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Small Bowel Carcinomas in Coeliac or Crohn's Disease: Clinico-pathological, Molecular, and Prognostic Features. A Study From the Small Bowel Cancer Italian Consortium

Alessandro Vanoli,[a,c,*] Antonio Di Sabatino,[b,*] Daniela Furlan,[c] Catherine Klersy,[d] Federica Grillo,[e] Roberto Fiocca,[e] Claudia Mescoli,[f] Massimo Rugge,[f] Gabriella Nesi,[g] Paolo Fociani,[h] Gianluca Sampietro,[i] Sandro Ardizzone,[j] Ombretta Luinetti,[a] Antonio Calabrò,[k] Francesco Tonelli,[l] Umberto Volta,[m] Donatella Santini,[n] Giacomo Caio,[m] Paolo Giuffrida,[b] Luca Elli,[o] Stefano Ferrero,[p] Giovanni Latella,[q] Antonio Ciardi,[r] Roberto Caronna,[s] Gaspare Solina,[t] Aroldo Rizzo,[u] Carolina Ciacci,[v] Francesco P. D'Armiento,[w] Marianna Salemme,[x] Vincenzo Villanacci,[x] Renato Cannizzaro,[y] Vincenzo Canzonieri,[z] Luca Reggiani Bonetti,[a] Livia Biancone,[ab] Giovanni Monteleone,[ab] Augusto Orlandi,[ac] Giuseppe Santeusanio,[ac] Maria C. Macciomei,[ad] Renata D'Incà,[ae] Vittorio Perfetti,[af] Giancarlo Sandri,[ag] Marco Silano,[ah] Ada M. Florena,[ai] Antonino G. Giannone,[ai] Claudio Papi,[aj] Luigi Coppola,[ak] Paolo Usai,[al] Antonio Maccioni,[am] Marco Astegiano,[an] Paola Migliora,[ao] Rachele Manca,[a] Michele Martino,[b] Davide Trapani,[c] Roberta Cerutti,[c] Paola Alberizzi,[a] Roberta Riboni,[a] Fausto Sessa,[c] Marco Paulli,[a] Enrico Solcia,[a] Gino R. Corazza[b]

Departments of [a]Molecular Medicine and [b]Internal Medicine, San Matteo Hospital Foundation, University of Pavia, Pavia, Italy [c]Department of Surgical and Morphological Sciences, University of Insubria, Varese, Italy [d]Biometry and Statistics Service, San Matteo Hospital, Pavia, Italy [e]Pathology Unit, Department of Surgical and Diagnostic Sciences, San Martino/IST University Hospital, Genova, Italy [f]Pathology Unit, Department of Medicine, University of Padua, Padua, Italy [g]Division of Pathological Anatomy, Department of Surgery and Translational Medicine, University of Florence, Florence, Italy Units of [h]Pathology, [i]IBD Surgery and [j]Gastroenterology, Luigi Sacco University Hospital, Milan, Italy Departments of [k]Experimental and Clinical Biomedical Sciences and [l]Surgery and Translational Medicine, University of Florence, Florence, Italy Divisions of [m]Gastroenterology and [n]Pathology, Sant'Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy [o]Center for Prevention and Diagnosis of Coeliac Disease, Fondazione IRCCS Ca' Granda, Milan, Italy [p]Division of Pathology, Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy [q]Gastroenterology Unit, Department of Life and Enviromental Sciences, University of L'Aquila, L'Aquila, Italy Departments of [r]Radiological, Oncological, Pathological Sciences and [s]Surgical Sciences, Umberto I Hospital, La Sapienza University, Rome, Italy Units of [t]General Surgery and [u]Pathology, Cervello Hospital, Palermo, Italy [v]Department of Medicine and Surgery, University of Salerno, Salerno, Italy [w]Department of Advanced Biomedical Sciences, Federico II University of

Naples, Naples, Italy [x]Pathology Section, Spedali Civili Hospital, Brescia, Italy Departments of [y]Gastroenterology and [z]Pathology, National Cancer Institute, Aviano, Italy [aa]Section of Pathology, Department of Diagnostic Medicine and Public Health, University of Modena and Reggio Emilia, Modena, Italy Departments of [ab]Systems Medicine and [ac]Biopathology and Image Diagnostics, University of Tor Vergata, Rome, Italy [ad]Pathology Unit, San Camillo-Forlanini Hospital, Rome, Italy [ae]Gastroenterology Section, Department of Surgery, Oncology and Gastroenterology, University of Padua, Padua, Italy [af]Internal Medicine, S.S. Annunziata Hospital, ASST-Pavia, Varzi, Italy [ag]Clinical Nutrition Unit, Sant'Eugenio Hospital, Rome, Italy [ah]Unit of Human Nutrition and Health, Istituto Superiore di Sanità, Rome, Italy [ai]Institute of Pathologic Anatomy, Giaccone University Hospital, University of Palermo, Palermo, Italy Units of [aj]IBD and [ak]Pathologic Anatomy, San Filippo Neri Hospital, Rome, Italy [al]Department of Internal Medicine, University of Cagliari, Cagliari, Italy [am]Pathology Unit, SS. Trinità Hospital, Cagliari, Italy [an]General and Specialistic Surgery, Città della Salute e della Scienza-Molinette Hospital, Turin, Italy [ao]Unit of Pathological Anatomy, Sant'Andrea Hospital, Vercelli, Italy

*Alessandro Vanoli and Antonio Di Sabatino are joint first authors.

Corresponding author: Prof. Antonio Di Sabatino, MD, Clinica Medica I, Fondazione IRCCS Policlinico San Matteo, Università di Pavia, Piazzale Golgi 19, 27100 Pavia, Italy. Tel.: +39 0382 501596; fax: +39 0382 502618; email: a.disabatino@smatteo.pv.it

Abstract

Background and Aims: An increased risk of small bowel carcinoma [SBC] has been reported in coeliac disease [CD] and Crohn's disease [CrD]. We explored clinico-pathological, molecular, and prognostic features of CD-associated SBC [CD-SBC] and CrD-associated SBC [CrD-SBC] in comparison with sporadic SBC [spo-SBC].

Methods: A total of 76 patients undergoing surgical resection for non-familial SBC [26 CD-SBC, 25 CrD-SBC, 25 spo-SBC] were retrospectively enrolled to investigate patients' survival and histological and molecular features including microsatellite instability [MSI] and KRAS/NRAS, BRAF, PIK3CA, TP53, HER2 gene alterations.

Results: CD-SBC showed a significantly better sex-, age-, and stage-adjusted overall and cancer-specific survival than CrD-SBC, whereas no significant difference was found between spo-SBC and either CD-SBC or CrD-SBC. CD-SBC exhibited a significantly higher rate of MSI and median tumour-infiltrating lymphocytes [TIL] than CrD-SBC and spo-SBC. Among the whole SBC series, both MSI—which was the result of MLH1 promoter methylation in all but one cases—and high TIL density were associated with improved survival at univariable and stage-inclusive multivariable analysis. However, only TILs retained prognostic power when clinical subgroups were added to the multivariable model. KRAS mutation and HER2 amplification were detected in 30% and 7% of cases, respectively, without prognostic implications.

Conclusions: In comparison with CrD-SBC, CD-SBC patients harbour MSI and high TILs more frequently and show better outcome. This seems mainly due to their higher TIL density, which at multivariable analysis showed an independent prognostic value. MSI/TIL status, *KRAS* mutations and *HER2* amplification might help in stratifying patients for targeted anti-cancer therapy.

Key Words: Inflammatory bowel disease; microsatellite instability; *MLH1* promoter methylation; tumour-infiltrating lymphocytes; survival

1. Introduction

Carcinomas of the small bowel [SBC] are relatively rare cancers; their incidence is, however, increasing.[1,2] Compared with colorectal cancer, SBC diagnosis remains a challenge despite significant progress in imaging and endoscopy, and SBC exhibit worse prognosis.[3] Although SBC are often sporadic, a number of predisposing conditions including hereditary syndromes—familial adenomatous polyposis, Lynch syndrome, and Peutz-Jeghers syndrome—and immune-mediated intestinal disorders—coeliac disease [CD] and Crohn's disease [CrD] —have been identified.[4]

In CD, which is a chronic enteropathy induced in genetically susceptible individuals by gluten ingestion,[5] SBC risk has been estimated to be 14-fold higher than that of the general population.[6] In CrD, which is a relapsing transmural inflammatory bowel disease [IBD] resulting from an inappropriate immune response to commensal microrganisms, SBC risk is 33-fold higher.[7] However, information concerning SBC associated with the two aforementioned disorders, especially in the case of CD, is limited to a few small series or case reports,[8–12] and both the histological features and the molecular landscape are largely unknown. SBC associated with CD [CD-SBC] were reported to harbour a high incidence of mismatch repair [MMR] deficiency[9,10] and to follow the CpG island methylator-microsatellite instability [MSI] pathway.[12] In contrast, MSI has rarely been observed in SBC associated with CrD [CrD-SBC].[11]

On this basis we aimed to comparatively assess survival, together with histopathological, molecular, and prognostic features, of SBC in a relatively large multicentre collection of patients with sporadic SBC and SBC associated with either CD or CrD.

2. Methods

2.1. Patients

This retrospective study included 51 patients with pathologically confirmed primary non-familial, non-ampullary SBC associated with either CD [*n* = 26] or CrD [*n* = 25], who had surgical resection and complete survival data from 20 tertiary referral Italian coeliac and/or IBD centres participating in the Small Bowel Cancer Italian Consortium, i.e. Pavia [Coordinating Centre], Aviano, Bologna, Brescia, Cagliari, Firenze, Genova, L'Aquila, Modena, Milano-Ca' Granda, Milano-Sacco, Napoli, Padova, Palermo-Cervello, Palermo-Giaccone, Roma-S. Filippo Neri, Roma-Tor Vergata, Roma-Umberto I, Roma S. Eugenio, Torino.

Demographic and clinical data of CD and CrD patients are reported in Table 1 and Table 2, respectively. CD diagnosis was based on serum IgA anti-endomysial and anti-tissue transglutaminase antibody positivity associated with typical duodenal histopathological lesions [Marsh type 3].[5] In five cases [19%], CD diagnosis was concomitant with that of SBC. The remaining 21 cases were under a strict gluten free-diet at SBC diagnosis except for three patients with poor compliance. Only one of 26 CD patients was diagnosed as refractory CD type 1.

CrD diagnosis was ascertained according to the usual clinical criteria,[13] and the site and extent of the disease were confirmed by endoscopy, histology, and imaging. In three patients [12%], CrD diagnosis was simultaneous with that of SBC. Sixteen out of 25 CrD patients [64%] had fibro-stenosing disease, five [20%] were predominantly inflammatory, and four [16%] had penetrating behaviour at SBC diagnosis. Only four CrD patients were under immunomodulatory therapy.

Twenty-five patients with sporadic SBC [spo-SBC], i.e. without a concomitant intestinal immune-mediated disorder, were included as a control group. In spo-SBC, CD was excluded [serum IgA anti-endomysial and anti-tissue transglutaminase antibody negativity with normal serum total IgA], and CrD was ruled out by the absence of classic clinical and biochemical features. Re-examination of the sporadic surgical specimens further confirmed the lack of histological lesions indicative of either CD or CrD. The main exclusion criteria for all SBC subgroups were Lynch syndrome, Peutz-Jeghers syndrome, familial adenomatous polyposis, and juvenile polyposis. These hereditary syndromes were excluded in all cases by negative family and personal history, colonoscopy, and histological evaluation of the surgical specimens, and, in MSI SBC cases, by the presence of MLH1 promoter methylation as well.

This study was approved by the Ethics Committee of the San Matteo Hospital Foundation of Pavia.

2.2. Histology and immunohistochemistry

Tissue samples were fixed in 4% formaldehyde and processed in paraffin wax; 4-µm-thick sections were stained with haematoxylin and eosin [H&E] for morphological evaluation. All cases were investigated for histological type and grade, according to the World Health Organization [WHO] classification,[14] lymphovascular invasion, and all the parameters required to fulfil the criteria of the American Joint Committee on Cancer [AJCC] staging system.[15] For immunohistochemistry, 4-µm-thick sections were incubated at 4°C for 18–20 h with the following antibodies: CD3 [polyclonal, Dako, Carpinteria, CA], CD8 [polyclonal, Dako], MLH1 [monoclonal, clone ES05, Dako], MSH2 [monoclonal, clone FE11, Dako], MSH6 [monoclonal, clone EP49, Dako], PMS2 [monoclonal, clone EP51, Dako], p53 [monoclonal, clone DO7, Dako], HER2 [monoclonal, Leica Biosystem, Newcastle, UK], and PD-L1 [monoclonal, clone E1L3N, Cell Signaling Technology, Danvers, MA]. Immunoreactions were developed using 0.03% 3,3' diaminobenzidine tetrahydrochloride and sections were then counterstained with Harris' haematoxylin. Tumour-infiltrating lymphocytes [TILs] were stained using CD3 and CD8 antibodies and counted in 10 consecutive high-power fields [HPFs], selecting areas containing the maximum number of neoplastic cells with minimal reactive stroma and necrosis and evaluating only lymphocytes in direct contact with tumour cells, i.e. 'intraepithelial' TILs. A tumour was also classified as having 'high TIL density' when the mean number of TILs per HPF was greater than 15 for CD3 or greater than 9.5 for CD8.[16] Immunostaining of MMR proteins [MLH1, MSH2, MSH6, and PMS2] in tumour cells was evaluated as positive [retained expression] or negative [absent expression]; only tumours showing absence of nuclear staining of all neoplastic cells in the presence of an internal positive control [intra-tumour stromal and inflammatory cells or non-tumour mucosa] were considered negative. Carcinomas were considered p53-positive when more than 50% of tumour cells showed strong nuclear p53 immunoreactivity, in line with previous studies.[17] Scoring of HER2 immunostaining was conducted according to published criteria for gastric cancer.[18] A central pathology review was performed by two surgical pathologists specialised in gastrointestinal pathology [AV and ES].

2.3. Microsatellite instability analysis

Tumour DNA was obtained from formalin-fixed and paraffin-embedded tissues using three representative 8-µm-thick sections of tumour samples. DNA was extracted after manual microdissection using a QIAamp DNA FFPE tissue kit according to the manufacturer's protocol [Qiagen, Hilden, Germany]. MSI analysis was performed using a pentaplex panel of monomorphic mononucleotide repeats [BAT25, BAT26, NR-21, NR-22, and NR-24].[19]

2.4. MLH1 methylation analysis

MLH1 methylation status was examined by pyrosequencing in SBC cases exhibiting loss of MLH1 immunohistochemical expression. Bisulphite modification of genomic DNA [300 ng] was performed with the EZ DNA Methylation-Gold™ Kit [Zymo Research, Irvine, CA] according to the manufacturer's recommendations. A region of 84 nucleotides inside the Deng C-region of *MLH1* promoter[20] was analysed by pyrosequencing according to the protocol previously reported.[21] Analytical sensitivity and linearity of the assay were assessed using a serial dilution of fully methylated DNA and unmethylated DNA [Chemicon International Inc., Billerica, MA]. A sample was classified as methylated when the mean of all the five cytosines was greater than 8%. Mono- or bi-allelic methylations of the *MLH1* promoter were also validated by MS-MLPA using the SALSA MS-MLPA ME011 MMR kit [MRC-Holland, Amsterdam, The Netherlands]. MS-MLPA analysis was performed according to the manufacturer's recommendations and a methylation ratio was calculated using Coffalyser V7 software [MRC Holland].

2.5. Gene mutation analysis

Mutation analysis of *KRAS, NRAS, BRAF*, and *PIK3CA* genes was performed using the Sequenom MassARRAY system [Diatech Pharmacogenetics, Jesi, Italy], based on matrix-assisted laser

Table 1. Clinical and pathological features of 26 patients affected by coeliac disease-associated small bowel carcinoma.

Pt	Sex	Age at CD dgn [yrs]	Age at SBC dgn [yrs]	CD duration at SBC dgn [mo]	Dietary status at SBC dgn	SBC location	Histological type	SBC grade	SBC stage	Follow-up [mo]	Outcome
1	M	56	60	48	Strict GFD	Jejunum	ADCA/usual	G2	IIB	107	Alive
2	F	66	68	22	Strict GFD	Jejunum	ADCA/usual	G3	I	163	Alive
3	F	38	39	12	Strict GFD [RCD type1]	Jejunum	ADCA/usual	G2	IIA	29	Alive
4	F	47	48	12	Strict GFD	Jejunum	ADCA/usual	G2	IIA	41	Alive
5	F	63	64	12	Strict GFD	Jejunum	ADCA/usual	G2	IIA	41	Alive
6	F	42	42	0	No GFD	Duodenum	ADCA/signet ring cell	G3	IIIB	20	Alive
7	F	68	72	43	Strict GFD	Jejunum	ADCA/usual	G2	IIA	123	Alive
8	F	34	59	300	Poor compliance to GFD	Jejunum	ADCA/usual	G2	I	208	Alive
9	F	46	46	5	Strict GFD	Jejunum	ADCA/usual	G2	IV	13	Dead
10	F	38	38	1	Strict GFD	Jejunum	ADCA/usual	G3	IIIB	27	Alive
11	F	7	28	252	Strict GFD	Jejunum	ADCA/usual	G2	IIA	167	Alive
12	F	42	42	0	No GFD	Ileum	ADCA/usual	G2	IIA	75	Alive
13	F	41	43	24	Strict GFD	Ileum	ADCA/usual	G2	I	210	Alive
14	F	53	55	31	Poor compliance to GFD	Jejunum	ADCA/usual	G2	IIB	31	Alive
15	M	51	51	0	No GFD	Ileum	ADCA/usual	G3	IIA	113	Alive
16	F	61	66	60	Strict GFD	Jejunum	Medullary	G3	IIIA	74	Alive
17	M	30	30	0	No GFD	Duodenum	ADCA/usual	G2	IIIB	21	Alive
18	M	43	54	132	Strict GFD	Jejunum	ADCA/usual	G2	IIA	22	Alive
19	F	56	67	132	Poor compliance to GFD	Jejunum	Medullary	G3	IIA	168	Alive
20	M	53	53	0	No GFD	Duodenum	ADCA/mucinous	G3	IIIB	34	Alive
21	M	79	80	12	Strict GFD	Jejunum	Medullary	G3	IIB	71	Alive
22	M	28	40	144	Strict GFD	Duodenum	ADCA/usual	G2	IIIA	31	Dead
23	M	31	32	7	Strict GFD	Duodenum	Medullary	G3	IIA	48	Alive
24	M	66	72	72	Strict GFD	Duodenum	ADCA/signet ring cell	G3	NA	12	Dead
25	M	65	66	12	Strict GFD	Jejunum	ADCA/usual	G2	IIB	54	Alive
26	F	52	54	24	Strict GFD	Jejunum	ADCA/usual	G3	IV	17	Dead

ADCA, adenocarcinoma; CD, coeliac disease; dgn, diagnosis; F, female; GFD, gluten-free diet; M, male; mo, months; NA, not applicable; Pt, patient; RCD, refractory coeliac disease; SBC, small bowel carcinoma; yrs, years.

Table 2. Clinical and pathological features of 25 patients affected by Crohn's disease-associated small bowel carcinoma

Pt	Sex	Age at CrD dgn [yrs]	Age at SBC dgn [yrs]	CrD duration at SBC dgn [mo]	CrD phenotype[a]	Previous therapy for CrD	SBC location	Histological type	SBC grade	SBC stage	Follow-up [mo]	Outcome
1	M	84	84	0	A3L1B3	No	Ileum	ADCA/signet ring cell	G3	IIIA	1	Dead
2	M	69	69	1	A3L1B2	5-ASA, CS	Ileum	ADCA/signet ring cell	G3	IIIB	3	Dead
3	M	43	59	312	A3L1B2	5-ASA, CS	Ileum	ADCA/usual	G2	IIIA	33	Dead
4	F	52	55	52	A3L1B2	AZA, Infliximab,5-ASA, CS	Ileum	ADCA/usual	G3	IIIB	7	Dead
5	M	27	33	72	A2L1B1	Infliximab, 5-ASA, CS	Ileum	ADCA/usual	G3	IV	21	Dead
6	F	40	44	48	A2L3B2	5-ASA, CS	Ileum	ADCA/usual	G2	IV	3	Dead
7	M	55	73	216	A3L1B3	5-ASA, CS, AB	Ileum	ADCA/usual	G3	IIIB	37	Dead
8	F	24	54	360	A2L1 B3	5-ASA, CS, AB	Ileum	ADCA/usual	G2	IIIA	61	Alive
9	M	27	68	492	A2L1 B1	5-ASA, CS	Ileum	ADCA/usual	G3	IV	2	Dead
10	M	37	54	204	A2L1 B3	5-ASA, CS, AB	Ileum	ADCA/signet ring cell	G3	IIIA	5	Dead
11	M	69	69	0	A3L1B2	No	Ileum	ADCA/usual	G2	IIA	155	Alive
12	F	58	59	1	A3L1B2	5-ASA, CS	Ileum	ADCA/signet ring cell	G3	IIA	30	Dead
13	M	27	50	276	A2L4B2	5-ASA, CS	Duodenum	ADCA/signet ring cell	G3	IIB	23	Dead
14	M	29	60	372	A2L1B2	5-ASA	Ileum	ADCA/usual	G2	IV	9	Dead
15	F	52	62	120	A3L1B1	AZA, 5-ASA, CS	Ileum	ADCA/usual	G3	IIB	72	Dead
16	M	56	56	3	A3L1B1	5-ASA, CS	Ileum	ADCA/usual	G3	IV	16	Alive
17	M	77	77	0	A3L1B2	No	Ileum	ADCA/usual	G2	IIA	117	Dead
18	M	50	77	324	A3L1B2	5-ASA, CS	Ileum	ADCA/usual	G2	IIIA	0	Dead
19	M	51	52	11	A3L1B2	AZA, 5-ASA, CS	Ileum	ADCA/usual	G2	IIA	12	Alive
20	F	52	77	300	A3L1B2	AZA, 5-ASA, CS	Ileum	ADCA/usual	G2	I	0	Dead
21	F	22	44	264	A2L3B2	5-ASA, CS	Ileum	ADCA/usual	G2	I	204	Alive
22	F	58	58	3	A3L1B2	5-ASA, CS	Ileum	ADCA/usual	G2	IIA	81	Alive
23	M	39	63	252	A2L1B2	AZA, 5-ASA, CS	Jejunum	ADCA/usual	G2	IIA	24	Alive
24	M	50	63	156	A3L1B2	5-ASA, CS	Ileum	Medullary	G3	IIIB	20	Alive
25	F	33	57	288	A2L3B3	AZA, Infliximab, 5-ASA, CS, AB	Ileum	ADCA/usual	G2	I	73	Alive

[a]Montreal classification.

AB: antibiotics; ADCA, adenocarcinoma; 5-ASA: 5-aminosacylic acid; AZA, azathioprine; CrD, Crohn's disease; CS: corticosteroids; dgn, diagnosis; F, female; M, male; mo, months; NA, not applicable; Pt, patient; SBC, small bowel carcinoma; yrs, years.

desorption/ionisation time-of-flight mass spectrometry, together with the Myriapod Colon Status Kit [Diatech Pharmacogenetics]. This kit includes a series of multiplexed assays designed to interrogate a total of 153 non-synonymous hotspot mutations in the four genes. DNA amplification was done in a 5-µl reaction mixture containing 10-20 ng of tumour DNA. Poly merase chain reaction [PCR], shrimp alkaline phosphatase reaction and single base pair extension steps were carried out following the protocols provided by Diatech Pharmacogenetics. Completed genotyping reactions were spotted in nanolitre volumes onto a matrix-arrayed silicon SpectroCHIP with 96 elements using the MassARRAY Nanodispenser [Diatech Pharmacogenetics]. SpectroCHIP was analysed using the Sequenom MassARRAYs Analyzer 4 spectrometer and the spectra were processed by the MassARRAY Typer Analyzer 4.0 software [Diatech Pharmacogenetics]. All automated system mutation calls were confirmed by manual review of the spectra. We investigated TP53 mutations at exons 5–8 which correspond to the core domain involved in protein-protein interaction [tetramerisation] and in binding to DNA, and represent the region where the vast majority of TP53 mutations are detected. Briefly, exons 5–8 were amplified by PCR using sets of primers reported in IARC TP53 database tools [http://p53.iarc.fr/ProtocolsAndTools.aspx]. In detail, we used primer pairs that amplify small [poor DNA quality] fragments [IARC code: P-312 and P-271 for exon 5; P-239 and P-240 for exon 6; P-237 and P-238 for exon 7; P-316 and P-319 for exon 8]. PCR products were subjected to automated sequencing by ABI PRISM 310 [Applied Biosystems, Foster City, CA]. All mutated cases were confirmed at least twice, starting from independent PCR reactions. In each case, the detected mutation was confirmed in the sequence as sense and antisense strands. All molecular analyses were performed at a central laboratory [Insubria University Molecular Pathology Laboratory, Varese, Italy].

2.6. Fluorescent in situ hybridisation

HER2 amplification by fluorescent in situ hybridisation [FISH] was performed with a PathVysion HER-2 DNA probe Kit [Abbott Laboratories, Des Plaines, IL] in SBC cases showing equivocal [weak-to-moderate, 2+] or positive [intense, 3+], circumferential, basolateral or lateral HER2 immunoreactivity in at least 10% of tumour cells. The *HER2* amplification scoring was performed by counting *HER2* and *CEN17* signals from 40 to 100 nuclei/tissue sample. Non-tumour [normal small bowel] mucosa was used as internal negative control. Samples with a *HER2*/CEN17 ratio ≥ 2.0, or ratio < 2 with > 10% of neoplastic cells showing *HER2* ≥ 6 signals/nuclei, or presence of a pattern of *HER2* signals in clusters, were considered amplified.

2.7. Statistical analysis

This is a retrospective, longitudinal study. The follow-up extended from the date of surgery to the date of death or last follow-up. Descriptive statistics were computed as median and 25th–75th percentiles for continuous variables and as counts and percentages for categorical variables. Median follow-up and its interquartile range [25th–75th] were computed by means of the inverse Kaplan-Meier method. The Kruskal-Wallis test and Fisher's exact test were used to compare continuous and categorical variables, respectively, between types of neoplasms. Cumulative survival was plotted according to the Kaplan-Meier method. Death rates per 100 person-years (95% confidence interval [CI]) were also computed as summary measures. The association between candidate prognostic factors and tumour death was estimated by means of Cox regression. Multivariable models including non-collinear variables with $P < 0.1$ at univariable analysis were fitted. Model discrimination was assessed with the Harrell's c statistic [the closer to 1, the better] and calibration with the shrinkage coefficient [the closer to 1, the better]. Hazard ratios [HR] and their 95% confidence intervals [95% CI] were computed. The proportional hazard assumption was tested, based on Schoenfeld residuals. A two-sided P-value < 0.05 was considered statistically significant. For post hoc comparisons, Bonferroni correction applies. Stata 14.1 [StataCorp., College Station, TX] was used for computation.

3. Results

In total, we analysed a cohort of 76 patients, 26 with CD-SBC, 25 with CrD-SBC, and 25 with spo-SBC [Table 3]. Median age at the time of cancer diagnosis among the CD-SBC subgroup was significantly lower than among the spo-SBC patients, and median duration of intestinal disease at SBC diagnosis was significantly lower in CD-SBC in comparison with CrD-SBC. A higher proportion of females was found in CD-SBC in comparison with the other two subgroups, although the difference did not reach statistical significance. As expected, the most common small bowel subsite was the ileum for CrD-SBC, whereas in both CD-SBC and spo-SBC it was the jejunum. No significant difference was observed among the three subgroups in terms of stage and presence of local lymph nodes or distant metastases. SBC diagnosis was suspected or obtained preoperatively in all CD-SBC and spo-SBC patients, in contrast to only seven of 25 CrD-SBC cases [28%]. Nevertheless, the proportion of SBC patients in stage I-II with more than seven lymph nodes assessed was comparable between the three subgroups.

Patients were followed-up for a median of 71 months [25th–75th IQR: range 30–123]. Overall survival curves in Figure 1A show the prognostic effect of the clinical subgroup at univariable analysis. A significantly worse overall survival was observed in CrD-SBC in comparison with CD-SBC [HR 6.29, 95% CI 2.10–18.85, $p = 0.003$] but not to spo-SBC [HR 1.75, 95% CI, 0.68–4.54, $p = 0.460$]. Spo-SBC showed a trend for worse overall survival in comparison with CD-SBC, although the difference did not reach statistical significance [HR 3.59, 95% CI 0.88–14.57, $p = 0.087$] [Figure 1A]. Median survival was 28 months for CrD-SBC and 72 months for spo-SBC, but it was not evaluable for CD-SBC as the cumulative survival was > 50%. Five-year overall survival rates were 83% [95% CI, 61–93], 38% [95% CI, 18–58], and 54% [95% CI, 29–73] for CD-SBC, CrD-SBC, and spo-SBC, respectively. No survival difference was found between CrD patients under immunomodulatory therapy or untreated patients [$p = 0.581$]. Stage I-II patients showed a significantly better overall survival in comparison with stage III-IV cases at univariable analysis [Supplementary Figure 1, available as Supplementary data at *ECCO-JCC* online]. Stage-, age,- and sex-adjusted multivariable analysis confirmed the significant prognostic power of both the clinical subgroup and the stage [Table 4, model 1]. Cancer-specific survival was significantly worse in CrD-SBC in comparison with CD-SBC [HR 5.65, 95% CI 1.86–17.18, $p = 0.007$] but not to spo-SBC [HR 1.56, 95% CI 0.59–4.17, $p = 0.829$]. Spo-SBC patients showed a trend towards a worse cancer-specific survival in comparison with CD-SBC cases, although the difference did not reach statistical significance [HR 3.64, 95% CI 1.16–11.46, $p = 0.082$, after Bonferroni correction].

Tumour WHO histotype and grade, as well as lymphovascular invasion, showed neither significant difference among the three

Table 3. Demographic and clinico-pathological features of all 76 small bowel carcinoma patients

	CD-SBC	CrD-SBC	Spo-SBC	Overall *p*-value	*p*-Value among subgroups*
Number	26	25	25		
Age at SBC diagnosis, median [25th–75th IQR], yrs	53 [42–66]	59 [54–69]	65 [62–72]	0.004	CD vs CrD: 0.102 CD vs Spo: 0.005 CrD vs Spo :0.491
Duration of intestinal disorder at SBC diagnosis, median [25th–75th IQR], mo	17 [5–60]	156 [3–288]	NA	0.024	
Female, *N* [%]	16 [62%]	9 [36%]	8 [32%]	0.074	
Site, *N* [%]				< 0.001	
Duodenum	6 [23%]	1 [4%]	2 [8%]		CD vs CrD: < 0.001
Jejunum	17 [65%]	1 [4%]	18 [72%]		CD vs Spo: 0.367
Ileum	3 [12%]	23 [92%]	5 [20%]		CrD vs Spo: < 0.001
Stage, *N* [%]				0.588	
I	3 [11%]	3 [12%]	1 [4%]		
II	14 [54%]	9 [36%]	13 [72%]		
III	6 [23%]	8 [32%]	9 [36%]		
IV	2 [8%]	5 [20%]	2 [8%]		
NA[a]	1 [4%]	0	0		
Local lymph node metastases, *N* [%]	8 [32%]	13 [52%]	11 [46%]	0.358	
Distant metastases, *N* [%]	2 [8%]	5 [20%]	2 [8%]	0.446	
Histological type, *N* [%]				0.343	
Medullary CA	4 [15%]	1 [4%]	1 [4%]		
ADCA/usual	19 [73%]	19 [76%]	19 [76%]		
ADCA/mucinous	1 [4%]	0	3 [12%]		
ADCA/signet ring cell	2 [8%]	5 [20%]	2 [8%]		
Histological grade, *N* [%]				0.197	
Low grade [G1-G2]	15 [58%]	13 [52%]	19 [76%]		
High grade [G3-G4]	11 [42%]	12 [48%]	6 [24%]		
Lymphovascular invasion, *N* [%]	17 [65%]	20 [80%]	13 [52%]	0.111	
CD3+ TIL/HPF, median [25th–75th IQR]	23.7 [7.9–65.8]	3.3 [1.7–7.0]	5.5 [1.4–19.9]	< 0.001	CD vs CrD: < 0.001 CD vs Spo: 0.002 CrD vs Spo: 0.528
CD8+ TIL /HPF, median [25th–75th IQR]	18.6 [5.7–43.1]	1.0 [0.5–6.0]	4.0 [1.7–22.8]	< 0.001	CD vs CrD: < 0.001 CD vs Spo: 0.020 CrD vs Spo: 0.053

[a]In one CD-SBC patient [case 24 in Table 1], who presented with a locally advanced cancer and died 12 months after surgery, incomplete data regarding lymph node status precluded assigning an AJCC stage.

ADCA, adenocarcinoma; CA, carcinoma; CD-SBC, coeliac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; HPF, high-power field; IQR, interquartile range; NA, not applicable; Spo-SBC, sporadic small bowel carcinoma; TIL, tumour-infiltrating lymphocyte.

*Significant if $p < 0.017$ according to Bonferroni correction.

subgroups [Table 3] nor prognostic value. The most common histological type in all cases was usual type, tubular adenocarcinoma. However, the signet ring cell type was more prevalent in CrD-SBC, and the medullary type was more common in CD-SBC. CD-SBC cases were more infiltrated by $CD3^+$ and $CD8^+$ lymphocytes in comparison with CrD-SBC or spo-SBC cases [Supplementary Figure 2A-F, available as Supplementary data at *ECCO-JCC* online]. The median number of $CD3^+$ and $CD8^+$ TILs was significantly higher in CD-SBC than in either CrD-SBC or spo-SBC [Table 3]. A strong correlation between $CD3^+$ and $CD8^+$ TILs [Spearman correlation coefficient $R = 0.91$, $P < 0.001$] was found. SBC patients having a number of $CD3^+$ TILs > 15/HPF showed a better overall survival in comparison with those with ≤ 15 $CD3^+$ TIL/HPF [Figure 1B and Table 5]. SBC cases having > 9.5 $CD8^+$ TIL/HPF showed a better overall survival in comparison with those with ≤ 9.5 $CD8^+$ TIL/HPF [HR 0.08, 95% CI 0.02–0.35, $p = 0.001$]. At multivariable analysis, stage, $CD3^+$ TIL and the clinical subgroup were independent prognostic factors [Table 4, model 2].

MSI was found in 25 out of 76 cases of SBC [33%], and no discordance was observed between immunohistochemical and molecular analyses. A significantly higher MSI frequency was found in CD-SBC compared with either CrD-SBC or spo-SBC [Table 5]. All MSI tumours showed a loss of both MLH1 [Supplementary Figure 2G-I] and PMS2 immunohistochemical expression while retaining MSH2 and MSH6 expression. *MLH1* promoter methylation was detected in all but one MSI cases. The patient with an MSI SBC lacking the *MLH1* promoter methylation was affected by CrD and had no history of familial cancer. Nineteen of 25 MSI cases [76%] showed > 15 TIL/HPF in contrast to nine of 51 non-MSI tumours [18%, $p < 0.001$]. At univariable analysis, MSI tumours showed a better overall survival in comparison with non-MSI tumours [Figure 2A and Table 5]. Moreover, among CD-SBC patients, MSI was able to separate two subgroups with a different stage [18% in stage III-IV for MSI in comparison with 75% of non-MSI, $p = 0.005$] and different overall survival [Figure 2B-D]. However, in a multivariable analysis inclusive of age, sex, stage, and clinical subgroup, MSI lost significant survival prognostic power

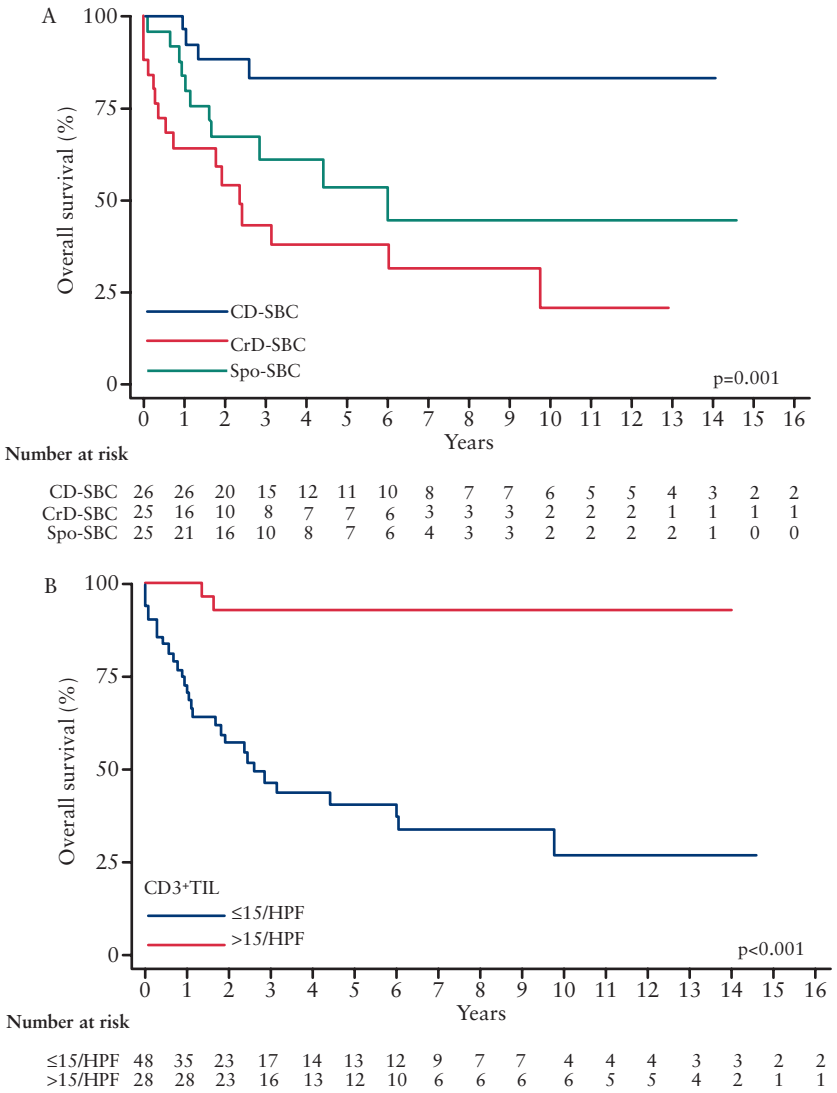

Figure 1. [A] Kaplan-Meier overall survival estimates for all patients by clinical subgroup. [B] Kaplan-Meier overall survival estimates for all patients by $CD3^+$ tumour-infiltrating lymphocytes [TIL] density; *p*-value is log-rank across subgroups. CD-SBC, coeliac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; HPF, high-power field; spo-SBC, sporadic small bowel carcinoma.

[Table 4, model 3], which was retained [HR 0.31, 95% CI 0.10–0.98, $p = 0.046$] when clinical subgroup was dropped from the model. It should be outlined that nine of 28 high-TIL SBC lacked MSI status, which may account for the higher prognostic power of TILs, as also suggested by the more favourable hazard ratios and *p*-values that high-TILs showed when compared with MSI status or MSI plus high-TIL cases at univariable overall survival analysis [Table 5].

No BRAF mutation was observed in any case of SBC [Table 6]. *KRAS, NRAS*, and *PIK3CA* mutations were detected in 23, three, and 10 out of 76 cases, respectively. As expected, *KRAS* and *NRAS* mutations were mutually exclusive, whereas six SBC cases [8%], including four spo-SBC, one CD-SBC, and one CrD-SBC, showed concurrent mutations in *PIK3CA* and *KRAS/NRAS* genes. Most of the *KRAS* mutations were in codons 12 and 13, and were pG12V or pG13D. The rare *NRAS* mutations were in codons 12 or 61, whereas *PIK3CA* mutations were equally distributed in codons 542, 545, 546, and 1047. *KRAS* mutations were more frequent in spo-SBC compared with CrD-SBC, but no difference was found between CD-SBC and the other two subgroups [Table 6].

P53 overexpression involving > 50% of tumour cells did not differ among the three subgroups [Table 6]. *TP53* mutations were found in 17 of 47 SBC cases investigated [six of 15 CD-SBC, three of 17 CrD-SBC, and eight of 15 spo-SBC]. *TP53* mutations were found in cases showing either p53 overexpression [16 cases] or complete lack of p53 immunostaining [one case]. *TP53* mutations and MSI proved to be mutually exclusive [$p = 0.038$]; however, despite their wild-type *TP53*, seven [28%] MSI tumours showed p53 overexpression at immunohistochemistry. Most [88%] *TP53* mutations were observed in exons 7 and 8, only two cases showed *TP53* mutations in exon 6, whereas no mutation was found in exon 5. In total, six of eight non-MSI CD-SBC cases harboured *TP53* mutations. Five [7%] SBC cases were $HER2^+$ at immunohistochemistry and revealed *HER2* gene amplification [Table 6 and Figure 3]. Histologically, all five *HER2* amplified cases were usual type, tubular adenocarcinomas. Three $HER2^+$ cases were jejunal tumours [two CD-SBC and one spo-SBC] and the remaining two were ileal CrD-SBC. Three *HER2* amplified SBC cases were *TP53* mutated, two were *KRAS* mutated and one had MSI. *KRAS, NRAS, PIK3CA*, and *TP53*

Table 4. Overall survival by multivariable Cox models of 75 patients with small bowel carcinoma.

	MODEL 1[a]		MODEL 2[b]		MODEL 3[c]	
	Hazard ratio [95% CI]	*p*-Value	Hazard ratio [95% CI]	*p*-Value	Hazard ratio [95% CI]	*p*-Value
Clinical groups		0.007		0.045		0.024
CD-SBC	1.00 [base]		1.00 [base]		1.00 [base]	
CrD-SBC	6.77 [1.84–24.94]	0.004*	4.36 [1.09–17.44]	0.037*	5.29 [1.34–20.90]	0.018*
Spo-SBC	2.92 [0.77–11.05]	0.115[d]*	2.06 [0.50–8.52]	0.316[e]*	2.39 [0.60–9.58]	0.218[f],*
Age at SBC diagnosis [as continuous variable]	1.01 [0.98–1.05]	0.399	1.02 [0.98–1.05]	0.337	1.01 [0.98-1-04]	0.529
Sex [male vs female]	1.13 [0.46–2.76]	0.786	0.66 [0.26–1.70]	0.392	0.46 [0.40–2.46]	0.981
SBC stage, III-IV vs I-II	7.84 [3.16–19.48]	< 0.001	9.08 [1.06–1.16]	< 0.001	8.38 [3.26–25.32]	< 0.001
$CD3^+$ TIL> 15/HPF vs ≤ 15/HPF	—	—	0.13 [0.03–0.58]	0.008	—	—
MSI	—	—	—	—	0.50 [0.15–1.67]	0.256

CD-SBC, coeliac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; CI, confidence interval; HPF, high-power field; MSI, microsatellite instability; Spo-SBC, sporadic small bowel carcinoma; TIL, tumour-infiltrating lymphocyte.

[a]Model 1: LR chi2[5] = 42.52, *p*-value < 0.001; Harrel's C = 0.82; shrinkage coefficient = 0.88.

[b]Model 2: LR chi2[6] = 53.29, *p*-value < 0.001; Harrel's C = 0.86; shrinkage coefficient = 0.89.

[c]Model 3: LR chi2[6]:43.94, *p*-value < 0.001; Harrel's C = 0.82; shrinkage coefficient = 0.86.

[d]Hazard ratio [95% CI]: 0.43 [0.19–0.96], *p*-value = 0.040 versus CrD-SBC.

[e]Hazard ratio [95% CI]: 0.47 [0.21–1.05], *p*-value = 0.067 versus CrD-SBC.

[f]Hazard ratio [95% CI]: 0.45 [0.20–1.10], *p*-value = 0.052 versus CrD-SBC.

*For post hoc comparisons, significance after Bonferroni correction set at 0.017.

Table 5. Microsatellite instability status and tumour-infiltrating lymphocytes: distribution among clinical subgroups and overall survival analysis on 76 small bowel carcinomas.

	Distribution among clinical subgroups			Total	Survival analysis	
	CD-SBC	CrD-SBC	Spo-SBC		HR [95% CI]	*p*-Value
MSI	17/26 [65%]*	4/25 [16%]	4/25 [16%]	25/76 [33%]	0.22 [0.08–0.64]	0.005
$CD3^+$ TIL > 15/HPF	16/26 [61%]**	4/25 [16%]	8/25 [32%]	28/76 [37%]	0.09 [0.02–0.36]	< 0.001
MSI plus $CD3^+$ TIL > 15/HPF	14/26 [54%]***	1/25 [4%]	4/25 [16%]	19/76 [25%]	0.26 [0.12–0.57]	< 0.001

CD-SBC, coeliac disease-associated small bowel carcinoma; CI, confidence interval; CrD-SBC, Crohn's disease-associated small bowel carcinoma; HPF, high-power field; HR, hazard ratio; MSI, microsatellite instability; Spo-SBC, sporadic small bowel carcinoma; TIL, tumour-infiltrating lymphocyte.

*p = 0.001 vs CrD-SBC or Spo-SBC; **p = 0.001 vs CrD-SBC; ***p < 0.001 vs CrD-SBC or Spo-SBC.

mutations, p53 overexpression, and *HER2* amplification showed no prognostic value.

PD-L1 staining was observed in membranes and/or cytoplasm of some stromal immune cells [mostly macrophages], usually restricted to the tumour invasive margin, in eight [six MSI and two non-MSI] of the 23 CD-SBC cases tested, in five of the 25 CrD-SBC cases, and in five of the 23 spo-SBC cases tested [Supplementary Figure 3, available as Supplementary data at *ECCO-JCC* online], without significant difference among the subgroups. However, only one SBC case, which was an MSI medullary cancer associated with CD, expressed PD-L1 in the tumour cell cytoplasm.

4. Discussion

This is the largest study of SBC in CD and the only one systematically comparing CD-SBC, CrD-SBC, and spo-SBC. We found that in patients undergoing surgery for SBC, the underlying immune-mediated disorder represents a stage-independent prognostic factor. Survival analysis showed a significantly better prognosis of CD-SBC in comparison with CrD-SBC. However, in agreement with Palaskak-Juif *et al.*,[22] we found no survival difference between CrD-SBC and spo-SBC. We observed a non-significant trend for improved overall survival of CD-SBC patients compared with an equally numerous spo-SBC subgroup. A significant survival improvement was observed by Potter *et al.*[9] by comparing a smaller CD-SBC group [n = 17] with a much higher 'control' group [n = 51] mostly, though not exclusively, composed of sporadic cases. Of note, the 5-year survival rate of our CD-SBC patients was as high as 83%, suggesting a relatively indolent behaviour of CD-SBC.

Among the molecular alterations with prognostic impact, MSI, which is a consequence of deficient MMR, was significantly more frequent among cases of CD-SBC in comparison with CrD-SBC and spo-SBC. The MSI prevalence we found in CD-SBC [17/26 cases, 65%] is in line with that of previous studies by Potter *et al.*[9] [8/11, 73%] and Diosdado *et al.*[10] [6/9, 67%]. With regards to CrD-SBC, the low percentage of MSI in our cases [16%] is in agreement with those of Rashid *et al.*[23] [14%] and Svrcek *et al.*[11] [3%].

We confirm, in a larger series, the favourable prognostic influence of MSI suggested by previous studies.[9,10] Due to its unequal distribution among clinical subgroups, MSI lost significant prognostic power in a subgroup-inclusive multivariable model. However high TIL density, despite its high correlation with MSI and prevalence in

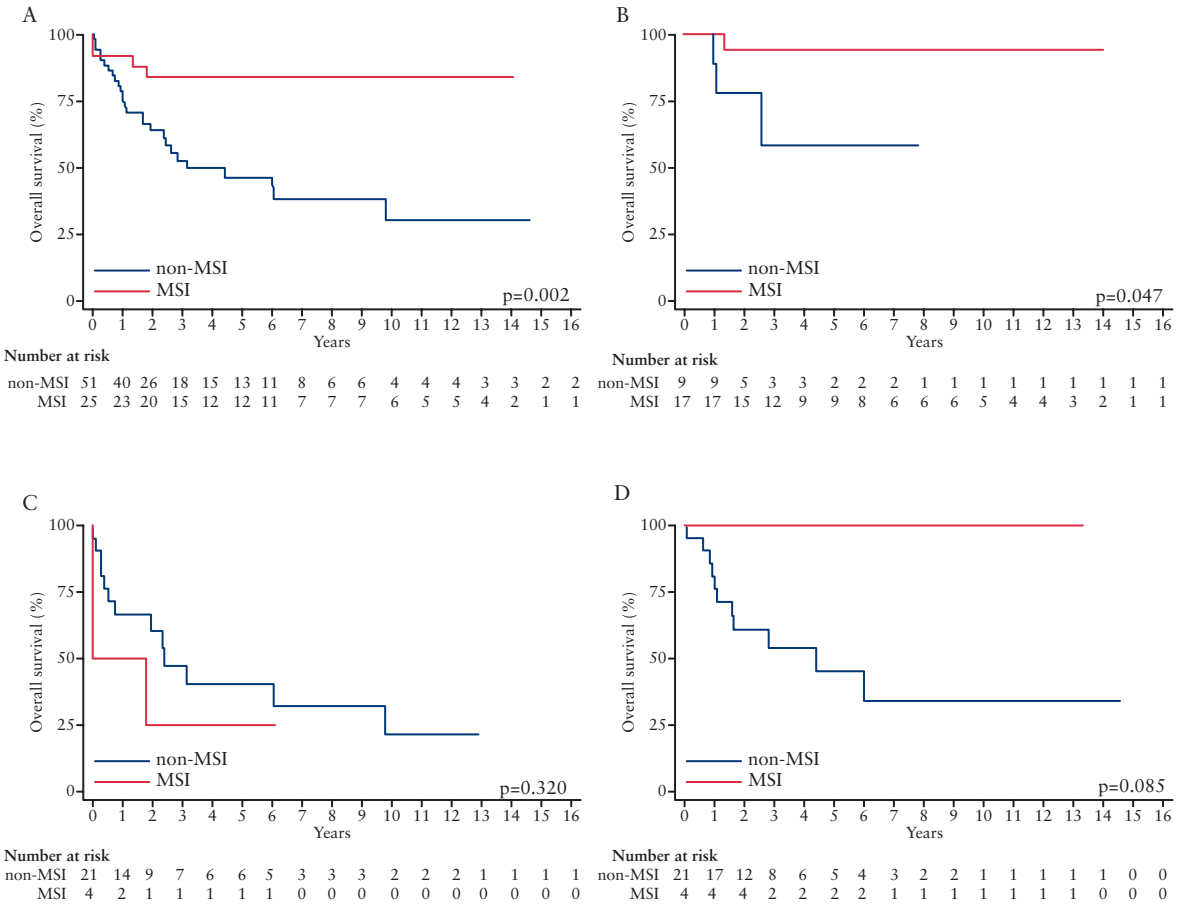

Figure 2. [A] Kaplan-Meier overall survival estimates for all patients. [B] Coeliac disease-associated small bowel carcinoma patients. [C] Crohn's disease-associated small bowel carcinoma patients. [D] Sporadic small bowel carcinoma patients, by microsatellite instability (MSI); p-value is log-rank across subgroups.

Table 6. Molecular alterations of the 76 small bowel carcinomas.

	CD	CrD	Spo	Overall *p*-value	*p*-Value among groups*
SBC	26	25	25		
KRAS mutation, *N* [%]	8 [31%]	3 [12%]	12 [48%]	0.021	CD vs CrD: 0.173 CD vs Spo: 0.258 CrD vs Spo: 0.012
NRAS mutation, *N* [%]	1 [4%]	1 [4%]	1 [4%]	1.000	
BRAF mutation, *N* [%]	0 [0%]	0 [0%]	0 [0%]	1.000	
PIK3CA mutation, *N* [%]	4 [15%]	2 [8%]	4 [16%]	0.759	
HER2 amplification, *N* [%]	2 [8%]	2 [8%]	1 [4%]	1.000	
p53 overexpression [> 50%], N [%]	12 [46%]	12 [48%]	13 [52%]	0.958	

CD, coeliac disease; CrD, Crohn's disease; Spo, sporadic; SBC: small bowel carcinoma.
*significant if $p < 0.017$ according to Bonferroni correction.

CD-SBC, retained significant power in such a model. This finding seems relevant as TILs are known from studies of other carcinomas to be the main effector of MSI-related prognosis improvement.[24,25] In addition, increased TILs can also be elicited by other agents besides MSI status, including oncogenic viruses and additional, presently undefined, causes.[26] In fact, in addition to a predominance of cases showing both high TIL density and MSI, nine SBC showing aetiologically unexplained high TILs in the absence of MSI were present in our series. This suggests that TIL assessment may prove to be an appropriate parameter for SBC prognostic evaluation. In this regard, the presence of PD-L1 reactive immune cells in a subset of SBC patients, and in particular in MSI-positive SBC cases, seems interesting and may deserve further investigation in the light of the potential therapeutic role of immune checkpoint inhibitors in SBC, as recently demonstrated for gastrointestinal MSI and/or γ-interferon-producing cancers.[27,28]

In spo-SBC we observed a higher prevalence [48%] of *KRAS* mutation than in CrD-SBC, which might be accounted for by the lower percentage we found in the latter subgroup [12%] in comparison with those reported by Rashid *et al.*[23] [43%] and Svrcek

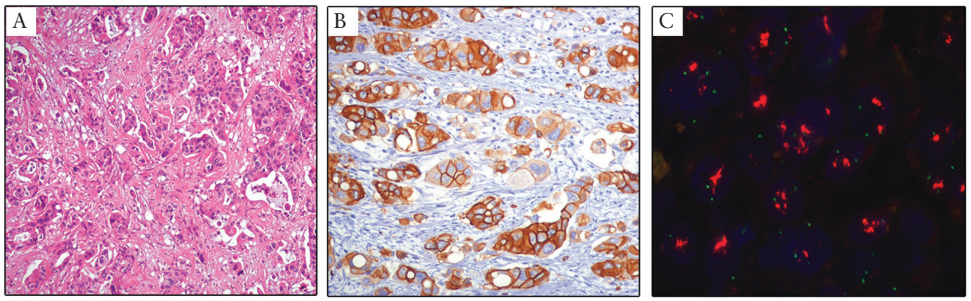

Figure 3. An example of HER2-positive SBC. Histology of a high-grade SBC [A, original magnification ×200], showing HER2-positive by immunohistochemistry [B, original magnification ×200] and revealing *HER2* gene amplification by FISH [C, original magnification ×200]. This SBC was found in the ileum of a patient with CrD. SBC, small bowel carcinoma; CrD, Crohn's disease.

et al.[11] [23%]. With regards to CD-SBC, our study is the first assessing the frequency of *KRAS* mutation which was found in 31% of cases. This finding, although by itself irrelevant for patient survival in our series, could be relevant in selecting patients in whom anti-EGFR targeted therapy could be beneficial.[29] *HER2* amplification, although restricted to only five cases, also seems worth consideration as a potential therapeutic target.[30]

Of note, there was no *BRAF* mutation in any case, including those harbouring MLH1 hypermethylation. *BRAF* mutations are reported to be absent or extremely rare in spo-SBC[31,32] and in CrD-SBC.[11] This finding seems to rule out *BRAF* mutation as a potential initiator of *MLH1* gene promoter methylation, which represents the almost exclusive cause of MSI in our non-familial SBC. This conclusion is in contrast with the role which *BRAF* mutation plays in the majority of MSI sporadic colorectal cancers.[33] Thus, the identification among SBC cases of a possible oncogene mutation activating a process of *MLH1* gene silencing remains an open issue. We first demonstrated the presence of *PIK3CA* mutation in a subset [16%] of CD-SBC cases. However, there was no significant difference in *PIK3CA* mutation rate among the three subgroups and no prognostic relevance. No significant difference was evident among the three subgroups for p53 changes regarding either protein overexpression or gene mutation. *TP53* mutations, as already reported in other cancers, proved mutually exclusive with MSI status.[34]

The role of chronic inflammation in the genesis of intestinal cancer is well known.[1] As both CD and CrD are T helper 1-mediated disorders, the prominent differences revealed in SBC arising in these two disorders are surprising. However, it should be recalled that the inflammatory process implicated in CD and CrD shows substantial differences in terms of types of inflammatory cells and cytokines involved.[35,36] Interestingly, all but one of our CD-SBC cases arose in non-refractory CD, a finding at variance with the origin of enteropathy-associated T-cell lymphoma.[37] However, the median age at CD diagnosis of our CD-SBC patients [49 years] was two decades higher than that reported for Italian adult cancer-free CD patients [28 years],[38] confirming that a delayed CD diagnosis may predispose to an increased risk of neoplastic complications in general and of SBC in particular. A delayed CD diagnosis may also contribute to the apparently low interval [17 months] between CD diagnosis and SBC detection.

We do acknowledge that the present study has some limitations, the most important being its inherently retrospective nature. However, the involvement of centres with long-term referral experience in the field, which were following agreed guidelines, was a guarantee of data quality.

In conclusion, although both CD-SBC and CrD-SBC arise from an inflammatory background, they differ substantially in survival, at least in part due to distinctive cellular/molecular changes with special reference to T-cell intra-tumour infiltration and MSI. The management of patients with SBC associated with immune-mediated intestinal disorders, including CD and CrD, should be integrated with the assessment of MSI/TIL status, *RAS* mutations, and *HER2* amplification, in order to stratify patients according to their prognosis and/or to identify possible candidates for targeted anti-cancer therapies.

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Conflict of Interest

No authors have any conflicts of interest to disclose.

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None.

Author Contributions

AV, ADS, DF, ES, and GRC designed the study. OL and ADS procured financial support. AV, ADS, DF, ES, FG, RM, MM, DT, RCe, PA, RR, and FS collected data. FG, RF, CM, MR, GN, PF, GSa, SA, OL, ACa, FT, UV, DS, GC, LE, SF, GL, RCar, ACi, GSo, AR, CC, FPD, MSa, VV, RCan, VC, LRB, LB, GM, AO, GSant, MCM, RD, PG, VP, GSandr, MSi, AMF, AGG, CP, LC, PU, AM, MA, PM, MP, ADS, and GRC provided material. All authors analysed and interpreted the data. CK did the statistical analyses. AV, ADS, DF, ES, FG, RF, and GRC wrote the manuscript. All authors approved the final report.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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