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PHD in EXPERIMENTAL AND TRANSLATIONAL MEDICINE

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CLINICO-PATHOLOGIC, HISTOPHENOTYPIC, MOLECULAR AND PROGNOSTIC CHARACTERIZATION OF SMALL BOWEL CARCINOMAS ASSOCIATED WITH CELIAC DISEASE OR CROHN'S DISEASE

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INDEX

1. INTRODUCTION

1.1 SMALL BOWEL CARCINOMA: EPIDEMIOLOGY

Small bowel carcinoma (SBC) is a remarkably rare neoplasm; its incidence is, however, increasing and is reported to be 7.3 cases per million people per year (1,2; **Figure 1**). Overall, only 2% of all malignant neoplasms of the gastrointestinal (GI) tract and 0.6% of all new cancer cases occur in the small intestine. SBCs represent the second most common neoplasm of the small intestine, accounting for about 40% of all cancers of this organ, whereas neuroendocrine neoplasms currently constitute the dominant histotype (2). The incidence rates vary with geographic regions, with higher rates in North America and Western Europe and lower rates in Asian countries (3). SBCs occur more frequently in men than in women and affect blacks more often than whites (3). The median age at SBC diagnosis ranges from 55 to 65 years (4,5).

More than half of all SBCs arise in the duodenum, even though this organ constitutes only 4% of the entire length of the small intestine (6). This finding suggests that bilio-pancreatic secretions may play a role in their etiopathogenesis. A smaller percentage of tumors arise in the jejunum, particularly in the first 30 cm distal to the ligament of Treitz. Ileal carcinomas are the least common. Occasionally, an SBC arises in a Meckel diverticulum.

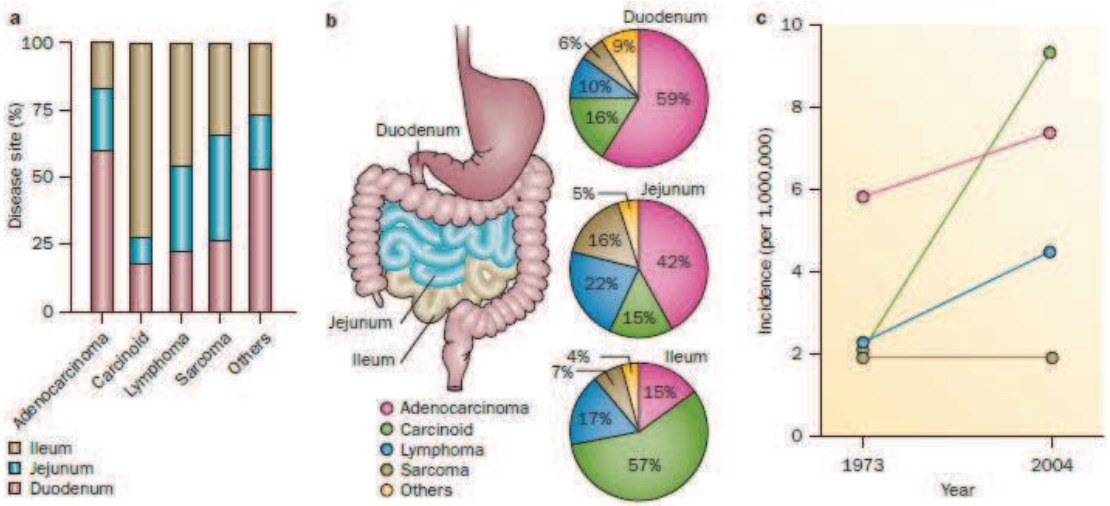

Figure 1. *Epidemiology of small bowel tumors from the NCDB (1985–2005) and US SEER (1973–2005) cohorts and Connecticut Tumor Registry 1980–2000.2,3,6* ***a*** *| Most adenocarcinomas and carcinoids are present in the duodenum and the ileum, respectively.* ***b*** *| The proportion of histological tumor subtypes found in the small bowel varies depending on the anatomic location of the small bowel.* ***c*** *| The incidence of small bowel tumors, especially carcinoids, adenocarcinomas and lymphomas has increased in the past few years. Abbreviations: NCDB, National Cancer Data Base; SEER, Surveillance, Epidemiology and End Results.* From Raghav, K. & Overman, M. J. *Nat. Rev. Clin. Oncol.* 10, 534–544 (2013).

Epithelial neoplasms develop far less frequently in the small intestine than in the colon, even though the small intestine has a larger epithelial surface area and a higher rate of cellular turnover. Several hypotheses have been proposed to explain the relative rarity of small bowel adenomas and carcinomas (7). First, the transit time of substances through the small intestine is relatively short compared with the colon, resulting in brief contact time between the mucosa

and the luminal contents. Second, unlike the colon, the small intestine does not contain a large quantity of bacteria, that are known to convert bile salts into potential carcinogens. Moreover, in the healthy small bowel, immune homeostasis prevails with a gut microbiota that is in balance with intestinal epithelial cells producing antimicrobial peptides and releasing immune modulatory cytokines that drive naïve dendritic cells to differentiate into tollerogenic dendritic cells. Third, the luminal contents are more liquid in the small bowel than in the colon. As a result, potentially carcinogenic luminal substances are diluted and the risk of mechanical trauma is reduced. Fourth, the small intestine is rich in lymphoid tissue, which provides a potentially high level of immune surveillance against neoplastic cells. Finally, the presence of Paneth cells, that are specialized secretory epithelial cells located in the small intestine on the bottom of the crypts of Lieberkühn, play a pivotal role in the maintenance of the intestinal barrier function by secreting large quantity of antimicrobial peptides such as defensins and lysozyme.

1.2 CLINICAL FEATURES OF SMALL BOWEL CARCINOMA

SBCs are generally asymptomatic in their early stages, but occult gastrointestinal bleeding may occur, leading to anaemia. Patients may have presenting symptoms of intestinal obstruction, intussusception, or perforation. Duodenal tumors may obstruct the bile duct and cause jaundice.

The development of newer techniques such as video capsule endoscopy, double-balloon enteroscopy, and computed tomographic (CT) enterography may explain the increasing incidence and allow a better localization of SBC, which may appear as an annular lesion, a discrete nodular mass, or an ulcerative lesion, often with corresponding narrowing of the lumen.

1.3 PATHOLOGIC AND MOLECULAR FEATURES OF SMALL BOWEL CARCINOMA

On macroscopy, SBC may have a flat, annular, stenotic, ulcerative, or polypoid gross appearance (**Figure 2**). Larger lesions tend to be found in the more distal portions of the small bowel, because lesions in that area often fail to produce symptoms until they are advanced. Direct spread may cause adherence to adjacent structures in the peritoneal cavity, usually a loop of small intestine. Lymphatic spread to regional lymph nodes is common.

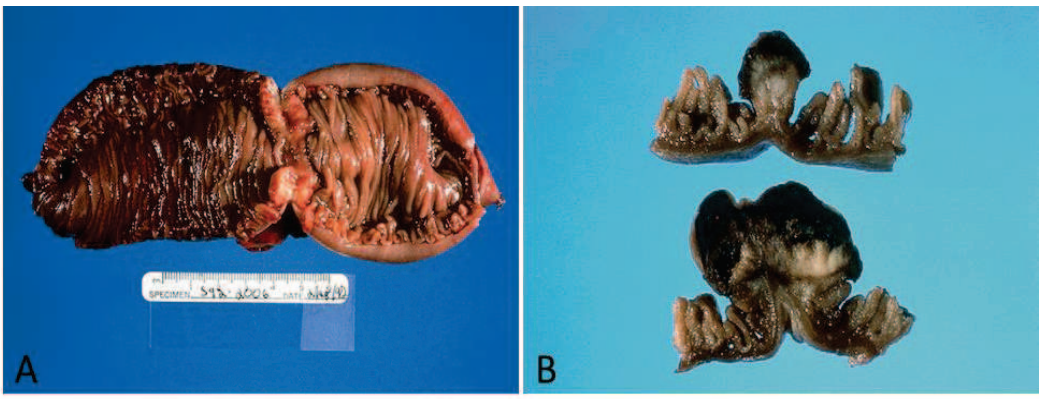

Figure 2. SBC *gross appearance. **A)** Jejunal resection specimen demonstrating a circumferential SBC constricting the small intestinal lumen. **B)** An SBC demonstrating a polypoid growth pattern. From: Odze and Goldblum "Surgical Pathology of the GI Tract, Liver, Biliary Tract and Pancreas" 3rd Edition*

The World Health Organization's (WHO) histologic classification of epithelial tumors of the small intestine is summarized in **Figure 3**. Tumors arising in the ampulla of Vater are separately classified from those arising elsewhere in the small intestine because significant treatment and prognostic differences exist for this group of tumors. Most SBCs are adenocarcinomas with variable degrees of mucin production. The grading system for SBC is identical to that used for the large bowel and the majority of SBCs are moderately differentiated (**Figure 4**). However, approximately 20% of SBCs are poorly differentiated. Rarer SBC histotypes include mucinous carcinomas, signet ring cell carcinomas, adenosquamous or squamous carcinomas and medullary carcinomas.

World Health Organization Classification of Epithelial Tumors of the Small Intestine

- Premalignant lesions
  - Adenoma
    - Tubular
    - Villous
    - Tubulovillous
  - Dysplasia (intraepithelial neoplasia), low grade
  - Dysplasia (intraepithelial neoplasia), high grade
  - Hamartomas
    - Juvenile polyp
    - Peutz-Jeghers polyp
- Carcinomas
  - Adenocarcinoma
    - Mucinous adenocarcinoma
    - Signet ring cell carcinoma
  - Adenosquamous carcinoma
  - Medullary carcinoma
  - Squamous cell carcinoma
  - Undifferentiated carcinoma
- Neuroendocrine neoplasms

Figure 3. *2010 WHO Classification of epithelial tumors of the Small Bowel.*

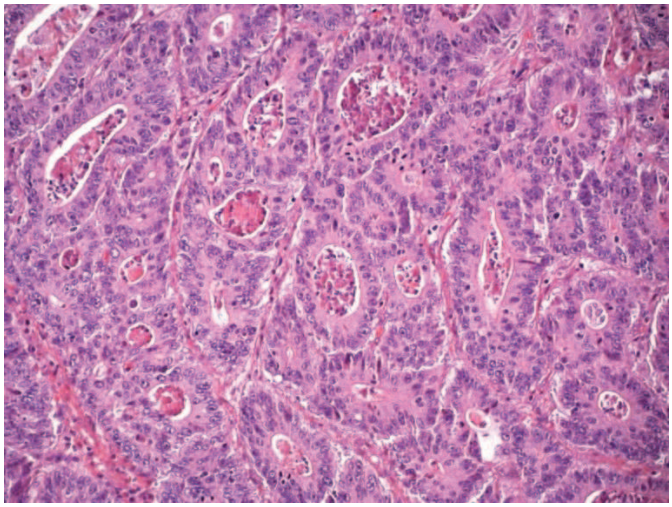

Figure 4. *Moderately differentiated adenocarcinoma of the small intestine.*

SBCs show more variable expression of cytokeratin 7 (CK7) than do colorectal carcinomas (CRC). In one study, diffuse positive CK7 immunoreactivity was

identified in 54% of nonampullary SBCs, and focal positivity was present in the remaining 46% of cases (8). In the same study, 67% of cases expressed CK20. Expression of MUC1, MUC2, and MUC5AC occurs in 53%, 57% and 40% of SBCs, respectively (9).

Most SBCs, like CRC, are believed to arise from an adenoma–carcinoma sequence in which genetic alterations progressively accumulate, leading to cancer development (**Figure 5**). Residual adenomatous tissue adjacent to foci of invasive SBC is seen in about 50% of duodenal SBCs. Histologically, small bowel adenomas are similar to those of the colon, but with a high propensity to be more villous or tubule-villous in architecture.

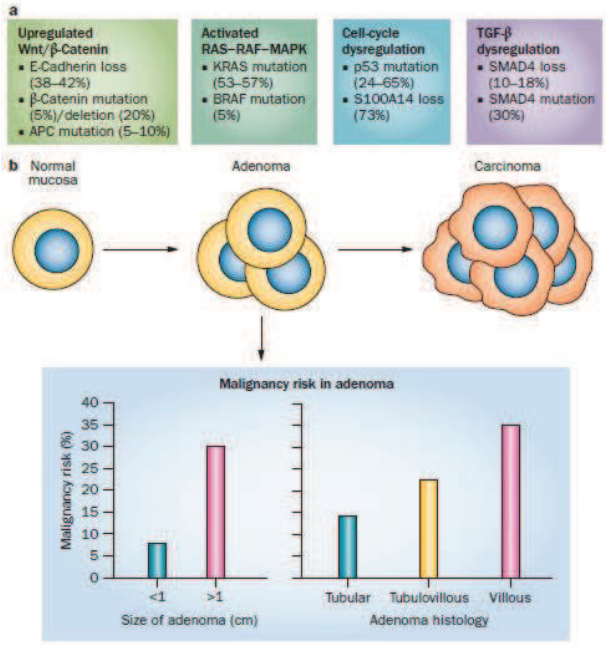

Figure 5. *The adenoma–carcinoma sequence in small bowel carcinoma.* ***a*** *| A number of molecular alterations are implicated in small bowel carcinogenesis.* ***b*** *| Risk of progression of adenoma to malignancy depends on the tumor size and histology. From Raghav, K. & Overman, M. J. Nat. Rev. Clin. Oncol. 10, 534–544 (2013).*

Several notable molecular similarities and differences between SBC and CRC exist. *KRAS* mutations, which occur commonly in CRC and are thought to represent an early change in the adenoma–carcinoma sequence in the colon, are also found in SBC, occurring in 14% to 83% of cases (10, 11). The reported wide variation in mutation frequency in different studies may be related to the fact that combined tumors from duodenal and other small-intestinal locations are often included in the analyses. In general, *KRAS* mutations are more frequent in duodenal neoplasms than in those that arise in other small bowel sites. *BRAF* V600E mutations are extremely rare in SBC (12). The most remarkable molecular finding in SBCs is that loss-of-function mutations in the *APC* tumor-suppressor gene, which is the most common event in the early development of CRC, do not have a pivotal role in the development of SBC (13). Although somatic mutations are found in 80% of sporadic CRC, only about 5% of sporadic SBCs (spo-SBCs) harbor this defect. Despite the absence of *APC* gene mutations, upregulation of the Wnt/β-catenin pathway as indicated by aberrant protein expression of β-catenin is still seen in 40–48% of SBCs (12, 13). Mutations in *CTNNB1* (the gene coding for β-catenin), have been reported in 14% of patients with SBC (six out of 42 tested cases) (14-16). Interestingly, the mutation spectrum is also different, with gain-of-function missense point mutations being common in CRC, but only large insertions or deletions reported

in SBC. p53 overexpression and *TP53* mutations are seen in 40% of SBCs, indicating the pivotal role of p53 in this disease (10).

Microsatellite instability (MSI) and loss of mismatch-repair (MMR) proteins are seen in 18–35% of SBCs compared to approximately 15% of CRCs (17, 18). Moreover, as with CRC, a SBC subset show methylation abnormalities. In a study of 37 SBCs, 24 tumors were found to show abnormal methylation patterns in at least one of the loci studied (17). Eleven tumors were classified as CpG island methylator phenotype-high (CIMP-H), and 13 were CIMP-low. As in the colon, CIMP-H status was strongly associated with the high-frequency MSI phenotype.

A recent genomic characterization of a large series of 7559 SBC patients found that the most common genetic alterations affected *TP53* (58.4%), *KRAS* (53.6%*),* *APC* (26.8%), *SMAD4* (17.4%), and *PIK3CA* (16.1%) and demonstrated distinct differences in comparison with either colorectal cancer or gastric carcinoma (19). In addition, genomic profiling identified potentially targetable genetic alterations in most SBC cases (91%). In this regard, Laforest *et al.* found *ERBB2* alterations in 12% of their SBCs, through mutations (7 cases) or amplifications (3 cases) (20).

1.4. TREATMENT AND PROGNOSIS OF SMALL BOWEL CARCINOMA

Surgery and systemic chemotherapy is the mainstay of therapy for locoregional and metastatic disease, respectively (3). Surgical resection with adequate lymph-node sampling is critical for long-term survival in resectable disease.

SBC prognosis is poor (21). In one study, the median overall survival time was 20.1 months, with a 5-year overall survival rate of 26% (22). The primary reason is that patients are often asymptomatic until late during the disease, and metastases are often present at the time of diagnosis. Tumor stage is the single most important prognostic factor in SBC (22). Other factors associated with poor prognosis include poor differentiation, positive margins, duodenal location, male sex, black ethnicity and older age. High lymph-node ratio (>50–75%) and a low number of assessed lymph nodes have been significantly associated with decreased survival (23).

1.5 SMALL BOWEL CARCINOMA PREDISPOSING CONDITIONS

Although SBCs are often sporadic, several predisposing conditions (**Figure 6**), including hereditary syndromes (e.g. familial adenomatous polyposis, Lynch syndrome, Peutz-Jeghers syndrome and juvenile polyposis syndrome) and

immune-mediated intestinal disorders, such as celiac disease (CD) and Crohn's disease (CrD), have been identified (3).

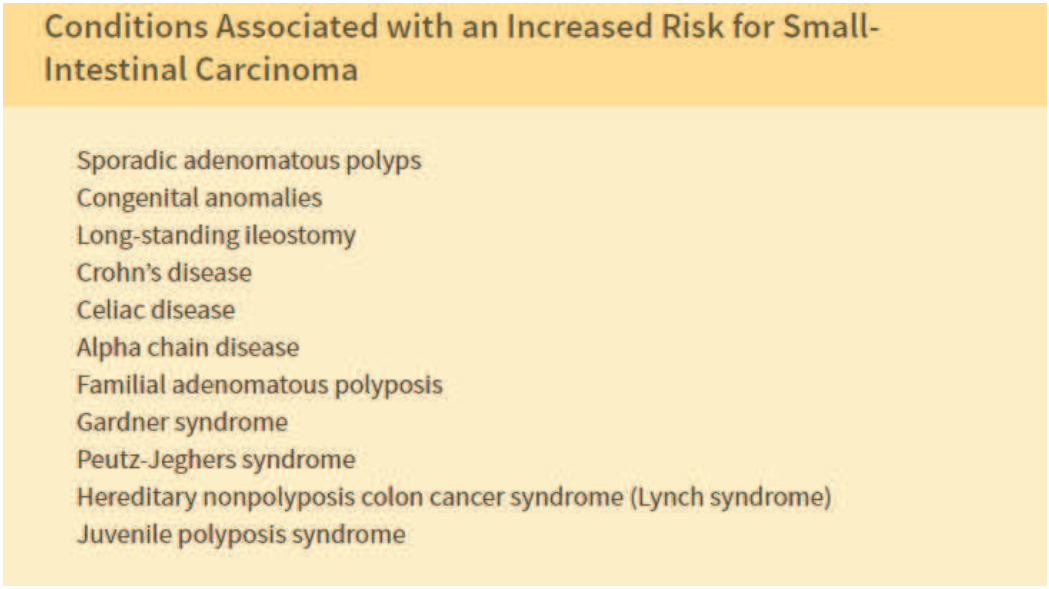

Figure 6. *Predisposing conditions for small bowel carcinoma development. From: Odze and Goldblum Surgical Pathology of the GI Tract, Liver, Biliary Tract and Pancreas, 3rd Edition.*

CD is a chronic enteropathy induced, in genetically susceptible individuals, by gluten ingestion (24) that affects 1% of western population (25). It is a predisposing condition for SBC as well as for many other malignant neoplasms, such as enteropathy-associated T-cell lymphoma, and esophageal carcinoma (26, 27). In a recent meta-analysis of eight studies including 79.991 CD patients and 75 SBCs associated with CD (CD-SBCs), SBC risk of CD subjects has been estimated to be 14-fold higher than that of the general population (27) and in one study of 175 patients with SBC, 13% had celiac disease (28). The

diagnosis of CD preceded that of SBC in 63% of these patients who followed a gluten-free diet in most cases (29). CD-SBCs frequently arise in the jejunum and may be multifocal. CD-SBCs were reported to harbor a high incidence of MMR deficiency (29,30) and to follow the CpG island methylator (CIMP) pathway (31).

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 (32) than that of the general population. SBCs associated with CrD (CrD-SBCs) are reported to account for 7% of all SBCs (32). Risk factors for development of SBC in individuals with CrD include surgically excluded loops of small bowel, chronic fistulous disease, male sex and a long CrD duration. In one study, adenocarcinoma risk was found to be lower in CrD patients who had undergone small bowel resection or who had used salicylates for longer than 2 years (33). In comparison with spo-SBC and CD-SBC, CrD-SBC tends to involve more frequently the ileum than the duodenal-jejunal tract. It may be very difficult to macroscopically identify the tumor in an area of severe CrD. In these patients, SBCs typically arise in the setting of intraepithelial neoplasia or dysplasia (flat or polypoid) rather than in preexisting adenomas. However, the diagnosis of dysplasia is sometimes difficult because of recurrent and persistent inflammatory changes associated with the underlying IBD. Histologically, the diagnosis of dysplasia is based on

identification of a combination of architectural and cytologic features. Architectural alterations may result in a configuration that resembles adenoma. Cytologic abnormalities consist primarily of cellular and nuclear pleomorphism, nuclear hyperchromasia, loss of polarity, and nuclear stratification. Most CrD-SBCs develop in areas of macroscopically identifiable IBD.

2. AIMS

Several studies are available concerning SBC clinical, histologic, phenotypic and molecular features (8, 17, 20, 34, 35). These studies, however, deal mostly with spo-SBC cases. Information concerning CD-SBC and CrD-SBC is limited to a few small series or case reports (29, 30, 31, 36, 37), and both the histologic features and the molecular landscape of these rare cancers are still largely unknown.

On this basis the primary aim of this thesis was to comparatively assess histopathological, phenotypic, 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.

Secondary aims were (a) to look for any prognostic influence of histopathological structure and tumor cell phenotype in our whole SBC series; b) to gain new insights into SBC histogenesis, with special reference to the carcinogenetic process at work in the inflamed non-tumor mucosa of CD-SBC or CrD-SBC; and c) to search for the presence of Epstein-Barr virus (EBV), a recognized oncogenic agent, in tumor cells of SBCs, especially of those associated with CrD, because EBV is detected at high frequency in non-neoplastic intestinal mucosa of IBD patients.

3. MATERIALS AND METHODS

3.1 STUDY POPULATION

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 Celiac and/or IBD Centres participating in the Small Bowel Cancer Italian Consortium, i.e. Pavia (Coordinating Centre), Aviano, Bologna, Brescia, Cagliari, Genova, Firenze, 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) (24). In 5 cases (19%), CD diagnosis was concomitant to 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 (38), and the site and extent of the disease were confirmed by endoscopy, histology and imaging. In three patients (12%), CrD diagnosis was simultaneous to that of SBC. Sixteen out of 25 CrD patients (64%) had fibro-stenosing disease, 5 (20%) were predominantly inflammatory while 4 (16%) had penetrating behavior at SBC diagnosis. Only 4 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 cases, CD was excluded (serum IgA anti-endomysial and anti-tissue transglutaminase antibody negativity with normal serum total IgA), while 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 histologic evaluation of the surgical specimens, and, in MSI SBC cases, by the presence of *MLH1* promoter methylation, as well. Neuroendocrine neoplasms were also excluded.

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

3.2 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Tissue samples were fixed in 4% formaldehyde and processed in paraffin wax. Four μm-thick sections were stained with haematoxylin–eosin (H&E) for morphological evaluation. All cases were investigated for histologic type and grade, according to the WHO classification (39), lymphovascular invasion and all the parameters required to fulfil the criteria of the AJCC staging system (40). In addition, SBCs were also classified, on the basis of their histologic structure, as a) glandular type (if >70% of the tumor exhibited glandular pattern), b) diffuse/poorly cohesive cell type (signet ring cell cancers or cancers showing

diffusely infiltrating, poorly cohesive cells, dispersed in a frequently desmoplastic stroma as single elements or as small aggregates with little to no gland formation, in >70% of the tumor), c) mixed type (characterized by a combination of both glandular and poorly cohesive cell/desmoplastic patterns, with at least 30% each, within the same tumor) and solid cancers (when they showed almost exclusively a solid or trabecular pattern) (41-43). Among solid cancers, d) medullary-type cancers (characterized by tumor cells exhibiting prominent infiltration by T lymphocytes and a pushing margin) were distinguished from the e) non-medullary solid cases lacking these features (42). All cases were also checked for the presence of adjacent dysplastic lesions and metaplastic features of the uninvolved mucosa.

For immunohistochemistry, four μm-thick sections were incubated at 4°C for 18-20 hours with specific antibodies directed against: the gastric foveolar cell marker MUC5AC (monoclonal, clone CLH2, Abcam), the pancreatobiliary duct marker cytokeratin (CK) 7 (monoclonal, clone OV-TL 12/30, Dako), the pyloric and Brunner's gland marker MUC6 (monoclonal, clone CLH5, Novocastra), as well as against intestinal differentiation markers including the caudal type homeobox transcription factor 2 (CDX2, monoclonal, clone DAK-CDX2, Dako), the goblet cell marker MUC2 (monoclonal, clone Ccp58, Santa Cruz Biotechnology), CK20 (monoclonal, clone Ks20.8, Dako) and the small bowel brush border marker CD10 (monoclonal, clone 56C6, Dako). In addition, immunoreactions for C-terminal β-catenin (monoclonal, clone 14/Beta-Catenin, BD), for the transcription factor Sex-determining Region Y-Box 9 (SOX-9, polyclonal, Millipore), for the mismatch repair proteins including MLH1 (monoclonal, clone ES05, Dako), MSH2 (monoclonal, clone FE11, Dako), MSH6 (monoclonal, clone EP49, Dako) and PMS2 (monoclonal, clone EP51,

Dako), for p53 protein (monoclonal, clone DO7, Dako), for the neuroendocrine marker chromogranin-A (monoclonal, clone LK2H10, Ventana), for CD3 (polyclonal, Dako), CD8 (polyclonal, Dako), for human epidermal growth factor receptor 2 (HER2, monoclonal, Leica Biosystem, Newcastle, UK) and for Programmed Death-Ligand 1 (PD-L1, monoclonal, clone E1L3N, Cell Signaling Technology, Danvers, MA) were also performed. Immunoreactions were developed using 0.03% 3,3' diaminobenzidine tetrahydrochloride and sections were then counterstained with Harris' hematoxylin.

For the assessment of tumor cell phenotype, only cases with at least 10% immunoreactive cells were regarded as positive with the exception of CDX2, for which a cut-off of 20% was applied (44). Cases with nuclear accumulation of C-terminal β-catenin in at least 10% of dysplastic/tumor cells was recorded as positive and were also tested for loss of nuclear expression of N-terminal β-catenin (monoclonal, clone E247, Abcam). Only relatively strong nuclear SOX-9 immunoreactivity, with the same intensity as the deepest intestinal crypt cells, was regarded as positive. Tumor-infiltrating lymphocytes (TILs) were stained using CD3 and CD8 antibodies and counted in ten 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 tumor cells, i.e. "intraepithelial" TILs. A tumor 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 (45). Immunostaining of MMR proteins (MLH1, MSH2, MSH6 and PMS2) in tumor cells was evaluated as positive (retained expression) or negative (absent expression): only tumors showing absence of nuclear staining of all neoplastic cells in the presence of an internal positive control (intra-tumor stromal and

inflammatory cells or non-tumor mucosa) were considered negative. Carcinomas or dysplastic lesions were considered p53 positive when more than 50% of tumor cells showed strong nuclear p53 immunoreactivity, in line with previous studies (46). Scoring of HER2 immunostaining was conducted according to published criteria for gastric cancer (47).

3.3 MICROSATELLITE INSTABILITY ANALYSIS

Tumor DNA was obtained from formalin-fixed and paraffin-embedded tissues using three representative 8 μm-thick sections of tumor samples. DNA was extracted after manual microdissection using a QIAamp DNA formalin-fixed, paraffin-embedded tissue kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Microsatellite instability analysis was performed using a pentaplex panel of monomorphic mononucleotide repeats (BAT25, BAT26, NR-21, NR-22 and NR-24) by the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) (48).

3.4 MLH1 METHYLATION ANALYSIS

MLH1 methylation status was examined by pyrosequencing in SBC cases exhibiting loss of MLH1 immunohistochemical expression. Bisulfite modification of genomic DNA (300 ng) was performed with 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 (49) was analysed by pyrosequencing according to the protocol previously reported (50). Analytical sensitivity and linearity of the assay was

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).

3.5 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 desorption/ionization 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 to 20 ng of tumuor DNA. 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 nanoliter 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 (tetramerization) 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.

3.6 HER2 FLUORESCENT IN SITU HYBRIDIZATION

HER2 amplification by fluorescent in situ hybridization (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 tumor cells. The *HER2* amplification scoring was performed by counting *HER2* and *CEN17* signals from 40 to 100 nuclei/tissue sample. Non-tumor (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.

3.7 EPSTEIN-BARR VIRUS ENCODED RNAS (EBER) IN SITU HYBRIDIZATION

As EBV is reported to be the most prevalent viral infection in non-neoplastic mucosa of IBD, especially in steroid-refractory cases (51), all CrD-SBCs and 11 CD-SBCs were also analysed for the expression of EBER, a marker of latent phase EBV infection. The formalin-fixed, paraffin-embedded tissue sections were pretreated with proteinase K (DAKO) for 30 min at room temperature, then hybridized with a FITC-labeled peptic nucleic acid probe complementary to EBV-encoded RNAs (EBER-1 and 2; DakoCytomation, Glostrup, Denmark) and incubated overnight at 55 °C. After washing in restricting conditions for 35 min, the hybridized cells were visualized with an *in-situ* hybridization detection kit (K5201; DAKO) according to the manufacturer's instructions. The sections were then counterstained with Kernechtrot, dehydrated through graded alcohols, immersed in xylene and mounted with a permanent medium. The present *in-situ* hybridization method stained the nuclei of EBV-infected cells dark blue, while the nuclei of non-infected cells appeared red. Specificity controls were performed by omitting the EBER probe and by running in parallel EBV positive and negative gastric cancers characterized in a previous investigation (42).

3.8 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 25^{th}-75^{th} percentiles for continuous variables and as

counts and percentage for categorical variables. Median follow-up and its interquartile range (25^{th}-75^{th}) were computed by means of the inverse Kaplan Meier method. The Kruskall Wallis test and the Fisher 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 year (95% CI) were also computed as summary measures. The association between candidate prognostic factors and tumor-related death was estimated by means of Cox regression. Multivariable models including non-collinear variables with $p<0.1$ at univariable analysis were fitted. The choice of variables to be included in the multivariable model was decided a priori and was based on the biological knowledge of the tumor. 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. 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). A 2-sided p-value <0.05 was considered statistically significant. For post-hoc comparisons Bonferroni correction was applied. Stata 14.1 (StataCorp, College Station, TX) was used for computation.

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

Pt	Sex	Age at CD dgn (yrs)	Age at SBC dgn(yrs)	CD duration at SBC dgn (mo)	Diet status at SBC dgn	SBC location	Histologic 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, celiac disease; dgn, diagnosis; F, female; GFD: gluten-free diet; M, male; NA, not applicable; Pt, patient; RCD: refractory celiac disease; SBC, small bowel carcinoma.

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*	Previoustherapy for CrD	SBC location	Histologic 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

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

4. RESULTS

4.1 CLINICO-PATHOLOGICAL CHARACTERIZATION OF SMALL BOWEL CARCINOMAS

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 in the CD-SBC subgroup was significantly lower than that of spo-SBC patients and median duration of intestinal disease at SBC diagnosis was significantly lower in CD-SBCs in comparison to CrD-SBCs. A higher proportion of females was found in CD-SBCs in comparison to 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-SBCs, while in both CD-SBCs and spo-SBCs 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 7 of 25 CrD-SBC cases (28%). Nevertheless, the proportion of SBC patients in stage I-II with more than 7 lymph nodes assessed was comparable between the three subgroups.

Patients were followed-up for a median of 71 months (25^{th}-75^{th}: 30-123). Overall survival curves show the prognostic effect of the clinical subgroup at univariable analysis. A significantly worse overall survival was observed in CrD-SBC cases in comparison to 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) cases. Spo-SBC patients showed a trend for worse overall survival in comparison to CD-SBC cases, although the difference did not reach statistical significance (HR 3.59, 95% CI 0.88-14.57, p=0.087) (**Figure 7A**). Median survival was 28 months for CrD-SBC

and 72 months for spo-SBC patients, while it was not evaluable for CD-SBC cases 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 not (p=0.581). Stage I-II patients showed a significantly better overall survival in comparison to stage III-IV cases at univariable analysis. Stage-, age- and sex- adjusted multivariable analysis confirmed the significant prognostic power of both the clinical subgroup and stage (**Table 4, model 1**). Cancer-specific survival was significantly worse in CrD-SBC patients in comparison to 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) cases. Spo-SBC patients showed a trend towards a worse cancer-specific survival in comparison to 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).

Tumor WHO histotype and grade, as well as lymphovascular invasion, showed neither significant difference among the three subgroups (**Table 3**) nor prognostic value. The most common WHO histotype in all cases was usual-type, tubular adenocarcinoma. However, the signet ring cell type was more prevalent in CrD-SBCs, while the medullary type was more common in CD-SBCs.

CD-SBC cases were more infiltrated by $CD3^+$ and $CD8^+$ lymphocytes in comparison to CrD-SBC or spo-SBC cases (**Figure 8**). The median number of

$CD3^+$ and $CD8^+$ TILs was significantly higher in CD-SBCs than in either CrD-SBCs or spo-SBCs (**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 to those with ≤15 $CD3^+$ TIL/HPF (**Figure 7B and Table 5**). SBC cases having >9.5 $CD8^+$ TIL/HPF showed a better overall survival in comparison to 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**).

4.2 HISTOLOGIC CLASSIFICATION OF SMALL BOWEL CARCINOMAS IN FIVE HISTOTYPES

Histologic analysis showed predominance of gland-forming tumors over diffuse, mixed glandular/diffuse, medullary, or solid non-medullary cases (**Table 6 and Figure 9**). Survival analysis of these five tumor histotypes gave a trend for worse outcome of diffuse, mixed and solid non-medullary compared to glandular or medullary cases (**Figure 10A**). When diffuse, mixed and solid histotypes were pooled in a single group of 28 cases (**Figure 10B**), a significant survival difference emerged for this group in comparison with glandular cases (HR: 4.93, 2.23-10.89, p<0.001). Multivariable analysis confirmed a significantly improved survival of patients with glandular type SBC, independently of clinical group, TIL density and stage (**Table 7**).

4.3 TUMOR CELL PHENOTYPE

The distribution of the intestinal phenotypic markers CDX2, MUC2, CK20 or CD10, the gastric marker MUC5AC and the pancreatobiliary duct marker CK7 among small bowel carcinoma subsets is outlined in **Table 8**, where 39 tumors with essentially intestinal phenotype (i.e. positive for CDX2, MUC2, CK20 and/or CD10 while negative for both MUC5AC and CK7) were separated from the remaining 37 tumors expressing a metaplastic gastro-pancreatobiliary phenotype, either alone or admixed with the intestinal one (i.e. positive for MUC5AC and/or CK7 while being positive or negative for CDX2, MUC2, CK20 and CD10). A general predominance of the intestinal phenotype among both celiac disease-associated and sporadic cases and of the non-intestinal (gastro-pancreatobiliary or mixed) phenotype among CrD-SBC cases was observed (**Figure 11**). Intestinal phenotype was more frequently found in glandular (32/42, 76%) compared to in diffuse/mixed/solid cases (6/28, 21%, $p<0.001$). In particular, 11 of 13 mixed, 4 of 7 diffuse and 7 of 8 solid SBCs showed gastro-pancreatobiliary markers expression. Of note, all of the 10 glandular cases with the non-intestinal phenotype occurred in CrD patients.

Survival analysis showed a favorable influence of individual intestinal markers (especially CDX2 and MUC2) and a worse influence of gastro-pancreatobiliary markers. The pyloric and Brunner's gland marker MUC6 was found in a small fraction (16%) of cases, including 2 CD-SBCs, 7 CrD-SBCs and 3 spo-SBCs,

without a significant difference among clinical groups (p=0.156) and without prognostic value (HR: 0.80, 0.30-2.13, p=0.654).

Cumulative markers analysis showed better survival for patients with tumors of essentially intestinal phenotype compared with those expressing non-intestinal markers (in at least 10% of cells) (**Table 8**). This finding may contribute to the poor outcome of CrD-SBC patients, most of which (20/25, 80%) showed non-intestinal marker expression. In a stage, sex and age inclusive survival analysis of 75 SBC patients, the favorable influence of intestinal phenotype was retained (hazard ratio: 0.30, 0.14-0.69; p=0.004), while it lost significance when the clinical group was also added to the model.

4.4 MICROSATELLITE INSTABILITY IN SMALL BOWEL CARCINOMAS

MSI was found in 25 out of 76 cases of SBC (33%), and no discordance was observed between immunohistochemical and molecular analysis. A significantly higher MSI frequency was found in CD-SBCs compared with either CrD-SBCs or spo-SBCs (**Table 5**). All MSI tumors showed a loss of both MLH1 (**Figure 8, G-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 9 of 51 non-MSI tumors (18%, p<0.001). At univariable analysis, MSI tumors showed a better overall survival in comparison to non-MSI tumors (**Figure 12 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 to 75% of non-MSI, p=0.005) and different overall survival. However, in a multivariable analysis inclusive of age, sex, stage and clinical subgroup, MSI lost significant survival prognostic power (**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 9 of 28 high-TIL SBCs were non-MSI cases. These cases may contribute to explain the higher prognostic power of high TIL density compared to MSI status, as also highlighted by univariable survival analysis (**Table 5**).

4.5 IMPACT OF MICROSATELLITE INSTABILITY AND TUMOR-INFILTRATING LYMPHOCYTES ON PROGNOSTIC POWER OF HISTOTYPE AND PHENOTYPE

When we found that both MSI and high TIL density were segregating among SBCs showing glandular or medullary histotype and intestinal phenotype, we investigated for any potential impact of MSI and/or high TILs on the prognostic power of histotype and phenotype. Separation of 25 cases showing MSI, 28 cases with high TILs or 19 cases showing both MSI and high TILs from the remaining SBCs gave highly significant survival differences (hazard ratio: 0.22, 0.08-0.64, p=0.005; hazard ratio: 0.09, 0.02-0.36, p<0.001 and hazard ratio: 0.26, 0.12-0.57; p<0.001, respectively). Survival analysis of the 51 non-MSI or the 48 low TIL cases confirmed the importance of histologic structure and phenotype with an improved survival of glandular versus diffuse/mixed/solid cases (HR: 0.28, 0.12-0.63, p=0.002 among microsatellite stable cases and HR: 0.40, 0.18-0.87, p=0.022 among low TIL cases, respectively), and of intestinal

versus non-intestinal phenotype SBC cases (HR: 0.50, 0.23-1.08, p=0.080 and HR: 0.41, 0.19-0.89, p=0.023, respectively).

4.6 PD-L1 IMMUNOHISTOCHEMICAL EXPRESSION IN SMALL BOWEL CARCINOMAS

PD-L1 staining was observed in membranes and/or cytoplasm of some stromal immune cells (mostly macrophages), usually restricted to the tumor invasive margin, in 8 (6 MSI and 2 non-MSI) of the 23 CD-SBCs tested, in 5 of the 25 CrD-SBCs, and in 5 of the 23 spo-SBC cases tested (**Figure 13**), without significant difference among the subgroups. However, only one SBC case, which was a MSI medullary cancer associated with CD, expressed PD-L1 in the tumor cell cytoplasm.

4.7 GENE MUTATIONS IN SMALL BOWEL CARCINOMAS

No BRAF mutation was observed in any case of SBC (**Table 9**). *KRAS, NRAS*, and *PIK3CA* mutations were detected in 23, 3 and 10 out of 76 cases, respectively. As expected, *KRAS* and *NRAS* mutations were mutually exclusive, while 6 SBC cases (8%), including 4 spo-SBCs, 1 CD-SBC and 1 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-SBCs compared with CrD-SBCs, while no difference was found between CD-SBCs and the other two subgroups (**Table 9**).

P53 overexpression involving >50% of tumor cells did not differ among the three subgroups (**Table 9**). *TP53* mutations were found in 17 of 47 SBC cases investigated (6 of 15 CD-SBCs, 3 of 17 CrD-SBCs and 8 of 15 spo-SBCs). *TP53* mutations were found either in cases showing p53 overexpression (16 cases) or complete lack of p53 immunostaining (1 case). *TP53* mutations and MSI proved to be mutually exclusive (p=0.038); however, despite their wild-type *TP53,* seven (28%) MSI tumors 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 8 non-MSI CD-SBC cases harbored *TP53* mutations.

Five (7%) SBC cases were $HER2^{+}$ at immunohistochemistry and revealed *HER2* gene amplification (**Table 9 and Figure 14**). Histologically, all 5 *HER2* amplified cases were usual-type, tubular adenocarcinomas. Three $HER2^{+}$ cases were jejunal tumors (2 CD-SBCs and 1 spo-SBC) and the remaining two were ileal CrD-SBCs. Three *HER2* amplified SBC cases were *TP53* mutated, two were *KRAS* mutated and one had MSI. *KRAS, NRAS, PIK3CA* and *TP53* mutations, p53 overexpression, and *HER2* amplification showed no prognostic value.

4.8 SMALL BOWEL CARCINOMA EXPRESSION OF NUCLEAR β-CATENIN AND SOX-9

As shown in **Table 10** and **Figure 15A-B**, nuclear β-catenin expression was significantly concentrated within the CD-SBC group, which differed significantly from the remaining SBCs. Interestingly, N-terminally directed antibodies failed to recognize nuclear β-catenin in 17/40 (42%) tumors where the protein was easily detected in the nucleus by C-terminal antibodies. Both N- and C- terminal antibodies recognized membranous β-catenin in normal non-tumor mucosa of the same cases, thus suggesting the possibility of a tumor-selective N-terminal β-catenin loss. This was found to be unrelated to patient background disease and survival (**Table 10**). Like nuclear β-catenin expression, the loss of MLH1 expression (which overlapped perfectly with molecularly assessed microsatellite instability status) was also highly and selectively concentrated among CD-SBCs ($p=0.001$ vs remaining SBCs). In fact, a correlation was found among the whole small bowel cancer series between the distribution of the two changes ($p<0.001$).

The nuclear expression of SOX-9, another Wnt-related transcription factor, was significantly correlated with that of β-catenin ($p=0.005$) and was also highly represented among CD-SBCs (**Figure 15C**), although less selectively than β-catenin. Indeed, no significant difference for SOX-9 expression was found among clinical groups. A trend for SOX-9 to be more extensively expressed in intramucosal or superficial than in deeply invasive tumor growths was noted.

Univariate survival analysis of the whole series showed a significantly better survival for patients bearing nuclear β-catenin positive vs negative SBC, while SOX-9 failed to reveal prognostic relevance. The association of nuclear β-catenin with more favorable outcome was retained in a stage, age and sex inclusive multivariable model (HR: 0.32, 0.13-0.75; p=0.010) or in a stage and histotype inclusive model (HR: 0.29, 0.13-0.65; p=0.003), while losing significance only when clinical groups were added to the model. However, when survival of 20 SBC patients showing both MSI and nuclear β-catenin was compared with that of 20 cases showing nuclear β-catenin in the absence of MSI, the latter group had a worse outcome (HR: 7.14, 2.00-25.00; p=0.002). In addition, among the 51 microsatellite stable cases, the 20 β-catenin positive SBC cases lacked any survival difference compared with 31 β-catenin negative SBC patients (HR: 0.66, 0.31-1.43; p=0.296).

4.9 DYSPLASIA AND PRENEOPLASTIC CHANGES

Evidence for residual adenomatous polypoid growth was obtained, within or adjacent to the superficial part of the neoplasm, in 10 of 25 spo-SBCs, 8 of 25 CrD-SBCs and only one of 26 CD-SBCs. Focal flat dysplasia, usually adjacent to the invasive cancer focus, was detected in 4 CD-associated, 1 sporadic and 5 CrD-associated SBCs (**Figure 15D**). Minute dysplastic foci were found, in addition, associated with mucosal metaplastic changes in 3 CrD-SBCs (see below). Total dysplastic lesions are summarized in **Table 11**, where it appears that nuclear SOX-9 (**Figure 15E**), found in 23/28 (82%) of cases investigated, is

the molecular marker most widely expressed in dysplasia, irrespective of clinical group, followed by p53 overexpression (15/29, 52%) and β-catenin (10/29, 34%) (**Figure 15F**), with only 2 of 29 (7%) dysplastic lesions showing MLH1 loss. On the contrary, the latter change occurred in 24/76 (32%) carcinomas, including 5 of the cases retaining MLH1 staining in the corresponding dysplasia (**Figure 15G**). This finding outlines a relatively late appearance of MSI status during carcinogenesis.

Diffuse or patchy CD lesions, i.e. goblet-cell poor crypt hyperplasia plus increased intraepithelial lymphocytes, either isolated (5/25) or associated with villous atrophy (17/25), were seen in the majority (22/25, 88%) of CD-SBCs, both in cancer-adjacent and cancer-distant small bowel mucosa. Notably, nuclear SOX-9 expression, normally restricted to the deep lower half of crypts (**Figure 15H**), showed a prominent expansion to involve the upper half of the crypts in non-tumor mucosa of all 19 CD-SBCs investigated, sometimes reaching the crypt/villous junction or even to the superficial epithelium covering the flattened mucosa resulting from villous atrophy (**Figure 15I**). Care was taken to discard chromogranin-A reactive cells, usually showing SOX-9 positive cytoplasm in the absence of nuclear staining and scattered as single elements along the whole crypt-villous unit (**Figure 15H**). Interestingly, direct continuity of SOX-9 reactive crypt hyperplasia with dysplasia and intramucosal neoplasia was consistently seen (**Figure 15E**). No relevant change of nuclear β-catenin expression, which remained restricted to very few cells interposed with Paneth cells within the deepest crypts, was observed in SOX-9 positive crypt

hyperplasia. However, intense nuclear β-catenin appeared in adjacent dysplastic or neoplastic lesions (**Figure 15F**).

While no preneoplastic change was identified in non-tumor mucosa of spo-SBCs, metaplastic changes showing expression of the gastric foveolar marker MUC5AC and/or the ductal pancreatobiliary marker CK7 were extensively represented (86%) in the chronically inflamed, non-neoplastic mucosa associated with CrD-SBC, sometimes coupled with minute dysplastic or neoplastic foci (**Figure 15J**). Notably, 93% of the overt dysplastic lesions associated with CrD-associated carcinomas showed expression of CK7 and/or MUC5AC; moreover, the gastro-pancreatobiliary markers were concordantly expressed in metaplastic, dysplastic and invasive components of all but one of the 15 CrD-SBCs investigated (**Figure 15K-M**).

4.10 IDENTIFICATION OF TWO EPSTEIN-BARR VIRUS (EBV)-POSITIVE SMALL BOWEL CARCINOMAS ASSOCIATED WITH CROHN'S DISEASE

A lympho-epithelioma-like carcinoma (LEC) arising on a background of long-standing CrD (corresponding to case #24 in **Table 2**) and showing prominent nuclear reactivity for EBER, high density of TILs (CD3+ TILs: 156 /HPF and CD8+ TILs: 100/HPF) and absence of MSI, was first identified (**Figures 16**). This cancer was diagnosed in the terminal ileum of a 63-year old male with a 13-year history of ileal, fibrostenosing, active CrD (**Figure 17**) under treatment with oral corticosteroids and mesalazine. Briefly, during elective surgery for

subocclusive symptoms, a 15-cm ulcerated mass was detected in the surgical specimen, showing a LEC infiltrating the intestinal wall up to the subserosal adipose tissue, with four lymph node metastases, classified as stage IIIB (T3N2M0). After intestinal resection, the patient was treated with oral mesalazine, and he is now cancer-free 20 months after resection.

As in the stomach, which is the most frequent site of EBV^+ carcinomas in the gastrointestinal tract, a substantial number of such neoplasms do not show LEC histology but present instead with conventional-type glandular morphology, we searched for the presence of EBV in tumor cells, using EBER *in situ* hybridization, in our SBC series. A conventional-type adenocarcinoma associated with CrD (corresponding to case #11 in **Table 2**) was found to be diffusely $EBER^+$ (**Figure 18A**). The newly found EBV^+ SBC case was diagnosed in the terminal ileum of a 69-year old male patient in whom a concomitant diagnosis of ileal, fibrostenosing, active CrD was clinically and pathologically confirmed. The patient underwent surgery and a 3.7-cm ileal tumor was found in the surgical specimen. Histologically, the SBC featured a conventional glandular histology, infiltrating the intestinal wall up to the subserosa with no lymph node metastases, classified as stage IIA (T3N0M0). Similar to the LEC case, this glandular case showed high-TIL density (CD3+: 15.2/HPF and CD8+: 14.9/HPF) and absence of MSI (**Figure 18B**). After surgery, the patient was treated with oral budesonide, and he is now in maintenance therapy with oral mesalazine and cancer-free 155 months after radical surgery.

None of the remaining CrD-SBCs or of the 11 CD-SBC cases investigated showed tumor cell positivity for EBER.

The glandular EBV$^+$ CrD-SBC showed a *Q546R* mutation of *PIK3CA* gene and no *KRAS*, *NRAS*, *BRAF* or *TP53* mutations, while the LEC case showed no mutation of *PIK3CA*, *KRAS*, *NRAS*, *BRAF* or *TP53* genes. Nuclear β-catenin expression was present in the LEC, but it was not found in the glandular case (**Table 12**).

The intestinal marker MUC2 was extensively expressed in both EBV$^+$ SBCs. The gastric foveolar cell marker MUC5AC was positive in a restricted minority of invasive tumor cells (<10%) limited to the superficial portion of the two EBV+ carcinomas, while it was lost in the more deeply invasive part of the SBCs.

Both EBV$^+$ SBCs were coupled with a smaller EBV$^+$ adenoma component, which showed positivity for both MUC5AC (**Figure 18C-D**) and MUC2. In the two EBV+ cases, EBER positivity was also seen in restricted foci of apparently non-dysplastic, goblet cell-poor immature mucosa contiguous to frankly dysplastic lesions. In the non-dysplastic epithelium, only partial overlapping was found between EBER and MUC5AC expression (**Figure 18E-F**).

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*
N.	26	25	25		
Age at SBC diagnosis, median (25^{th}-75^{th}), 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 (25^{th}-75^{th}), months	17 (5-60)	156 (3-288)	NA	0.024	
Female, N (%)	16 (62%)	9 (36%)	8 (32%)	0.074	
Site, N (%)					
Duodenum	6 (23%)	1 (4%)	2 (8%)		
Jejunum	17 (65%)	1 (4%)	18 (72%)	<0.001	CD vs CrD:<0.001 CD vs Spo: 0.367 CrD vs Spo:<0.001
Ileum	3 (12%)	23 (92%)	5 (20%)		
Stage, N (%)					
I	3 (11%)	3 (12%)	1 (4%)		
II	14 (54%)	9 (36%)	13 (72%)		
III	6 (23%)	8 (32%)	9 (36%)	0.588	
IV	2 (8%)	5 (20%)	2 (8%)		
NA§	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	
Histologic type, N (%)					
Medullary CA	4 (15%)	1 (4%)	1 (4%)		
ADCA/usual	19 (73%)	19 (76%)	19 (76%)	0.343	
ADCA/mucinous	1 (4%)	0	3 (12%)		
ADCA/signet ring cell	2 (8%)	5 (20%)	2 (8%)		
Histological grade, N (%)					
Low grade (G1-G2)	15 (58%)	13 (52%)	19 (76%)	0.197	
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 (25^{th}-75^{th})	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 (25^{th}-75^{th})	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

*significant if p<0.017 according to Bonferroni correction. §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, celiac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; HPF, high power field; NA, not applicable; Spo-SBC, sporadic small bowel carcinoma; TIL, tumor-infiltrating lymphocyte.

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

	MODEL 1[@]		MODEL 2^		MODEL 3[&]	
	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[♣]*	2.06 (0.50-8.52)	0.316[▲]*	2.39 (0.60-9.58)	0.218°*
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

[@]Model 1: LR chi2(5)=42.52, p-value<0.001; Harrel's C=0.82; shrinkage coefficient=0.88. ^Model 2: LR chi2(6)=53.29, p-value<0.001; Harrel's C=0.86; shrinkage coefficient=0.89. [&]Model 3: LR chi2(6):43.94, p<0.001; Harrel's C=0.82; shrinkage coefficient=0.86.*For post-hoc comparisons, significance after Bonferroni correction set at 0.017. [♣]Hazard ratio (95% CI): 0.43 (0.19-0.96), P value = 0.040 versus CrD-SBC. [▲]Hazard ratio (95% CI): 0.47 (0.21-1.05), P value = 0.067 versus CrD-SBC.°Hazard ratio (95% CI): 0.45 (0.20-1.10), P value = 0.052 versus CrD-SBC. CD-SBC, celiac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; HPF, high power field; MSI: microsatellite instability; SBC, small bowel carcinoma; Spo-SBC, sporadic small bowel carcinoma; TIL, tumor-infiltrating lymphocyte.

Table 5. Microsatellite instability status and tumor-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

[£]p=0.001 vs CrD-SBC or Spo-SBC; [$]p=0.001 vs CrD-SBC; ^p<0.001 vs CrD-SBC or Spo-SBC; Abbreviations: CD-SBC, celiac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; HPF: high-power field; HR: hazard ratio; CI: confidence interval; , MSI: microsatellite instability; Spo-SBC, sporadic small bowel carcinoma; TIL: tumor-infiltrating lymphocyte.

Table 6. Histologic classification of the 76 small bowel carcinomas investigated.

Histotype	Distribution among clinical groups			Total
	CD-SBC (n=26)	**CrD-SBC** (n=25)	**Spo-SBC** (n=25)	
Glandular	14 (54%)	13 (52%)	15 (60%)	42 (55%)
Medullary	4 (15%)	1 (4%)	1 (4%)	6 (8%)
Solid	3 (11.5%)	2 (8%)	3 (12%)	8 (11%)
Diffuse	2 (8%)	4 (16%)	1 (4%)	7 (13%)
Mixed	3 (11.5%)	5 (20%)	5 (20%)	13 (23%)

No significant histotype distributive difference among clinical groups was found. Abbreviations: CD-SBC, celiac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; Spo-SBC, sporadic small bowel carcinoma.

Table 7. Overall survival by a multivariable Cox model of the 75 small bowel carcinomas patients with complete stage data.

	Hazard ratio (95% CI)	p value
Clinical group		0.033
CD-SBC	1.00 (base)	
CrD-SBC	4.98 (1.35-18.34)	0.016*
Spo-SBC	2.48 (0.66-9.29)	0.176♣*
Stage III-IV vs stage I-II	9.31 (3.30-26.26)	<0.001
$CD3^+$ tumor-infiltrating lymphocytes >15/high-power field vs ≤15	0.15 (0.03-0.69)	0.015
Glandular histotype vs non-glandular[$]	0.37 (0.16-0.84)	0.018

Model: LR chi2(5)=56.72, p-value<0.001; Harrell's C=0.87; shrinkage coefficient=0.91. *For post-hoc comparisons among clinical groups, significance after Bonferroni correction set at 0.017. ♣Hazard ratio (95% CI): 0.50 (0.22-1.14), p-value =0.098 versus Crohn's disease- associated small bowel carcinomas. [$]Medullary histotype included among non-glandular cases. Abbreviations: CD-SBC, celiac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; Spo-SBC: sporadic small bowel carcinoma.

Table 8. Expression of phenotypic markers in 76 small bowel carcinomas: their distribution among clinical groups and prognostic value

	Distribution among clinical groups			Total	Survival analysis	
	CD-SBC (n=26)	**CrD-SBC (n=25)**	**Spo-SBC (n=25)**		**Hazard ratio (95% confidence interval)**	**p-value**
Cytokeratin 7	5 (19%)[^]	15 (60%)[$]	4 (16%)	24 (32%)	2.72 (1.18-6.29)	0.019
MUC5AC	5 (19%)	13 (52%)	5 (20%)	23 (30%)	2.54 (1.08-5.98)	0.032
CDX2	21 (81%)[£]	9 (36%)	17 (68%)	47 (62%)	0.28 (0.13-0.63)	0.002
MUC2	18 (69%)	12 (48%)	12 (48%)	42 (55%)	0.25 (0.12-0.53)	<0.001
Cytokeratin 20	13 (50%)	9 (36%)*	20 (80%)	42 (55%)	1.19 (0.57-2.50)	0.635
CD10	8 (31%)	3 (12%)	11 (44%)	22 (29%)	0.66 (0.30-1.65)	0.302
Intestinal phenotype	17 (65%)[§]	5 (20%)°	17 (68%)	39 (51%)	0.39 (0.18-0.81)[&]	0.012

^p=0.004 vs CrD-SBC; [£]p=0.002 vs CrD-SBC ; [§]p=0.002 vs CrD-SBC ; [$]p=0.003, vs Spo-SBC; *p=0.004 vs Spo-SBC; °p=0.001 vs Spo-SBC; [&]versus remaining 37 cases expressing gastro-pancreatobiliary markers. Abbreviations: CD-SBC, celiac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; Spo-SBC: sporadic small bowel carcinoma.

Table 9. 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	

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

Table 10. β-catenin and SOX-9 expression in small bowel carcinomas: distribution among clinical groups and survival analysis.

	Distribution among clinical groups			Total cases	Survival analysis	
	CD-SBC	**CrD-SBC**	**Spo-SBC**		**Hazard ratio (95% confidence interval)**	**p value**
Nuclear β-catenin, C-terminal antibody	24/26 (92%)^	6/24 (25%)	10/25 (40%)	40/75 (53%)	0.30 (0.14-0.63)	0.002
Nuclear N-terminal β-catenin loss	10/26 (38%)	2/24 (8%)	5/25 (20%)	17/75 (23%)	0.49 (0.22-1.12)$	0.091
among C-terminal β -catenin positive cases	10/24 (42%)	2/6 (33%)	5/10 (50%)	17/40 (42%)	0.85 (0.24-2.97)§	0.796
SOX-9 expression	20/23 (87%)	11/22 (50%)	14/23(51%)	45/68 (66%)	0.70 (0.31-1.56)	0.380
Microsatellite instability°	17/26 (65%)£	4/25 (16%)	4/25 (16%)	25/76 (33%)	0.22 (0.08-0.64)	0.005
among C-terminal β–catenin positive cases	17/24 (71%)^	1/6 (17%)	2/10 (20%)	20/40 (50%)	0.14 (0.04-0.50)	0.002

^p<0.001 vs CrD-SBC or Spo-SBC; £p=0.001 vs CrD-SBC or Spo-SBC; *p<0.001 vs CrD-SBC and p=0.008 vs Spo-SBC; $versus remaining SBCs; §versus remaining C-terminal β-catenin positive cases;°All 17 MSI cases also had nuclear β -catenin among CD-SBC; as against only one of 4 among CrD-SBCs and 2 of 4 among Spo-SBCs. Abbreviations: CD-SBC, celiac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; Spo-SBC: sporadic small bowel carcinoma.

Table 11. Analysis of dysplastic and metaplastic changes associated with small bowel carcinomas.

		CD-SBC	CrD-SBC	Spo-SBC	Total
Dysplasia		5/26 (19%)[$]	16/25 (64%)[£]	11/25 (44%)[&]	32/76 (42%)
	Metaplastic phenotype°	0/5 (0%)	14/15 (93%)	2/10 (20%)	16/30 (53%)
	β-catenin nuclear expression	5/5 (100%)	0/13 (0%)	5/11 (45%)	10/29 (34%)
	SOX-9 expression	5/5 (100%)	10/13 (77%)	8/10 (80%)	23/28 (82%)
	MLH1 loss	1/5 (20%)	1/14 (7%)	0/10 (0%)	2/29 (7%)
	p53 overexpression	4/5 (80%)	7/15 (47%)	4/9 (44%)	15/29 (52%)
Non-dysplastic mucosa					
	Metaplastic phenotype°	2/25 (9%)	19/22 (86%)[§]	0/24 (0%)	21/71 (30%)

[$]polypoid in one and flat in 4 cases; [£]polypoid in 8, flat in 5 and minute foci in metaplasia in 3 cases; [&]polypoid in 10 and flat in 1 case. [§]$p<0.001$ vs either celiac disease-associated carcinomas or sporadic carcinomas. °Metaplastic phenotype was defined as extensive MUC5AC and/or cytokeratin 7 expression. Abbreviations: CD-SBC, celiac disease-associated small bowel carcinoma; CrD-SBC, Crohn's disease-associated small bowel carcinoma; Spo-SBC: sporadic small bowel carcinoma.

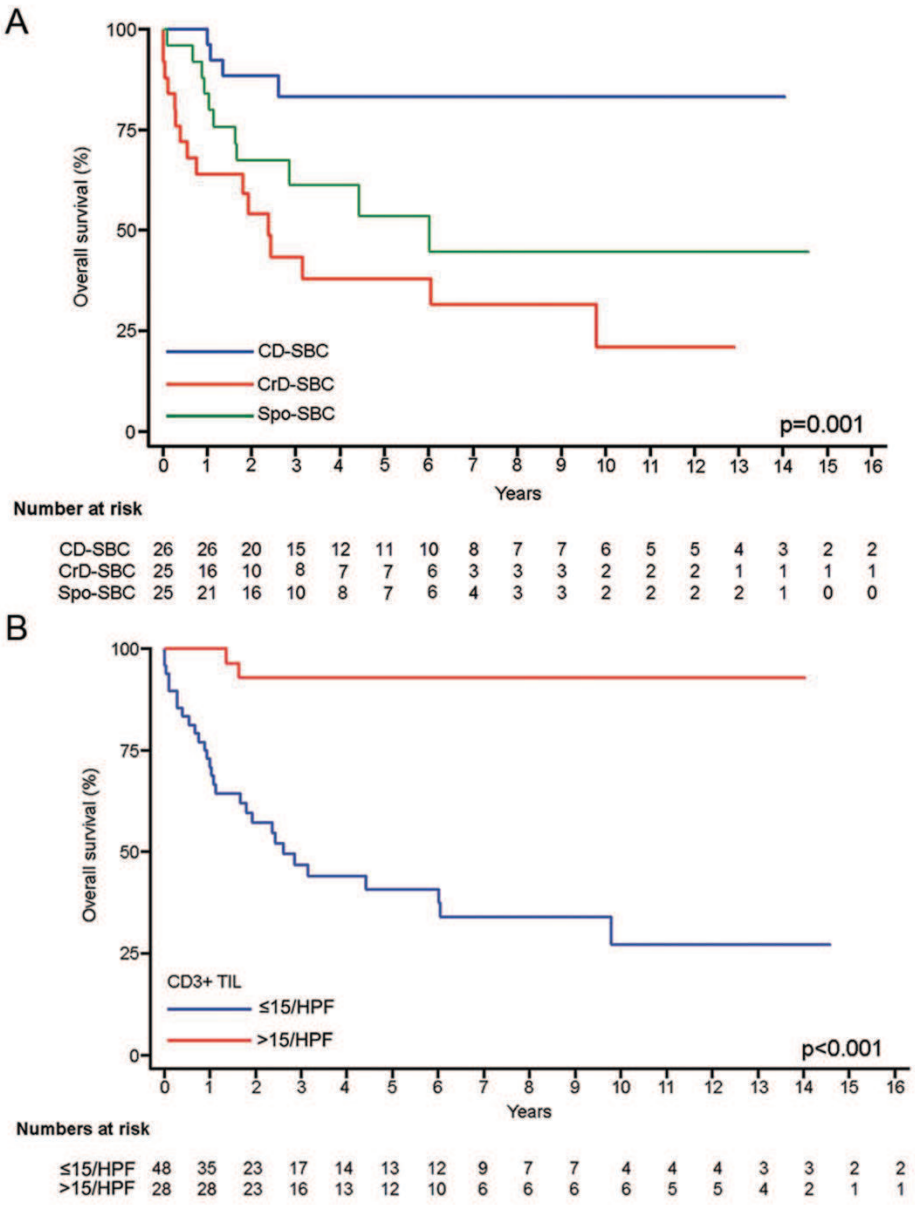

Figure 7. ***A)*** *Kaplan-Meier overall survival estimates for all patients by clinical subgroup.* ***B)*** *Kaplan-Meier overall survival estimates for all patients by $CD3^+$ tumor-infiltrating lymphocytes (TIL) density. P value is log-rank across subgroups.*

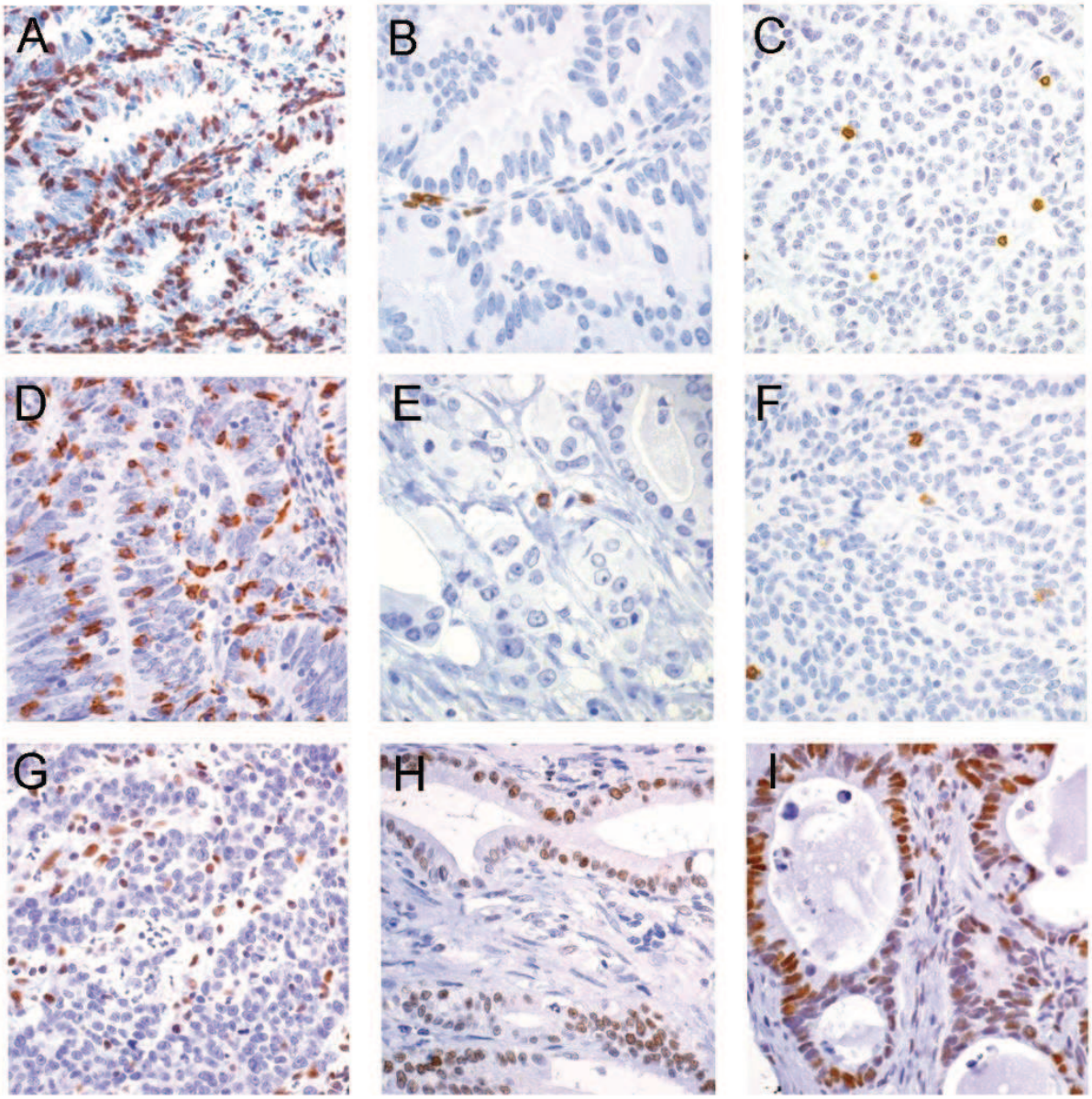

Figure 8. *Immunohistochemical detection of CD3, CD8 and MLH1. Immunohistochemical detection of CD3 showed numerous CD3-positive TILs in a CD-SBC (**A**). On the other hand, CD3 positivity was limited to a few cells in a CrD-SBC (**B**, original magnification x400) and a spo-SBC (**C**). Similarly, immunohistochemical detection of CD8 showed numerous CD8-positive TIL in a CD-SBC (**D**), in contrast to isolated CD8-positive cells in a CrD-SBC (**E**) and a spo-SBC (**F**). Immunohistochemical detection of MLH1 showed loss of expression in a CD-SBC (**G**), while MLH1 expression was retained by both a CrD-SBC (**H**) and a spo-SBC (**I**). Data are representative of staining performed in 26 CD-SBCs, 25 CrD-SBCs and 25 spo-SBCs.*

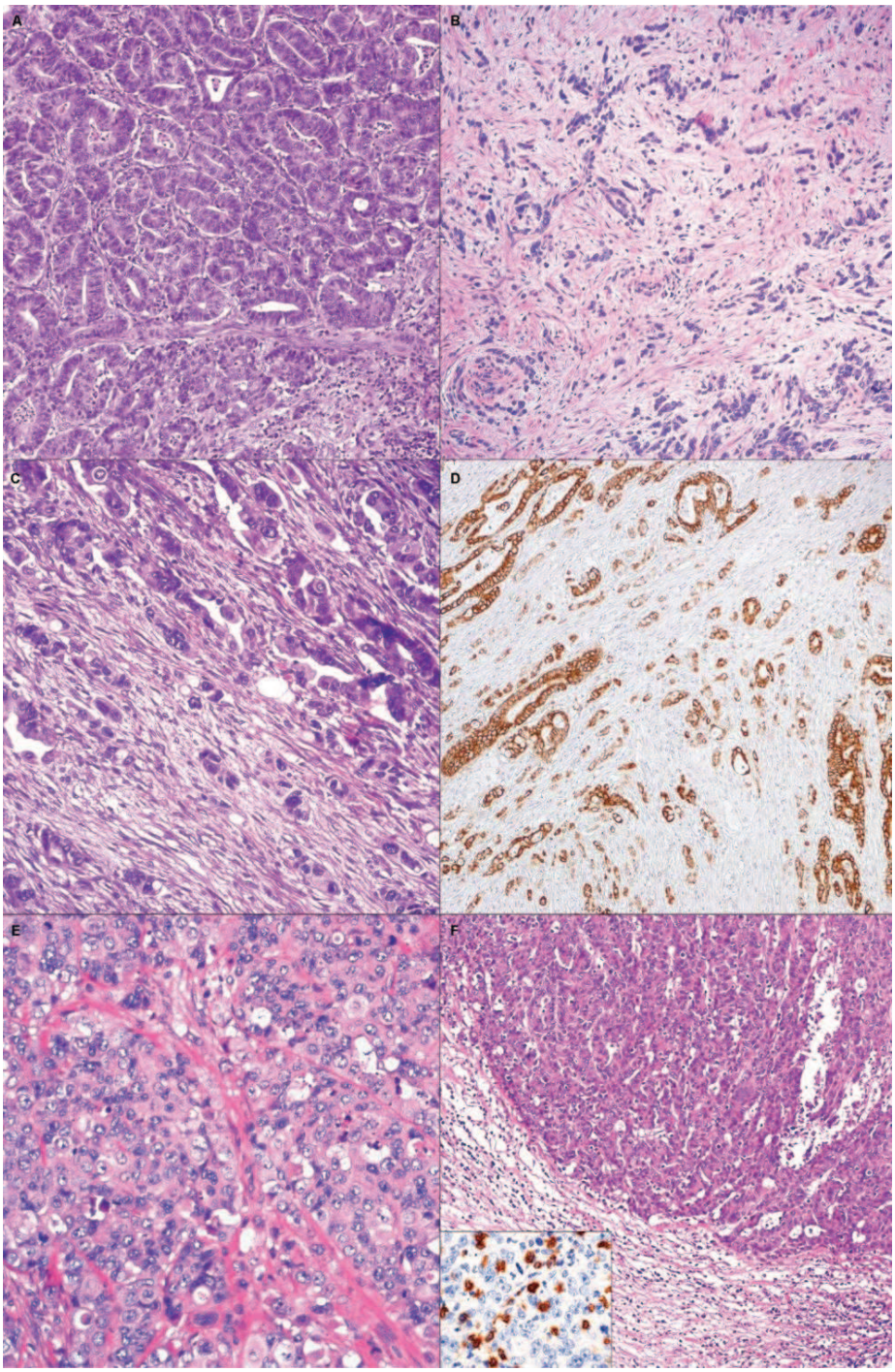

Figure 9. *SBC histotypes.* ***A)*** *This glandular type CD-SBC is predominantly composed of well-formed tubular structures.* ***B)*** *This diffuse type CD-SBC is characterized by poorly cohesive cells, dispersed in a desmoplastic stroma as single elements or as small aggregates.* ***C-D)*** *A mixed type Crohn's disease-associated ileal carcinoma characterized by a combination of both glandular and diffuse patterns within the same tumor. In* ***D,*** *the same CrD-SBC expressing the gastric marker MUC5AC in both components.* ***E****) A non-medullary solid type CD-SBC composed of nests of large, eosinophilic and atypical cells without intratumor T cell infiltration.* ***F****) This medullary-type CD-SBC is characterized by solid/trabecular growth of cells, a pushing margin, and prominent infiltration by numerous T lymphocytes (see CD3 immunostaining in the inset).*

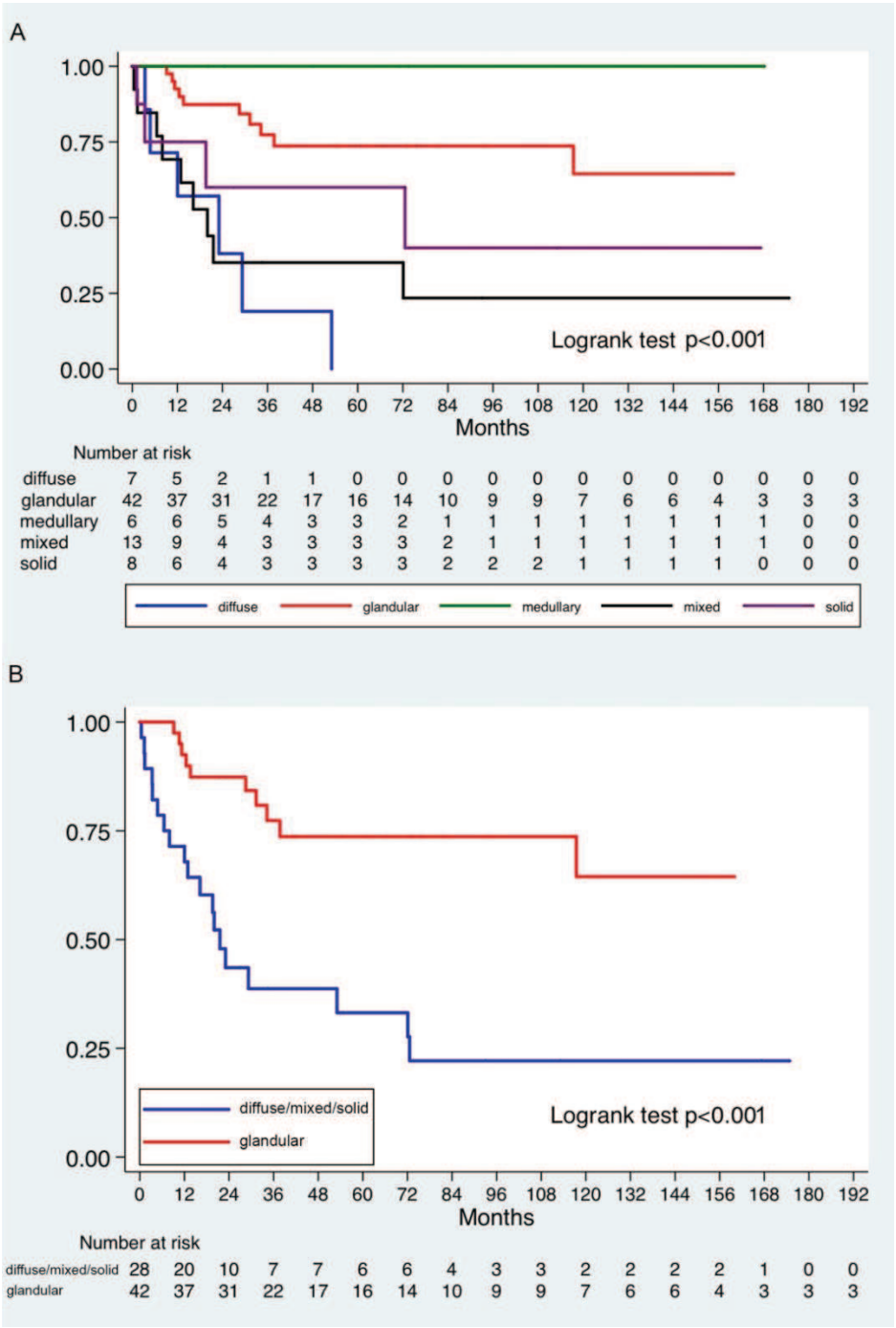

Figure 10. ***A)*** *Kaplan-Meier survival estimates for all 76 SBC patients by histotype. Post-hoc comparisons (significance after Bonferroni correction set at p<0.005): a) significant differences: diffuse vs glandular: p<0.001; diffuse vs medullary: p=0.003; glandular vs mixed: p<0.001; b) non-significant trends: medullary vs mixed: p=0.013; medullary vs solid: p=0.064; glandular vs solid: p=0.088; c) non-significant differences: diffuse vs mixed: p=0.544; diffuse vs solid: p=0.170; mixed vs solid: p=0.401; glandular vs medullary: p=0.207. Notably, no difference was found between diffuse, mixed and solid histotypes, which were pooled in Figure 10B.* ***B)*** *Kaplan-Meier survival estimates for the 70 non-medullary SBC patients by glandular versus diffuse/mixed/solid histotype.*

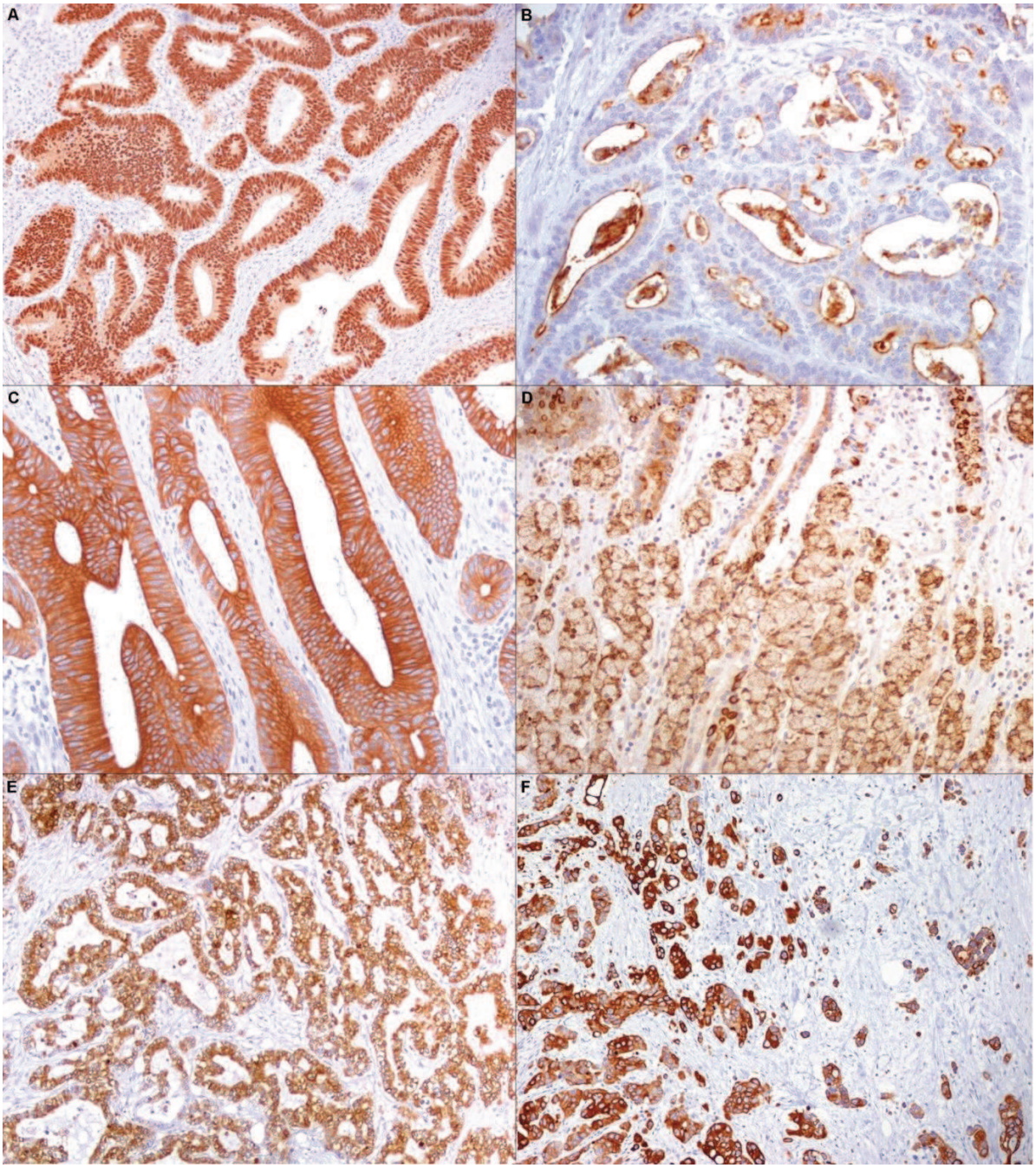

Figure 11. *Phenotypic marker expression in SBC.* ***A)*** *Uniform and intense nuclear positivity for the intestinal transcription factor CDX2 in a glandular type CD-SBC.* ***B)*** *Luminal surface expression of the brush border marker CD10 in a CD-SBC.* ***C)*** *A sporadic SBC reactive for the intestinal marker CK20.* ***D)*** *Signet ring cell CrD-SBC reactive for the goblet cell mucin MUC2.* ***E)*** *A glandular CrD-SBC extensively expressing the gastric foveolar marker MUC5AC.* ***F)*** *A mixed type CrD-SBC reactive for the pancreatobiliary duct marker CK7.*

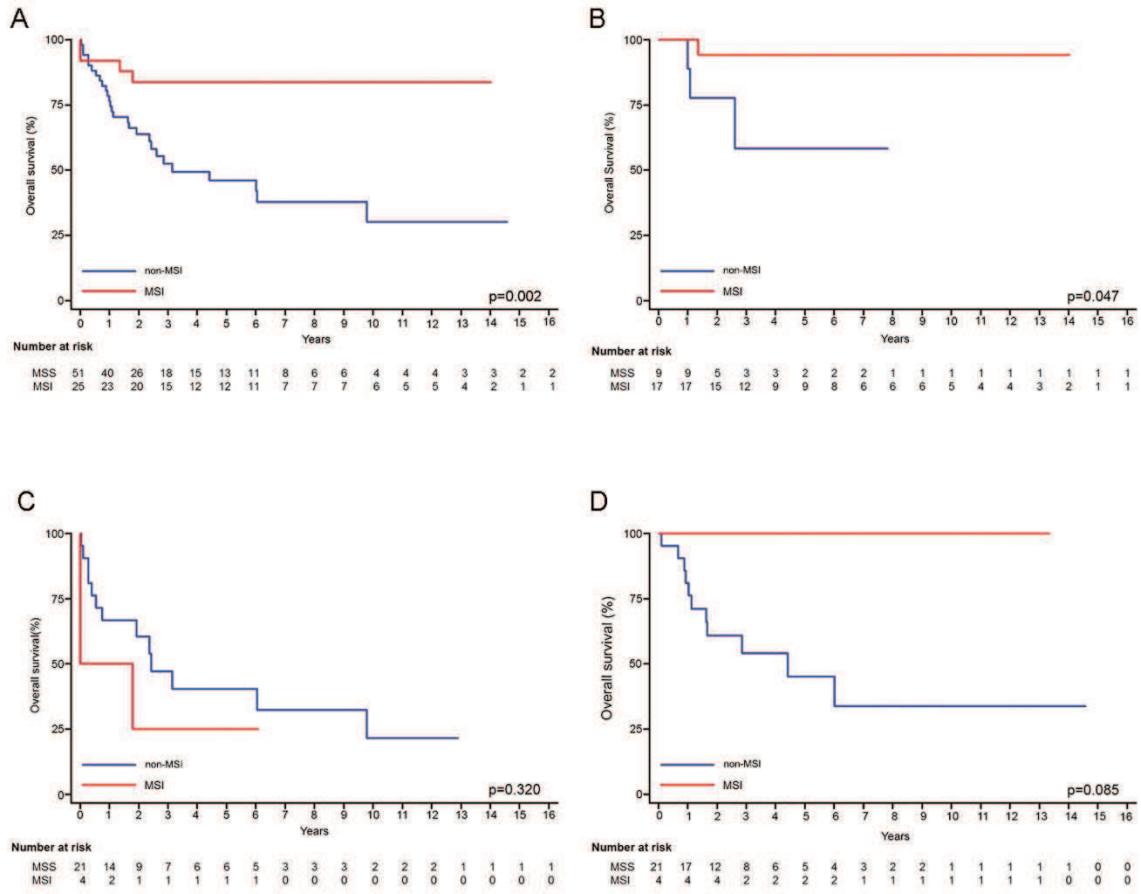

Figure 12. *Kaplan-Meier overall survival estimates for all patients **(A)**, CD-SBC patients **(B)**, CrD-SBC patients **(C)** and spo-SBC patients **(D)** by microsatellite instability (MSI). P value is log-rank across subgroups.*

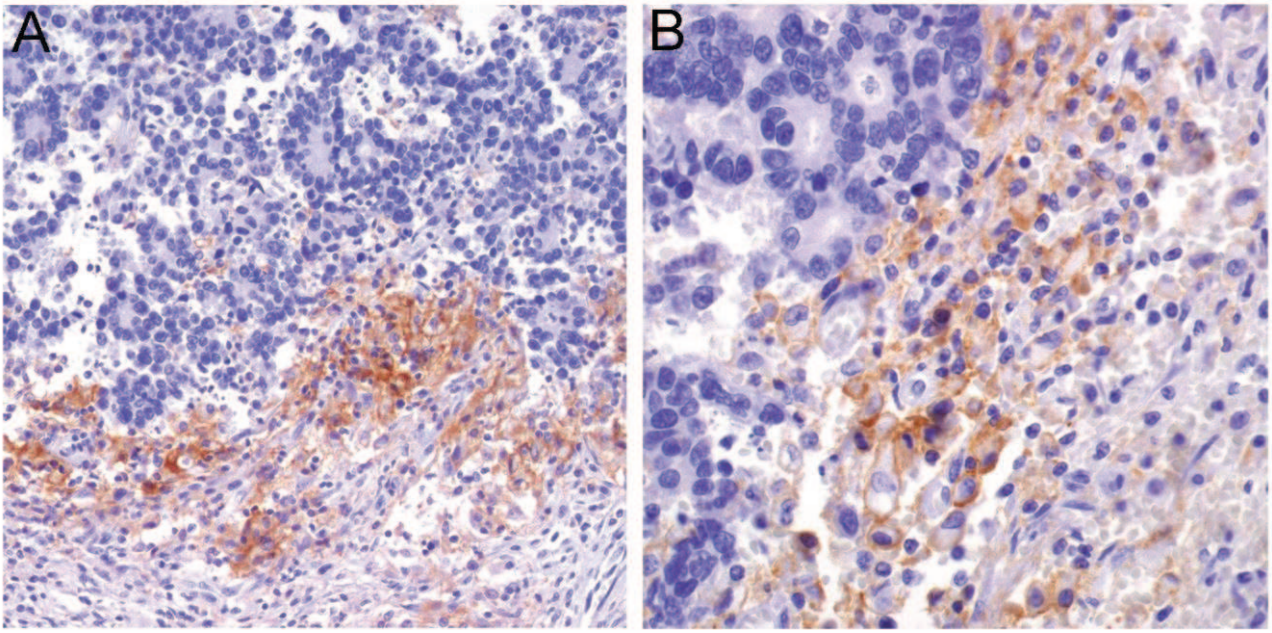

Figure 13. *An example of PD-L1 immunohistochemical detection. PD-L1 immunohistochemical detection showing PD-L1 expression in membranes and/or cytoplasm of some immune cells (mostly macrophages), while tumor cells are negative (**A**, original magnification, x200 and enlarged in **B**, x400). This SBC was found in the jejunum of a patient with CD and harbored MSI.*

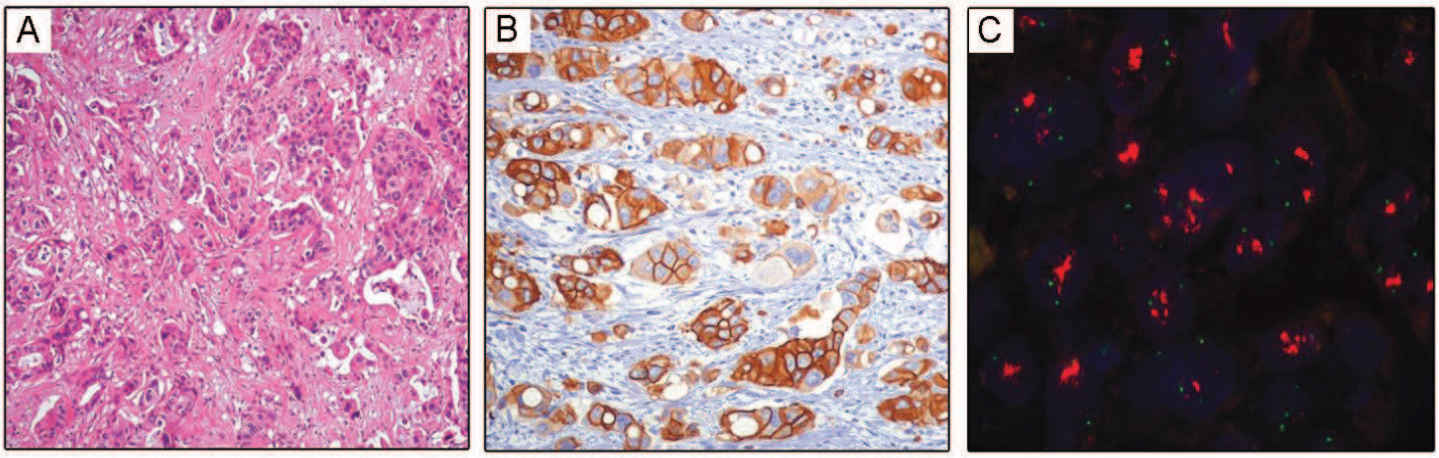

Figure 14. *An example of HER2 positive SBC. Histology of a mixed-type SBC (**A**), showing HER2 positive by immunohistochemistry (**B**) and revealing HER2 gene amplification by FISH (**C**). This SBC was found in the ileum of a patient with CrD.*

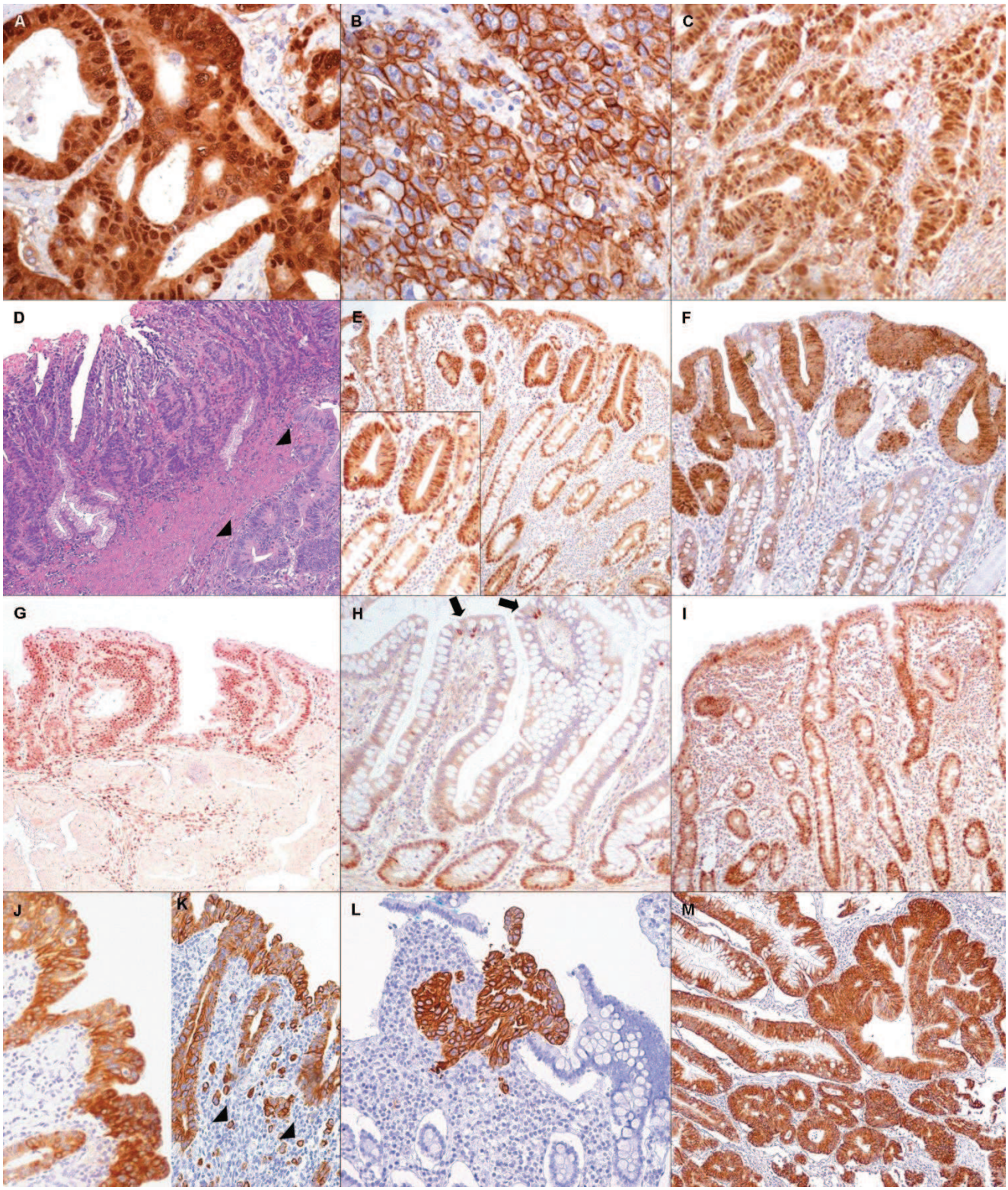

Figure 15. *Molecular/cellular marker expression and preneoplastic changes.* ***A-B)*** *Nuclear accumulation of β-catenin in a CD-SBC* ***(A)****, to be compared with a membranous/cytoplasmic reactivity of a CrD-SBC* ***(B). C)****. Intense SOX-9 expression in the tumor cell nuclei of a CD-SBC.* ***D)*** *Evidence for residual high grade dysplasia overlying the superficial part of an invasive CD-SBC (arrowheads).* ***E-F)*** *Nuclear SOX-9* ***(E)*** *and β-catenin (****F****) expression in a flat, low-grade, dysplastic lesion of a CD-SBC case. Note the villous atrophy and, in the inset of* ***E),*** *the extensive nuclear SOX-9 reactivity of hyperplastic crypts adjacent to dysplasia.*

G) *Same CD-SBC as in* ***(D)*** *showing discrepancy in MLH1 expression between the dysplastic (positive) and the underlying, invasive (negative) components.* ***H)*** *A normal jejunal mucosa adjacent to a sporadic SBC with nuclear SOX-9 expression restricted to the deep-half of the crypts. Note a few enteroendocrine cells with SOX-9 reactive cytoplasm and unreactive nucleus (arrows).* ***I)*** *SOX-9 nuclear expression in the atrophic mucosa distant from a CD-SBC, showing a prominent SOX-9 expansion to involve the upper half of the crypts, the crypt/villous junction and even the surface epithelium.* ***J)*** *A focus of MUC5AC-positive, high-grade dysplasia of a CrD-SBC case.* ***K)*** *A dysplastic lesion and an underlying small focus of diffuse type CrD-SBC (arrowheads), both concordantly reactive for CK7.* ***L)*** *An isolated, "microscopic" CK7-positive neoplastic lesion with focal stromal invasion in the small bowel mucosa distant from a "macroscopic" CrD-SBC.* ***M)*** *Diffuse and intense MUC5AC positivity of a polypoid adenomatous dysplasia adjacent to a CrD-SBC.*

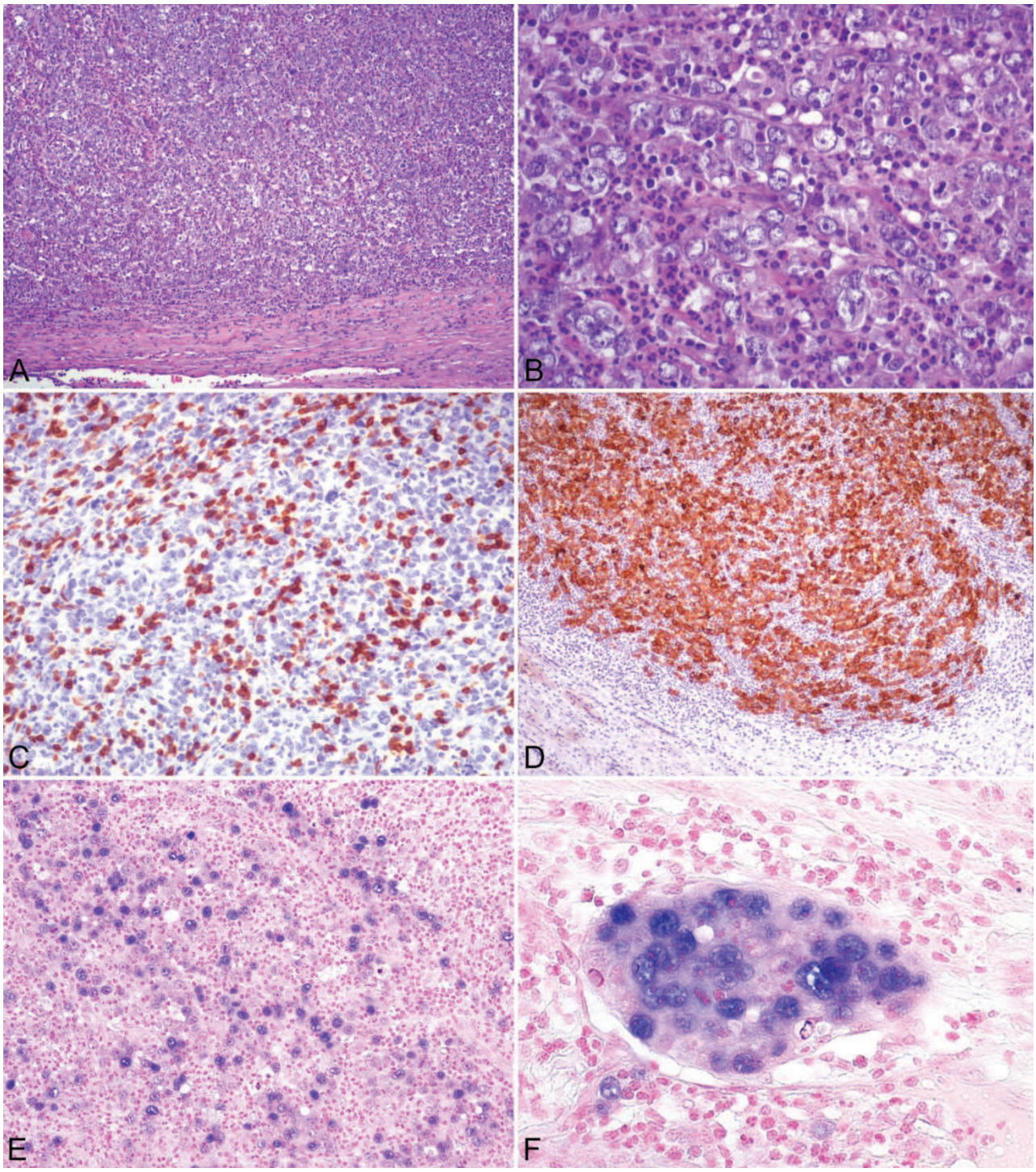

Figure 16 Lymphoepithelioma-like small bowel carcinoma associated with Crohn's disease. **A**, Low-power histology revealing a solid poorly differentiated tumor. **B**, High-power histology showing polygonal tumor cells, with vesicular nuclei, prominent nucleoli and eosinophilic cytoplasm, mixed with numerous lymphocytes and granulocytes. **C**, CD3 immunostain confirming the high number of intratumoral T lymphocytes. **D**, Tumor cells immunoreactive for cytokeratin 8/18. **E**, Epstein–Barr virus (EBV)-encoded RNA (EBER) in-situ hybridization demonstrating EBV infection in neoplastic cells. **F**, EBER positive tumor cells in ametastatic lymph node.

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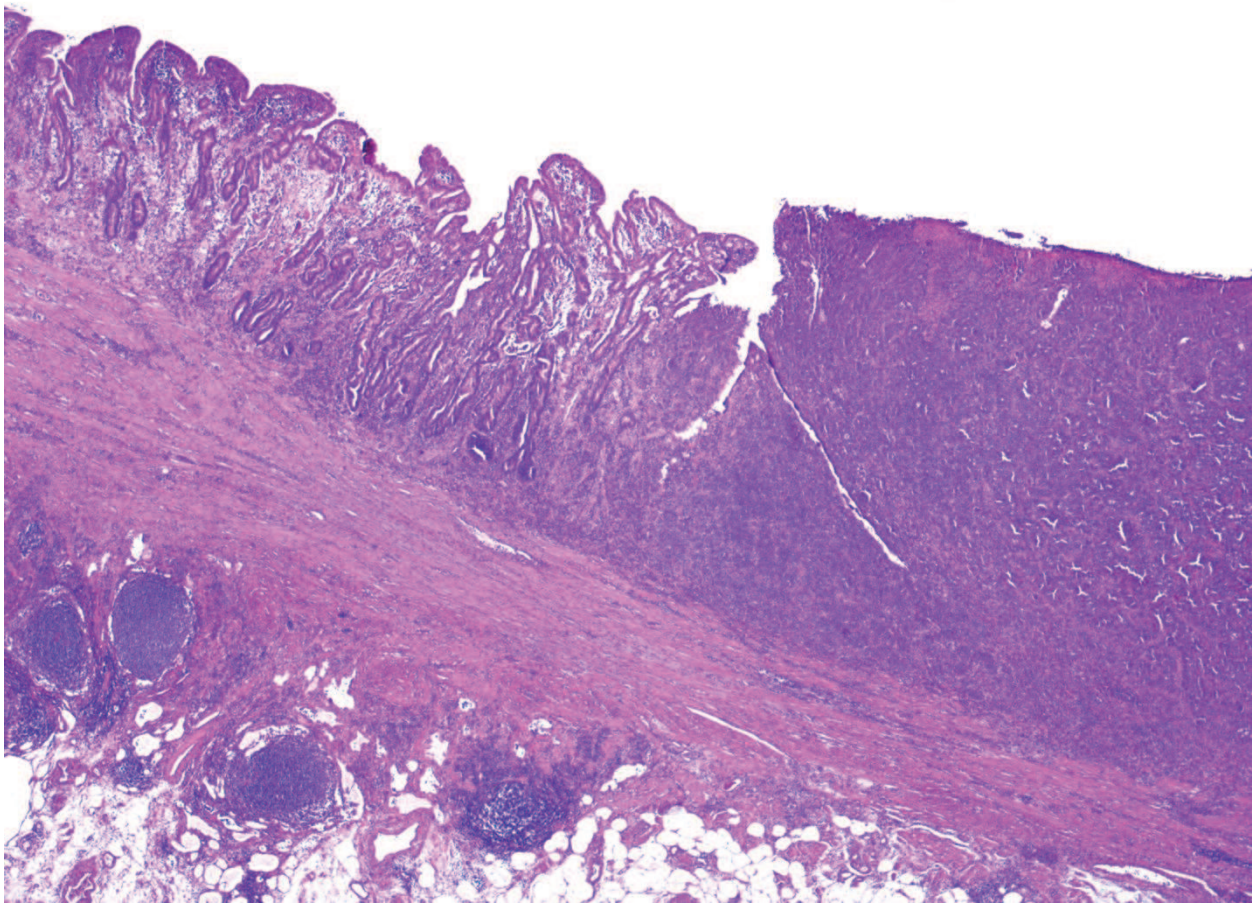

Figure 17. Small bowel adjacent to the carcinoma showing histologic features consistent with active Crohn's disease

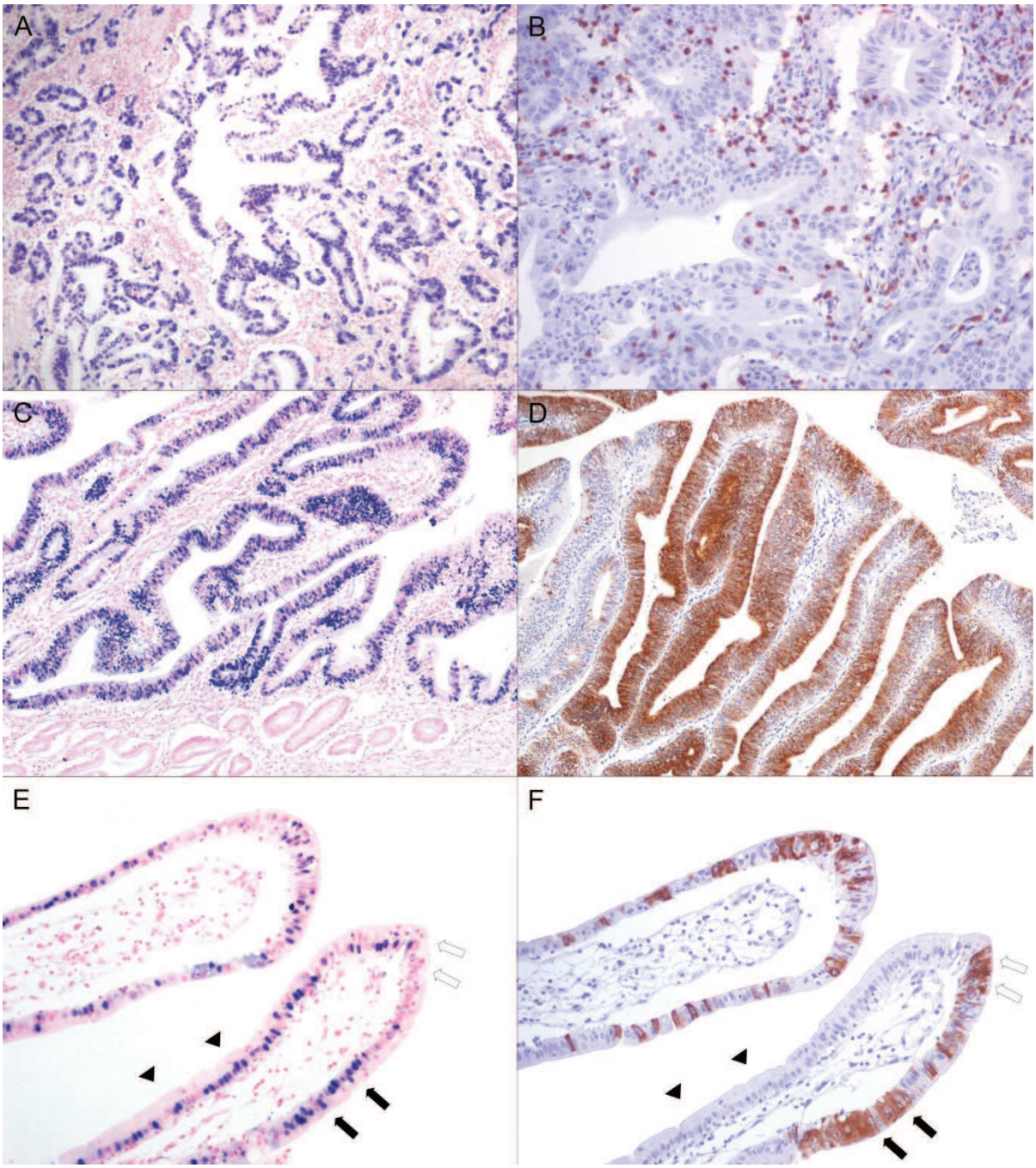

Figure 18. A) Diffuse EBER positivity (blue) of neoplastic nuclei in the glandular cancer (adenocarcinoma). **B**) Numerous $CD8^+$ TILs (brown) scattered within the cancerous epithelia. **C**) Extensive EBER positivity of an adenomatous growth adjacent to the invasive cancer. Note the lack of EBER positivity of normal crypts in the lower part of the micrograph. **D**) Heavy cytoplasmic immunostaining of the same villous adenoma as of **C)**, for the gastric foveolar marker MUC5AC. **E, F**) Consecutive sections of two villi in tumor-adjacent non-neoplastic mucosa showing many $EBER^+$ epithelial cells (**E**). These $EBER^+$ villous cells include both MUC5AC-reactive (black arrows) and unreactive (arrowheads) cells; in addition, $EBER^-$ $MUC5AC^+$ cells (empty arrows) are also found (**F**).

5. DISCUSSION

5.1 SURVIVAL DIFFERENCE BETWEEN CELIAC AND CROHN'S DISEASE CARCINOMAS

This is the largest study of SBC in CD and the only one systematically comparing CD-SBCs, CrD-SBCs and spo-SBCs. 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. (52) 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 to an equally numerous spo-SBC subgroup. A significant survival improvement was observed by Potter et al. (30) 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 five-year survival rate of our CD-SBC patients was as high as 83%, suggesting a relatively indolent behavior of CD-SBC.

5.2 PREVALENCE AND PROGNOSTIC INFLUENCE OF MICROSATELLITE INSTABILITY AND TUMOR-INFILTRATING LYMPHOCYTES

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 to 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. (30) (8/11, 73%) and Diosdado et al. (29) (6/9, 67%). With regards

to CrD-SBC, the low percentage of MSI in our cases (16%) is in agreement with those reported by Rashid et al. (10) (14%) and Svrcek et al. (37) (3%).

We confirm, in a larger series, the favorable prognostic influence of MSI suggested by previous studies (29,30). 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 CD-SBC, retained significant power in such a model. This finding seems relevant as TILs are known from CCR studies to be the main effector of MSI-related prognostic improvement (53,54). 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 (25%) of our SBCs, and in particular in MSI-positive SBC cases, seems interesting and is in line with the recently published data by Thota et al. (55), who observed PD-L1 expression on tumor cells and immune cells in 17% and 43% of their SBC cases, respectively. This findings may deserve further investigation in the light of the potential role of therapies targeting the programmed cell death (PD)/PD-L1 immune checkpoint pathway in SBC, as already demonstrated for colorectal or other solid cancers with MMR deficiency/MSI (56, 57).

5.3 PROGNOSTIC POWER OF SBC HISTOTYPE AND PHENOTYPE

Retention of intestinal phenotype and gland-forming capacity by SBC tumor cells was associated with better patient survival compared to acquisition of non-intestinal phenotype or non-glandular histologic structure. The prognostic power of glandular histology was found to retain significance in a multivariable model inclusive of stage, clinical subgroup and TIL density. When substituted

for glandular histology in the same model, the intestinal phenotype lost prognostic power, which, however, was retained when the clinical subgroups were eliminated from the model. It should be noted that the subgroup-independent behavior of the glandular histotype fits with its relatively uniform distribution among the clinical subgroups, while the group-sensitive behavior of the phenotype couples with the high, selective expression of non-intestinal markers, associated with worse prognosis, among CrD-SBCs. The latter finding may contribute to the poor survival shown by CrD-SBC patients. The histotype and phenotype approaches proposed here offer new tools for SBC prognostic evaluation, and these should be added to the clinical subgroup characterization and MSI status.

5.4 RAS, BRAF, PIK3CA, TP53 AND HER2 GENE ALTERATIONS

In spo-SBCs we observed a higher prevalence (48%) of *KRAS* mutation than in CrD-SBCs, which might be accounted for by the lower percentage we found in the latter subgroup (12%) in comparison to those reported by Rashid et al. (10) (43%) and Svrcek et al. (37) (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-EGRF targeted therapy could be beneficial (58). *HER2* amplification, although restricted to only five cases, also seems worth being considered as a potential therapeutic target (20).

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, either regarding protein overexpression or gene mutation.

Of note, there was no *BRAF* mutation in any case, including those harboring MLH1 hypermethylation. *BRAF* mutations are reported to be absent or extremely rare in spo-SBC (35,59) and in CrD-SBC (37). This finding seems to rule out *BRAF* mutation in inducing *MLH1* gene promoter methylation, which represents the almost exclusive cause of MSI in our non-familial SBCs. This conclusion is in contrast with the role which *BRAF* mutation plays in the majority of MSI sporadic colorectal cancers (60). Thus, the identification of a possible oncogene mutation activating a process of *MLH1* gene silencing in SBC cases remains an open issue.

5.5 β-CATENIN NUCLEAR EXPRESSION IS A HALLMARK OF CELIAC DISEASE-ASSOCIATED SMALL BOWEL CARCINOMAS

Like MSI, the nuclear accumulation of β-catenin was also found to be highly prevalent among CD-SBCs and to be apparently associated with improved survival. This seemed surprising as, unlike MSI, nuclear β-catenin expression has been reported by studies of other neoplasms to imply a less than favorable prognostic influence (61-63). However, when among β-catenin-positive SBCs, MSI and microsatellite stable cases were compared, or among microsatellite stable carcinomas, β-catenin-positive and negative cases were compared, the lack of favorable prognostic influence of β-catenin itself, in the absence of MSI, became evident. The distributive association we found, especially among CD-SBCs, between nuclear β-catenin expression and the prognostic favorable MSI status, might account for this misleading prognostic influence of β-catenin. Notably, such an association is at variance with CRC

findings, where nuclear β-catenin expression, highly prevalent among microsatellite stable cases, due to *CTNNB1* or *APC* point mutations, has been reported to be rare in MSI cases (64). Instead of point mutations, large *CTNNB1* N-terminal deletions have been reported to stabilize β-catenin in 20% of sporadic SBCs (16), a finding we confirmed by selective N- versus C-terminal β-catenin immunohistochemistry in our spo-SBCs and extended to about 40% of CD-SBCs. Whether additional stabilization mechanisms may account for the very high CD-SBC rate of nuclear β-catenin (around 90%) and for its association with MSI status, it remains to be further investigated.

5.6 SOX-9 EXPRESSION IN SMALL BOWEL CANCER AND NON-NEOPLASTIC MUCOSA

We found that another Wnt-related protein, the SOX-9 transcription factor, was also highly expressed in SBCs, with a distribution comparable to that of nuclear β-catenin, at least among CD-SBCs. This finding seems relevant, as SOX-9 high expression has been reported in several cancers, including colorectal, gastric, pancreatic, hepatocellular, brain, lung and prostate cancers (65). In some of these cancers, SOX-9 expression was found to be associated with tumor progression, invasion and metastasis, a pattern not seen in our SBCs. However, an important SOX-9 role in carcinogenesis has also been proposed (65), at least in part through Wnt activation via frizzled and LRP receptor overexpression (66, 67). In keeping with this hypothesis, SOX-9 overexpression has also been reported in some precancerous conditions, including metaplastic and dysplastic lesions of chronic H. pylori gastritis (68, 69), chronic bladder injury (70) and early stages of colorectal tumorigenesis (65) as well as in ‘acute’ untreated adult CD (71). This led us to investigate

non-tumor mucosa of SBC-bearing patients for SOX-9 expression. In most CD-SBC cases we frequently observed signs of persistent mucosal damage, including atrophy of the villi, excessive intraepithelial T lymphocyte infiltration and goblet cell-poor, relatively immature, crypt hyperplasia, not unlike those reported in biopsies of some non-neoplastic adult CD patients, even when under gluten-free diet (72). Notably, multifocal extension of nuclear SOX-9 expression, usually involving the upper half of the hyperplastic crypts was found in most CD cases investigated, even in the mucosa at a distance from the neoplasm. By itself, the topographic continuity we observed at some foci between SOX-9 positive crypt hyperplasia and intramucosal dysplastic or cancerous growths may be suggestive for a histogenetic link between such lesions.

A role for SOX-9 in the mucosa repair process of persistent CD lesions seems conceivable considering its known role in intestinal mucosal repair from other types of damage (73). The SOX-9 activating role of NF-kB transcription factor, as ascertained, for instance, in experimental *H. pylori* gastritis (69), seems especially important. Indeed, NF-kB activity, in turn activated by pro-inflammatory cytokines, has been shown to be enhanced in many immune-inflammatory processes, including IBD and related carcinogenesis (74) as well as CD (75). In fact, a switch from the known SOX-9 role in adult stem cell modulation to cancer stem cell activation, often chronic injury promoted and involving constitutive oncogene activation or oncosuppressor gene silencing, has been suggested by several studies (65, 70, 76-78).

5.7 DISTINCT HISTOGENESIS OF CELIAC DISEASE-ASSOCIATED AND CROHN'S DISEASE-ASSOCIATED SMALL BOWEL CARCINOMAS

Our evidence of polypoid adenomatous remnants in the superficial part of CrD-SBCs and spo-SBCs supports the hypothesis that the adenoma-carcinoma sequence, well known from colorectal neoplasms, is also operative for these two types of SBC (37, 79, 80). In CD-SBC we obtained evidence for a possible adenomatous polyp origin in only a single case, thus confirming the previous finding (36, 81) of a substantial lack of such lesions in Cd cases, while we found four CD-SBCs with flat dysplasia reactive for both nuclear β-catenin and SOX-9.

Metaplastic changes showing the same gastric and/or pancreatobiliary phenotype as found in the associated cancer were frequent in dysplastic or non-dysplastic mucosa adjacent to CrD-SBCs, though not to CD-SBCs or spo-SBCs. This finding may suggest a precancerous role of such lesions in CrD. Thus, two distinct histogenetic processes seem at work for CD-associated and CrD-associated SBCs, starting from immature crypt hyperplasia or epithelial metaplasia, respectively. Although a more extensive, prospective and systematic search for hyperplastic, metaplastic and dysplastic lesions is warranted for a better understanding of SBC histogenesis in the two types of immune-inflammatory conditions we studied, the present findings may indicate promising lines of investigation to identify preneoplastic lesions of potential help in early cancer diagnosis.

5.8 EPSTEIN-BARR VIRUS POSITIVE ILEAL CARCINOMAS

A small group of medullary-type cancers, i.e. a solid structure with well demarcated “pushing” borders, found to have a good prognosis in accordance with previous studies (82-84), were distinct from solid non-medullary cancers. As expected, all SBCs with medullary-type histology had high TILs and five of the six cases also harbored MSI. The single medullary-type microsatellite stable case was tested with EBER *in situ* hybridization, finding it to be diffusely positive. Therefore, we searched for the presence of EBV, using EBER *in situ* hybridization, in tumor cells of all CrD-SBCs and a second case of EBV^+ ileal SBC was detected. Unlike the first case, which was suspected because of a LEC histology, this case showed a conventional, non-distinctive, gland-forming structure. Both EBV^+ cases turned out to have increased TILs despite the lack of MSI status, the main cause of high TILs in SBCs. In keeping with the molecular changes known to be involved in EBV^+ cancers (85), we detected a *PI3KCA* gene mutation in the glandular histology case, nuclear β-catenin expression in the LEC case, and the absence of *TP53* mutations in both cases. Both EBV^+ SBC patients had a favourable post-operative prognosis. This is an interesting finding considering the generally poor prognosis of CrD-SBC patients, with a median survival of 28 months in our series.

In both EBV^+ SBC cases we detected a clear-cut EBER signal also in adenomatous dysplastic (non-invasive) lesions associated with the cancer growth as well as in small foci of iuxta-tumoral epithelium apparently lacking signs of dysplasia, a finding which may support the hypothesis of an initiating or very early direct role of EBV in the carcinogenetic process (85-88). The reactivity of EBV^+ adenomatous components for the gastric foveolar cell

marker MUC5AC, known to be expressed in about half of EBV^+ gastric cancers (42, 85, 87, 88), and shown to be also largely expressed in CrD-SBCs (89 and present study), is also particularly interesting. However, our detection of EBV in both $MUC5AC^+$ and $MUC5AC^-$ non-dysplastic epithelium, likely the earliest site of viral infection during the carcinogenetic process, does not support MUC5AC expression or a foveolar-type metaplastic change of ileal enterocytes as a needed prerequisite for latent viral infection. Interestingly, EBV was reported to be the most prevalent intestinal viral infection in inflammatory bowel disease, and it has been shown to spread from immune to epithelial cells (90). However, in infected intestinal epithelial cells of cancer-free inflammatory bowel disease patients, EBV usually establishes a productive, lytic phase of its life cycle without expression of latency-associated EBER (90), known to have oncogenic properties by promoting cellular growth and modulating innate immunity in EBV-associated cancers (85). Therefore, EBV^+ carcinomas may occur in the ileum of CrD patients and are characterised by increased TILs in the absence of MSI, either with LEC or with a more conventional glandular histology. EBV infection should thus be investigated in such cancers irrespective of their histologic type, considering the better prognosis these neoplasms seem to show.

5.9 CONCLUSIONS

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 SBCs 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 (91,92). 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 (93). 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) (26), 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 prognosis, tumor cell phenotype, MSI/TIL status, Wnt pathway activation, and mucosal precursor lesions.

5.10 FUTURE PERSPECTIVES

This PhD study identified specific histopathological, molecular and prognostic features of SBCs arising in celiac patients that suggest biological and clinical similarities with those described in CRC showing MSI and CpG Island Methylator Phenotype (CIMP). Recently, the Consensus Molecular Subtype (CMS) Consortium proposed the current best description of CRC heterogeneity at the gene-expression level, after analysis of 18 different CRC gene expression datasets (94). This transcriptomic classification enables the categorization of most CRCs into one of the four robust subtypes. Most CRCs with MSI cluster in the CMS1 group (MSI immune subtype, 14%), which is characterized by hypermutation, hypermethylation, enrichment for BRAFV600E mutations and evidence of strong immune activation (immune response, PD1 activation, natural killer (NK) cell, T helper 1 cell and cytotoxic T cell infiltration signatures), consistent with pathological descriptions of prominent CD8+ TILs. Next, CRCs with chromosomal instability can be classified into three groups based on gene expression signals: CMS2 (canonical subtype, 37%); CMS3 (metabolic subtype, 13%); and CMS4 (mesenchymal subtype, 23%).

Therefore, the next step of this project will be to evaluate the presence of CIMP in our series of CD-SBCs collected by the Small Bowel Cancer Italian Consortium and, for the same samples, to generate RNA sequencing data in order to compare the transcriptional signatures of CD-SBC with the four CMS of CRC. The final goal of the project will be to give new insights of clinical and therapeutic interest to guide CD-SBC management.

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