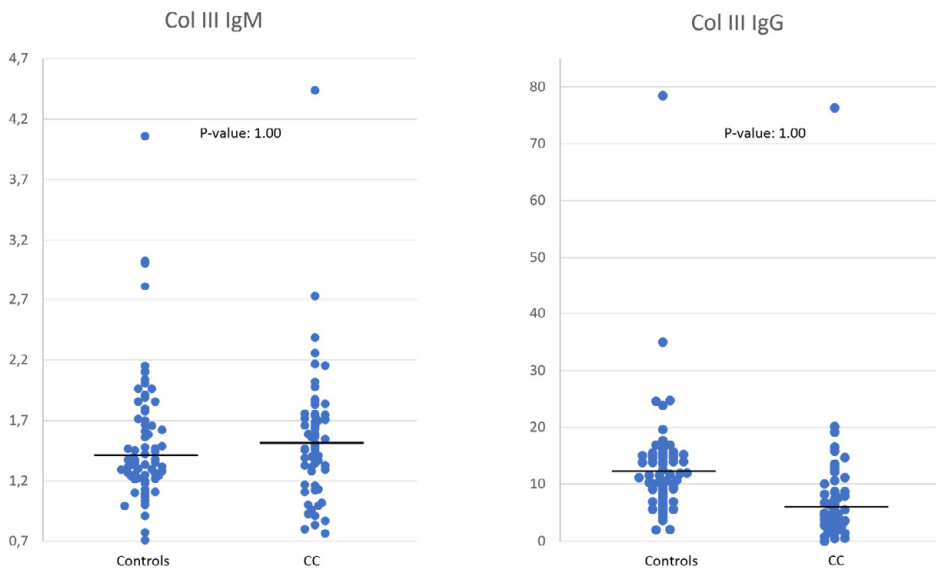
In the study, 66 women with CC were included. At inclusion, the mean age was 60 years (range 31-74 years) and mean age at diagnosis was 55 years (range 28-69 years). The mean symptom duration was 11 years, and the mean disease duration was 6 years at inclusion. More than two-thirds were smokers or formers smokers. Hypertension (30%), rheumatic disorders (15%) and asthma and cancer (both 14%) were the most common concomitant diseases. The most frequent used medication was budesonide (38% of the patients).

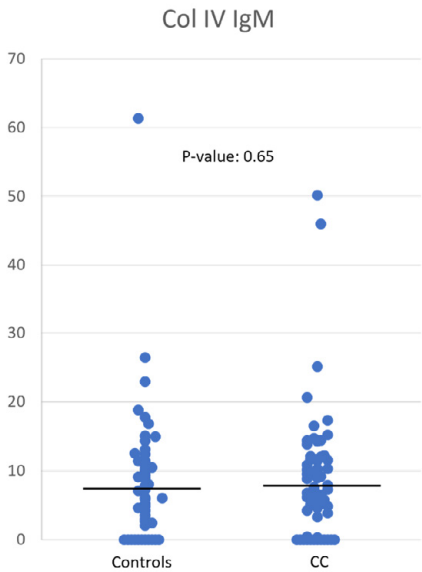
Controls

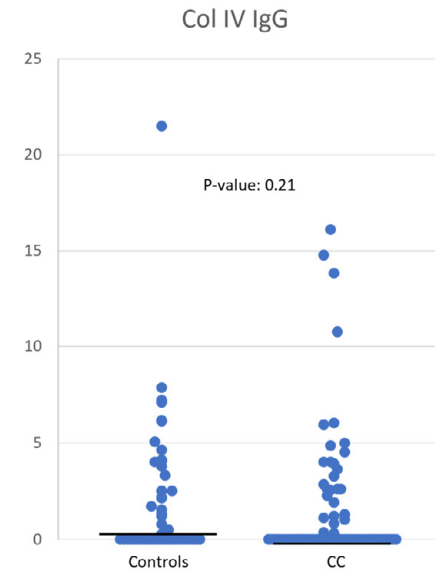
The control groups were selected from the same cohort consisting of 100 healthy female blood donors. The mean age of the control group was 41.7 years (range 19-69).

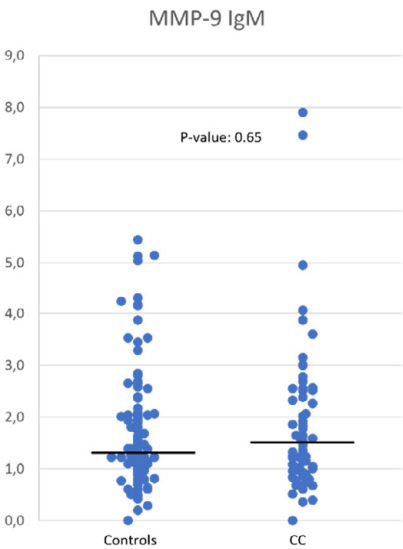
Presence of antibodies

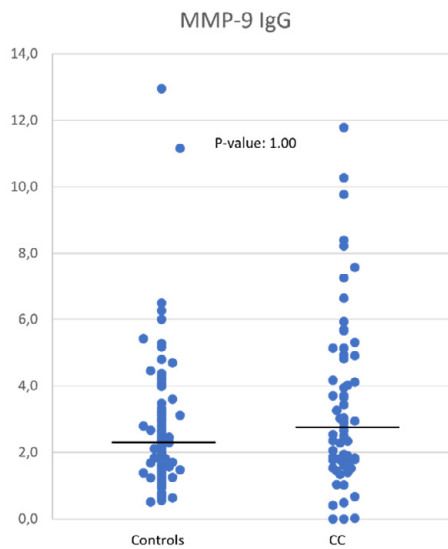
Between CC patients and controls, no difference in prevalence of collagen-associated autoantibodies was observed (Figure 5 a-e). In patients who expressed autoantibodies (n=18), the mean disease duration was significantly lower compared to those CC patients who did not (3.7 years vs 6.4 years, p=0.03). Autoantibodies against TPO and ASCA were slightly more present in CC patients compared to controls, but not significantly. Levels of total IgE did not differ between the two groups.




Col IV IgM
70
60
50
40
30
20
10
0
P-value: 0.65
Controls
CC


Col IV IgG
25
20
15
10
5
0
P-value: 0.21
Controls
CC


MMP-9 IgM
9,0
8,0
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
P-value: 0.65
Controls
CC


MMP-9 IgG
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
P-value: 1.00
Controls
CC

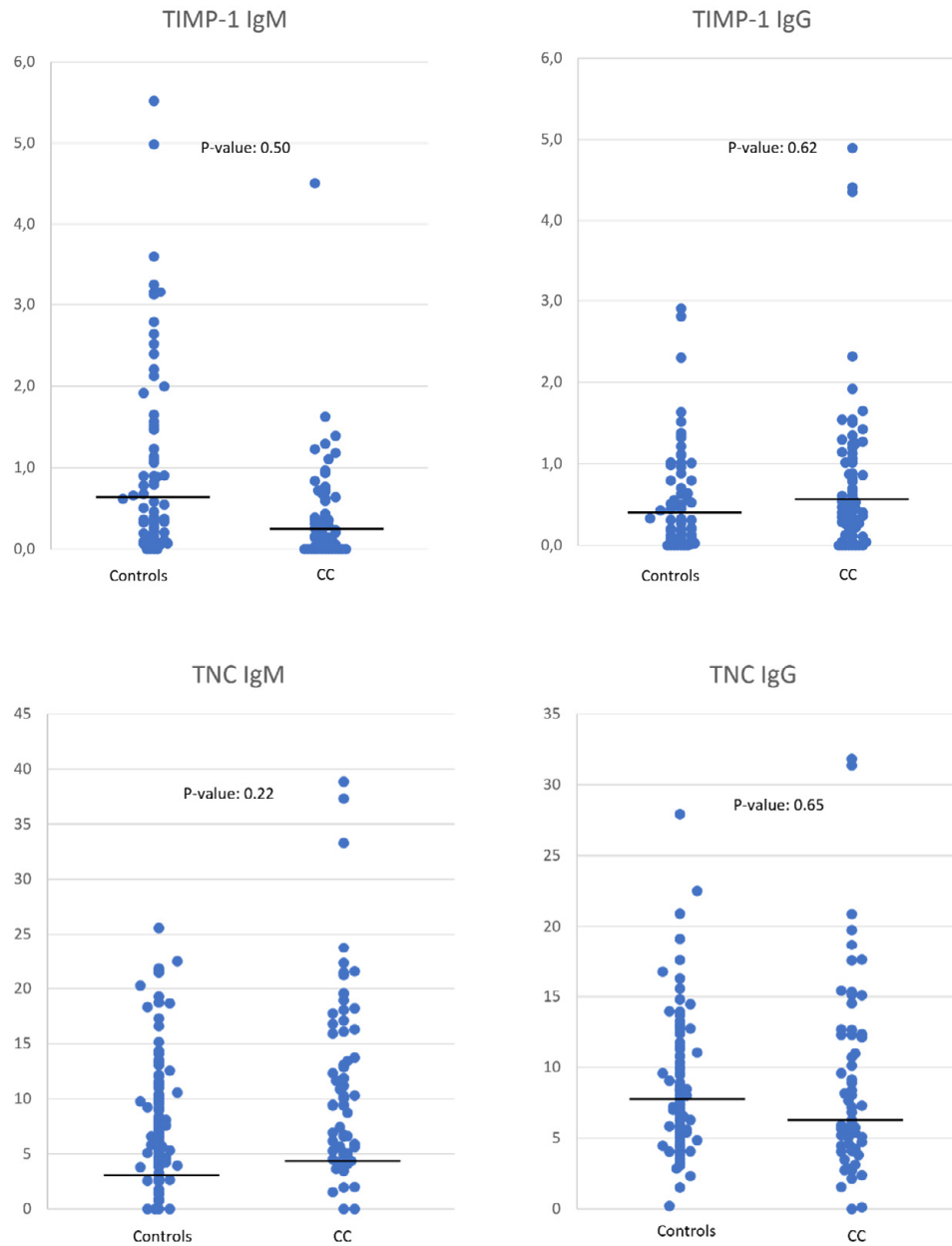


Figure 5 a-e, Levels of collagen-associated antibodies in patients with collagenous colitis and controls

CC, collagenous colitis; Col III, collagen type III; Col IV collagen type IV; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitors of metalloproteinases-1; TNC, tenascin-C. For CC n=66 and controls n=70, 51, 100, 65, 100, respectively. Y-axis: Antibody levels in relative units (RU) (absorbance values after subtracted background). Cut-off value set at the 97.5 th percentile. Black bars state median values. Fischer's exact test was used to calculate the differences between controls and CC patients.

Treatment with budesonide was not associated with any of these autoantibodies and no difference in prevalence of autoimmune/immune-mediated diseases between those who expressed autoantibodies and those who did not were seen (Table 6).

Table 6, Differences in patient characteristics between CC-patients who expressed collagen-associated autoantibodies and those who did not.

	Antibodies, n = 18	No antibodies, n = 48	p-value
Mean age at inclusion (range)	59 (31-73)	61 (31-74)	0.36
Mean disease duration, years (range)	3.7 (1-14)	6.4 (1-22)	***0.03***
Smokers or former smokers (n, %)*	15 (83.3)	32 (66.7)	0.23
Budesonide treatment (n, %)	4 (22.2)	21 (43.4)	0.16
Past or current diseases: (n, %)			
Reumatic disorder	3 (16.7)	7 (14.6)	1.0
Cancer	2 (11.1)	7 (14.6)	1.0
Asthma	2 (11.1)	7 (14.6)	1.0
Thyroid disorder	3 (16.7)	5 (10.4)	0.67
Celiac Disease	1 (5.6)	2 (4.2)	1.0

CC, Collagenous colitis; Collagen-associated autoantibodies, antibodies against collagen type III, collagen type IV, matrix metalloproteinase-9, tissue inhibitors of metalloproteinases-1, and tenascin-C. Student's t-test was used to calculate differences between mean values. Fisher's exact test was used to calculate other differences in frequency between groups. *, missing value: 6.

Study III

First stage

In total, 738 patients were included, of whom 395 (53%) were from Edinburgh and 344 from Malmö. The median age in the group was 68 years (interquartile range 58-77). In Edinburgh and Malmö, 268 (68%) and 285 (83%) were females, respectively. The female:male (F:M) ratio in the whole group was 3.0:1. Seventy-one patients were diagnosed with extra-colonic cancer, with an average time interval of three years between CC diagnosis and cancer. For expected and observed cases, RR and p-values, see table 7.

Table 7, Observed and expected cancers in the Scottish/Swedish cohort (stage 1).

Cancer type	Cases	Expected	RR (95% CI)	p-value
Skin (NMSC)	16	1	15.0 (2.6–87.1)	***0.001***
Bladder	6	1	9.2 (1.1–75.0)	***0.019***
Lung	18	2	3.9 (1.6-9.3)	***0.001***

NMSC, Non-melanoma skin cancer; RR, Relative risk; CI, Confidence interval.

The observed numbers of bladder and lung cancer were evenly distributed between Edinburgh and Malmö. However, 15 of the 16 cases of non-melanoma skin cancer (NMSC) were from Malmö which resulted in a second stage investigation with an expanded cohort collected only in Sweden.

Second stage

Above the already 344 included patients from Malmö in stage 1, an additional number of 797 CC patients from Skåne, Linköping and Örebro were included, which resulted in a total number of patients of 1141, see table 8.

Table 8, Patient characteristics, second stage

	Linköping	Örebro	Skåne	Total
Total (n)	130	133	878	1141
Age, median (IQR)	66 (57-75)	64 (53-74)	68 (58-76)	67 (57-76)
Gender (% female)	75 %	84 %	74 %	76 %

IQR, interquartile range

Average follow-up time was 8 years (range 2-15), and average time interval between CC and cancer diagnosis was 4 years (range 0-14 years). Besides the BCC and cutaneous squamous cell carcinoma (cuSCC) cases, the total number of cancer cases was 98. However, five cases of rare cancer types were excluded since it was considered not applicable for data calculation, leaving 93 cancer cases for analysis. Patients with CC had a significantly increased risk to be diagnosed with cuSCC (SIR 3.27; 95% CI 2.42-4.32) and a significantly decreased risk for CRC (SIR 0.23; 95% CI 0.04-0.75). Numbers of observed and expected cases and SIR for each cancer are presented in Figure 6. SIR for each cancer diagnosis were also calculated separately for each region (data not shown). The risk of lung cancer was increased in Skåne (SIR 1.85; 95% CI 1.05-3.03) but since there were no other cases in the other regions, this did not become significant for the whole group. For cuSCC, the mean time interval between CC diagnosis and cancer diagnosis was 5.4 years (range 0.6-12.1 years). In a sensitivity analysis, we excluded the cuSCC-cases diagnosed within two years after CC-diagnosis, resulting in 34 observed cases and SIR 2.42; 95% CI 1.70-3.34.

Cancer site	Observed	Expected	SIR	mid-p
Eye	1	0.16	6.07	0.164
Oesophagus	3	0.79	3.82	0.054
Cervix	2	0.61	3.28	0.15
cuSCC	46	14.08	3.27	0.001
Vulva	1	0.37	2.70	0.364
CNS	4	1.65	2.42	0.113
Kidney	4	1.69	2.37	0.121
Stomach	3	1.54	1.94	0.273
Bladder/Ureter	10	5.16	1.94	0.055
Rectal/Anus	7	4.03	1.74	0.167
Pancreas	3	1.96	1.53	0.449
Lung	14	9.30	1.51	0.142
Prostate	10	9.68	1.03	0.878
Leukemia/Myeloma	4	3.69	1.08	0.816
Melanoma	5	5.10	0.98	1.000
BCC	94	95.89	0.98	0.847
Breast	16	21.48	0.74	0.233
Lymphoma	2	3.03	0.66	0.611
Uterus	2	3.66	0.55	0.411
Colon	2	8.81	0.23	0.009

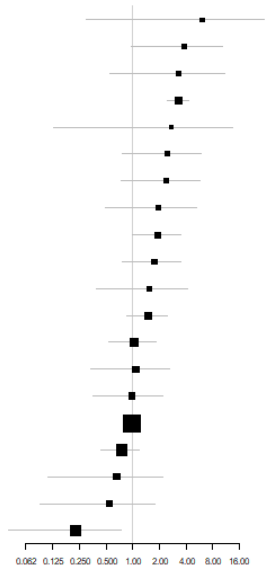


Figure 6, Observed and expected cancers in the entire Swedish cohort (stage 2).

cuSCC, Cutaneous squamous cell carcinoma; BCC, Basal cell carcinoma; SIR, Standardized incidence ratio. SIR with 95% Confidence intervals depicted in a forest plot.

Study IV

Cases

During the entire study period 2010-20, 1985 cases with first-time diagnosis of MC were included, of which 71% were women. Fifty-six percent were diagnosed with LC (n=1105). In the cohort, 61% of all men and 53% of all women were diagnosed with LC. For age and gender distribution, see table 9.

Table 9, Patient characteristics

		MC		CC		LC	
Total (n)		1985		880		1105	
Age (years)	mean (SD)	62.9	(15.7)	65.3	(14.1)	61.0	(16.7)
	median (IQR)	66	(52–75)	68	(57–75)	64	(50–74)
Men (n)		570		221		349	
Age (years)	mean (SD)	64.4	(15.8)	68.1	(13.0)	62.1	(16,9)
	median (IQR)	68	(57–76)	71	(63–77)	66	(51,5–75)
Women (n)		1415		659		756	
Age (years)	mean (SD)	62.3	(15.7)	64.3	(14.4)	60.5	(16.6)
	median (IQR)	65	(52–74)	66	(55–75)	63	(49–73)

MC, Microscopic colitis; CC, Collagenous colitis; LC, Lymphocytic colitis; SD, Standard deviation; IQR, Interquartile range.

Incidence of MC in Skåne in general

For the whole study period, the total ASR in Skåne was 14.2 cases per 100 000 person-years (95% CI 13.6-14.9). LC was significantly more common (ASR 7.9 per 100 000 person-years; 95% CI 7.4-8.4) than CC 6.3 (ASR 6.3 per 100 000 person-years; 95% CI 5.9-6.8), resulting in a CC:LC ratio on 0.8:1 (95% CI 0.7-0.9).

Age

The mean age at diagnosis of MC for the entire study period was 62.9 years, range 4-95. CC-patients were slightly older at diagnosis than LC-patients (mean age 65.3 versus 61.0 years) and male patients were significantly older than women at diagnosis of MC ($p < 0.01$). When calculating age-specific incidence rates by 5-year age groups, the incidence peaked in age class age class 80-84. In a subgroup analysis, new cases of LC were most common in age class 80-84, and in CC age class 74-80. See Figures 7 a, b and c.

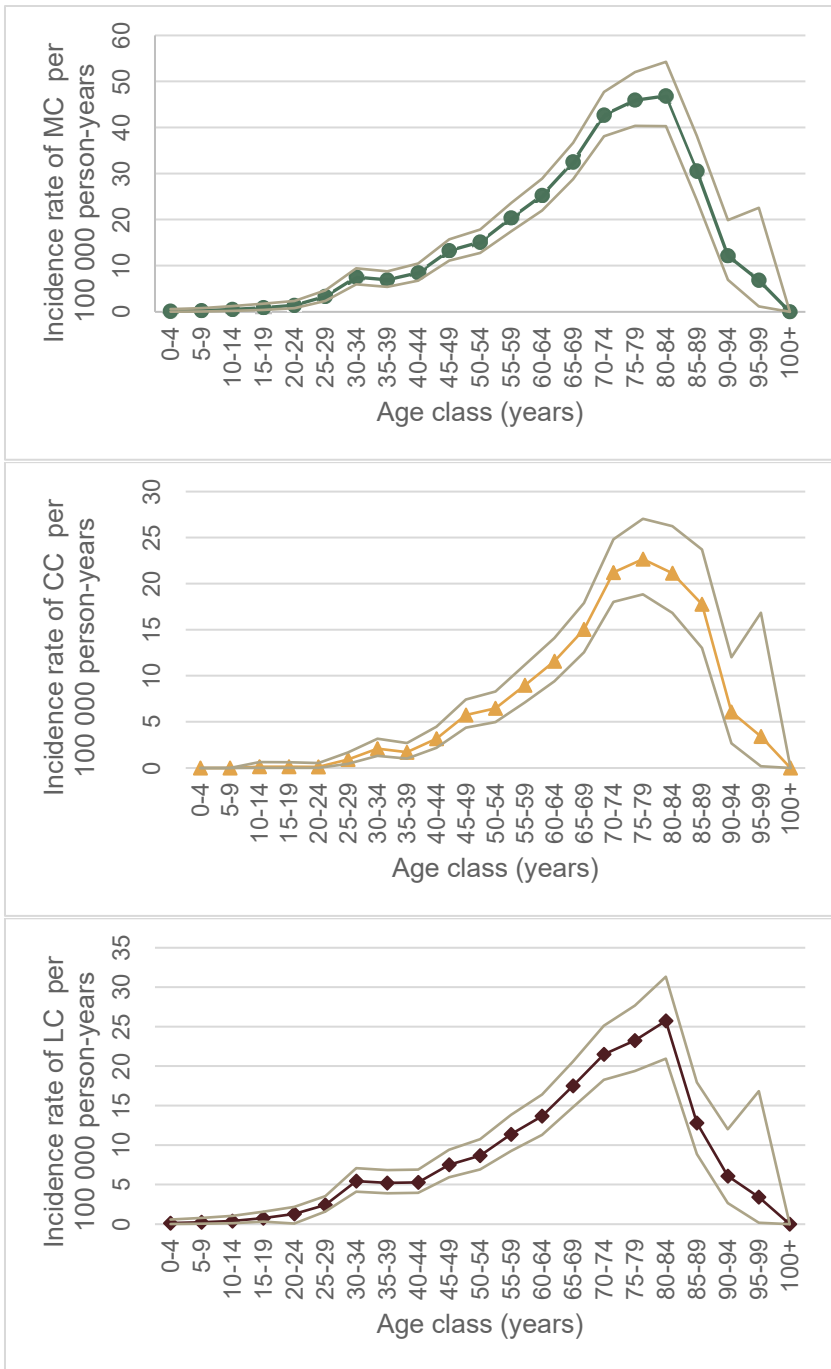


Figure 7a (MC), b (CC) and c (LC). Age-specific rate per 100 000 person-years with 95% confidence interval in microscopic colitis, collagenous colitis and lymphocytic colitis, respectively.

MC, Microscopic colitis; CC, Collagenous colitis; LC, Lymphocytic colitis

Gender

The incidence of MC including both subtypes was overrepresented in females. The MC F:M SRR was 2.3:1 (95% CI 2.1-2.5). LC had a slightly lower F:M ratio (SRR 2.0:1; 95% CI 1.8-2.3) in comparison to CC (SRR 2.7:1; 95% CI 2.3-3.1).

Calendar year of diagnosis

The CC incidence was relatively stable through the entire study period with a total ASR per 100 000 person-years of 6.3 (range 4.6-8.1). The incidence of LC increased significantly 2015-20 reaching 12.1 per 100 000 person-years (95% CI 11.2-12.9) in comparation to 2010-2014 (4.8 per 100 000 person-years; 95% CI 4.2-5.8) (SRR 2.5; 95% CI 2.2-2.9). Linear regression analysis showed that the LC incidence increased significantly with 1.0 case per 100 000 person-years for each year (p=0.009). No significant change in ASR for CC was seen over the years. The ASR pattern was similar between women and men (Figures 8 a and b and 9).

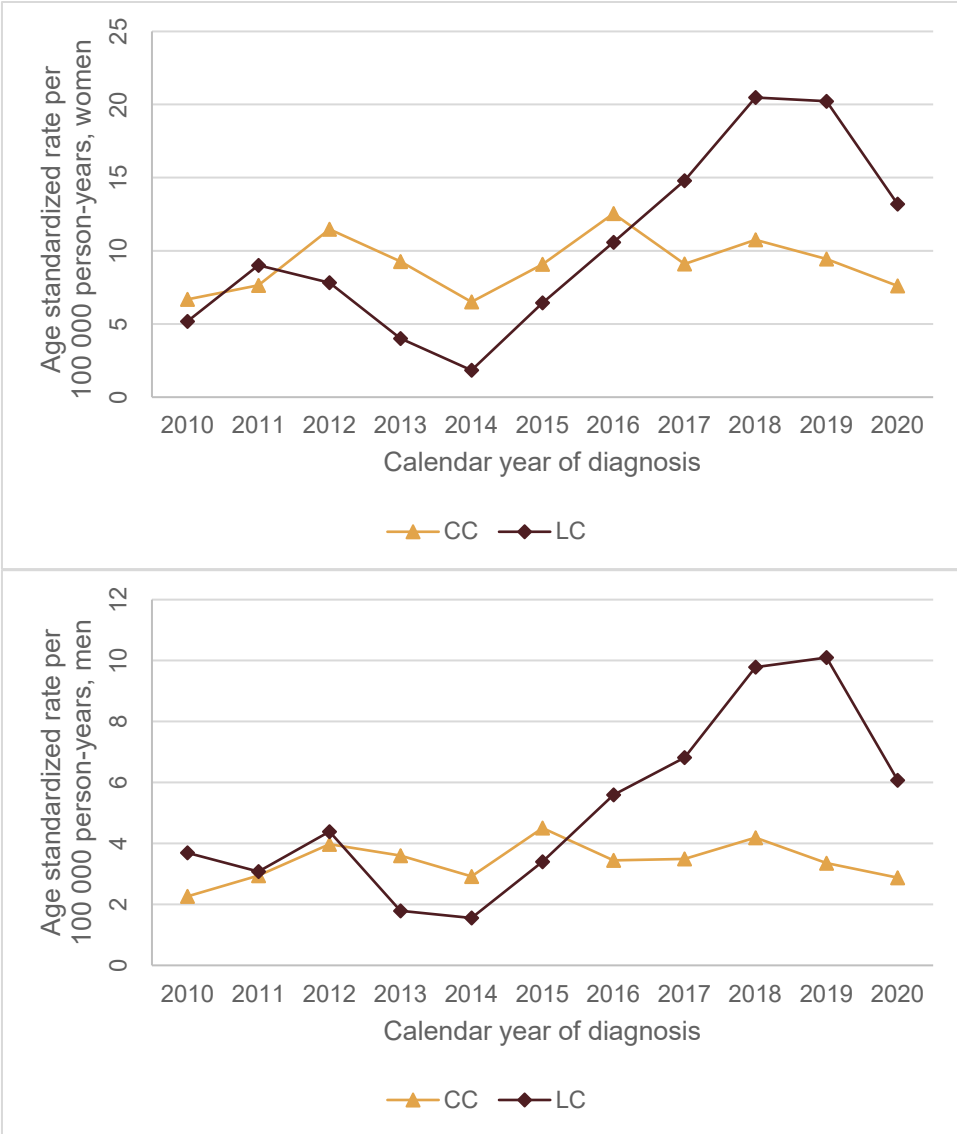


Figure 8 a (women) and b (men). Age-standardized rate per 100 000 person-years in collagenous colitis and lymphocytic colitis during the period 2010-20 in women and men, respectively.

CC, Collagenous colitis; LC, Lymphocytic colitis.

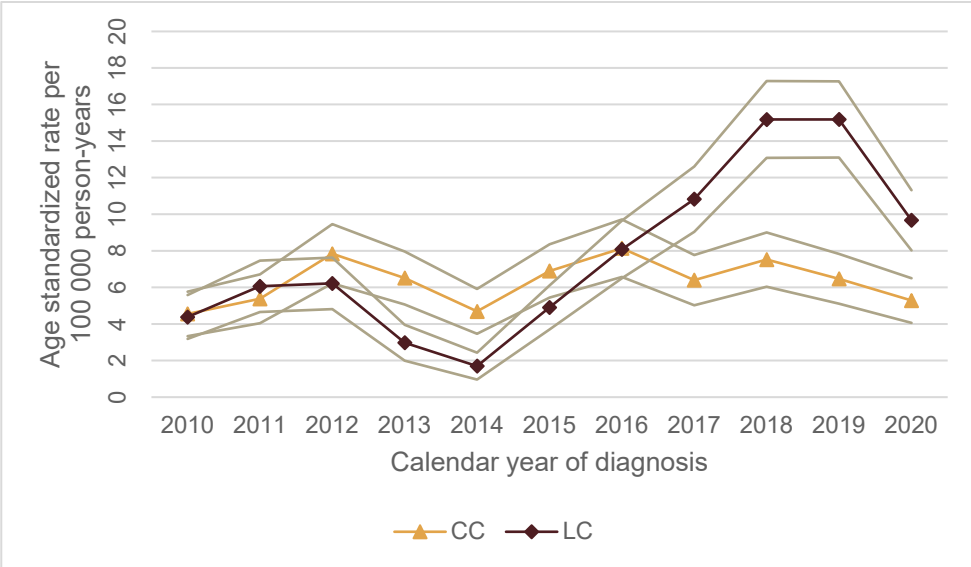


Figure 9 Age-standardized rate per 100 000 person-years for collagenous colitis and lymphocytic colitis with 95% confidence interval during the period 2010-2020.

CC, Collagenous colitis; LC, Lymphocytic colitis.

Population density

No differences in incidence rates of MC including the subtypes were observed when the population was divided in groups by population concentration (Table 10).

Table 10, Cases with MC, CC and LC divided into three main categories based on population concentration: A (rural), B (mixed, e.g., small towns) and C (urban areas).

	MC			CC			LC		
	Cases	**ASR**	**SE**	**Cases**	**ASR**	**SE**	**Cases**	**ASR**	**SE**
DSA A	235	13.35	0.90	110	6.17	0.60	125	7.19	0.66
DSA B	297	13.92	0.82	126	5.77	0.52	171	8.15	0.63
DSA C	1423	14.20	0.38	629	6.35	0.25	794	7.85	0.28
	Ratio	**95% CI**		**Ratio**	**95% CI**		**Ratio**	**95% CI**	
A/B	0.96	0.81-1.14		1.07	0.82-1.39		0.88	0.70-1.11	
B/C	0.98	0.86-1.11		0.91	0.75-1.10		1.04	0.88-1.23	
C/A	1.06	0.93-1.22		1.03	0.84-1.26		1.09	0.91-1.32	

MC, Microscopic colitis; CC, Collagenous colitis; LC, Lymphocytic colitis; ASR, Age-standardized rate; SE, Standard error; DSA, Demographical Statistical Areas.

Municipality of residence

For the entire study period, the ASR for MC varied between the municipalities. The maximum ASR was observed in Båstad municipality (at the coast in NW Skåne) reaching 20.1 per 100 000 person-years, and a minimum level in Bjuv municipality (inland in NW Skåne) at 8.2 per 100 000 person-years, see Figure 10. As illustrated in Figure 9, the increasing ASR for MC is mainly dependent on the marked increase of new cases of LC during the second part of the decade.

When stratifying LC by geographic location and diagnosis year-groups (2010-2014 and 2015-2020), high and significantly increasing crude incidence numbers were observed mainly in the north-west coastline in Skåne, see Figure 11 a (crude) and b (age-standardized). In 2019, where the LC incidence peaked, Helsingborg municipality had 50 cases as compared to two per year during 2010-2015. Båstad municipality had the highest incidence reaching 79.6 per 100 000 the same year. However, since Båstad is a small municipality, the number of cases was only 12.

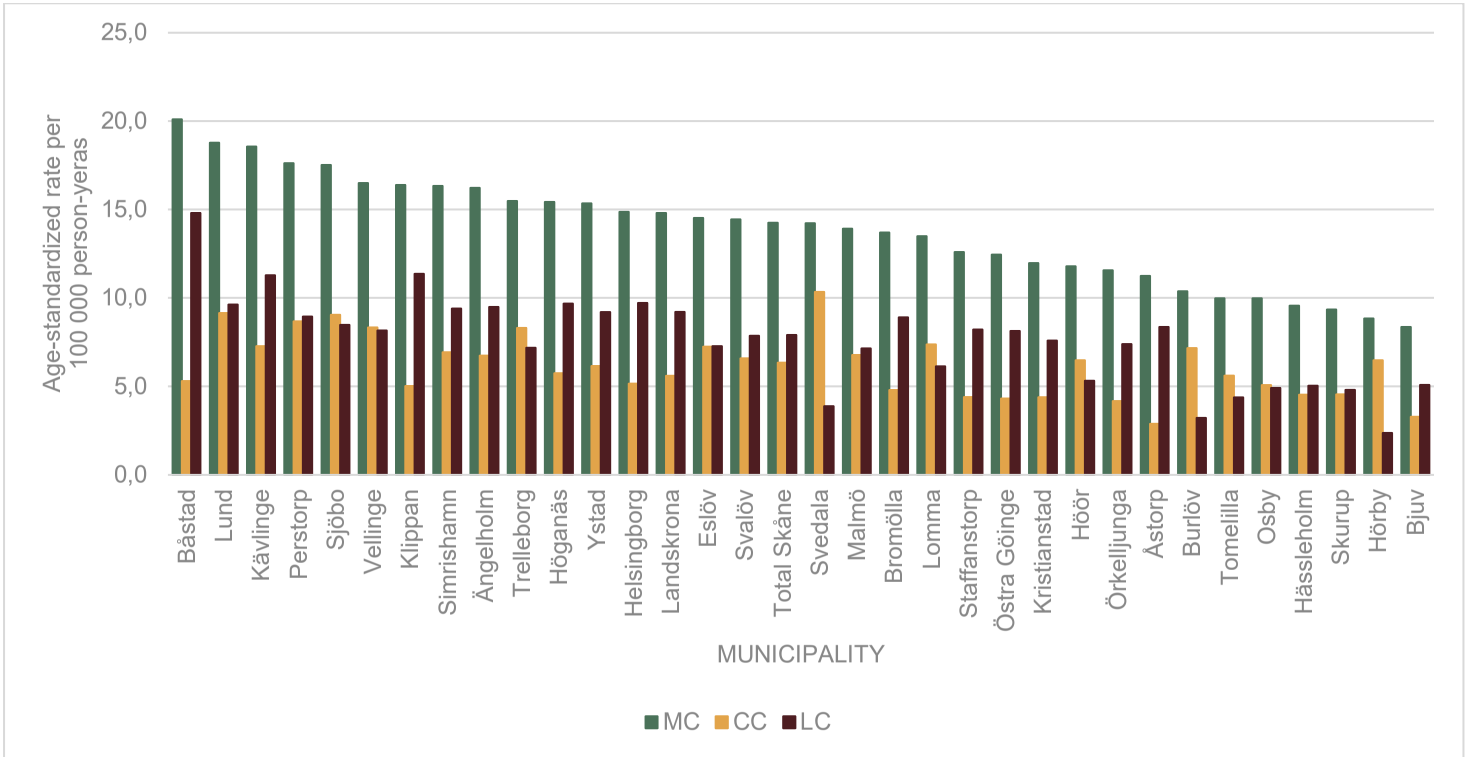


Figure 10, Age-standardized rate during the period 2010-20 divided in the 33 municipalities in Skåne.

MC, Microscopic colitis; CC, Collagenous colitis; LC, Lymphocytic colitis

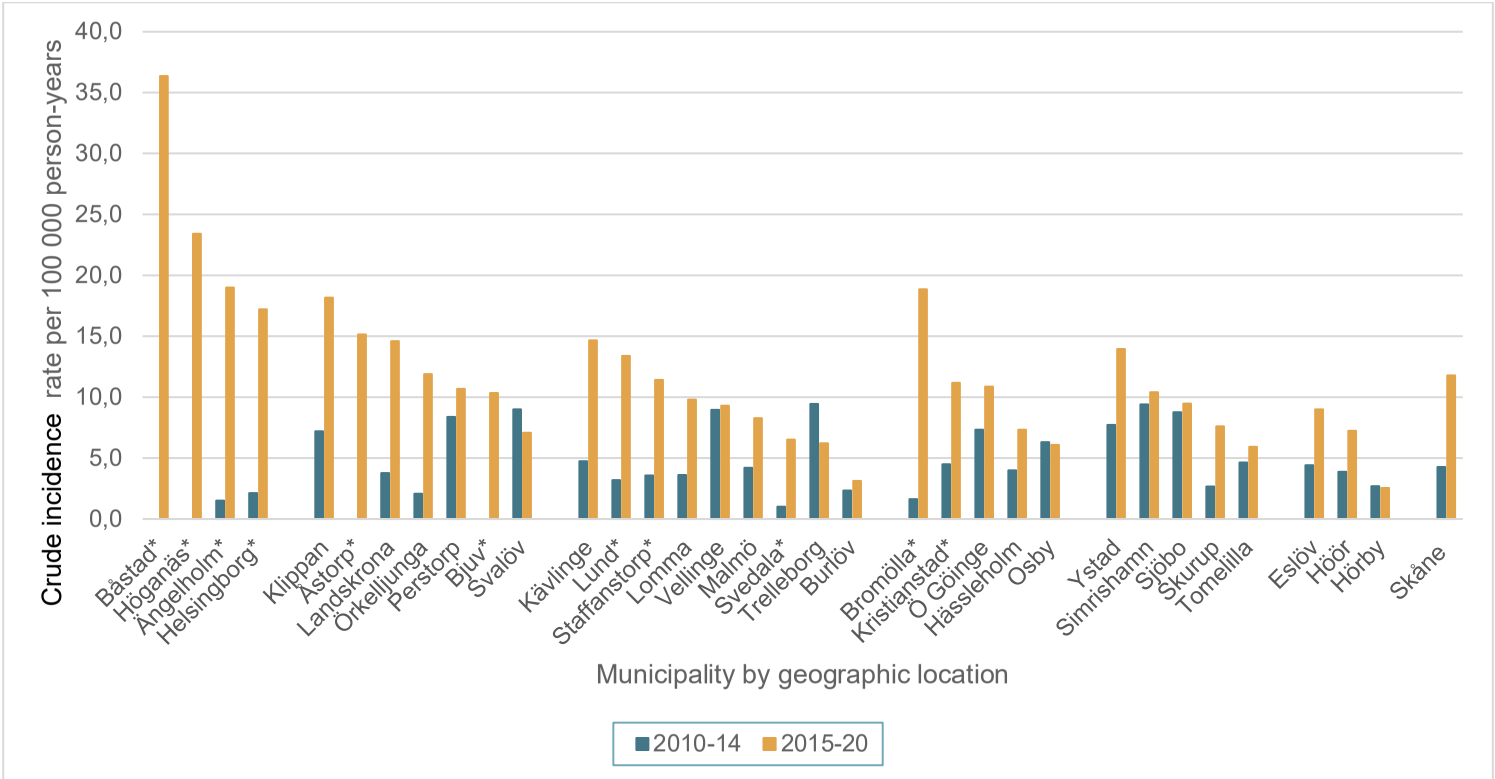


Figure 11 a, Crude incidence rate per 100 000 person-years for lymphocytic colitis divided into six parts of Skåne; north-west at the coast, north-west inland, south-west, north-east, south-east and central

North-west at the coast, Båstad, Höganäs, Ängelholm, Helsingborg; north-west inland, Klippan, Åstorp, Landskrina, Örkelljunga, Perstorp, Bjuv, Svalöv; south-west, Kävlinge, Lund, Staffanstorp, Lomma, Vellinge, Malmö, Svedala, Trelleborg, Burlöv; north-east, Bromölla, Kristianstad, Östra Göinge, Hässleholm, Osby; south-east, Ystad, Simrishamn, Sjöbo, Skurup, Tomelilla and central, Eslöv, Höör, Hörby. *, significantly different age-standardized rates between the two periods ($p < 0.05$).

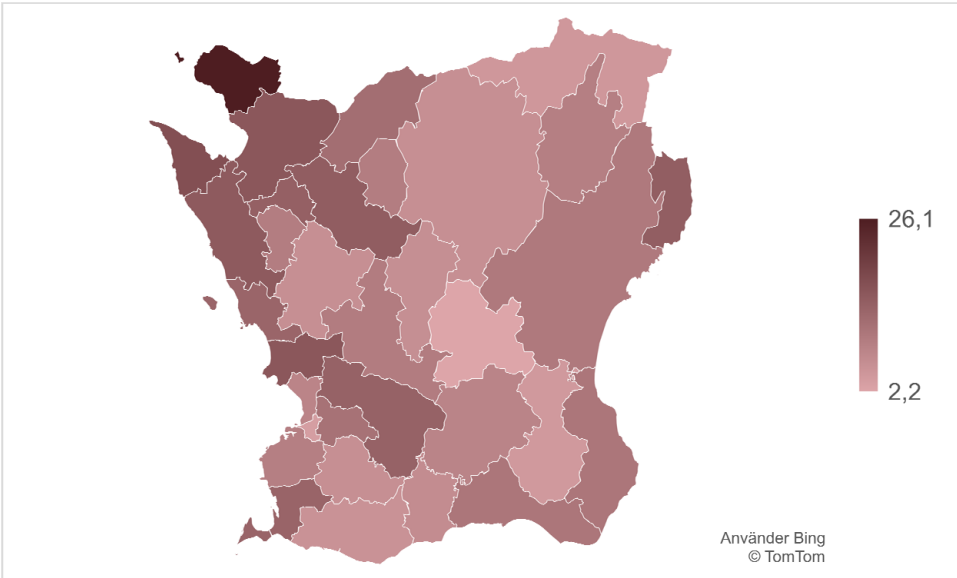


Figure 11 b, Age-standardized rates per 100 000 for lymphocytic colitis 2015-2020, illustrating the variation in incidence between different parts of Skåne.

Colonoscopies

The total number of colonoscopies with biopsies in Skåne during the follow-up were 88955. The mean number per year was 10102 (range 9589-10839). During the pandemic year 2020, 9589 colonoscopies were performed, which is the lowest number during the time period. During the whole period, 22.3 MC-cases were discovered per 1000 colonoscopies. The lowest rate was found during 2014, were only 7.3 cases per 1000 colonoscopies were found. This is in line with the low number of the MC cases that year. See Figure 12.

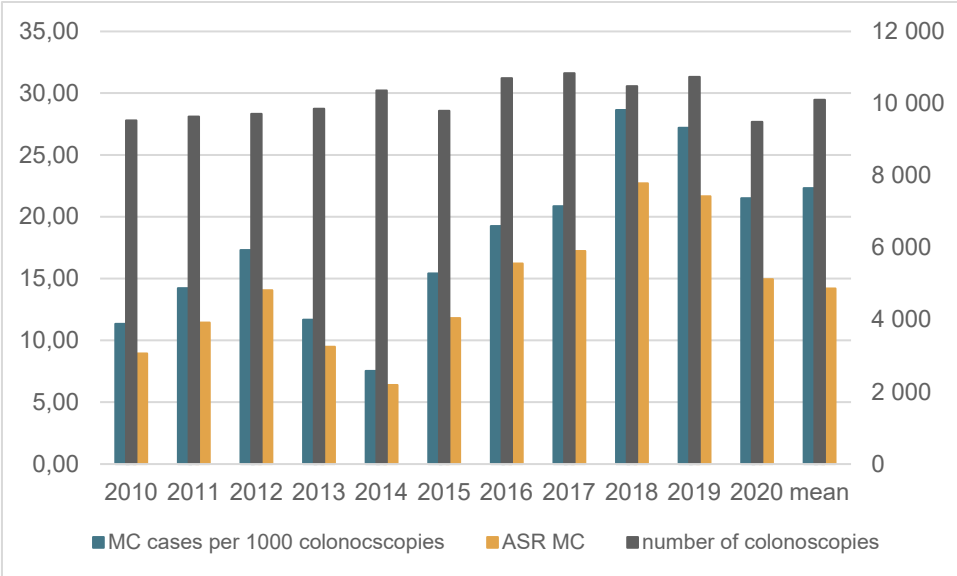


Figure 12, Left y-axis, cases of microscopic colitis per 1000 colonoscopies in Skåne in relation to age-standardized rate per 100 000 person-years of microscopic colitis 2010-2020. Right y-axis, total number of colonoscopies with biopsies in Skåne.

MC, microscopic colitis; ASR, age-standardized rate.

Discussion

Methodological considerations

Randomized controlled trials are generally regarded as the most proper method of hypothesis testing in epidemiology (84). In an experimental study setting, the effects of a particular intervention or therapeutic procedure is studied. The subjects in the study population are randomly allocated to intervention and control groups, and the outcome is assessed by comparing the results. If the initial randomization is done properly, differences between the groups depend on chance unaffected by biases of the investigators (85).

On the other hand, in observational studies, the study population involved is from the natural environment. The researcher measures but does not intervene, both considering exposure (*e.g.* intake of specific dietary factors) and outcome (microscopic colitis). In that case, potential **confounding factors** need to be considered and managed in all stages of the investigation- if possible.

This thesis is not based on interventional studies. Instead, epidemiology, and occurrence of putative autoantibodies have been scrutinized. **Study I**, a prospective **cohort study**, is based on all individuals (almost 30.000) recruited to the MDC. Exposures to different factors were measured (*e.g.* intakes of certain macronutrients and life-style factors) and the development of outcome (MC or not MC) could be evaluated. The data collection concerning exposures was done during the nineties, and was extensive which enabled us to test for various potential confounding factors within the study, for example smoking, physical activity etc. However, the MDC study was designed many years ago by another research team and we did not have any influence on which exposures that were studied. In MDC, 40 % of those that were invited, participated. Therefore, **selection bias** (systematic differences between participants and non-participants) is important to take under consideration. For example, Manjer *et al.* could conclude that the mortality rate during recruitment and follow-up of MDC was higher in non-participants, indicating that this group may have had an unhealthier lifestyle than the participants (86). The statistical analysis method of choice in this study was Cox proportional hazard risk model. The method analyses the effect of several variables (risk factors) upon the time it takes for a specified event (in this case; MC) to occur.

Study II was conducted in a laboratory setting with an ELISA method to identify antibodies in sera from CC patients and blood donors. In ELISA, the workflow and

protocols are complex and include several wash steps. This is challenging and can of course imply mistakes that influence the result. Plate wash instrumentation, buffers and procedure are often causes when an ELISA test goes wrong. Consequently, precision, double-check procedures etc are important. Despite these measures an intra- and inter- assay variability up to 10% may occur.

In study III and IV, incidence rates were calculated. A frequently occurring problem in incidence studies involves comparison of incidence rates for a particular diagnosis between two different populations. A false picture is given if only the crude rates between two populations are calculated, since the age structure may differ. For example, even though if the age-specific rates are the same, an older population would end up with more cases than in the younger group. Hence, when comparing incidence of a disease between groups or areas, one must take the age structure in consideration by performing age-standardization. This can be ruled out in two ways, one so-called direct and another indirect. By the direct method, the crude age-specific rates are multiplied by the weights from a set Standard Population, for example "the World Standard Population" or, as in the case in study IV, from Sweden's population in 2020. By this calculation, crude rates are age-standardized and thereby the values will be comparable with each other, even though the rates origin from different demographic regions or municipalities. In the indirect method of standardization, a standard set of age-specific rates (for example, the actual rates in a region) are used to calculate the expected numbers of a particular disease, e.g., cancer. The SIRs can then be calculated by comparing the observed numbers of cases (for example, actual numbers of CRC in the CC-population) with the expected.

The direct standardization method is generally considered to be preferred when it comes to absolute risk calculations and to achieve descriptive data, why this method was suitable for study IV. However, in study III, we aimed to rule out the relative risk for cancer between patients with CC and those without. In that case, the indirect method is the most suitable method of choice. Study III was conducted in two phases, first with the cohorts from Edinburgh and Malmö where direct age-standardization was conducted. From the ASRs, the relative risk (RR) for each specific cancer in the CC-population was calculated by dividing the CC-ASRs with the ASR from the background population (in this case, Lothian). In the second stage, where three different regions in Sweden were included, an indirect age-standardization was accomplished. This resulted in SIRs where the expected numbers of cases were calculated from the specific cancer incidence rates in respective areas for each age-group and gender. The method chosen in the second stage probably resulted in more valid data than that in stage 1. The choice to carry on with Stage II was based on the fact that 15 out of 16 of the NMSC cases came from Sweden. This unexpected skewness is not in accordance with other NMSC incidence studies where Sweden and Scotland show comparable rates (87). This indicated that the registries of NMSC we had access to in Scotland were not

complete and the validity of the result concerning the NMSC-risk in stage 1 was questionable. A concern in indirect standardization in study III is that the reference population was contaminated. In other words, the CC-cases were also included in the reference group. Since the CC-population was very small in comparation to the background population it did probably not cause a significant influence on the background population's incidence rates for different cancer forms.

A general limitation in incidence calculations from register studies is that it is not possible to adjust for possible confounding factors, and the result can only be considered as observational. However, the descriptive data can be hypothesis-generating and causal associations may be discovered in studies with another design that are based on the results from a descriptive study.

Study-specific discussion

Study I

In this study, no associations between intake of dietary factors and MC could be found. Lifestyle factors, such as physical activity level, educational level and BMI did not seem to influence the risk of MC either. However, female gender, increased alcohol consumption and smoking increases the risk for MC.

To the best of our knowledge, this is the first study that investigates the association between diet and MC. However, diet as a risk factor has been studied previously in IBD where high sugar intake and low intake of vegetables were associated with an increased risk of IBD (88). In this study, exceeded alcohol intake seem to enhance the risk (p for trend=0.032). However, the aHR did not increase significantly why we cannot draw any conclusions concerning on what level the intake of alcohol begins to be harmful. In a review concerning alcohol intake and cardiovascular disease, intake over 100 gram alcohol per week (approx. 8 standard drinks [SD]) is associated with an increased risk for morbidity (83). In our study, the quartile with the highest intake of alcohol consumed nine or more SDs per week. This group did not have a significantly increased risk compared to zero consumers in our data, but the amount consumed could serve as a relevant threshold. The association between high intake of alcohol and onset of MC is probably multifactorial and could be similar to the effect it causes on patients with IBD, namely increased permeability and epithelial cell damage in colon (89). Furthermore, a high alcohol intake is associated with dysbiosis and a reduction of *Akkermansia muciniphila* (90). This bacterium is of importance for the turnover of the mucin layer (91, 92).

Up until 2015, two studies had investigated the association between MC and alcohol intake. Yen *et al.* described a significantly increased trend between weekly and daily alcohol intake and the MC population compared to controls, whereas Roth *et al.* did not observe any differences between MC patients and controls (93, 94).

Study II

In this study we were not able to identify presence of any autoantibodies against Col III, Col IV, MMP-9, TIMP-1 or TNC to a larger extent in CC patients compared to healthy blood donors. The presence of antibodies against TPO, ASCA and total IgE did not differ significantly from the background population either. However, the collagen-associated autoantibody-positive patients had a significant shorter disease duration compared to those without. This may be caused by a general increased immunological activation at the time of disease onset including development of non-disease-specific autoantibodies that can still be detected some years after onset. As time goes by, a contemporary decrease in non-specific immune activation results in a decrease in circulating antibodies, something that has previously been described in CeD (95). In conclusion, autoantibody prevalence may reflect time from disease onset.

Despite the Th1-dominated cellular immune response in CC (45), the symptoms are mainly local and extra-intestinal manifestations are scarce which indicate that the systemic immunological impact in CC is significantly lower than in other immune-driven diseases in the same organ system, such as IBD. In CC, activation of genes involved in presentation of luminal antigens to bacteria and viruses has been found in tissue samples. A bacterial origin for CC could thus be contemplated (96). Furthermore, the only effective treatment, budesonide, mediates its effect locally in the large intestine, while systemic corticosteroid treatment has lower impact. Consequently, in CC, inflammatory markers may only be seen in the intestinal wall rather than in the systemic circulation.

Based on our results any autoimmune pathogenetic mechanism with an origin from the investigated structures in the collagen layer in this study is less plausible. However, the many autoimmune features described in CC talks for an autoimmune pathogenesis which can exist via other antigens.

Study III

The first stage of this study indicated that the risk for lung and bladder cancer was increased in patients with CC. Also, the risk of NMSC was increased in this cohort. In the second stage, which consisted of a large Swedish cohort we could confirm the decreased risk of colon cancer in patients with CC, something that has been reported in previous studies (66, 72). Also, we could confirm the increased risk of NMSC that could be observed in stage 1 and discovered that this increase was caused by a high incidence of the skin cancer type cuSCC.

A few studies that have investigated risk of CRC in CC patients. However, these studies either included prevalent cases of CRC or comprised a limited number of CC-patients (66, 72). In this study, we estimate that the risk of being diagnosed with colon cancer is reduced at least 4 times (SIR 0.23; 95% CI 0.04-0.75). In patients

with longstanding albeit low-grade inflammatory response in the colon, one would expect an increased risk of CRC. However, in this case the modest inflammation in CC patients may be protective. For instance, frequent watery diarrhoea reduces the transit-time, something that may also reduce the potential impact from toxic agents. One hypothesis presented by Yen *et al*. is that the increased number of lymphocytes in the colonic mucosa in MC patients may have a protective function against carcinogenesis through recruitment of γδ T-cells that kill DNA-damaged or stressed cells (66).

In the second stage of the study with patients from Sweden, the calculated risk increase for cuSCC in patients with CC was more than three-fold (SIR 3.27; 95% CI 2.42-4.32). UV-light exposure and immunosuppressive treatment related to organ transplantation are risk factors for cuSCC with high level of evidence (97, 98). Also, smoking seems to increase the risk (99), as well as glucocorticoids, but with lower evidence (100, 101). In IBD, the incidence of NMSC (both cuSCC and BCC) is increased in patients, but likely related to the exposure of immunosuppressive treatment (thiopurines and biologics) (102). However, Singh *et al*. also described an increased risk of BCC in men with Crohn's disease not treated with immunosuppression (103). This can indicate that the inflammation *per se,* not only the immune-suppressive treatment, also is a risk factor for NMSC, probably as a result of dysregulation of the immune system. In CC, Günaltay *et al*. described a decreased production of IL-37 in CC patients, indicating a disturbed immune response (46). Thus, the elevated risk of cuSCC in CC may be related to risk factors both IBD and MC share, such as smoking, a malfunctioning immune system caused by the disease itself, medication or other not yet known procarcinogenic factors. Interestingly, in coeliac disease that is associated with MC, the risk for NMSC is also increased (77).

The association between CC and smoking habits is already well described (58). Smoking is related to cancer in both lung and bladder which is probably the reason why these incidence rates were elevated in the first stage of the study (104, 105).

Study IV

The results in this study verifies the ongoing increase of MC incidence over the world. However, our Swedish data is still not at the same levels as recently reported from Denmark (MC 20.7 cases per 100 000 person-years, 2001-2016) (8) and Olmsted, USA (25.8 cases per 100 000 person-years, 2011-2019) (14). It remains to be seen whether the incidence in Sweden will end up at the same level in the next decade. In this study, the CC incidence remained stable while a substantial, significant increase was apparent in LC. Actually, we report the highest rates in LC this far described in Sweden (15.2 cases per 100 000 person-years 2018-2019). In our data, LC is far more common than CC during the last part of the decade (2015-2020) which is in line with another Swedish nationwide study (25) as well as data

reported overseas in USA and Canada (13, 14, 16). In contrast, nationwide studies from other parts of Europe (Iceland, the Netherlands and Denmark) present data where CC is more common than LC (5, 8, 9). The explanation behind these differences is unclear. Since the number of biopsies that is taken during colonoscopy vary in different countries, some cases of CC could be missed or mistaken as LC in view of the fact that the thickness of the collagenous band may vary in different parts of the large intestine. However, since LC incidence seem to vary more than CC over this limited time span another and more likely interpretation is that a yet unidentified environmental risk factor probably influences LC to a higher extend than CC.

This study confirms the risk factor of female gender with a more than double relative risk for MC in females compared to men, which is in line with previous studies (8, 9, 25). The association between MC and female gender is somewhat stronger in CC (SRR 2.7:1) than in LC (SRR 2.0:1), which may be a result of the fact that CC is more dependent on hormonal factors.

Population density as a risk factor for MC is to date not studied to any larger extent. However, this factor could be studied by comparing incidence rates from countries with different population concentrations. In Iceland, a country with three inhabitants per square kilometre, the incidence rate for MC 1995-1999 was 9.2 per 100 000 person-years in comparation to Netherlands (409 inhabitants per square kilometre) with 3.4 cases of MC per 100 000 person-years (5, 9). In line with this, a nationwide study from Denmark demonstrated higher incidence of MC in less populated regions compared to densely populated (8). However, the categorization by region in the latter investigation does not exclude that all MC cases actually lived in urban areas within the less populated regions or in areas with a higher mean age. In our study, we sorted each case of MC by population concentration resulting in three groups (dense, cities; medium, small villages and low populated, rural areas). By organising the groups in this way instead of region of residence, the risk of a misclassification could be minimized. Despite this, no differences could be found between the groups, and MC incidence including the subtypes CC and LC were evenly distributed across the groups. Therefore, population density is probably not a risk factor for MC. We also divided the cohort based on municipality of residence. Interestingly, some municipalities presented sharp increases in incidence rates, especially in LC and particularly during the last part of the decade. For example, Båstad municipality in north-west Skåne, peaked at 79.6 LC cases per 100 000 person-years during 2019, whereas for the whole region of Skåne the age-standardized rate for LC 2019 was 15.2 cases per 100 000 person-years. In view of this, some environmental factor that affects disease onset must be suspected.

The number of colonoscopies with biopsies was stable during the follow-up time. Least examinations were performed during 2020, probably as a consequence of the covid pandemic. However, the number of MC cases per 1000 colonoscopies during 2020 (21.5) was almost of the same magnitude as the number for the whole period

(22.3). Consequently, the sharp increase of LC cases in north-west Skåne is probably not due to an increased number of endoscopies. Furthermore, if that was the case also the number of CC cases would increase during the same time span.

Strengths and limitations

Study I

Assessing dietary habits is complicated and has historically been criticized and questioned. It is complicated to measure diet with validity and precision and there is always a risk of **recall bias**. However, the MDC dietary assessment method was comprehensive and included food dairy, questionnaires and interviews. Also, it has been studied and assessed to have a relatively high validity (106). Another limitation in this study is that the dietary habits were assess only at study start. Hence, there is a possibility that the individuals have changed habits during follow-up. In this study, we made a sensitivity analysis were those that had declared change in food habits at data collection were excluded. However, no difference in the results were seen. However, since we have no data on change of food habits during follow-up, this is a factor to take in consideration when interpreting the results. On the other side, Golbohm *et al.* evaluated the stability of food habits among participants in a Dutch cohort study, describing that the change of mean intake for most nutrients was limited in a five-year follow-up (107). A strength of this study is the prospective design where assessments of dietary habits and lifestyle were performed prior to disease onset. Diagnosis of a gastrointestinal disease would most probably result in dietary changes and a prospective design ought to limit that factor as well as risk for recall bias.

Study II

Since the pathogenetic mechanisms associated with the collagen layer was our main focus in this study, we only selected cases with verified active and pronounced CC. This resulted in a rather small cohort with 66 women which may lead to difficulties for significant outcome, but on the other hand it could increase the validity since no cases with mild disease or uncertain diagnosis were included. In addition, if any autoantibody should have any relevance, at least a significant minority ought to have it. The strict selection of this homogenous group should have increased the probability to identify any type of antibody but failed to do so, which increases the likelihood that our results are generalizable. The control group was not age matched, which may be considered as a flaw. However, the cut-off value used to assess presence of antibodies must be determined from a healthy control group, otherwise there is a risk of a **type II error**. If the control group would consist of older women

(which often are affected by inflammatory diseases/processes) there would have been an increased risk of presence of autoantibodies in that group, too (108).

Study III

Up until 2019, this was the largest study concerning the risk of cancer in patients with a previous diagnosis of CC including 1141 patients in stage II. In this stage, the studied regions and its population are well defined. Since the cancer registry at the National Board of Health and Welfare covers more than 96 % of the cancer cases in Sweden and the control groups consisted of all cancer cases in the same regions as our cohorts, the data can almost be considered as complete (109). Because the data achieved for this study comes from registries, no information about confounding factors (*e.g.* smoking status, medication use etc.) were available. The associations are therefore not necessarily causal and have to be considered as observational. Another limitation is that we did not get information about location of the skin tumours since this is not available in the National Cancer Registry. One explanation behind the high numbers of cuSCC may be caused by detection bias, that is that patients are coincidently diagnosed with other conditions when they are in frequent contact with health care services. However, in a sensitivity analysis, where all cuSCC-cases diagnosed within the first two years after CC diagnosis were excluded, the result still indicated an increased risk (SIR 2.42; 95% CI 1.70-3.34).

Study IV

This study was registry-based, and information about comorbidity, medication, or lifestyle related risk factors such as smoking was not possible to retrieve, which all may contribute to the demonstrated variations. A strength is that Skåne is a well-defined region where almost all patients attend their local hospital. All biopsies are analysed at the Department of Pathology and can be collected through available data bases. Also, we are, to the best of our knowledge, the first research group that has investigated population density as a possible risk factor for MC.

General discussion

The main aim of this thesis was to investigate unexplored pathogenetic mechanisms, epidemiologic factors and prognosis of microscopic colitis to achieve a better understanding of the disease in particular but also about gastrointestinal immune-mediated diseases in general. With this knowledge it can be possible to establish the relationship between the other major types of gastrointestinal immune-driven diseases IBD and CeD as well as increase the understanding behind these diseases' behaviour in general.

One question I wanted to answer in this thesis was whether MC is an autoimmune disease. We could not demonstrate presence of autoantibodies in study two, which strengthens the hypothesis that MC, in line with IBD, demonstrates more characteristics of an immune-driven disease rather than autoimmune. However, in recent studies autoantibodies against immunological components (granulocyte macrophage-colony stimulating factor and anti-integrin) have been identified in Crohn's disease (CD) and ulcerative colitis, respectively (110, 111). Since their role in the pathogenetic process has not yet been clarified, it cannot be concluded that they are true autoantibodies. CeD on the other hand, demonstrates presence of autoantibodies against tissue transglutaminase, which indicates that this disease can be defined as autoimmune. However, an external factor (gluten) is required; which not is regarded as an established prerequisite for autoimmunity.

These three major types of immune-driven diseases differ in many ways, but they also share many characteristics. Also, the diseases covaries to some extent. Patients with MC are often diagnosed with CeD as well (74) and transitional forms from IBD to MC and vice versa have been reported (112). Furthermore, from an epidemiological point of view, all three disease groups have increased during the last decades. As demonstrated in the fourth study, MC and in particular LC is increasing in incidence in Skåne. In Scandinavia, the incidence rate of CeD is 50 cases per person-years (113) and during the last decade worldwide, the rate has been increasing with 7,5% each year (114). Concerning IBD, the incidence in Denmark and especially Faroe Islands is the highest in the world (ASR 2010: 81,5 cases per 100 000 person-years in Faroe Islands). Globally, the IBD incidence is increasing and mostly because of a sharp increase in newly industrialized countries. In Europe on the other hand, the increase seems to have levelled out (115-117). This pattern can also be seen in MC and CeD where the incidence has stabilized during the last years in the most endemic regions, such as parts of northern Europe (8, 113, 118).

The immune-driven gastrointestinal diseases all seem to follow the same pathogenetic pattern: In genetically predisposed individuals (1) exposed to specific environmental risk factors (2), a luminal antigen (3) triggers an abnormal immune response in the mucosal layer (4). The luminal antigen requires some kind of barrier dysfunction (5) to penetrate to the mucosa. The local immune response also activates a systemic response (6) to some degree.

1. The genetic predisposition differs between the three disease groups. In IBD more than 200 genes for epithelial function and immune function have been identified as factors that contribute to increased disease risk (119). In MC and CeD much of the genetic risk is mediated through the HLA system, *i.e.*, predominantly HLA DQ2 (37).

2. Environmental risk factors are present in all these groups. Environmental factors influence gut microbiota, barrier integrity and immune function. Factors of importance are diet, smoking, alcohol consumption, vitamin D and

medication. In this study, we could not verify any association between diet and MC, which is present in particularly in CeD (gluten) but also in IBD where excessive consumption of sugar, animal fat and linoleic acid is considered as risk factors for disease development, whereas a high fibre diet and citrus fruit consumption may play a protective role (120). Smoking exceeds the risk for MC and CD while it has a protective effect on ulcerative colitis (58, 121). The mechanisms behind the contradictory effects of smoking are not investigated to a larger extend but may be associated with an altered microbiota and barrier function (42, 122). Alcohol has associations with both MC and IBD, probably caused by the effect on the microbiota as well as the harmful effect on the epithelial layer but have not been investigated in CeD (89). Vitamin D deficiency has been linked to abnormal immune function and dysbiosis in CeD and IBD (123) but no association was found in MC (124). Medication is a well described risk factor for MC, especially PPI, SSRI and NSAID (61, 62, 125). The latter is also associated with IBD (126). In summary, the environmental risk factors mentioned above influence to a large extent the natural luminal milieu where the microbiota plays a key role.

3. The luminal antigen gluten in CeD is required for disease activation (127). However, in IBD and MC, no such crucial agent has yet been discovered. Concerning the effect on the intestinal microbiota of the environmental risk factors discussed above, the plausible antigen in IBD and MC could be a result of metabolites caused by the dysbiosis, or at least, the dysbiosis could be required for disease onset. In line with this – in all three disease groups, an altered microbiota with reduced diversity is evident (128-130), for example reduced levels of *Akkermansia mucinophilia* (41, 91). In dysbiosis, beneficial species producing short chain fatty acids are reduced while those producing hydrogen sulphide are increased in numbers (131). The dysbiosis harms the mucin layer and bacteria can than penetrate and reach the epithelium (91, 132) and this may in turn result in translocation of toxins and bacteria that trigger the mucosal immune cells.

4. The immune response in the mucosal layer is present in all diseases but have different profiles. Both the innate and the adaptive system are involved. In MC, faecal levels of eosinophil markers are increased (133) and faecal calprotectin (a stable protein associated with activation of neutrophils) increases in IBD relapses (134). In MC and CD, a Th1 cytokine profile is present. MC, especially CC patients, also have an enhanced presence of immune regulatory foxp3 cells (47). The immune response is probably influenced directly or indirectly by exposure to environmental risk factors.

5. The epithelial dysfunction may be a consequence of the altered microbiota but can probably also be caused by external pathogens. Onset of CeD may be triggered by infections and gastroenteritis, particularly *Clostridium difficile*,

is associated with an increased risk of subsequent MC (73, 135). The pathogens activate cells from the innate immune system such as macrophages and dendritic cells which activates cytokines and interferons, causing epithelial destruction. This also leads to an increased permeability which increase the interaction between luminal factors and the mucosal immune system, such as gluten peptides and tissue transglutaminase in CeD (127).

6. The systemic and extraintestinal effects are in particular present in CeD and IBD. However, skin manifestations can be observed in all three diseases with dermatitis herpetiformis and BCC in CeD (77, 136), erythema nodosum and pyoderma gangrenosum in IBD (137) and, as demonstrated in this thesis, cuSCC in MC.

In conclusion, the three disease groups share many characteristics, which at least to some extent, indicate common pathogenetic pathways. In genetically predisposed individuals, exposure for different risk factors will probably determine which disease the patient eventually will suffer from.

Conclusions

- Intakes of protein, carbohydrates, sucrose, total fat, SFA, MUFA, n-3 PUFA, n-6 PUFA, dietary fiber and zinc were not associated with MC
- Female gender is a risk factor for MC
- Active smoking and former smoking are both risk factors for MC
- Increased alcohol consumption increases the risk for MC.
- Lifestyle factors such as BMI, physical activity and education are not associated with MC.
- Patients with CC neither express autoantibodies against collagen III, collagen IV, MMP-9, TIMP-1 and TNC to a larger extent than healthy persons, nor ASCA, TPO or total IgE.
- The risk of cuSCC is elevated more than 3 times in patients with CC. Hence, clinicians must be extra aware of this association.
- CC patients have a decreased risk of colon cancer.
- The age-standardized incidence of MC 2010-2020 in southern Sweden is 14.2 cases per 100 000 person-years.
- LC is significantly more common than CC in Skåne, especially in some parts.
- A cluster of new cases of LC was identified in north-west Skåne between 2015-2020 and some environmental risk factor – or factors – must be suspected.

Future perspectives

The observations made in the articles in this thesis immediately lead to new hypotheses. In the first study, different macronutrients' association with MC was studied. As has been proposed in IBD, micronutrients as well as food additives could also be factors of importance for MC disease onset (138). One of the factors of special interest is emulsifying agents, which are suggested to dissolve the mucus layer, something that will contribute to development of inflammation in the gastrointestinal tract. Especially CMC and polysorbate-80 have been suggested to promote colitis in mice (139). Whether such food components may play a role in disease onset in MC remains to be clarified.

In the second study, we investigated presence of autoantibodies against relevant proteins and structures in the collagen layer. However, not all of the potential autoantigens were investigated, and they deserve to be mentioned. The levels of collagen type I and VI and procollagen type I and IV are elevated in the epithelium in patients with CC and are thus candidates for further studies (49, 51, 53). MMP-1, -2, -8, -13 and -18 all degrade collage and could also be potential targets for autoantibodies (140).

In the third study, we described an elevated risk for cuSCC in patients with CC. A dysregulated immune defence may be a contributing factor for this association. A study comprising CC patients with and without skin cancer is initiated but delayed due to the covid pandemic. This investigation aims at a better understanding of the immune system and skin microbiota in CC based on measurements of the immune system with flow cytometry and furthermore investigation of skin permeability, skin microbiota and prevalence of HPV.

The unexpected finding that the incidence of LC increased markedly in the north-west part of Skåne during a few years in the fourth study also deserves attention. In Helsingborg municipality, the increase in incidence was rather sharp and went from two observed cases in the beginning of the follow-up, to 50 cases in 2019. Influences of one or more environmental factors are a likely explanation behind this increase; *e.g.*, medication, prevalence of gastroenteritis or water quality. In order to find the yet unidentified cause to this temporal and spatial fluctuation in the incidence of MC, a further study is indicated where potential environmental factors are scrutinized.

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Paper I

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ORIGINAL ARTICLE

The association between the intake of specific dietary components and lifestyle factors and microscopic colitis

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BACKGROUND/OBJECTIVES: The incidence of microscopic colitis (MC) has increased over the previous decades. In addition to smoking and drugs, currently unidentified environmental factors may have a role. The aim of this study was to determine whether specific dietary or other lifestyle factors were associated with the development of MC.

SUBJECT/METHODS: The population-based cohort Malmö Diet and Cancer Study of 28 095 individuals was examined. Information about dietary habits was collected by a modified diet history method. Data on anthropometry were measured, and socio-economic and lifestyle factors were collected by questionnaires. Cases of MC were identified in medical registers. Associations were estimated using Cox regression analysis.

RESULTS: During a 22-year period, 135 patients were diagnosed with MC. Intakes of protein, carbohydrates, sucrose, saturated fat, monounsaturated fat, polyunsaturated fat, omega-3 or omega-6 fatty acids, fibre and zinc were not associated with MC. We could verify the previously reported association between MC and smoking (hazard ratio (HR): 2.29; 95% confidence interval (CI): 1.66–3.84) and the female gender (HR: 3.57; 95% CI: 2.22–5.74). High alcohol consumption was associated with an increased risk for MC (HR: 1.89 for the highest quartile; 95% CI: 0.82–4.33, *P* for trend = 0.032). In a *post hoc* analysis, alcohol intake including all patients independently of consumption seemed to reduce the smoking-related risk.

CONCLUSIONS: Despite a large cohort and a long follow-up period, we could not detect any dietary risk factors for MC. The aetiological mechanisms behind the positive impact of smoking and alcohol on MC risk should be investigated.

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INTRODUCTION

Microscopic colitis (MC) is an inflammatory disorder in the colonic mucosa that causes chronic, non-bloody diarrhoea. Because of histopathological differences, MC is divided into two subtypes: collagenous colitis (CC) and lymphocytic colitis (LC).[1] Age, female gender, smoking and drugs are known risk factors for the development of MC.[2–6] Data on alcohol consumption are not conclusive, but a possible association could exist.[4,7]

In accordance with the classic inflammatory bowel diseases (IBDs), ulcerative colitis (UC) and Crohn's disease (CD), the global incidence of both CC and LC has increased during the last decades.[1,8,9] An increased awareness of the disease improved diagnostic procedures, and yet unidentified environmental factors could contribute to this change.

MC has many features in common with classic IBD. Environmental factors, for example, certain dietary factors, smoking and alcohol have been suggested to have a role in IBD. There is a lack of investigations on the impact of dietary factors on the occurrence of MC. To anticipate the possible effect of dietary factors on MC, one possibility is to look at the observations made in IBD. A high intake of n-6 polyunsaturated fatty acids, which are found in red meat and margarine, increases the risk for developing both UC and CD.[10–12] A high total intake of fat also seems to result in an increased risk for IBD.[11] In contrast, fish consumption, particularly fish containing high concentrations of n-3 polyunsaturated fatty acids, seems to be negatively associated with UC and CD in some studies,[10,12,13] whereas another study found a positive association between omega-3 and CD.[11] High consumption of fibre and fruit has been associated with a decreased risk of developing CD,[11] and high vegetable intake was associated with a decreased risk of developing UC.[14]

Increased carbohydrate intake, as well as increased intake of plant polysaccharides (fibres), influences bacterial metabolism in the large intestine.[15,16] The gut microbiota could protect us from pathogens and is believed to influence gut inflammation through several different mechanisms.[10,11]

Some minerals influence the immune response. Zinc has been suggested to have a great impact on immune function in general,[17] and a lower concentration of zinc in the drinking water increases the risk of type 1 diabetes.[18] Zinc deficiency has been shown to increase symptoms in an experimental colitis model in rats.[19]

There is a known association between MC and coeliac disease.[20] The immune response in the gut in MC is dominated by a Th1 mucosal cytokine profile, which is in many ways similar to that in coeliac disease, suggesting a response to luminal antigens in MC.[21]

Our hypothesis was that lifestyle factors such as dietary factors, smoking, alcohol, obesity, education and physical activity are involved in the development of MC. To address this, the influence of lifestyle factors on incident MC was studied in a population-based cohort of 28 095 participants.[22–24] The aim of this

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prospective study was to examine whether lifestyle factors were associated with the development of MC.

MATERIALS AND METHODS

Study population

The Malmö Diet and Cancer (MDC) study is set in Malmö, Sweden's third largest city. The population was identified through the Swedish National Population Registry. All men born between 1923 and 1945 and all women born between 1923 and 1950 living in Malmö during the screening period (1991–1996) were invited to participate. Recruitment was conducted through advertisements in the local media and through invitation by mail. The only exclusion criteria were inadequate Swedish language skills or mental incapacity.

A total number of 74 138 inhabitants were invited to participate. With a participation rate of ~40%, the final cohort consisted of 28 098 individuals. A comparison of participants and non-participants has been previously presented.[22,23] In the present study, we excluded all participants with prevalent MC ($n=3$). This left 28 095 study subjects available for analysis. Of these, 11 062 were men (39%) and 17 033 were women (61%), with a mean age of 58 years (range 44–74). A more detailed description of the cohort is described elsewhere.[23,24]

Data collection

The participants visited the MDC centre twice. During the first visit, the study objects obtained information about the background and aim of the project and detailed instructions about the dietary assessment and other procedures of the study, and received an extensive lifestyle questionnaire to complete at home (including education, socio-economic variables, reproduction, alcohol habits, smoking, physical activity, household activity, substantial dietary change and medical history). The season of the data collection was registered. Height, weight and blood pressure were measured. At the second visit, a dietary interview was performed by trained interviewers, and the lifestyle questionnaire was checked for incomplete answers.

Diet assessment methodology

The dietary assessment method, specifically developed for the MDC study,[25,26] collected information on dietary intake by using a combination of the following:

A 7-day food diary where information about lunch and dinner meals, cold beverages including alcohol, pharmaceutical drugs, natural medication and nutrient supplements was registered.
A 168-item dietary questionnaire for the assessment of foods not covered in the 7-day food diary consumed during the last year, including information about portion size (assessed using photographic aids) and frequencies. The participants also described the overall meal pattern during weekdays and weekends.
A 1-h-long interview where participants were asked questions about portion size (assessed using photographic aids), food choices and food preparation practises for the foods collected in the food diary. The interviewer checked the food diary and dietary questionnaire so that the reported food consumption did not overlap and was in concordance with the reported meal pattern by the participant.

The average daily intake of foods (grams per day) was calculated based on the frequency and portion size estimates from the questionnaire and food diary (and interview). The food intake was converted to nutrient data using the MDC food and nutrient database, originating from PC KOST2-93 of the Swedish National Food Administration.[25] Data on the relative validity and reproducibility for the diet method have been described in detail.[23,26,27] Energy-adjusted validity correlations were as follows: protein, 0.54/0.53; carbohydrates, 0.66/0.70; fat, 0.64 (men)/0.69 (women); and fibre, 0.74/0.69. In September 1994, the routines for the coding dietary data were slightly altered to reduce the interview time.[25] A method version variable was created to control for undue influences.

Dietary factors

The dietary factors examined in this study were the average daily intakes from foods and supplements: protein, carbohydrates, total fat, saturated fatty acids (SFAs), monounsaturated fatty acids, polyunsaturated fatty acids (PUFAs), n-3 PUFA, n-6 PUFA, dietary fibre and zinc. All factors were adjusted for total energy intake, that is, energy percentage (E%) for macronutrients, g/MJ for fibre and mg/MJ for zinc.

We also used a diet quality index score developed within the MDC study to reflect the adherence to current Swedish nutrition recommendations and dietary guidelines.[28] The participants were given one point for reaching the recommended intake level for each of the following six dietary factors: SFAs (⩽14 E%), PUFAs (5–10 E%), sucrose (⩽10 E%), fibre (⩾2.4 g/MJ), fruits and vegetables (⩾400 g/day), and fish and shellfish (⩾300 g/week). The cutoff for SFAs was increased to 14 E% (that is, approximately one standard deviation (s.d.) increase) because few of the participants had an intake below the recommended level (10 E%). The score was divided into three categories: low (0–1 points), medium (2–4 points) and high (5–6 points).

Other variables

Smoking was categorized into never smokers, ex-smokers and smokers. Alcohol habits were categorized into five categories. Zero consumers reported no consumption during the past year in the questionnaire and no consumption in the 7-day food diary. The other individuals were divided into gender-specific quartiles based on their consumption in the food diary. Body mass index (BMI) was categorized into quartiles. The education level was divided into elementary school or below, primary and secondary school, upper secondary school, further education without a degree and university degree. Leisure-time physical activity was obtained from the estimated minutes per week spent on 17 different activities. A score was constructed taking into account the intensity and duration of the activity; the score was divided into quartiles.[29]

Definition of MC

The diagnosis of MC is based on the presence of gastrointestinal symptoms in combination with a macroscopic normal endoscopy and microscopic inflammation. The established histopathological criteria for CC are (i) a chronic inflammatory infiltrate in the lamina propria with (ii) a thickened subepithelial collagen layer ⩾ 10 μm and (iii) epithelial damage such as flattening and detachment. To fulfil the criteria for LC, an intraepithelial lymphocyte count ⩾ 20 per 100 surface epithelial cells must also be present.[30]

Cases

Patients who had undergone colonoscopies through the end of 2013 in Malmö owing to the occurrence of chronic gastrointestinal symptoms, in which the histopathological criteria for MC were fulfilled according to gastrointestinal pathologists with a special interest in and knowledge of MC,[30] were initially selected for this study. Only those who were included in the MDC study before disease development were finally included. The diagnosis of MC was set by the physician in charge, and all medical registers were scrutinised to confirm the diagnosis.

Statistical analyses

Patients with MC were compared with non-cases within the total cohort. The associations between dietary factors (divided into quartiles with the first quartile used as the reference) and lifestyle factors and MC risk were estimated through the Cox proportional-hazards regression models with time from baseline until diagnosis, death or end of follow-up as the underlying time variable. The analyses were adjusted for potential confounding factors (age, gender and smoking habits). Analyses with dietary factors were also adjusted for total energy intake, season for inclusion in the study and version of the dietary method. We also tested *P* for trend, that is, the linear association between lifestyle and dietary factors and MC risk by using these variables as continuous variables (for example, 1–4 for the dietary factors). This analysis tested whether increasing amounts of exposure had a positive or a negative association with MC risk. All dietary parameters were also tested in a multiple logistic regression model but with the same non-significant outcome (data not shown). In a sensitivity analysis, we excluded individuals with a reported dietary change in the past.[31,32] In the calculation of the association between smoking, alcohol and MC risk, both smoking and alcohol intake were set as reference. Values are given as hazard ratios (HRs) and 95% confidence interval (CI) or mean and range. A *P*-value below 0.05 was considered

statistically significant. For all statistical analyses, SPSS Version 22 (IBM Corp., Armonk, NY, USA) was used.

Ethics

The Ethics Committee at Lund University approved the design of the original MDC study protocol, which complied with the Declaration of Helsinki (LU-51-90). The study protocol for the present investigation was approved separately (LU 650/13).

RESULTS

In the total cohort, 135 patients with incident MC were identified during the follow-up period of 22 years. Of these, 115 were women (85%) with a mean age of 58 years (range 45–73), and 20 were men (15%) with a mean age of 61 years (range 49–73). Females had a higher risk of developing MC compared with men (HR 3.57; 95% CI 2.22–5.74; $P < 0.001$). Of the 135 patients with MC, 73 had CC (54%) and of these, 63 were women and 10 were men with a mean age of 57 years (range 46–73). Further, 62 had LC (46%) and of these, 52 were women and 10 were men with a mean age of 59 years (range 45–73). Baseline characteristics at the time for screening are given in Table 1. The age at the time of screening did not differ between cases and non-cases (HR 1.02; 95% 0.99–1.04; $P = 0.19$). The mean age at MC diagnosis was 69 years (range 49–86) for women and 72 years (range 52–85) for men.

The mean intake of different dietary factors separated into cases and non-cases is shown in Table 2. Neither the intake of protein, carbohydrates, sucrose and fat, including SFA, monounsaturated fatty acid and PUFA, nor that of fibre or zinc was associated with risk for MC (Table 3). Subgroup analysis of patients with CC and LC did not reveal any significant differences between cases and non-cases (data not shown). Furthermore, a diet quality index score based on adherence to the recommended intake of SFAs, PUFAs, sucrose, fibre, fruit and vegetables, and fish and shellfish was applied to determine whether a food pattern was associated with risk. No statistically significant associations could be found (Table 4). In a sensitivity analysis, those that had changed their dietary habits were excluded. This procedure did not influence the results (data not shown).

Lifestyle factors are depicted in Table 5. There was a strong association between smoking and MC risk (P for trend < 0.001).

Table 1. Baseline characteristics for the studied population

	Non-cases N = *27 960*		*Cases* N = *135*	
Gender (n, *%*)				
Males	11 042	39.5	20	14.8
Females	16 918	60.5	115	85.2
Mean age (years) (range)	58.1	(44.5–73.6)	58.2	(44.9–72.8)
Median age (years) (IQR)	57.8	(51.3–64.2)	58.2	(51.3–64.0)
Smoking habits (n, *%*)				
Never smokers	10 605	37.9	39	28.9
Ex-smokers	9465	33.9	41	30.4
Smokers	7878	28.2	55	40.7
Sum	27 948		135	
Alcohol habits (g/day) (n, *%*)				
Zero consumers	1806	6.5	7	5.2
⩽2.55	6585	23.6	33	24.4
2.55–7.93	6499	23.2	24	17.8
7.93–15.97	6533	23.4	38	28.1
15.98–194	6537	23.4	33	24.4
Sum	27 960		135	
BMI (kg/m^2) (n, *%*)				
⩽23.01	6999	25.0	37	27.4
23.02–25.28	7020	25.1	37	27.4
25.29–27.97	6932	24.8	32	23.7
⩾27.98	7004	25.1	29	21.5
Sum	27 955		135	
Education				
Elementary school or below	11 720	42.0	57	42.2
Primary and secondary school	7300	26.2	32	23.7
Upper secondary school	2472	8.9	17	12.6
Further without degree	2427	8.7	15	11.1
University degree	3970	14.2	14	10.4
Sum	27 889		135	
Physical activity (points) (n, *%*)				
⩽3950	6943	25.0	31	23.0
3951–6750	6942	25.0	40	29.6
6751–10 670	6941	25.0	34	25.2
⩾10 671	6943	25.0	30	22.2
Sum	27 769		135	

Abbreviations: BMI, body mass index; IQR, interquartile range. Leisure-time physical activity was obtained from the estimated minutes per week spent on 17 different activities. A score was constructed taking into account the intensity and duration of the activity; the score was divided into quartiles.

Table 2. Dietary intake among non-cases and microscopic colitis patients

	Non-cases N = *27 960*		*Cases* N = *135*	
	Mean	*Range*	*Mean*	*Range*
Protein (E%)	15.7	6.3–30.9	15.9	9.3–24.8
Carbohydrates (E%)	44.9	16.1–76.0	45.2	32.3–57.3
Fat (E%)	37.6	4.3–69.8	37.0	23.6–54.1
Saturated fatty acids (E%)	16.2	0.8–38.3	16.1	8.5–29.2
Monounsaturated fatty acids (E%)	13.1	0.5–26.2	12.7	6.6–18.2
Polyunsaturated fatty acids (E%)	6.0	1.7–24.8	5.9	3.0–10.9
Omega-3 (E%)	1.0	0.2–4.8	1.0	0.4–2.3
Omega-6 (E%)	4.8	0.7–24.4	4.7	2.4–9.1
Fibre (mg/MJ)	2.2	0.5–8.0	2.2	1.0–4.4
Zinc (mg/MJ)	1.4	0.5–26.8	1.6	0.7–21.0
Zinc (mg)	12.9	1.9–158.2	12.8	5.8–95.6

Abbreviations: E% = Energy percent. Range = min–max.

Increased alcohol intake was associated with an increased risk of developing MC (*P* for trend = 0.032). BMI, physical activity and education level did not differ between cases and non-cases.

A *post hoc* analysis was performed where the risk for MC was estimated by taking both alcohol (non-drinkers/drinkers) and smoking habits (non-smokers/current smokers) into account. Among non-drinkers/non-smokers 5 cases/100 000 person-years were observed compared with 83 cases/100 000 person-years among non-drinkers/smokers and 42 cases/100 100 person-years among drinkers/smokers. Compared with drinkers/smokers, a reduced risk for MC could be seen in both non-drinkers/non-smokers and drinkers/non-smokers. In non-drinkers/smokers, an increased risk was observed, although not statistically significant (Table 6).

DISCUSSION

The results in this study could not verify the hypothesis that intake of certain dietary factors that is, protein, carbohydrates, sucrose, fat, SFAs, monounsaturated fatty acids, PUFAs, n-3 PUFA, n-6 PUFA, fibre or zinc should be significant risk factors for the development of MC. A diet quality index based on adherence to dietary recommendations failed to reveal any differences between cases and non-cases. We could, however, verify the previously reported association between MC and smoking and the female gender. High alcohol consumption seemed to be associated with an increased risk for MC. However, in a *post hoc* analysis, alcohol intake *per se* seemed to—at least to some degree—reduce the increased smoke-related risk. In contrast, BMI, education or physical activity was not associated with any significant risk for the development of MC.

To the best of our knowledge, this is the first prospective study on the impact of dietary factors and lifestyle factors on the occurrence of MC. The principal strength of this study is the prospective design where pre-illness assessment and registration of dietary intake before disease onset data were performed. Because diagnosis of the disease may introduce dietary changes, prospective information of dietary habits may limit the influence of recall bias. In addition, the methodology of dietary assessment has a relatively high validity.[27,33] In view of the long time span, there is a possibility that the participants may have changed their dietary habits during follow-up. Especially in the Western world, the use of prepacked, salted and processed food has increased substantially over the last decades—based on the population level.[34] However, a sensitivity analysis failed to show any alterations. Onset of a significant disease such as cardiovascular disease may also influence a person's preferences in a healthier direction.[35] Of course, the long time span between interview and disease onset is a limitation. This is, however, an obstacle that is apparent in most large epidemiological population-based studies. For example, the investigation on 203 193 European individuals with a maximum follow-up time of 11 years from which 126 people with UC could be identified is based on single food recordings.[12] However, studies with repeated measurements—based on the individual level—indicate that most people tend to stick to their meal choices over time.[36,37] Furthermore, any changes in meal preferences over time should have an impact on both patients and non-cases. The cohort consisted of a large number of individuals, almost 30 000, who also had an age profile similar to the typical MC patient. Because follow-up occurred during a long time span (between 17 and 22 years; inclusion period 1991–1996 with follow-up until 2013), a fairly high number of people developing MC ($n = 135$) could be identified. On the basis of data from the same region on patients with CC, the incidence rate during the years 2001–2010 is known ($5.4/10^5$ inhabitants and year).[38] The age-adjusted number of patients who could be expected to develop CC during that decade was 35, which is the same number of patients who were identified in the present investigation. Although this estimation is restricted to only CC and not LC, and not the whole period, it gives an indication that the present investigation included a fairly representative disease population. Some limitations still have to be considered. Of all those invited to participate (74 138 inhabitants), only ~ 40% accepted the invitation (that is, 28 098 individuals). However, it is still regarded to be a fairly representative cohort.[22,23]

In a review article comprising 41 reports on diet in IBD, some correlations were reported. High intake of sugar and low intake of fruits and/or vegetables were associated with both CD and UC, whereas no definitive conclusions could be drawn for the intake of protein or unsaturated fatty acids.[39] Seven articles noted that a high sugar intake and five articles noted that a low fruit and/or vegetable intake were associated with UC. In a multicentre study comprising 126 UC patients, including four matched controls from a total cohort of 203 193 individuals, a significant association with intake of linoleic acid (an essential n-6 PUFA; odds ratio 2.49 for the highest quartile; 95% CI 1.23–5.07, *P* for trend = 0.02) was noted.[12] Despite the fact that these articles all found associations with diet and comprise a comparable number of patients, we could not find any statistically verified association in the present cohort. Consequently, diet probably influences MC to a lesser extent than classic IBD.

High alcohol intake seems to have a positive impact on the risk for MC. This is in line with a prospective cohort study on UC from United Kingdom (UK), where high intake of alcohol was associated with an increased risk for disease activity, whereas medium intake did not confer any risk.[40] In a Japanese case–control study, alcohol intake had a protective effect against UC compared with no alcohol intake.[41] The mechanisms behind these somewhat contradictory associations are unclear. As the reports come from different parts of the world, several confounding factors could contribute to this discrepancy. However, colitis, at least in mice, can be triggered by alcohol.[42] Ethanol and its metabolite acetaldehyde have several effects on the gastrointestinal tract. In particular, an observed increase in the permeability both in the small and large intestine is worth mentioning. Activation of cell signalling pathways, oxidative stress, remodelling of the cytoskeleton and modulation of the microbiota have been suggested as possible mechanisms.[43] Increased permeability is associated with several gastrointestinal diseases, and this effect may very well contribute to the disease course in MC as well.

In addition to alcohol, certain additives in spirits may also have a role. In the article from UK,[40] the authors speculate that—besides alcohol—sulphur and sulphate might also have a role, as

Table 3. Risk estimates for the intake of different dietary factors and microscopic colitis

Quartiles	*Quartile range*	*Crude HR*	*95% CI*	*Adjusted HR*	*95% CI*
Protein (E%)					
1	⩽14.01	1.00		1.00	
2	14.02–15.53	1.24	(0.76–2.04)	1.22	(0.74–2.00)
3	15.54–17.20	1.31	(0.80–2.14)	1.23	(0.75–2.03)
4	⩾17.21	1.25	(0.76–2.06)	1.26	(0.69–1.88)
P for trend		0.372		0.641	
Carbohydrates (E%)					
1	⩽41.11	1.00		1.00	
2	41.12–44.90	0.85	(0.52–1.38)	0.87	(0.53–1.41)
3	44.91–48.66	0.87	(0.54–1.41)	0.89	(0.55–1.45)
4	⩾48.67	1.09	(0.69–1.71)	1.14	(0.72–1.81)
P for trend		0.700		0.565	
Sucrose (E%)					
1	⩽6.08	1.00		1.00	
2	6.09–8.04	0.87	(0.53–1.44)	0.81	(0.49–1.35)
3	8.05–10.34	1.12	(0.70–1.80)	1.03	(0.63–1.66)
4	⩾10.35	1.17	(0.73–1.87)	1.06	(0.65–1.71)
P for trend		0.345		0.782	
Fat (E%)					
1	⩽33.56	1.00		1.00	
2	33.57–37.54	0.75	(0.47–1.20)	0.75	(0.47–1.20)
3	37.55–41.57	0.80	(0.50–1.27)	0.80	(0.50–1.28)
4	⩾41.58	0.82	(0.52–1.30)	0.82	(0.51–1.30)
P for trend		0.433		0.454	
Saturated fatty acids (E%)					
1	⩽13.59	1.00		1.00	
2	13.60–15.73	0.86	(0.54–1.38)	0.82	(0.51–1.32)
3	15.74–18.32	0.90	(0.56–1.43)	0.83	(0.52–1.33)
4	⩾18.33	0.90	(0.56–1.40)	0.80	(0.50–1.29)
P for trend		0.699		0.394	
Monounsaturated fatty acids (E%)					
1	⩽11.64	1.00		1.00	
2	11.65–13.09	0.78	(0.49–1.24)	0.77	(0.48–1.23)
3	13.10–14.53	0.87	(0.55–1.37)	0.89	(0.56–1.40)
4	⩾14.54	0.71	(0.44–1.15)	0.75	(0.46–1.22)
P for trend		0.232		0.346	
Polyunsaturated fatty acids (E%)					
1	⩽4.85	1.00		1.00	
2	4.86–5.77	0.98	(0.61–1.55)	1.05	(0.66–1.66)
3	5.78–6.83	0.82	(0.51–1.32)	0.93	(0.57–1.50)
4	⩾6.84	0,85	(0.53–1.36)	1.00	(0.62–1.63)
P for trend		0.380		0.899	
Omega-3 (E%)					
1	⩽0.77	1.00		1.00	
2	0.78–0.92	0.71	(0.44–1.14)	0.70	(0.44–1.13)
3	0.93–1.12	0.71	(0.44–1.15)	0.71	(0.44–1.15)
4	⩾1.13	0.90	(0.58–1.41)	0.89	(0.57–1.41)
P for trend		0.630		0.625	
Omega-6 (E%)					
1	⩽3.80	1.00		1.00	
2	3.81–4.63	0.89	(0.56–1.40)	0.85	(0.61–1.51)
3	4.64–5.60	0.74	(0.46–1.19)	0.84	(0.52–1.34)
4	⩾5.61	0.69	(0.43–1.11)	0.84	(0.51–1.36)
P for trend		0.091		0.383	
Fibre (mg/MJ)					
1	⩽1.78	1.00		1.00	
2	1.79–2.15	1.36	(0.85–2.17)	1.32	(0.82–2.11)
3	2.16–2.59	0.79	(0.47–1.34)	0.74	(0.44–1.27)
4	⩾2.60	1.04	(0.64–1.69)	0.93	(0.57–1.54)
P for trend		0.573		0.320	

Table 3. (Continued)

Quartiles	*Quartile range*	*Crude HR*	*95% CI*	*Adjusted HR*	*95% CI*
Zinc (mg/MJ)					
1	⩽1.11	1.00		1.00	
2	1.12–1.25	0.77	(0.46–1.28)	0.80	(0.48–1.34)
3	1.26–1.45	1.12	(0.71–1.78)	1.19	(0.75–1.90)
4	⩾1.46	1.07	(0.67–1.71)	1.01	(0.66–1.68)
P for trend		0.455		0.493	
Total zinc[a] *(mg)*					
1	⩽9.18	1.00		1.00	
2	9.19–11.37	1.10	(0.71–1.71)	1.30	(0.83–2.03)
3	11.38–14.70	0.65	(0.40–1.08)	0.98	(0.59–1.64)
4	⩾14.71	0.75	(0.46–1.21)	1.12	(0.68–1.82)
P for trend		0.074		0.912	

Abbreviations: CI, confidence interval; E% = Energy percent; HR, hazard ratio. The HR was estimated through Cox proportional-hazards regression models with corresponding 95% CIs. HR (95% CI) is stated for quartiles of intake. Variables were adjusted for age, sex, smoking, season and method version. *N* cases: 135; *N* non-cases: 27 960. A general linear model was used to calculate *P* for trend. The quartiles were used as continuous variables. [a]Including supplementary zinc intake.

Table 4. Risk estimates for the intake of different foods based on a diet score

Diet index	*Non-cases (n)*	*Cases (n)*	*Crude HR*	*95% CI*	*Adjusted HR*	*95% CI*
Diet score						
0	815	8	1.00		1.00	
1	3455	16	0.47	(0.20–1.10)	0.52	(0.22–1.21)
2	7433	35	0.46	(0.21–1.00)	0.56	(0.26–1.20)
3	7553	33	0.42	(0.19–0.90)	0.52	(0.24–1.12)
4	4952	25	0.46	(0.21–1.01)	0.56	(0.25–1.26)
5	2794	11	0.35	(0.14–0.87)	0.43	(0.17–1.07)
6	958	7	0.61	(0.22–1.67)	0.76	(0.27–2.11)
Sum		27 960	135			
P for trend			0.32		0.59	

Abbreviations: CI, confidence interval; HR, hazard ratio. The HR was estimated through Cox proportional-hazards regression models with corresponding 95% CIs. HR (95% CI) is stated for a diet index based on the intake of saturated fat, polyunsaturated fat, sucrose, fibre, vegetables and fruit and fish, where each variable is given a score of 0 or 1. On the basis of these parameters, the group has been divided into six categories, where 0 is the unhealthiest and 6 is the healthiest food pattern. Variables were adjusted for age, sex, smoking, season and method version. A general linear model was used to calculate *P* for trend. The diet index scores were used as continuous variables.

many alcoholic drinks contain large amounts of sulphates. Sulphites are used as preservatives in beer, white and red wine (especially in bag-in-box wines) and soft drinks, which were all found to be related to increased disease severity in UC. Sulphite degrades thiamine, and foods high in thiamine (which is required by the probiotic bacteria lactobacilli) are reported to be associated with clinical improvement in UC. Furthermore, sulphide (that is derived from sulphite) is toxic to colonocytes.[44] However, it was not within the scope of the present investigation to explore the impact of different types of alcohol.

Non-drinkers/non-smokers had the lowest MC risk compared with drinkers/smokers. Smokers who did not consume alcohol had the highest risk, higher than in smokers/drinkers, although not significant. In an investigation in UC, a protective effect by a moderate alcohol intake disappeared when adjusted for smoking.[45] Despite the association between high alcohol intake and MC risk, alcohol intake *per se* in non-smokers was associated with a risk reduction compared with drinkers/smokers. This somewhat puzzling outcome has been observed in another study as well.[4] The contradictory results may depend on the fact that all alcohol consumers were put together in one group in the *post hoc* analysis. As the number of high consumers was fewer compared with the number of low/medium consumers, the harmful effect of high alcohol volumes was hidden. A protective effect by alcohol against disease onset has previously been described for rheumatoid arthritis and multiple sclerosis.[46,47] The unifying factor in MC and multiple sclerosis is that the risk by smoking was reduced by alcohol, regardless of the initial alcohol-related risk level. The mechanisms underlying this phenomenon are unclear.

BMI, education level and physical activity were not associated with any risk increase for MC. BMI is associated with several immune-mediated diseases. According to a large Danish study based on 75 000 women followed for 11 years, there is a relationship between BMI and CD but not with UC.[48] Consequently, the relationship that seems to exist between obesity-mediated inflammation (based on the amount of visceral fat tissue) and CD[49] has not been proven in MC. High education seems to protect from CD[50] and rheumatoid arthritis,[51] indicating that socio-economic factors may have an impact on the risk at least in some other immune-mediated diseases. Physical activity has anti-inflammatory effects.[52] In view of this phenomenon, a reduced risk for MC in exercising individuals could hypothetically be expected. However, this was not the case in the present cohort. In other words, these lifestyle-related factors, which directly or

Table 5. Lifestyle factors in microscopic colitis

	Quartile range	*Crude HR*	*95% CI*	*Adjusted HR*	*95% CI*
Smoking					
Never smokers		1.00		1.00	
Ex-smokers		1.24	(0.80–1.92)	1.61	(1.03–2.51)
Smokers		2.10	(1.39–3.16)	2.53	(1.66–3.84)
P for trend		< 0.001		< 0.001	
Alcohol habits (g/day)					
Zero consumers		1.00		1.00	
Q1	⩽2.55	1.22	(0.54–2.76)	1.27	(0.56–2.88)
Q2	2.55–7.93	0.88	(0.38–2.04)	0.95	(0.41–2.22)
Q3	7.93–15.97	1.36	(0.61–3.05)	1.62	(0.72–3.65)
Q4	15.98–194	1.21	(0.54–2.75)	1.89	(0.82–4.33)
P for trend		0.522		0.032	
BMI (kg/m^2)					
Q1	⩽23.01	1.00		1.00	
Q2	23.02–25.28	0.99	(0.62–1.56)	1.1	(0.70–1.74)
Q3	25.29–27.97	0.89	(0.55–1.43)	1.1	(0.66–1.73)
Q4	⩾27.98	0.83	(0.51–1.35)	0.94	(0.57–1.53)
P for trend		0.395		0.903	
Education					
Elementary school or below		1.00		1.00	
Primary and secondary school		0.87	(0.56–1.34)	0.83	(0.54–1.28)
Upper secondary school		1.37	(0.80–2.36)	1.77	(1.02–3.08)
Further without degree		1.26	(0.71–2.22)	1.41	(0.79–2.50)
University degree		0.70	(0.39–1.26)	0.80	(0.44–1.45)
P for trend		0.709		0.776	
Physical activity (points)					
Q1	⩽3950	1.00		1.00	
Q2	3951–6750	1.24	(0.78–1.99)	1.28	(0.80–2.04)
Q3	6751–10 670	1.06	(0.65–1.72)	1.10	(0.67–1.79)
Q4	⩾10 671	0.96	(0.58–1.59)	1.01	(0.61–1.66)
P for trend		0.706		0.852	

Abbreviations: BMI, body mass index; CI, confidence interval; HR, hazard ratio. The HR was estimated through Cox proportional-hazards regression models with corresponding 95% CIs. HR was adjusted for sex, age and smoking, except for smoking that was adjusted for sex and age. A general linear model was used to calculate *P* for trend. Physical activity was obtained from estimated minutes per week spent on different activities. A score was constructed taking into account the intensity and duration of the activity; the score was divided into quartiles (Q). For smoking, 15 are missing in non-cases, and in education, 74 are missing in non-cases.

Table 6. Associations between intake of alcohol and/or smoking and microscopic colitis

Alcohol	*Smoking*	n	*Cases (n)*	*Cases/10^5 years*	*Crude HR*	*95% CI*	*Adjusted HR*	*95% CI*
−	−	1307	1	4.8	0.11	(0.02–0.83)	0.09	(0.01–0.63)
+	−	18 843	79	25.4	0.59	(0.41–0.84)	0.55	(0.38–0.79)
−	+	501	6	83.3	2.07	(0.89–4.84)	1.65	(0.71–3.87)
+	+	7432	49	41.9	1.00	(ref)	1.00	(ref)

Abbreviations: CI, confidence interval; HR, hazard ratio. − denotes no intake of alcohol the last year or no current smoking; + denotes intake of alcohol the last year or current smoking. The HR was estimated through Cox proportional-hazards regression models with corresponding 95% CIs. HR was adjusted for sex and age. Cases/10^5 years denotes the number of cases in that group (both non-cases and cases) divided by the total amount of person-years/100 000 years.

indirectly may influence inflammatory responses, did not associate with MC. Consequently, factors influencing the risk for MC probably have to be sought elsewhere.

The chosen dietary factors were selected based on previous data on other gastrointestinal diseases with an immune-mediated mechanism. Although the diet quality score did not reveal any significant association, a combined effect or other environmental factors cannot be ruled out. Food additives could be one such possibility,[53] as, for example, the addition of microbial transglutaminase in food makes gluten more immunogenic.[54] This increased immune stimulus could hypothetically facilitate the development of coeliac disease and MC. An altered microbiota has also been observed in MC with a decreased number of *Akkermansia*, involved in mucin regulation,[55] and artificial sweeteners are deranging the microbiota, leading to disrupted glucose homoeostasis.[56] Another possible factor is ordinary salt (NaCl), which in multiple sclerosis has been shown to influence the disease course negatively through induction of Th17 cells.[57] Recent reports have also found Th17 to be an important factor in chronic gastrointestinal inflammation.[58]

Yet, other environmental factors could of course be responsible for the onset of MC. Infectious agents could be one such possibility. Infection with *Helicobacter pylori* is inversely associated to MC.[59] The decrease in the prevalence of *Helicobacter Pylori* in the Western world is paralleled with an increase in MC;[1,9] the rationale for this hypothesis is well worth exploring further. Furthermore, a seasonal pattern with more cases of LC during the summer has been observed, something that could have many explanations.[60] The authors speculate that infections could have a role in the seasonal variation.

Consequently, despite a large cohort with a long follow-up, no association could be found between intake of protein, carbohydrates, different types of fat, fibre or zinc and occurrence of MC. BMI, education or physical activity did not confer any increased risk either. The previously observed risk increase in smoking and the female gender could be verified. Furthermore, high intake of alcohol seems to increase the risk for MC, although the smoking-related risk was modified by alcohol *per se*. The mechanisms behind these effects of alcohol have to be studied further in an investigation aimed at exploring this phenomenon.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Paper II

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Lack of autoantibodies against collagen and related proteins in collagenous colitis

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Abstract

Introduction: Collagenous colitis (CC) is a common cause of chronic diarrhea and is characterized by a subepithelial thickened collagen layer in the colonic mucosa.

It shares many of the characteristics found in autoimmune diseases, but no autoantibodies have been identified. In CC, an imbalance in collagen turnover is evident. The purpose of the present study was to investigate whether any collagen-associated autoantibodies or other antibodies such as TPO and ASCA were present, and if levels of total IgE were increased.

Methods: Sera from women with active CC were analysed with ELISA for detection of autoantibodies against collagen type III and IV (Col III and IV), matrix metalloproteinase-9 (MMP-9), tissue inhibitors of metalloproteinase-1 (TIMP-1) and tenascin-C (TNC). Sera were also analysed for TPO, ASCA and total IgE. Healthy female blood donors served as controls. The cut-off value in the control group was defined as relative units > 97.5th percentile.

Results: Sixty-six women were included (mean age 60 years; range 31–74, mean disease duration 6 years; range 1–22). No autoantibody was significantly overexpressed in the CC population compared to controls. The mean disease duration was lower ($p = 0.03$) in the subjects who expressed collagen-associated autoantibodies (3.7 years; range 1–14), compared to those who did not (6.4 years; range 1–22). Treatment with budesonide was not associated with any of these autoantibodies.

Conclusion: No increased presence of the investigated antibodies could be found in the present study of CC. Neither could antibodies against ASCA or TPO, or elevated levels of IgE, be found. Consequently, no association was found between CC and these proteins, even though this may not be generalizable to other compounds in the collagen layer.

Keywords: Autoantibodies, Autoimmunity, Collagenous colitis, Collagen type III, Collagen type IV, MMP-9, Tenascin, TIMP-1

Introduction

Collagenous colitis (CC) is an inflammatory disorder in the colonic mucosa that predominantly affects elderly women and causes chronic, non-bloody diarrhoea with normal or close to normal endoscopic findings. The condition was first described 1976 in Malmö, Sweden, by Lindström [1]. The histological criteria for CC are a thickened subepithelial collagen layer (> 10 μm) in the extracellular matrix (ECM) of the mucosa, epithelial damage and presence of an inflammatory infiltrate in the lamina propria [2] (Fig. 1). The excessive subepithelial collagen deposition is located underneath the basement membrane and seems to be caused by myofibroblast dysfunction, leading to collagen overproduction with ECM-remodelling, as well as an imbalance between

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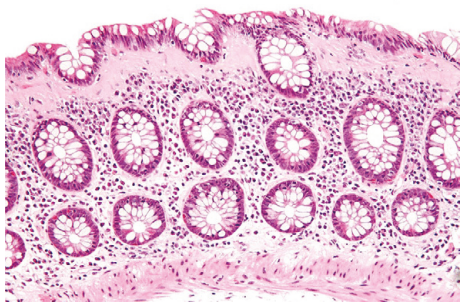

Fig. 1 Histological features of collagenous colitis. High magnification micrograph of collagenous colitis, H&E stain (Collagenous colitis—high mag—Collagenous colitis—Wikipedia) by Michael Bonert, MD, FRCPC. Copyright © 2011 Michael Bonert, MD, FRCPC (https://commons.wikimedia.org/wiki/User:Nephron / https://fhs.mcmaster.ca/pathology/contact_us/faculty/faculty_bios/Bonert.html. Licensed under CC BY-SA 3.0 (https://creativecommons.org/licenses/by-sa/3.0/legalcode)

fibrogenesis and fibrinolysis which results in an impaired degradation of ECM proteins [3, 4].

Whether CC is an autoimmune disease is yet not clear, although some characteristics suggest an autoimmune association. First, patients with CC are to a large extent HLA DQ2-carriers, a HLA-haplotype associated with autoimmune diseases [5–7]. In line with this, celiac disease (CeD), thyroid disorders and other autoimmune diseases are often identified in patients with CC [8]. Second, an inflammatory Th1-dominated mucosal cytokine profile has been observed [9, 10]. Third, the disease is prevalent mainly in a certain age and gender group, something that is typical for autoimmune diseases in general [11, 12].

A typical prerequisite for a classical autoimmune disorder is presence of autoantibodies [13]. Previous studies have suggested an increased presence of some antibodies in patients with CC, such as anti-nuclear antibodies (ANA) [14–16], serum IgM [14], thyroid peroxidase (TPO) [16] and anti-Saccharomyces cerevisiae antibodies (ASCA) [15, 16]. However, the sensitivity of these antibodies is too low to be able to consider them as diagnostic tools in CC. Antibody response in autoimmunity is often mediated via IgG, but also other subclasses are involved. Increased total IgE levels have been reported in patients with inflammatory bowel disease (IBD) [17, 18].

While general autoantibodies such as ANA are present in many different types of autoimmune diseases, the majority of antibodies in autoimmune disease have a defined target organ directed against a key enzyme and/or substrate in that organ, such as in transglutaminase autoantibodies in CeD (transglutaminase and gliadin) and anti-citrullinated protein antibodies in rheumatoid arthritis (RA; peptidylarginine deiminase and citrulline) [19, 20]. The central mechanism is that these enzymes and their substrates are active and involved in the inflamed tissue, something that might increase the risk that these proteins also could be targets in the immune response. In other words, any presence of autoantibodies against central enzymes and their substrates should predominantly be found in the active site, i.e. where the inflammation is most active. In CC, a disrupted collagen turnover is evident. If the same mechanisms are present in CC as in CeD or RA, autoantibodies against active enzymes involved in collagen turnover could be expected.

Collagen

The thickened collagen layer in CC is believed to reflect a local disturbance of ECM turnover, resulting in the formation of a disrupted ECM. Previous studies have shown increased levels of collagen type III (Col III) [3, 21] and both Col III and collagen type IV (Col IV) [22] in patients with CC. Col IV is a major component of basement membrane collagens and Col III is active in wound healing [23, 24].

Matrix metalloproteinase (MMP)

MMP is a family of zinc-dependent enzymes involved in the degradation of different components of the ECM. These proteinases play a central role in tissue remodelling during inflammation [25]. The MMP genes are responsive to several factors including cytokines such as TNF alfa and IL-1 [26]. MMP-9 is involved in inflammatory and remodelling processes in IBD and its levels are elevated in patients with ulcerative colitis [25, 27–30]. An allelic variation of MMP-9 has been suggested as a risk factor [31], and enhanced mRNA expression levels of the gene has been described in CC-patients [32].

Tissue inhibitors of metalloproteinase (TIMP)

The activity of MMPs is regulated by TIMPs TIMPs are typically induced by inflammatory cytokines such as IL-6 that is related to the Th1 mediated pathway [33]. Previous studies have shown increased transcripts of TIMP-1 in patients with CC [3, 32].

Tenascin-C (TNC)

Tenascins are a family of four extracellular matrix glycoproteins TNC is expressed in wound healing and inflammation [34, 35]. TNC also interacts with MMPs [35]. Previous studies have shown that TNC is increased in the collagen layer in patients with CC [3, 4, 36, 37]. This glycoprotein is regulated by cytokines, and it seems as if both the Th1 (TNF, IL-1 and IFN) and the Th2 (IL-4 and

IL-13) pathways are involved. Exogenous TNC stimulates collagen gene expression via TLR4 signaling [38] which induces a Th1-response [39].

In view of this aspect, we have primarily focused on the frequency of autoantibodies directed against specific endogenous proteins that are active in the turnover of the collagen layer in the intestinal mucosa in patients with CC, namely, Col III and Col IV, MMP-9, TIMP-1 and TNC. Furthermore, we have scrutinized the occurrence of other putative antibodies active in the immune-driven process behind CC, namely TPO and ASCA, and total levels of IgE.

Patients and methods

Patients

Patients with CC were diagnosed according to established clinical and histopathological criteria [40]. They were recruited from the out-patient clinics at the hospitals in Region Skåne. The procedure has been described in detail in a previous study [16].

To reassure that only patients with more pronounced and verified CC were included, only patients with at least two flare-ups or two histological examinations with positive findings in accordance to the histopathological criteria of CC were included. Patient characteristics (including clinical information) were retrieved from a questionnaire sent to the participants, and in some cases, completed with patient files. Blood samples were collected according to standardized methods.

Control group

Serum from healthy, female blood donors served as controls for all immunological analyses. The criteria for being a blood donor is strict and these individuals do not take any medication of relevance [41]. For the collagen-associated antibody-analyses, one hundred female blood donors served as controls. The size of the groups differed slightly due to shortage of blood samples, but they were all picked from the same cohort. Blood donors also served as controls for analyses of ASCA, TPO and total IgE, but these were selected by the Department of Immunology and Department of Clinical Chemistry at Skåne University Hospital, Malmö according to their standardized methods.

Immunological analyses

Collagen type III and IV

In-house enzyme-linked immunosorbent assays (ELISAs) were set up for analysis of anti-IgM and IgG antibodies against Col III and IV. The microtiter plates (82.158.001, Sarstedt, Nümbrecht, Germany) were coated with a recombinant Col III (Merck CC054 lot nr 2,861,783, 2,943,191) or Col IV (ab7536 lot no GR281135-14 Abcam, Cambridge, UK), 1 µg/mL in phosphate buffer saline (PBS), pH 7.4, pre-incubated 15 min in 50 °C, 100 µl/well in PBS or buffer only (to provide an internal blank). After overnight incubation at 4 °C the plates were washed three times with PBS with 0.05% Tween 20 (MP Biomedicals 02,194,724.5) (PBS-T) and blocked with 0.5% bovine serum albumin (A7030, Sigma) (BSA) in PBS-T. Dilutions of serum from patients and blood donors of 1:800 (IgG and IgM) and rabbit IgG anti-human Col III antibody (PA136061 lot no 2861783 Fisher Scientific, Göteborg Sweden) or Col IV antibody (ab6585 lot no GR322984-8, Abcam) in serial dilution (to construct a standard curve) with BSA in PBS-T were then added to the plates in triplicate (two wells coated with Col III or IV and one well coated with PBS) and incubated for 1 h at room temperature (RT). The washing procedure was repeated and deposition of autoantibodies directed to Col III and IV was detected using HRP-conjugated rabbit anti-human IgG or IgM (P0214 and P0216, respectively, DAKO Glostrup, Denmark), or goat anti-rabbit IgG (P0448, DAKO) appropriately diluted in PBS-T. To develop a color reaction, a tetramethylbenzidine (TMB) peroxidase substrate system (2-C) (KPL50-76-00, Gaitheraburg, USA) 1:1 was used. The absorbance at 450 nm was measured after 15 min of incubation at RT. Antibody levels are presented as relative units (RU) (absorbance values after subtracted background) and the concentration in each doublet is interpolated from the standard curve.

The cut-off value to determine presence of antibodies in the control group of 70 (Col III) and 51 (Col IV) healthy blood donors was defined as RU > 97.5th percentile.

The intra-assay correlation coefficient of variation (CV) of Col III IgG and IgM antibodies was 12.5% (n = 10) and 8.1% (n = 8), respectively, and inter-assay CV was 8.5% (n = 6) and 6.9% (n = 4), respectively. The intra-assay correlation CV of Col IV IgG and IgM antibodies was 6.6% and 3.2%, respectively (n = 6), and inter-assay CV was 13.5% and 6.2%, respectively (n = 10).

MMP-9 and Tenascin

Analyses of antibodies against MMP-9 and TNC were conducted by in-house ELISAs as previously described in detail [42]. Briefly, IgM-and IgG- antibodies against MMP-9 were analysed on microtiter plates (442,404, Nunc) coated with a recombinant MMP-9 (Pierce RP-75655 lot no. QA 1,951,751, Thermo Scientific, Rockford, IL, USA) in PBS-T or in PBS-T only. IgM and IgG autoantibodies against TNC were analysed on microtiter plates (442,404, Nunc, Roskilde, Denmark) coated with recombinant TNC (MBS1265425, Mybiosource, San Diego, CA, USA) in PBS-T, or in PBS-T only (to provide an internal blank), and incubated at 4 °C overnight. The absorbance at 450 nm was measured after 30 min of

incubation at RT. Antibody levels are presented as RU, and the concentration in each doublet is interpolated from the standard curve. The cut-off value to determine presence of antibodies in the control group of 100 healthy blood donors was defined as RU > 97.5th percentile.

The intra-assay CV of MMP-9 IgM and IgG antibodies was 10.1% (n = 5) and 6.9% (n = 8), respectively, and inter-assay CV was 14% and 8.5%, respectively (n = 4). The intra-assay correlation CV of TNC IgM and IgG antibodies was 9.1% and 7.6%, respectively (n = 8), and inter-assay CV was 20.3% and 13.6%, respectively (n = 4).

TIMP-1

Another in-house ELISA was set up for analysis of IgM and IgG antibodies against TIMP-1. The microtiter plates (82.158.001 Sarstedt) were coated with a recombinant TIMP-1 (MBS8249878 lot no JA1F10A, MyBiosource, San Diego, USA) in Carbonate buffer pH 9.6 or buffer only (to provide an internal blank). After overnight incubation at 4 °C the plates were washed three times with PBS-T and blocked with 0.5% BSA (A7030, Sigma) in PBS-T. Dilutions of serum from patients and blood donors of 1:100 (IgG and IgM) or rabbit IgG anti-human TIMP-1 antibody (MBS2544120 lot no AC5441, MyBiosource) in serial dilution (to construct a standard curve) with BSA in PBS-T were then added to the plates in triplicate (two wells coated with TIMP-1 and one well coated with Carbonate buffer) and incubated for 1 h at RT. The washing procedure was repeated and deposition of autoantibodies directed to TIMP-1 was detected using HRP-conjugated rabbit anti-human IgG or IgM (DAKO P0214 and P0216 respectively), or goat anti-rabbit IgG (P0448, DAKO) appropriately diluted in PBS-T. To develop a color reaction, a TMB Peroxidase substrate system (2-C) (50-76-00 KPL) 1:1 was used. The absorbance at 450 nm was measured after 30 min of incubation at RT. Antibody levels are presented as RU and the concentration in each doublet is interpolated from the standard curve.

The cut-off value to determine presence of antibodies in the control group of 65 healthy blood donors was defined as RU > 97.5th percentile.

The intra-assay CV of TIMP-1 IgG and IgM antibodies was 5.1% and 22%, respectively (n = 8), and inter-assay CV was 18% and 8.6%, respectively (n = 10).

ASCA, TPO and total IgE

ASCA and total IgE were both analysed at the Department of Immunology at Skåne University Hospital, Malmö. ASCA IgG were analysed by a fluorescent enzyme immunoassay method (FEIA) for which the reference value is set > 10 U/mL in accordance with the manufacturer's recommendation (Orgentec Diagnostika, AlegriaH, Mainz, Germany). Of 50 healthy blood donors tested at our laboratory, 10% were positive for ASCA IgG, whereas other studies have found 0.6%–3.1% positive among healthy blood donors by the same method [43]. Total IgE were also analysed by FEIA method. The cut-off value to determine increased titers of total IgE was set to > 129 kU/L. Out of 100 healthy blood donors, 14% had increased titers at our laboratory.

TPO antibodies were analysed by a chemiluminescence enzyme immunological method (Atellica IM, Siemens Healthcare GmbH, Erlangen, Germany) at the Department of Clinical Chemistry at Skåne University Hospital, Malmö. TPO antibodies are found in 10% of blood donors when the cut-off level is set to 60 kIU/L.

Statistical analyses

Fisher's exact test was used to calculate differences in antibody prevalence between controls and patients. Student's *t*-test was used to calculate differences between mean values of continuous variables. A p-value < 0.05 was considered statistically significant.

Results

Patient characteristics

In total, 66 women with CC were included in the study. The mean age at inclusion was 60 years (range 31–74 years) and mean age at diagnosis was 55 years (range 28–69 years). At inclusion, the mean symptom duration was 11 years and mean disease duration was 6 years. More than one-third of the patients were smokers, and another third were former smokers. The most common concomitant diseases were hypertension (30%), RA (15%) and asthma and cancer (14%). Budesonide was used by 38% of the patients, and high blood pressure therapy (HBPT), proton pump inhibitors (PPI) and levothyroxine were used in 20–30% of patients (Table 1).

Control group

The size of the control groups differed but they were selected from the same cohort consisting of 100 healthy female blood donors. The mean age of the total control group was 41.7 years (range 19–69).

Presence of antibodies

Levels of IgM and IgG autoantibodies against Col III, Col IV, MMP-9, TIMP-1 and TNC are illustrated in Fig. 2a–e. There was no difference in prevalence of these collage-associated autoantibodies between CC patients and controls (Fig. 3a). Sixteen patients expressed one type of autoantibody, whereas two patients expressed two different types. The mean disease duration was significantly lower ($p = 0.03$) in the subjects who expressed collagen-associated autoantibodies (3.7 years), compared

Table 1 Patient characteristics, n = 66

Age and duration	**Mean (years)**	**Range (years)**
Age at inclusion	60	31–74
Age at diagnosis	55	28–69
Disease duration at analysis	6	1–22
Symtom duration at analysis*	11	1–41
	N	**%**
*Smoking^*		
Current smoking	26	39
Former smoking	21	32
Prevalence past and current diseases		
Hypertension	20	30
Reumatic disorder	10	15
Cancer	9	14
Asthma	9	14
Thyroid disorder	8	12
Celiac disease	3	5
Diabetes	3	5
Current medication		
Budesonide	25	38
HBPT	20	30
PPI	18	27
Levothyroxine	13	20
Statins	11	17
SSRI	11	17
ASA	11	17
NSAID	5	8
IST	4	6
ICS	3	5
HRT	3	5

HBPT High blood pressure therapy, *PPI* Proton pump inhibitors, *SSRI* Selective serotonin reuptake inhibitors, *ASA* Acetylsalicylic acid, *NSAID* Nonsteroidal anti-inflammatory drugs, *IST* Immunosuppressive therapy, *ICS* Inhaled corticosteroids, *HRT* Hormone replacement therapy

Missing values: * = 4, ^ = 6

to those who did not (6.4 years). Treatment with budesonide was not associated with any of these autoantibodies and no difference in prevalence of autoimmune/immune-mediated diseases between the two groups were seen (Table 2).

The presence of autoantibodies against TPO and ASCA was slightly increased compared to controls, but not statistically significant (Fig. 3b) (p-values 0.78 and 0.36, respectively). Out of the nine TPO positive patients, six did neither declare thyroid disease nor levothyroxine treatment. However, when comparing the levels of the TPO titers between patients that were treated with levothyroxine/declared thyroid disease with those who did not, no differences were observed (data not shown).

(See figure on next page.)

Fig. 2 Levels of collagen-associated antibodies in patients with collagenous colitis and controls. Col III = collagen type III, Col IV = collagen type IV, MMP-9 = matrix metalloproteinase-9, TIMP-1 = tissue inhibitors of metalloproteinases-1, TNC = tenascin-C. CC = collagenous colitis (n = 66); treated = current budesonide treatment, controls = healthy blood donors (n = **a** 70, **b** 51, **c** 100, **d** 65, **e** 100). Black bars state median values. Y axis: Antibody levels in relative units (RU) (absorbance values after subtracted background). Cut-off value set at the 97.5 th percentile. Fischer's exact test was used to calculate the differences between controls and CC pat (including both treated and untreated)

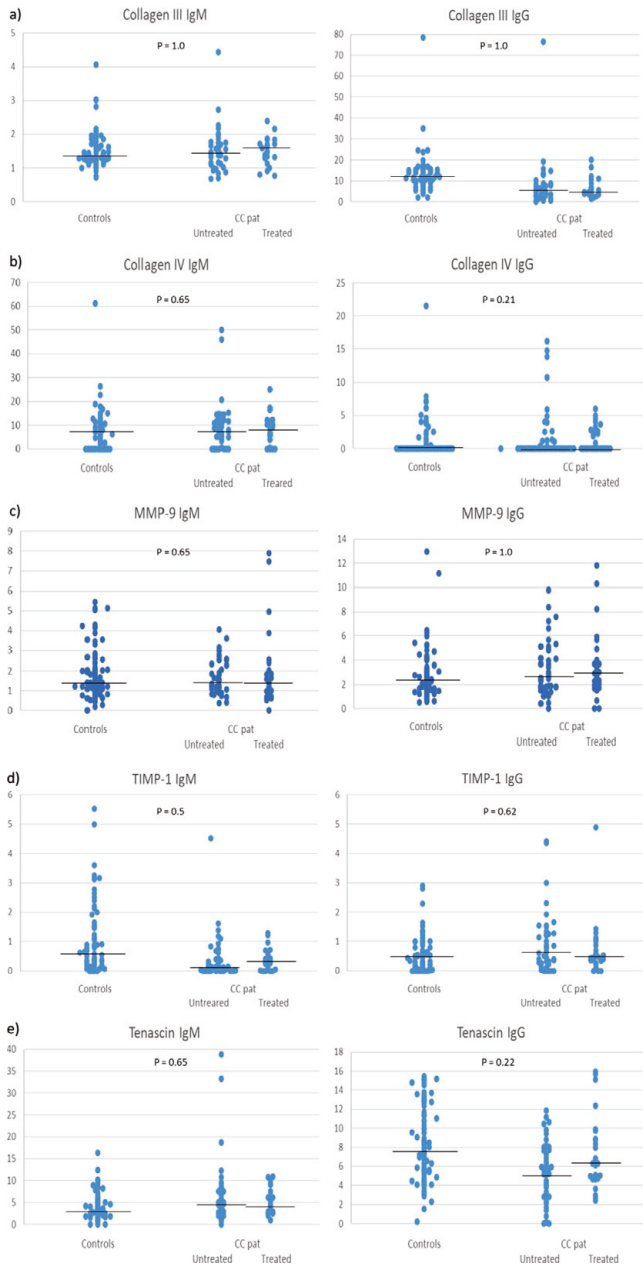

Fig. 2 (See legend on previous page.)

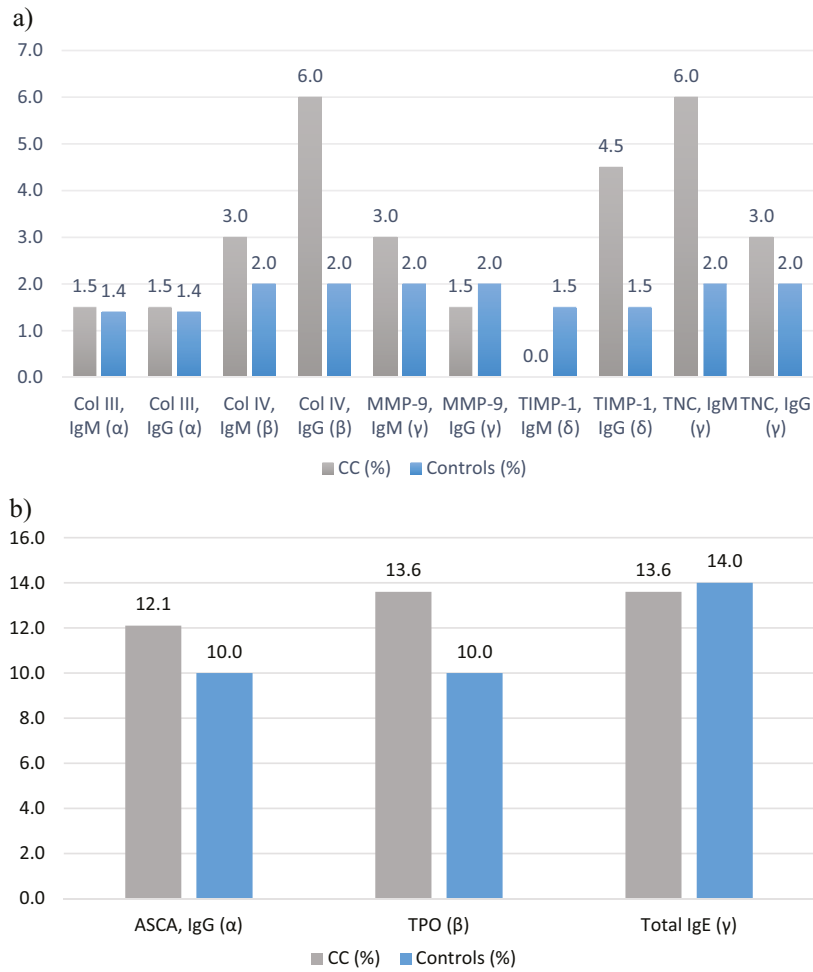

Fig. 3 **a** Presence of collagen-associated autoantibodies. Col III = collagen type III, Col IV = collagen type IV, MMP-9 = matrix metalloproteinase-9, TIMP-1 = tissue inhibitors of metalloproteinases-1, TNC = tenascin-C, CC = collagenous colitis. Relative units > 97.5 percentile in a cohort of healthy blood donors were considered as presence of antibodies. Controls consisted of α = 70, β = 51, γ = 100, δ = 65 healthy blood donors. Fischer's exact test was used to calculate the differences between groups. None of the differences between groups were statistically significant ($P < 0.05$). **b** Presence of antibodies against saccharomyces cerevisiae (ASCA), thyroid peroxidase (TPO) and increased total serum IgE. CC = Collagenous colitis. Controls consisted of α = 50 healthy blood donors, β = 254 healthy blood donors, γ = 100 healthy blood donors. Fischer's exact test was used to calculate the differences between groups. None of the differences between groups were statistically significant ($P < 0.05$)

Increased levels of total IgE were not more present in the CC patients than in the control group (p-value 1.00) and there could not be observed any correlation between total IgE-positivity and presence of any autoantibodies (data not shown).

Discussion

The present study has not been able to identify presence of autoantibodies against Col III, Col IV, MMP-9, TIMP-1 or TNC to a larger extent in CC patients compared to healthy blood donors. In accordance with this, the presence of antibodies against TPO, ASCA as well

Table 2 Differences in patient characteristics between CC-patients who expressed collagen-associated autoantibodies and those who did not

	Antibodies, n = 18	No antibodies, n = 48	*p*-value
Mean age at inclusion (range)	59 (31–73)	61 (31–74)	0.36
Mean disease duration, years (range)	3.7 (1–14)	6.4 (1–22)	*0.03*
Smokers or former smokers (n, %)*	15 (83.3)	32 (66.7)	0.23
Budesonide treatment (n, %)	4 (22.2)	21 (43.4)	0.16
Past or current diseases: (n, %)			
Reumatic disorder	3 (16.7)	7 (14.6)	1.0
Cancer	2 (11.1)	7 (14.6)	1.0
Asthma	2 (11.1)	7 (14.6)	1.0
Thyroid disorder	3 (16.7)	5 (10.4)	0.67
Celiac disease	1 (5.6)	2 (4.2)	1.0

CC Collagenous colitis, * = missing value: 6, Collagen-associated autoantibodies = antibodies against collagen type III, collagen type IV, matrix metalloproteinase-9, tissue inhibitors of metalloproteinases-1, and tenascin-C. Student's *t*-test was used to calculate differences between mean values. Fisher's exact test was used to calculate the differences in frequency between groups

as the levels of total IgE did not differ significantly from what could be expected in the background population. Even though we have tried to select specific antigens of relevance for CC there are a large number of other possible candidates.

Potential autoantigens

Since Col III and IV seem to be crucial in the collagen turnover in CC and it would have been logical if they had been involved in development of autoantibodies. However, previous studies have also shown increased presence of both collagen type I and VI in the tissue [3, 36] as well as increased transcripts of procollagen type I and IV [26] in patients with CC. Increased presence as well as degradation of collagen type I is also described in Crohn's disease [44] which make these collagens possible candidates for future studies.

A pathogenetic role of MMP-9 could have been plausible, since this MMP seem to be active in CC. Despite these circumstances we could not find any increased presence of autoantibodies against MMP-9. However, several different MMP types are involved in collagen turnover and autoantibodies could be directed towards other MMPs, most likely against MMP-1, -2, -8, -13 and -18, since they all degrade collagen [45]. In CC, the expression of MMP-1, 7 or 13 was not elevated, thus indicating that development of autoantibodies against these structures is less probable [3, 31].

Despite that both TIMP-1 and TNC seem to be active players in the pathogenic process in CC, no autoantibodies against these glycoproteins could be found.

Even though there was no association between CC and the collagen-associated autoantibodies, the autoantibody-positive patients had a significant shorter disease duration compared to those without autoantibodies. This subgroup did not have more concomitant diseases. Instead, this may be explained by a general increased immunological activation at the time of disease onset, which can cause development of non-disease-specific autoantibodies that can still be detected a few years after onset. As time goes by, a decrease in circulating antibodies may reflect an contemporary decrease in non-specific immune activation, something that has previously been described in CeD [46]. In conclusion, autoantibody prevalence reflects time from disease onset.

CC and thyroid diseases are related to each other, which in this study can be confirmed by the higher levothyroxine consumption among the CC patients (20%). This can be compared with 10–12% which is the proportion levothyroxine consumers among all women between 55 and 64 years in Skåne [47]. Despite this, no significant increase in occurrence of TPO antibodies could be observed in CC in the present study. This is in line with a previous investigation from our group [16]. The difference in design in the present compared to the former study [16] was that the present cohort consisted of cases with verified two flare-ups or two histological specimens indicating a more active and continuous disease. However, this did not make any difference in the results. Somewhat surprising, two thirds of those with CC that had positive TPO neither declared thyroid disease nor levothyroxine consumption. Positive TPO has been described as an early predictor of hypothyroidism so it is plausible that some of these individuals may develop thyroid disorder within a few years [48].

Even though previous studies indicated a possible association between CC and ASCA, we could not find any increased frequency in the present cohort. ASCA is

observed in Crohn's disease [49], which is a disease with a more severe disease course with organ damage. The different clinical characteristics could indicate a different pathogenetic background, something that could explain the negative results in CC.

Despite the increased levels of total IgE in IBD [17, 18], there was no such increase in CC. Different mechanisms are probably at hand in the different diseases.

Even though MC is driven by a Th1-dominated cellular immune response [10], the local symptoms and the low frequency of extra-intestinal manifestations indicate that the systemic immunological impact in MC is significantly lower than in other immune-driven diseases in the same organ system, such as classical IBD. In tissue samples activation of genes involved in presentation of luminal antigens to bacteria and viruses has been found. A bacterial origin for CC could thus be contemplated (51). Furthermore, the only effective treatment, budesonide, mediates its effect locally in the large intestine, while systemic corticosteroid treatment has lower impact. Consequently, in CC, inflammatory markers may only be seen in the intestinal wall rather than in the systemic circulation. On the other hand, MC is related to coeliac disease where the inflammatory activity is present locally in the intestine, but systemic antibodies are still present.

To the best of our knowledge, this is the first study that has investigated autoantibody prevalence against defined structures in the collagen layer in patients with CC. No such association could be found. Furthermore, the prevalence of antibodies against ASCA and TPO and the levels of total IgE were not increased. This talks against any autoimmune pathogenetic mechanism based on these structures. On the other hand, the many autoimmune features found in CC are still apparent why the possibility of an autoimmune pathogenesis via other antigens cannot yet be ruled out.

Strengths and limitations

A strength of this study was that the cohort consisted of 66 women with verified active and pronounced disease that demonstrated the typical characteristics for CC patients in general, *i.e.,* postmenopausal age of disease onset, an increased number of smokers and concomitant autoimmune disorders [8, 12]. The controls were not age matched. However, since the cut-off value to assess presence of antibodies in the CC patients was determined from a control group, this selection of individuals must come from a healthy cohort, otherwise there is a risk of a type II error. In contrast, in a control group consisting of older women that commonly are affected by inflammatory diseases/processes, there would have been an increased risk of presence of autoantibodies [11]. The sample size in this study was rather small. However, if any autoantibody should have any relevance, at least a significant minority ought to have it. The strict selection of this homogenous group should have increased the probability to identify any type of antibody but failed to do so, which increases the likelihood that our results are generalizable. Five individuals with CC and levothyroxine consumption declared neither thyroid dysfunction nor medication in the questionnaires, something that highlights the weakness with questionnaires without possibilities to validate the outcome.

Conclusion

In conclusion, Col III, Col IV, MMP-9, TIMP-1 or TNC are all proteins related to the collagen layer and its turnover and could thus have been expected to be targets for an immunological reaction and thereby prove that CC is of an autoimmune origin. Such autoantibodies could also have served as a diagnostic tool. Nevertheless, no increased presence of these autoantibodies could be found in the present study of CC. Neither could antibodies against ASCA or TPO, or elevated levels of IgE, be found. Consequently, no association was found between CC and these proteins, even though this may not be generalizable to other compounds in the collagen layer.

Abbreviations

ANA: Anti-nuclear antibodies; ASCA: Anti-Saccharomyces cerevisiae antibodies; BSA: Ovine serum albumin; CC: Collagenous colitis; CeD: Celiac disease; ColIII: Collagen type III; ColIV: Collagen type IV; CV: Coefficient of variation; ECM: Extracellular matrix; ELISA: Enzyme-linked immunosorbent assay; FEIA: Fluorescent enzyme immunoassay; HBPT: High blood pressure therapy; IBD: Inflammatory bowel disease; MMP: Matrix metalloproteinase; PBS: Phosphate buffer saline; PBS-T: Phosphate buffer saline with 0.05% Tween 20; PPI: Proton pump inhibitor; RA: Rheumatoid arthritis; RT: Room temperature; RU: Relative units; TIMP: Tissue inhibitors of metalloproteinase; TMB: Tetramethylbenzidine; TNC: Tenascin-C; TPO: Thyroid peroxidase.

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Author contributions

Conceptualization, JKL, BR and KS; methodology, JKL, BR, BO and KS; validation, JKL, BR, BO, KS; formal analysis, JKL and BR; investigation, JKL, BR, BO and KS; resources, JKL and KS; data curation, JKL, BR, BO and KS; writing—original draft preparation, JKL and KS; writing—review and editing, JKL, BR, BO and KS; visualization, JKL and KS; supervision, BO and KS; project administration, JKL, BR, BO and KS; funding acquisition, KS. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets generated and analysed during the current study are not publicly available due to a rather extensive amount of data files of which some furthermore requires some explanations, but will be available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved by the Regional Ethical Committees in Lund (2009/565 and 2011/209) and Stockholm (2016/271-31/1). All participants provided written informed consent to participate in the study. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Paper III

Errata

p 5, Table 4, line 7 "r" should read "Kidney"

p 5, Table 4, line 18 "r" should read "Lymphoma"

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Article

Cancer Risk in Collagenous Colitis

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Abstract: Data on malignancy in patients with collagenous colitis (CC) is scarce. We aimed to determine the incidence of cancers in patients with CC. In a two-stages, observational study, data on cancers in patients diagnosed with CC during 2000–2015, were collected from two cohorts. The risk was calculated according to the age-standardized rate for the first cohort and according to the standardized incidence ratio for the second cohort. The first cohort comprised 738 patients (394 from Scotland and 344 from Sweden; mean age 71 ± 11 and 66 ± 13 years, respectively). The incidence rates for lung cancer (RR 3.9, $p = 0.001$), bladder cancer (RR 9.2, $p = 0.019$), and non-melanoma skin cancer (NMSC) (RR 15, $p = 0.001$) were increased. As the majority of NMSC cases (15/16) came from Sweden, a second Swedish cohort, comprising 1141 patients (863 women, mean age 65 years, range 20–95 years) was collected. There were 93 cancer cases (besides NMSC). The risk for colon cancer was decreased (SIR 0.23, $p= 0.0087$). The risk for cutaneous squamous cell carcinoma was instead markedly increased (SIR 3.27, $p = 0.001$).

Keywords: colon cancer; cancer risk; collagenous colitis; lung cancer; microscopic colitis; skin cancer; squamous cell carcinoma

1. Introduction

Microscopic colitis (MC) is an inflammatory disorder of the colon that causes chronic, watery and non-bloody diarrhoea, occasionally associated with abdominal pain and weight loss. With a predilection for those ≥60 years of age and for females, MC has an incidence rate of approximately 10/100.000 per year [1–3]. Macroscopic findings are rare and the diagnosis is confirmed through

histopathology [4–6]. MC comprises two main histologic subtypes; collagenous colitis (CC) and lymphocytic colitis (LC). Histopathological features of CC include a continuous, thickened sub-epithelial fibrous band (>10 μm) and associated chronic mucosal inflammation. The collagen band contains entrapped capillaries, red blood cells, as well as inflammatory cells. Moreover, damaged epithelial cells appear flattened, mucin-depleted, and irregularly-oriented. Focally, small strips of surface epithelium may lift-off the basement membrane [7]. Patients with CC are considered to have a more symptomatic and long-lasting disease course than those with LC [8].

Chronic inflammation is considered a risk factor for cancer development. Moreover, chronic inflammation may result in cancer development in sites other than the affected organ/system. For instance, the risk of lymphoma in rheumatoid arthritis (RA) is increased by 60% [9]. In patients with inflammatory bowel disease (IBD), there is an increased risk of colorectal cancer (CRC), at least in some subgroups, as well as extra-intestinal cancers such as haematological, bladder, lung as well as skin cancers [10–12]. Patients with coeliac disease have a reported increased risk of non-Hodgkin lymphoma, small-bowel cancer, CRC and basal cell carcinoma (BCC) [13]. *Helicobacter pylori* itself contributes to many neoplasias, but studies have shown that the inflammatory response per se contributes to the carcinogenesis as well [14].

Data on the incidence of metachronous cancer(s) in MC is scarce. Although the inflammation is limited as compared to classical IBD the condition may be active for several years; furthermore, it often affects elderly individuals who have already an increased cancer risk. Additionally, many patients with CC smoke [15]. Chan et al described an increased risk of lung cancer in a small cohort of patients with CC with a mean follow-up time of 7 years. The study included 117 patients, and no cases of CRC were described [16]. A negative association has actually been suggested between CRC and MC (including both CC and lymphocytic colitis) in a cohort comprising 647 patients with MC and a mean follow-up time of five years. Twelve MC patients had CRC compared to 27 in a control group of similar size ($p = 0.015$) [17]. Therefore, the aim of the present study was to determine the incidence of metachronous cancer in patients with CC.

2. Patients and methods

The investigation was carried out as a two-stage, observational, international, multicentre, cohort-study, comprising two sizeable cohorts of patients diagnosed with CC. See Figure 1.

Figure 1. Participating centres: Series 1: 1 = Edinburgh, Scotland, 2 = Malmö; Series 2: 3 = Linköping, 4 = Örebro, 5 = Skåne region.

2.1. First Stage—Scotland and Sweden

In an international, retrospective, two-centre observational study; data on extra-colonic cancer in patients with CC were collected for a 14-year period (2000–2013) from Edinburgh, Scotland and Malmö, Sweden. The CC diagnosis was set according to established criteria i.e. symptoms of chronic, non-bloody diarrhoea and histopathological findings of thickened sub-epithelial collagen layer ≥10 μm, associated with chronic inflammation in the lamina propria and with an increased number of intraepithelial lymphocytes [18]. Data were obtained from the pathology department, Edinburgh and Malmö with a catchment area of 750,000 and 320,000 inhabitants, respectively. The records of those with CC were manually searched for data on metachronous, extra-colonic cancers.

2.2. Second Stage—Sweden

Due to an unexpectedly skewed distribution of cancer cases in the first stage of the study we decided to re-do the study and focus on Swedish data. Patients with CC in three different regions (Skåne, Linköping and Örebro) were included. In Sweden, all patients that are diagnosed with CC according to the established criteria are registered at the Departments of Pathology and are given a specific code number. All specimens taken during colonoscopy in the three regions are regularly sent to specific Pathology Departments. All patients with a CC diagnosis from 2000 in Skåne and Örebro and from 2008 in Linköping until the end of 2015 were included.

For each patient with CC, the follow-up period began at the time of CC diagnosis and continued until whichever of the following occurred first: death or the end of the observation period (31st December, 2015). Patients were not excluded after their first diagnosis of cancer, since we wanted to examine incidence risk for all cancers developed during follow up. The CC cohort was linked up with the National Cancer Register in each region. Cancers preceding the diagnosis of CC were not included in the cancer data.

2.3. Statistical Analysis

2.3.1. First Stage

Person-years at risk was calculated according to age-specific categories up to 85 years. The standard error (Se) was calculated using the Poisson approximation. Confidence interval (CI) of the age-standardised rate (ASR) was compared to public data, available from UK´s National Cancer Intelligence Network. The relative risk (RR) for ASR was calculated and compared to ASR in Lothian region, Scotland. The standardised cancer incidence rates (IR) were also compared to the ones of Lothian under the assumption that populations at the same latitude share the same IR.

2.3.2. Second Stage

Person-years at risk were calculated by gender and 5-year age groups, separately for the 3 geographical areas (Skåne, Linköping, and Örebro). Standardized incidence ratios (SIR) were calculated for each of the reported cancers. Because patients in this cohort came from three different Swedish regions and in light of the known national variations of cancer incidence, the expected numbers of cancers were calculated by pooling the patients and linking each area to existing cancer registries. The expected numbers of cases of cancers and specific cancer types were calculated by multiplying the number of person-years for each gender, age and area group by the corresponding specific cancer incidence rates in the respective areas. SIR and their 95% CI were calculated assuming that the observed number of cases followed a Poisson distribution. Mid-P exact test was applied and values below 0.05 were considered significant. For non-melanoma skin cancer (NMSC), both BCC and cutaneous squamous cell carcinoma (cuSCC) that can occur several times in one individual, the number of tumours was recorded—instead of individual cases—in both the CC cohort and in the control group.

This study was approved by the institutional review board at Lund University (The local Ethics Committee at Lund University, date: 6th November 2013; decision LU 2013/650 and date: 27th

October, 2016; decision LU 2016/788) and Lothian NHS. Since it was a retrospective registry study the Ethical Board approved that written informed consent was not necessary to obtain prior to the collection of data. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

3. Results

3.1. First Stage

The demographics of the first cohort can be seen in Table 1. Of the 738 included, 71 (50 women and 21 men) were affected by some form of extra-colonic, metachronous malignancy following the diagnosis of CC. The remainder of this cohort (n = 667) did not develop any extra-colonic cancer during the follow-up period. The average follow-up duration was 7 years (range 2–15 years), while the average time interval between CC and cancer diagnosis was 3 years (range 0–11 years). Of these 71 cases, 14 developed any cancer during the first year, 25 during the following two years and 32 from the third year.

Table 1. Characteristics of the collagenous colitis (CC)-cohort in Scotland and Sweden (Series 1).

	Edinburgh	Malmö	Total
N	394	344	738
Age, median (IQR)	68 (57–76)	69 (59–77)	68 (58–77)
% women (n)	68% (268)	83% (285)	75% (553)
IQR = interquartile range			

The RR for all cancers, lung, bladder cancer and NMSC, as well as the ASR in patients with CC were higher compared to those of the general population. The RR for lung cancer was 3.88 (CI: 1.62–9.31), for bladder cancer 9.23 (CI: 1.14–75.03) and for NMSC 14.96 (CI: 2.57–87.08). See Table 2.

Table 2. Observed and expected cancers in the Scottish/Swedish cohort (series 1). RR, relative risk.

Cancer type	Cases	Exp	RR	*P*-value
Skin (NMSC)	16	1	15.0	0.001
Bladder	6	1	9.2	0.019
Lung	18	2	3.9	0.001

NMSC = non-melanoma skin cancer.

The cases with bladder and lung cancer were evenly distributed, but in contrast 15/16 cases with NMSC were addressed from the Malmö cohort. Because of this a decision was taken to proceed to a second stage by including more regions in Sweden. Furthermore, in most countries the number of NMSC is difficult to determine because BCC and cuSCC cases are not reported to national cancer registries, or they are reported as one heterogeneous group [19]. However, in Sweden these cancer types are reported separately and consequently it is possible to obtain data on the occurrence of BCC and cuSCC.

3.2. Second Stage

In this stage, a total of 1141 patients with diagnosis of CC were identified. It should be noted that 344 out of those from Skåne were included also in stage one. The characteristics of the patients are shown in Table 3. The average follow-up duration was 8 years (range 2–15 years), while the average time interval between CC and cancer diagnosis was 4 years (range 0–14 years). Of the 93 solid cancers 17 were diagnosed during the first year, 25 during the following two years and 51 thereafter. The expected and observed cancer cases are presented in Table 4 and Table 5. The risk of lung cancer was increased in Skåne (SIR 1.85 CI: 1.053–3.029, $p = 0.034$) but since there were no other cases of lung cancer in the other two Swedish regions, this did not become significant in the whole group. The total number of cancer cases besides NMSC was 98. However, five rare cancer cases were considered not applicable for data calculation and thus excluded, leaving 93 cancer cases (61 women

and 32 men). The total number of NMSC was 140. The mean time interval between CC diagnosis and cuSCC was 5.4 years, range 0.6–12.1 years. Of the 46 cases of cuSCC, four developed cuSCC during the first year, eight during the following two years, and 34 after three years or more.

Table 3. Characteristics of the CC-cohort Sweden, three regions (Series 2).

	Linköping	Örebro	Skåne	Total
N	**130**	**133**	**878**	**1141**
Age, median (IQR)	**66 (57–75)**	**64 (53–74)**	**68 (58–76)**	**67 (57–76)**
% women (n)	**75% (98)**	**84% (112)**	**74% (653)**	**76% (863)**
IQR = interquartile range				

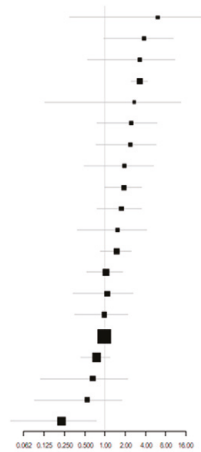
Table 4. Observed and expected cancers in the Swedish cohort, divided into Skåne, Linköping and Örebro (Series 2).

Cancer site	Skåne			Linköping			Örebro		
	obs	exp	SIR	obs	exp	SIR	obs	exp	SIR
Eye	1	0.13	7.64	0	0.023	0.00	0	0.011	0.00
Oesophagus	3	0.65	4.60	0	0.066	0.00	0	0.068	0.00
Cervix	2	0.46	4.33	0	0.07	0.00	0	0.079	0.00
CuSCC	36	11.58	3.11	6	1.44	4.18	4	1.060	3.78
Vulva	1	0.29	3.46	0	0.033	0.00	0	0.049	0.00
CNS	2	1.29	1.55	2	0.19	10.30	0	0.17	0.00
r	3	1.3	2.31	1	0.19	5.14	0	0.20	0.00
Stomach	1	1.27	0.79	1	0.14	7.14	1	0.14	7.27
Bladder/Ureter	8	4.29	1.87	0	0.5	0.00	2	0.38	5.30
Rectal/Anus	1	3.20	0.31	4	0.39	10.19	2	0.44	4.55
Pancreas	3	1.50	2.00	0	0.25	0.00	0	0.21	0.00
Lung	14	7.57	1.85	0	0.89	0.00	0	0.84	0.00
Prostate	8	7.56	1.06	1	1.56	0.64	1	0.56	1.78
Leukemia/Myeloma	3	2.93	1.02	0	0.37	0.00	1	0.40	2.52
Melanoma	3	3.92	0.76	2	0.63	3.18	0	0.55	0.00
BCC	83	82.08	1.01	7	7.62	0.92	4	6.19	0.65
Breast	11	17.61	0.62	3	1.89	1.59	2	1.98	1.01
r	1	2.47	0.40	0	0.30	0.00	1	0.26	3.78
Uterus	2	2.76	0.72	0	0.38	0.00	0	0.52	0.00
Colon	2	7.11	0.28	0	0.84	0.00	0	0.86	0.00

SIR = Standard Incidence Ratio, BCC = Basal cell carcinoma, cuSCC = Cutaneous squamous cell carcinoma.

Table 5. Observed and expected cancers in the whole Swedish cohort in a forest plot (Series 2).

Cancer site	Observed	Expected	SIR	mid-p
Eye	1	0.16	6.07	0.164
Oesophagus	3	0.79	3.82	0.054
Cervix	2	0.61	3.28	0.15
cuSCC	46	14.08	3.27	0.001
Vulva	1	0.37	2.70	0.364
CNS	4	1.65	2.42	0.113
Kidney	4	1.69	2.37	0.121
Stomach	3	1.54	1.94	0.273
Bladder/Ureter	10	5.16	1.94	0.055
Rectal/Anus	7	4.03	1.74	0.167
Pancreas	3	1.96	1.53	0.449
Lung	14	9.30	1.51	0.142
Prostate	10	9.68	1.03	0.878
Leukemia/Myeloma	4	3.69	1.08	0.816
Melanoma	5	5.10	0.98	1.000
BCC	94	95.89	0.98	0.847
Breast	16	21.48	0.74	0.233
Lymphoma	2	3.03	0.66	0.611
Uterus	2	3.66	0.55	0.411
Colon	2	8.81	0.23	0.009



BCC = Basal cell carcinoma, cuSCC = Cutaneous squamous cell carcinoma.

4. Discussion

This is the largest study published to date on the risk of metachronous malignancies in patients diagnosed with CC. In the first stage, it was noted that the risk for lung and bladder cancer was increased in patients with CC diagnosis. Furthermore, the risk of NMSC was also increased in this cohort. In the second stage of this observational, multicentre study comprising a large Swedish cohort we could confirm the decreased risk of colon cancer in patients with CC, as reported in previous studies [16,17]. However, previous studies either included prevalent cases of CRC (15) or comprised a fairly limited number of CC-patients (14). Analysis of the current large cohort from three counties in Sweden indicated that the risk of getting colon cancer was reduced at least four times (from 8.8 expected cases to two observed). Since we do not have information about previous colonoscopies in this elderly population with gastrointestinal complaints it cannot be excluded that this reduced risk hypothetically could be due to pre-emptive polypectomies preceding the diagnostic endoscopy. However, in patients with longstanding albeit low-grade, inflammatory response in the colon, one would instead expect to observe an increased risk of colon cancer. Nevertheless, not only the inflammation is modest, but it may also be protective. For instance, frequent watery diarrhoea reduces the transit time and likely any potential impact from toxic agents. Yen et. al suggest that elevated intraepithelial lymphocytes in the colonic mucosa in patients with MC may have a protective function against carcinogenesis through recruiting delta-gamma T-cells that kill cells undergoing DNA-damage or cell stress [17].

Data from the second series revealed a more than three-fold increase in cuSCC in patients with CC. Except UV-light exposure and immunosuppressive treatment related to organ transplantation, little is known about risk factors contributing to cuSCC [19–21]. Evidence that glucocorticoids enhance the risk of cuSCC is limited, but has been described previously [22,23]. The incidence of NMSC (both cuSCC and BCC) is increased in patients with IBD, but likely related to the exposure of immunosuppressive treatment (thiopurines and biologics) [24]. However, Singh *et. al* also described an increased risk of BCC in men with Crohn's disease not treated with immunosuppression [25]. Therefore, not only immunosuppressive treatment is related to an elevated risk of NMSC but also the inflammation per se, probably as a result of dysregulation of the immune system. In CC, Günaltay et al have described a decreased production of IL-37 in such patients, indicating a disturbed immune

response [26,27]. Thus, the elevated risk of cuSCC in CC may be related to a malfunctioning immune system caused by the disease itself, medication or other not yet known procarcinogenic factors.

The incidence rate of lung cancer was increased in the first stage cohort but not in the second. However, the incidence was increased also in the second series in Skåne but not for the whole Swedish cohort. In Skåne 878 out of the 1141 cases with CC were identified and 14 cases with lung cancer was found (7.57 expected). In contrast to this large cohort the expected incidence of lung cancer in the cities Örebro and Linköping (based on 150,000 inhabitants in each location) were one case in each location. Consequently, the observed incidence with no cases in these two minor cities must be interpreted with caution. The incidence rate in Skåne with around 1.3 million inhabitants is of course more reliable. The risk of urinary bladder and/or ureteric cancer was increased in the first cohort and showed a positive trend in the second, something that strengthens this observation. The association between CC and smoking habits is already well described [15]. Smoking is related to cancer in both lung and bladder which is probably the reason why these incidence rates are elevated in our CC cohort [28,29].

The risk of cancer in oesophagus also showed a positive trend in the second series. Risk factors of oesophagus cancer are among others, smoking, alcohol, hot liquids and HPV-infection [30]. As can be seen, some of the risk factors are shared with CC such as smoking and alcohol [31]. Furthermore, oesophageal cancer is often of squamous cell origin just as in the skin. In this study we did not get detailed information if the oesophagus cancer cases were of squamous cell or adenomatous origin. However, in view of the low number of cases with oesophageal or bladder cancer, despite a fairly large number of CC patients, a definitive conclusion cannot be drawn regarding putative relationship between CC and cancer in oesophagus or bladder.

Some strengths and limitations should be noted; this is the largest study to date concerning the risk of metachronous cancer in patients with a previous diagnosis of CC. In the second cohort, 1141 patients could be included. The studied regions are well defined, and patients are referred to specific hospitals within these regions. All common cancers types in the western world were represented in the present study. In other words, no common cancer type was totally absent. The control group in the Swedish cohort consisted of all cancer cases in the same regions as our cohorts, adjusted for year of onset, gender and age group. This procedure is necessary in order to obtain more reliable results. Furthermore, the cancer registry at the National Board of Health and Welfare covers more than 96% of the cancer cases in Sweden [32]. Since we wanted to investigate if CC can be considered as a risk factor for cancer, we included only incident cases of cancer diagnosed after the CC diagnosis. The outcome of cancer was assessed from day one after the CC-diagnosis. This may cause a risk of including prevalent cancers, but since we know that the time lapse between disease onset and diagnosis of CC may be long, we believe that these factors level out. A majority of the cancers was diagnosed after more than three years making detection bias less probable.

This study also has some limitations that merit consideration. First, nationwide registers do not contain information about lifestyle habits like smoking, alcohol consumption, family history, exercise or other possible confounding factors. We neither had detailed data of disease severity nor medication in our study population. Information about sun exposure and consequently also about the location of the skin tumours would have been valuable. Furthermore, the associations found are not necessarily causal; one disease could lead to another or a not yet known factor besides the studied could lead to both CC and cancer. The retrospective design also limits the conclusions that can be drawn, although a study with a prospective design would have been difficult to finalize.

Consequently, this study could confirm the previously described negative association between CC and colon cancer, an observation of unknown cause. Even though significance was not achieved for cancers in lung, bladder and oesophagus there was a trend indicating that there could be a correlation anyway. A new, so far, unknown association between CC and cuSCC has also been revealed. There are reports about an increase incidence of BCC in coeliac disease and of NMSC in IBD. Consequently, we have to be extra cautious when examining patients with gastrointestinal inflammation in order to reveal any incident skin cancers.

Author Contributions: Conceptualization, J.K.L., E.T., J.N.P., A.K. and K.S.**;** methodology, J.K.L., P.H., A.G., A.K., K.S.; software, P.H.; validation and formal analysis, J.K.L., P.H., A.G., A.K., K.S.; investigation, resources and data curation, J.K.L., K.J.D., J.B., A.M., A.G., A.N., G.W.J., E.T., J.N.P., P.F., A.K. and K.S.; writing- original draft preparation, J.K.L., A.K. and K.S.; writing- review and editing, J.K.L., K.J.D., P.H.; J.B., A.M., A.N., G.W.J., E.T., J.N.P., A.K., K.S.; supervision, P.H., A.K., K.S.; project administration, J.K.L., A.K., K.S.; funding acquisition, J.K.L., K.S.

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Paper IV

Regional variations in the incidence of microscopic colitis in the region Skåne 2010-2020

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Short title

Incidence of microscopic colitis in southern Sweden, 2010-2020.

Key words: Collagenous colitis; epidemiology; incidence; lymphocytic colitis; microscopic colitis; Skåne

Abstract

Background

In microscopic colitis (MC), the incidence has increased over the last decades. The aim of the present study was to determine the incidence of lymphocytic (LC) and collagenous colitis (CC) in Skåne, southern Sweden, during the period 2010-20 with focus both on the temporal and spatial variations.

Methods

The MC diagnosis was retrieved from the biopsy registries at the Departments of Pathology. Established diagnostic criteria (increased lymphocyte count, inflammation in lamina propria and in CC a collagen band) were used for diagnosis. Age, gender, date for diagnosis and municipality of residence were retrieved for all patients.

Results

In total 1985 patients could be identified with a mean age of 62.9 years (SD 15.7) whereof 1415 were women. The incidence for CC was stable with a total age-standardized rate (ASR) per 100 000 person-years of 6.34, (range 4.6-8.1). In LC the ASR was 7.90 (range 1.7-15.2) but increased markedly 2015-20 reaching 15.2 in 2019. The north-west parts of Skåne had the highest incidence with a peak incidence in Båstad municipality reaching 79.6 per 100 000 person-years in 2019.

Conclusions

The incidence of CC was stable during the period while LC differed substantially in a way that indicates that it most probably must be two different disease entities. In LC, in view of the marked and rapid increase, causative agents such as water contamination or infectious agents could be contemplated, why further studies are indicated.

Introduction

Microscopic colitis (MC) is a chronic inflammatory colonic disease that causes non-bloody diarrhoea mainly in women over 60 years (1). MC includes two subtypes: Lymphocytic colitis (LC) and collagenous colitis (CC). The two types are almost undistinguishable clinically but differs slightly histopathologically in the colonic mucosa; except for an inflammatory infiltrate in lamina propria and increased intraepithelial lymphocytes (LC), CC also demonstrates a thickened subepithelial collagenous layer (2).

CC was first described in Malmö 1976 by Lindstrom and LC was introduced a decade later by Lazenby (3, 4). Initially, MC was considered rare. Epidemiological studies from the 80s and 90s from Europe and the USA demonstrate incidence rates less than 10 cases per 100 000 person-years. Thereafter, increasing numbers of both CC and LC have been reported. A recent nationwide study from Denmark reported the highest incidence numbers worldwide in 2011 (32.3 MC cases per 100 000 person-years) (5). An increased awareness of the disease and increased endoscopic activity as well as increased exposure of known and yet unidentified risk factors is probably the reasons for this sharp increase in incidence.

The etiology and pathophysiology of MC remains largely unknown. Emerging evidence suggests a multifactorial process involving an abnormal mucosal immune response to luminal factors in genetically predisposed individuals (6). Well described risk factors are gender, age, smoking and drug exposure, *i.e.,* NSAID, PPI and SSRI (1). Alcohol (7, 8), HRT consumption (9, 10) and previous gastrointestinal infections (11) seem to be risk factors as well, but with weaker evidence. One study has also suggested an inverse association to population density, but this has not been validated in other studies yet (5).

The increasing incidence and geographical variations indicate that environmental factors contribute to the pathogenesis. A mapping of these fluctuations in time and space could give some information about possible contributing factors. The purpose of this study was to investigate the overall incidence of MC in Skåne 2010-2020, as well as the spatial and temporal variations in incidence.

Materials and methods

Catchment area/population demographics

Region Skåne with its 33 counties is the southernmost part of Sweden and is a mixed urban rural region with a total population approx. of 1,4 mill people year 2020. The population concentration is highest in the western part, 53 % of the Skåne population live in one of the ten counties adjacent to the western coastline. Thirty percent is of foreign background (born abroad or born in Sweden with both parents born abroad) (12). Life expectancy in Skåne is similar to Sweden in total, 84.3 years for females and 80,8 years for men. Forty percent of the females and 30% of the men between 16-74 years have a college degree or higher which is the third highest numbers in Sweden (13). As in the rest of Sweden, the healthcare system in Skåne is tax-funded and offered to the whole population and is assumed to offer equal access to health care for all residents.

Identification of MC cases

All residents in Sweden have their own Personal Identity Number (PIN), given at birth or at immigration. The numbers correspond to date of birth and is also encoded for gender. The PIN can be used to identify individuals within the health care registries. This identification can be used together with the Systematized Nomenclature of Medicine (SNOMED) system for biopsies at the Dept of Pathology to identify all patients with LC and CC through their specific codes (M40600 for CC and M47170 for LC).

All new MC-cases in Skåne 2010-2020 were identified. The validity of MC cases in the Swedish Pathology registries has recently been evaluated with a positive predictive value of 95% (14). The 2010-2020 data set was compared with data from 2000-2009, and duplicates were eliminated to assure that only new cases were included.

Identification of population and population density

Statistics Sweden (SCB) offers an open data base were population by municipalities, regions and the whole country as well as age and sex can be achieved. SCB also hosts data concerning populations density by organization the population in Demographical Statistical Areas (DSA). DSA is divided in three main categories based on population concentration: A (rural), B (mixed, eg small towns) and C (urban areas). Skåne comprises 789 DSAs, where 15% constitute category A, 14% B and 71% C (12).

Statistics

Crude and age-specific incidence rates for gender, age, calendar year, municipality of residence and DSA-category were calculated by dividing the total number of cases by the total number of person-years of observation, multiplied by 100 000. Two-sided exact confidence intervals (CI) were based on a Poisson distribution. To account for different demographics in the different municipalities, age-standardized rates (ASR) were calculated by multiplying the age-specific rates with weights from the 2020 Swedish population. For ASR, standard error (SE) and two-sided CI were calculated based on binominal approximation. In a sensitivity analysis, SE and CI also were calculated on Poisson approximation, with no different results.

To calculate the relative risk of disease between two ASRs, standardized rate ratio (SRR) was calculated by dividing ASR1 by ASR2. 95% CIs were calculated with Smiths formula (15), based on the SEs retrieved from the previous calculations on ASR for each group.

Differences in age at diagnosis was calculated with Student's T-test.

Linear regression analysis was used to determine whether an increase in ASR was significant. Statistical calculations were performed with IBM SPSS Statistics editor version 25.

A p-value below 0.05 was considered to be significant.

Ethics

This study was approved by the Swedish Ethical Review Authority 2020-06631 and 2021-01554. Since this was a registry-based study, no approval from participants was retrieved.

Results

Cases

In the registry at the Dept of pathology 2 000 cases with MC could be found during the period 2010-20. However, 15 cases could not be identified in the patient data bases in Region Skåne. Consequently, 1985 cases with first-time diagnosis of MC during the entire study period (2010-2020) could be included, of which 71% were women. LC was more common (n=1105, 56%), than CC (n=880, 44%). Of the total cohort 53% of the female cases and 61% of the male cases had LC. The demographics can be seen in Table 1.

Table 1, Demographic characteristics of the MC cohort.

		MC		CC		LC	
Total (n)		1985		880		1105	
Age (years)	mean (SD)	62.9	(15.7)	65.3	(14.1)	61.0	(16.7)
	median (IQR)	66	(52–75)	68	(57–75)	64	(50–74)
Men (n)		570		221		349	
Age (years)	mean (SD)	64.4	(15.8)	68.1	(13.0)	62.1	(16,9)
	median (IQR)	68	(57–76)	71	(63–77)	66	(51,5–75)
Women (n)		1415		659		756	
Age (years)	mean (SD)	62.3	(15.7)	64.3	(14.4)	60.5	(16.6)
	median (IQR)	65	(52–74)	66	(55–75)	63	(49–73)

MC=Microscopic colitis, CC= Collagenous colitis, LC= Lymphocytic colitis, SD= Standard deviation, IQR= Interquartile range.

Incidence of MC in Skåne in general

The total age-standardized incidence rate (ASR) for Skåne for the whole period was 14.2 cases per 100 000 person-years (95% CI 13.6-14.9). LC was significantly more common than CC. For CC, ASR was 6.3 (95% CI 5.9-6.8) and for LC 7.9 (95% CI 7.4-8.4) cases per 100 000 inhabitants, which results in a CC:LC ratio of 0.8:1 (95% CI 0.73-0.88).

Incidence of MC based on age

For the entire study period, the mean age at diagnosis of MC was 62.9 years, range 4-95. For CC, the mean age was slightly higher than in LC (65.3 versus 61.0 years). Male patients were significantly older than women at diagnosis of MC ($p < 0.01$)

The mean age for MC differed slightly between the years (highest 2013 64.2 years, lowest 2011 61.3 years) (data not shown). When calculating age-specific incidence rates, the age class 80-84 years had the highest risk of diagnosis. When stratifying for the subtypes, new cases of LC is most common in age class 80-84, and in CC age class 74-80 See Figures 1 a, b and c.

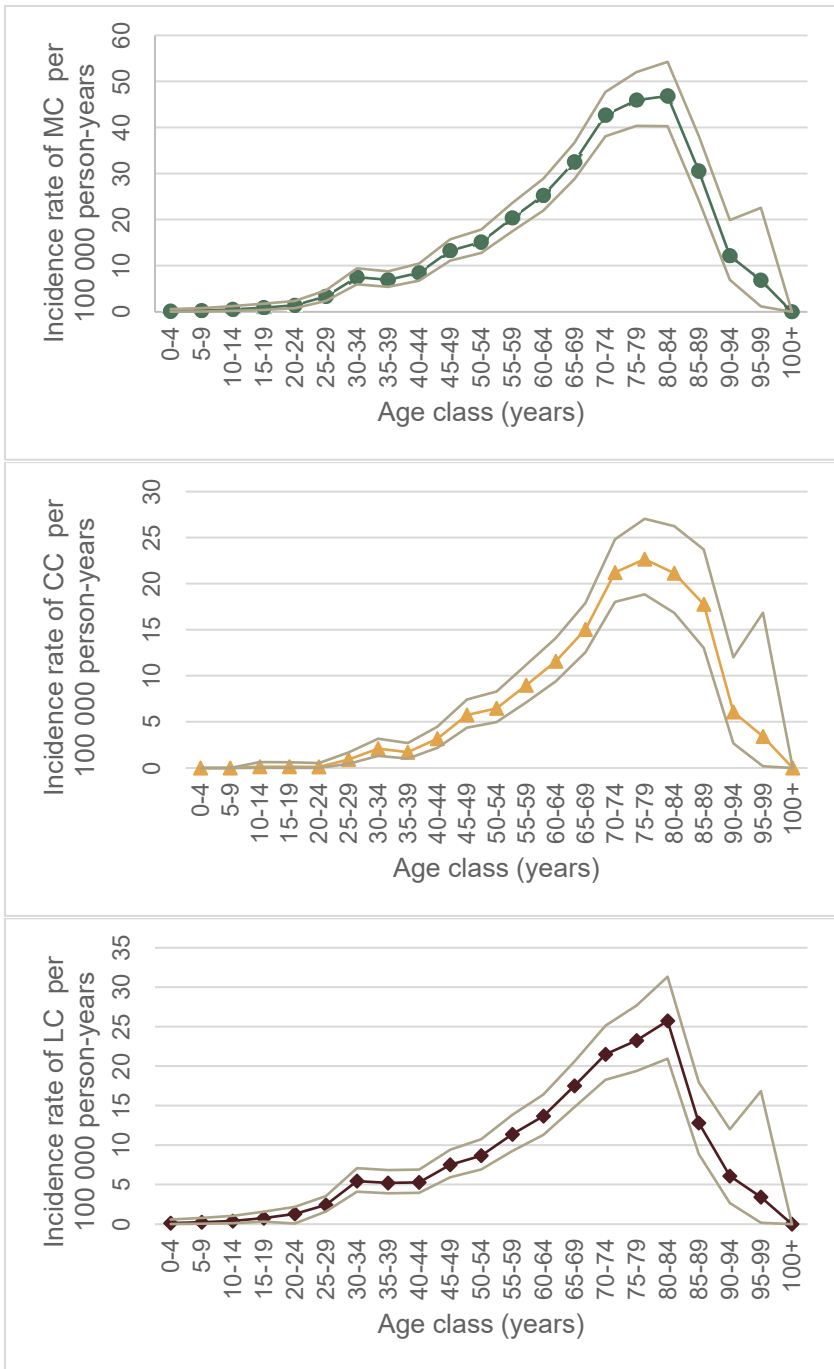

Figure 1a (MC), b (CC) and c (LC). Age-specific rate per 100 000 person-years with 95% confidence interval in microscopic colitis, collagenous colitis and lymphocytic colitis, respectively.

Incidence of MC based on gender

The incidence of MC was, as expected, higher in women. The MC female:male standardized rate ratio (SRR) was 2.3:1 (95% CI 2.1-2.5). Female dominance included both subtypes, even though LC had a slightly lower ratio (SRR 2.0:1; 95% CI 1.8-2.3) in comparison to CC (SRR 2.7:1; 95% CI 2.3-3.1).

Incidence of MC based on calendar period

The incidence for CC was stable with a total ASR per 100 000 person-years of 6.3, (range 4.6-8.1). In LC the ASR for the whole period was 7.9 per 100 000 person-years (range 1.7-15.2) but increased significantly 2015-20 reaching 12.1 per 100 000 person-years (95% CI 11.2-12.9) in comparation to 2010-2014 (4.8 per 100 000 person-years, 95% CI 4.2-5.8) (SRR 2.5, 95% CI 2.2-2.9). Linear regression analysis showed that the ASR for LC increased significantly for each year with a B value of 1.0. For CC, no significant change in ASR was seen over the years. The ASR pattern was similar for both sexes (Figures 2a and b and 3).

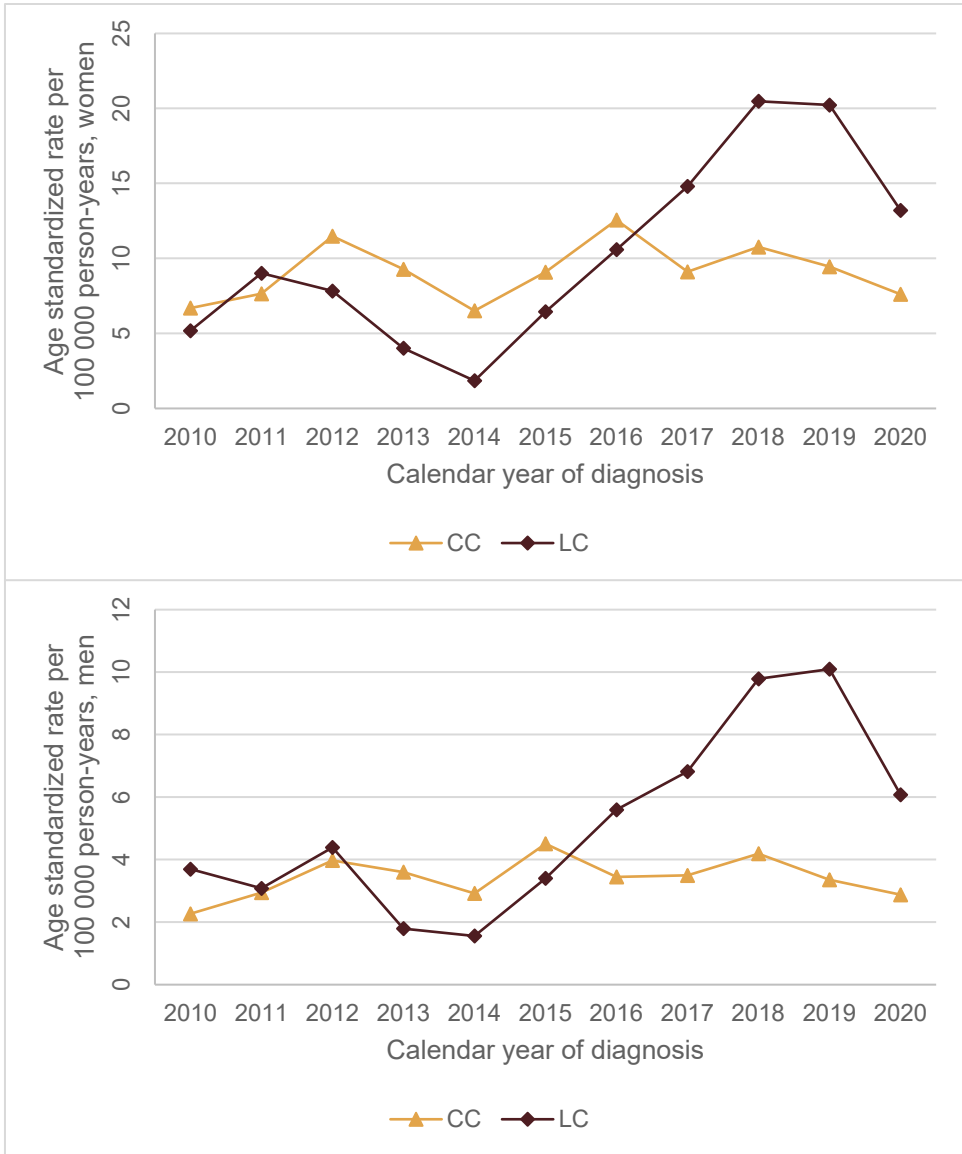

Figure 2 a (women) and b (men). Age-standardized rate per 100 000 person-years in collagenous colitis and lymphocytic colitis during the period 2010-20 in women and men, respectively.

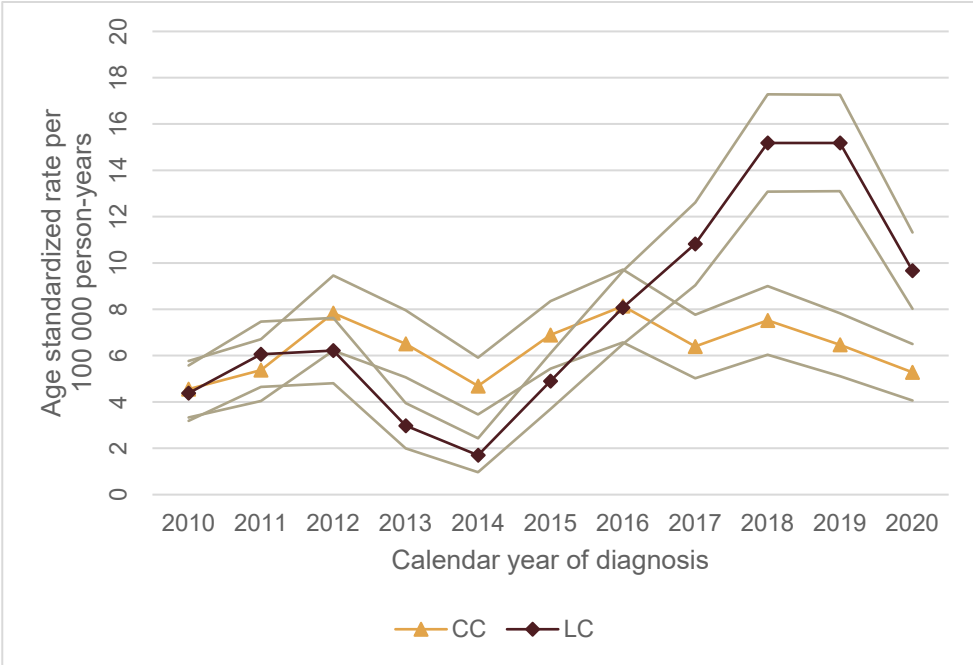

Figure 3, Age-standardized rate per 100 000 person-years for collagenous colitis and lymphocytic colitis with 95% confidence interval during the period 2010-2020.

Incidence of MC based on population density

The incidence of MC including the subtypes did not differ significantly when the population was divided into different population density-groups (Table 2).

Table 2, Cases with MC, CC and LC divided into three main categories based on population concentration.

	MC			CC			LC		
	Cases	**ASR**	**SE**	**Cases**	**ASR**	**SE**	**Cases**	**ASR**	**SE**
DSA A	235	13.35	0.90	110	6.17	0.60	125	7.19	0.66
DSA B	297	13.92	0.82	126	5.77	0.52	171	8.15	0.63
DSA C	1423	14.20	0.38	629	6.35	0.25	794	7.85	0.28
	Ratio	**95% CI**		**Ratio**	**95% CI**		**Ratio**	**95% CI**	
A/B	0.96	0.81-1.14		1.07	0.82-1.39		0.88	0.70-1.11	
B/C	0.98	0.86-1.11		0.91	0.75-1.10		1.04	0.88-1.23	
C/A	1.06	0.93-1.22		1.03	0.84-1.26		1.09	0.91-1.32	

A (rural), B (mixed, *e.g.,* small towns) and C (urban areas). MC=Microscopic colitis, CC= Collagenous colitis, LC= Lymphocytic colitis, ASR= Age-standardized rate, SE= Standard error, DSA= Demographical Statistical Areas.

Incidence of MC based on municipality of residence

The ASR for MC for the whole decade varied between the municipalities with a maximum level in Båstad (at the coast in NW Skåne) reaching 20.1 per 100 000 person-years, and a minimum level in Bjuv (inland in NW Skåne) at 8.2 per 100 000 person-years. See Figure 4. As can be seen in Figure 3 the incidence in MC was mainly dependent on a marked increase in LC during the second part of the decade. As can be seen in Figure 4 some specific municipalities such as Båstad had a double incidence compared to low-risk municipalities. In 2019, Helsingborg municipality had 50 cases as compared to two per year during 2010-15. Båstad municipality had the highest incidence reaching 79.6 per 100 000 the same year. However, since Båstad is a small municipality, the number of cases was only 12.

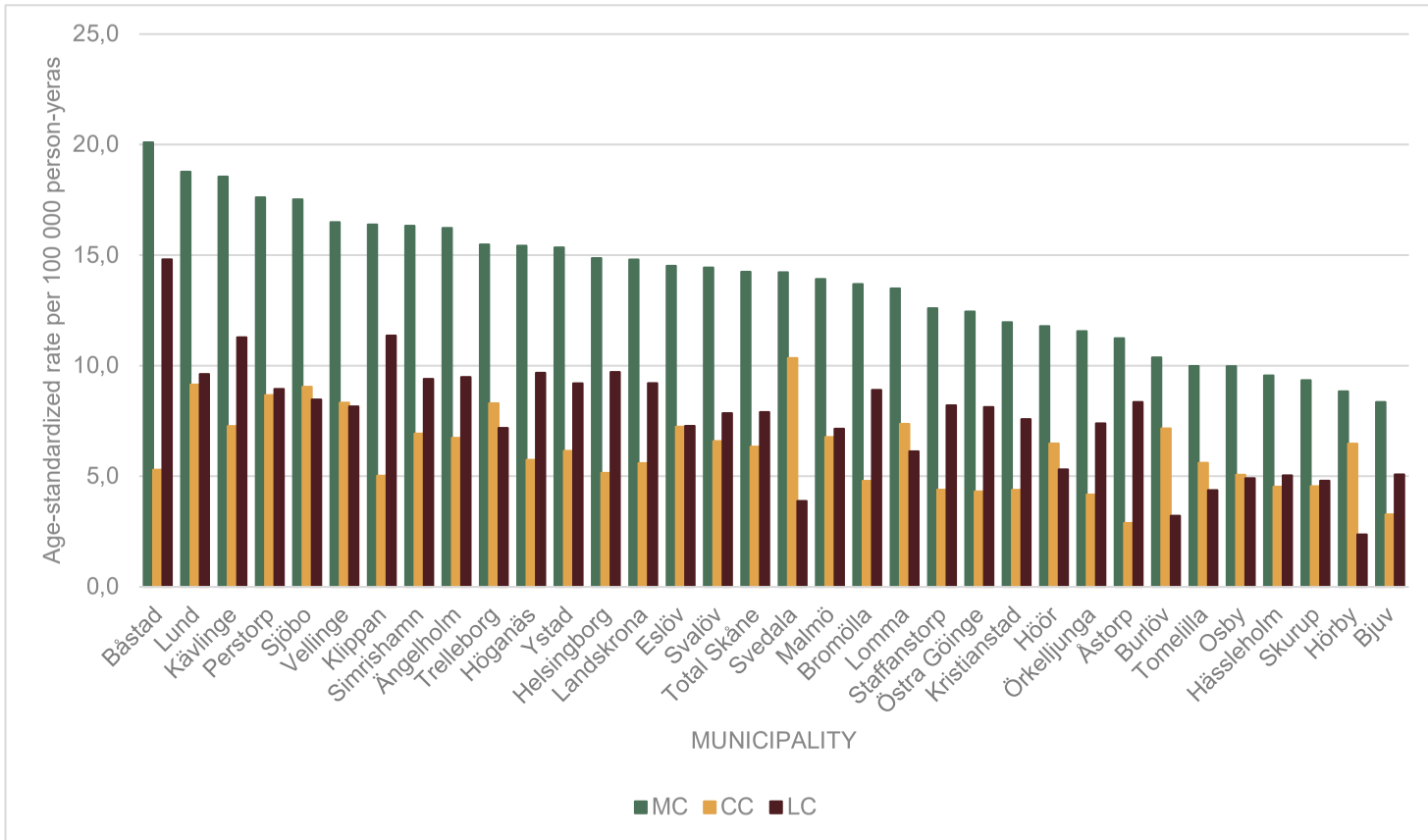

Figure 4 Age-standardized rate per 100 000 person-years during the period 2010-20 divided in the 33 municipalities in Skåne.

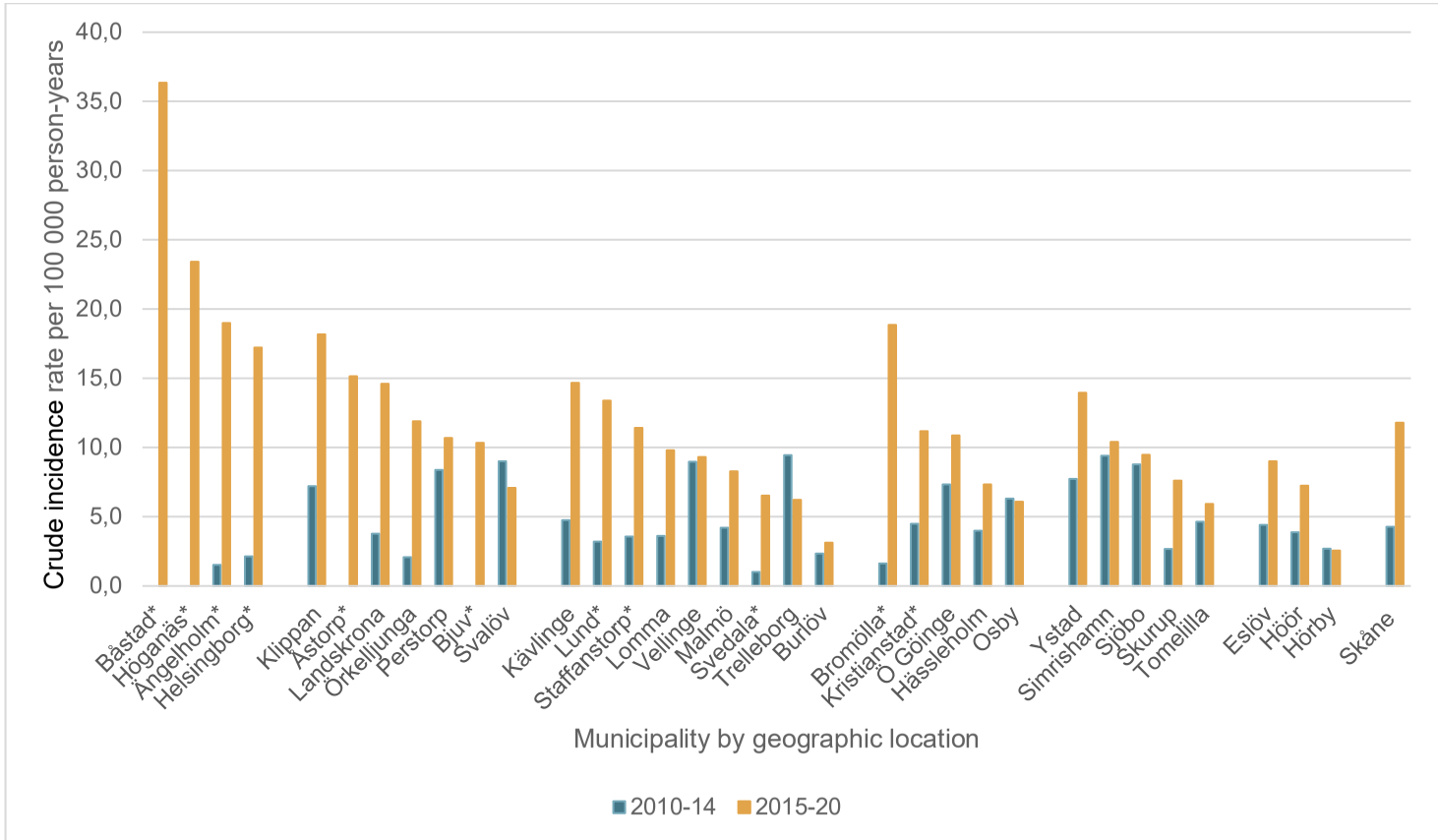

Figure 5 Crude incidence rate per 100 000 person-years for lymphocytic colitis divided into six parts of Skåne; north-west at the coast, north-west inland, south-west, north-east, south-east and central. *, significantly different age-standardized rates between the two periods ($p < 0.05$).

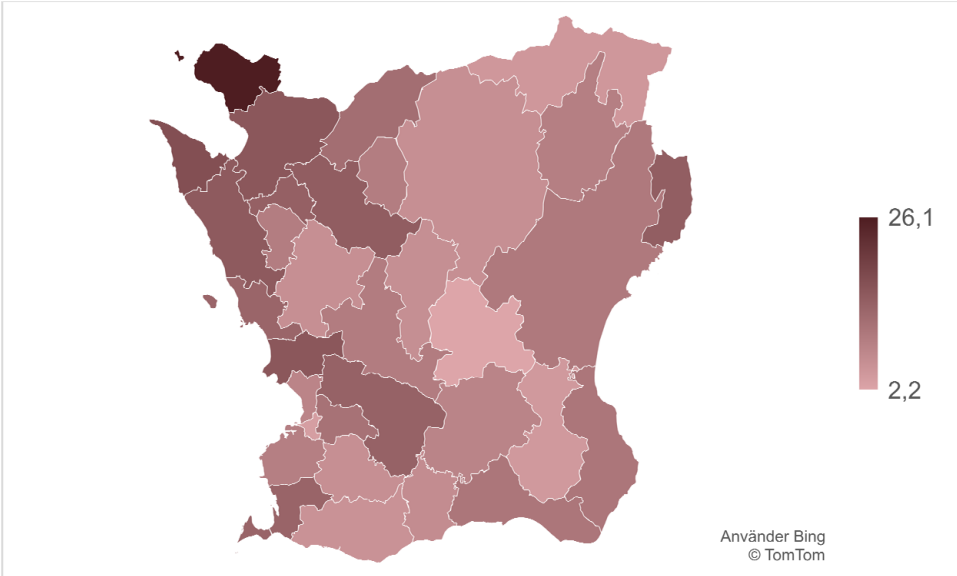

Figure 6, Age-standardized rates per 100 000 for lymphocytic colitis 2015-2020, illustrating the variation in incidence between different parts of Skåne.

Colonoscopies with biopsies in Skåne

The total number of colonoscopies with biopsies during the follow-up were 88955. The mean number per year was 10102 (range 9589-10839). During the pandemic year 2020, 9589 colonoscopies were performed, which is the lowest number during the time period. During the whole period, 22.3 MC-cases were discovered per 1000 colonoscopies. The lowest rate was during 2014, where only 7.3 cases per 1000 colonoscopies were found. This is in line with the low number of the MC cases that year. See Figure 7.

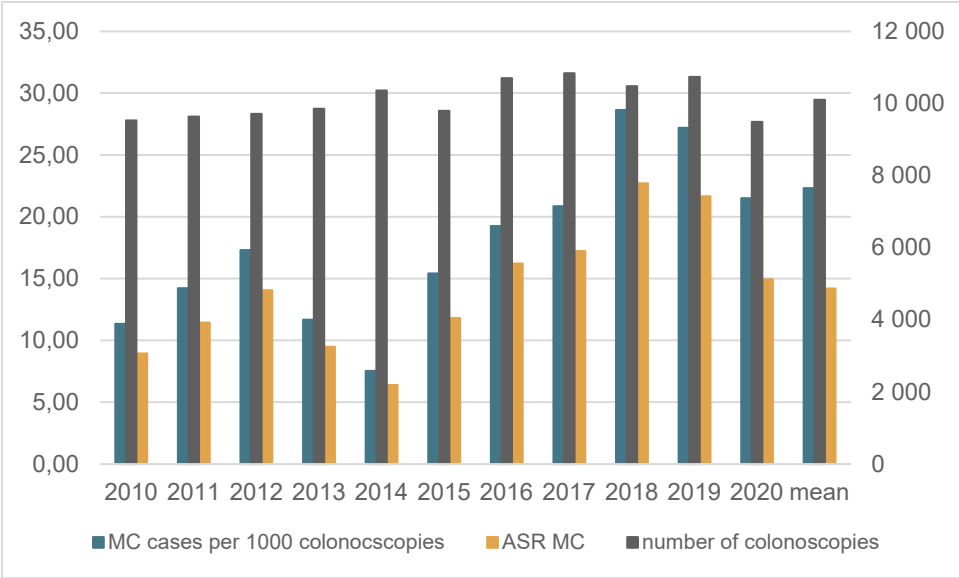

Figure 7, Cases of microscopic colitis per 1000 colonoscopies in Skåne in relation to age-standardized rate per 100 000 person-years of microscopic colitis 2010-2020.

Discussion

The present study verifies the ongoing increase of MC incidence that is reported from epidemiological studies over the world. Our results demonstrate current incident rates of MC in Skåne that is lower than reported from Denmark (MC 20.7 cases per 100 000 person-years 2001-2016 (5) and overseas in Olmsted, USA (25.8 cases per 100 000 person-years, 2011-2019 (16) but confirm the ongoing national increase in Sweden, something that Bergman et al demonstrated in a nationwide incidence study with observation time 1995-2015 (17). They observed steady increasing incidence rates for the whole observation time, where ASR for MC 2010-2015 was 10.7 cases per 100 000 person-years. In our study, the incidence in CC remained stable while LC increased substantially and significantly. Actually, we found the highest incidence rates in LC this far described in Sweden (15.2 cases per 100 000 person-years 2018-2019).

Our data indicate that LC is more common than CC, which is in line with the incidence studies from America, where Olmsted (2002-2010 and 2011-2019) and Calgary (2004-2008) reported data similar to ours (CC:LC 0.8:1, 0.6:1 and 0.5:1) (16, 18, 19) as well as the previous Swedish nationwide study (67% LC) (17). Also, a meta-analysis from 2015 showed a higher rate of LC than in CC (1). On the other side, nationwide studies from Denmark, Netherlands and Iceland all describe CC as the predominant subtype (5, 20, 21). The explanation of these differences remains

unclear, but the traditions on number of biopsies taken at colonoscopies may have an impact since the thickness of the collagenous band may vary in different parts of the large intestine. Hence, some cases of LC may be a "hidden" CC. However, more likely, LC that in general and in particular in our study, shows more varied incidence rates is probably influenced by a yet unidentified environmental risk factor in a higher extend compared to CC.

Some previous investigations in the region have been carried out. In a smaller study with 198 CC patients, the incidence in Malmö of CC 2001-2010 was slightly less than ours (CC 5.4 per 100 000 inhabitants) (22). However, in a geographically nearby region, but with another nationality, incidence rate of MC from Zealand (Denmark) was estimated 27.5 cases per 100 000 person-years in 2010-2016 (23). Several putative risk factors were contemplated. Smoking and consumption of PPIs were both more frequent in Zealand in that study.

The age-specific rate was highest in age classes between 70 and 80 which emphasizes the well documented association between MC and high age. However, for the oldest inhabitants, the incidence rate decreases. This could at least partly be an effect of the fact older patients more seldom is investigated with endoscopy sometimes due to comorbidity that prevents such measures. Otherwise, this could indicate that there really could be a risk maximum at 70-80 years of age.

We can in this study confirm the risk factor of female gender with a more than double relative risk for MC in females compared to men. This is in line with previous studies (5, 17, 21). The association between MC and gender is stronger in CC (SRR 2.7:1) than in LC (SRR 2.0:1), which may be a result of that LC is more associated with environmental risk factors and CC could be more dependent on hormonal factors.

Few studies have investigated population concentration as a potential risk factor for MC. However, the difference in incidence rates between Iceland 1995-1999 (MC 9.2 cases per 100 000 person-years, 3 inhabitants per square kilometer) and Netherlands 2000-2012 (MC 3.4 cases per 100 000 person-years, 409 inhabitants per square kilometer) may indicate that (20, 21). In line with this, the nationwide study from Denmark demonstrated higher incidence of MC in rural areas compared to densely populated (5). In Parkinson's disease this skewed distribution has also been observed (24). Exposure for chemical substances from farming has been suggested as trigger in that case. Even though these diseases probably do not share pathogenetic mechanisms, toxic compounds could influence inflammatory processes and thereby contribute to disease onset. In order to investigate whether we could confirm this skewed distribution, we divided the cohort with MC in Skåne into three different groups; urban, small villages and rural areas based on statistical information from Statistics Sweden. However, no differences could be found in our cohort. Instead, the MC cases including the subtypes were evenly distributed across the three groups which indicates that population concentration does not seem to be a risk factor for MC.

Based on previous observations in Skåne regarding inflammatory bowel disease (IBD) where some municipalities had a high incidence of ulcerative colitis and low of morbus Crohn and vice versa we also divided the cohort based on the 33 municipalities in Skåne (25). Interestingly, even though many municipalities presented stable age-standardized rates some increased markedly during the last part of the 2010s, especially LC cases. For example, in the north-west part of Skåne several municipalities showed exceptionally high incident rates. Båstad peaked at 79.6 LC cases per 100 000 person-years during 2019, which to the best of our knowledge is the highest number reported in LC. The only resemblance could be the incidence of IBD in the Faroe Islands that also was shown to have a magnitude of almost 80 cases per 100 000 person-years 2010-2014 (26). In view of the rapid change in a few years some environmental effect that has an impact over that time span must be suspected. Pollution in the water could be a possible explanation. An epidemic with gastroenteritis should most probably have a shorter time span. However, all age-standardized rates instead declined during 2020.

The number of colonoscopies with biopsies were stable during the follow-up time. Least examinations were performed during 2020, probably as a consequence of the covid pandemic. However, the number of MC cases per 1000 colonoscopies during 2020 (21.5) was almost of the same magnitude as the number for the whole period (22.3). Consequently, the sharp increase of LC cases in north-west Skåne is probably not due to an increased number of endoscopies. Furthermore, if that was the case also CC would increase during the same time span.

The study has some limitations. Because it is a registry-based study it has not been possible to retrieve information about other diseases, medication, or lifestyle related risk factors such as smoking on an individual basis. All these factors may contribute to the demonstrated variations. There are also some strengths. Skåne is a well-defined region where almost all patients attend their local hospital All biopsies are analyzed at the department of Pathology and can be collected through available data bases. General information about the background population is freely available from open registries.

In conclusion, the incidence of CC was stable during the period while LC differed substantially in a way that indicates that it most probably must be two different disease entities with different pathogenetical mechanisms and/or dependence on different risk factors. The incidence rate of LC exceeds previous investigations and whether the incidence will continue to increase remains to be seen. In LC, in view of the marked and rapid increase, causative environmental risk factors such as water contamination or infectious agents could be contemplated, while further studies are indicated.

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