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Identifying pathogenic, prognostic and theragnostic factors in cancer-associated gastrointestinal inflammation

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Dedicated to Benim Askim

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Abstract

Background and aims

Small bowel adenocarcinomas (SBAs) are frequently associated with severe prognosis and have restricted therapeutic options. Programmed cell death protein-1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway blockade is an effective treatment in many microsatellite instability-high (MSI-H) solid tumours. Additionally, a minority of Crohn's disease-associated SBAs (CrD-SBAs) show a relatively favourable behaviour, thus highlighting the need to improve the histopathologic prediction of CrD-SBA prognosis. We aimed at investigating PD-L1 and PD-1 expression in non-hereditary, non-ampullary SBAs, associated with coeliac disease (CoeD), Crohn's disease (CrD) or sporadic, recruited through the Small Bowel Cancer Italian Consortium. Secondary aim was to assess the invasive front markers tumor budding (Tb) and poorly differentiated clusters (PDCs) on CrD-SBAs investigated also for the primary aim.

Methods

We evaluated PD-L1 and PD-1 by immunohistochemistry in a cohort of 121 surgically resected SBAs, i.e. 34 CoeD-SBAs, 49 CrD-SBAs, and 38 sporadic SBAs. PD-L1 and PD-1 expression was correlated with several clinico-pathological features, including the aetiology, microsatellite instability status and tumour-infiltrating lymphocyte (TIL) density. We then systematically analysed the Tb and PDCs in the invasive front of 47 CrD-SBAs.

Results

The prevalence of PD-L1 positivity according to combined positive score (CPS) was 25.6% in the entire cohort of SBAs, with significantly ($p=0.001$) increased percentage (35%) in both CoeD-SBAs and CrD-SBAs compared to sporadic SBAs (5%). CPS≥1 SBAs were significantly ($p=0.013$) more frequent in MSI-H cases (41%) than in non-MSI-H ones (18%); however, 15 CPS≥1 microsatellite stable SBAs were also found. CPS≥1 SBAs displayed higher TIL and PD-1$^+$ immune cell density, more often medullary histotype, as well as a better outcome compared to CPS<1 cases. Both Tb and PDC analyses proved highly effective in prognostic assessment of CrD-SBA. In addition, they retained prognostic power when combined with two other parameters, i.e. glandular histology and stage I/II, both known to predict a relatively favourable SBA behaviour. In particular, association of Tb and PDCs in a combined invasive front score allowed to find a minor

subset of cancers (12/47, 25%), characterised by combined invasive front-low grade associated with a glandular histology and a low stage (I or II) and displaying no cancer-related death over a median follow-up of 73.5 months.

Conclusions

This study demonstrates an increased proportion of PD-L1$^+$ cases in both CoeD-SBAs and CrD-SBAs in comparison with sporadic SBAs. In addition, the identification of a subset of PD-L1$^+$ microsatellite stable SBAs supports the need to ascertain additional biomarkers of response to immune checkpoint inhibitors along with MSI-H. The improved separation of lower from higher grade CrD-SBAs provided by invasive front analysis should represent an additional help in choosing appropriate therapy for these rare and frequently ominous cancers.

Introduction

Small bowel adenocarcinomas: introduction

Small bowel adenocarcinomas (SBAs) are remarkably uncommon neoplasms, frequently sporadic. However, there are several predisposing conditions including hereditary syndromes, namely familial adenomatous polyposis, Lynch syndrome, Peutz-Jeghers syndrome, and juvenile polyposis syndrome, and chronic immune-mediated intestinal disorders, i.e. coeliac disease (CoeD) and Crohn's disease (CrD) (Table 1) [1]. The underlying gut disorder, that is CoeD or CrD, has been shown to be a stage-independent prognostic factor in patients undergoing surgery for SBA [2]. Although both CoeD and CrD are sustained by analogous immune-mediated mechanisms, namely T helper 1 and 17 responses [3], CoeD-associated SBA (CoeD-SBA) and CrD-associated SBA (CrD-SBA) represent distinct cancers in terms of clinical, histopathological, and molecular features (Table 2). The jejunum is the most frequent location for SBA in CoeD [2], an immune-mediated enteropathy triggered by dietary gluten in genetically susceptible individuals [4]. CoeD-SBA exhibits a high frequency of microsatellite instability (MSI), increased tumour-infiltrating T lymphocytes (TIL), a glandular histotype, and an intestinal phenotype [2,5–7]. Conversely, SBA often localises in the inflamed ileum in CrD [2,8], one of the two main forms of inflammatory bowel disease due to an excessive immune response towards commensal microbiota [9]. In particular, SBA always arises in CrD patients with small bowel involvement [8,10]. Unlike CoeD-SBA, most CrD-SBAs are microsatellite stable, have low TILs and frequently show a non-glandular histotype associated with a non-intestinal phenotype [2,7,8,10–12].

Table 1. Risk factors for small bowel adenocarcinoma.

Risk Factor
Inherited Tumour Syndromes
Familial adenomatous polyposis
Peutz-Jeghers syndrome
Hereditary nonpolyposis colon cancer syndrome (Lynch syndrome)
Juvenile polyposis syndrome
MUTYH-associated polyposis
Other Genetic Disorders
Cystic fibrosis
Immune-Mediated Intestinal Disorders
Coeliac disease
Crohn's disease
Other Causes
Small bowel sporadic adenomatous polyps
Long-standing ileostomy

Table 2. Clinical, histopathological and molecular features of small bowel adenocarcinomas (SBAs) according to the aetiologic group.

Feature	CoeD-SBA	CrD-SBA	Sporadic SBA
Age at diagnosis	53–62 yrs [2,5,6,13,14]	42–73 yrs [2,8,10–12,15–22]	56.5–72.1 yrs [2,5,6, 10,17,20,22]
Site	Jejunum and duodenum [2,5,6,13,14]	Ileum [2,8,10–12,15–17,19-22]	Jejunum and duodenum [2,5,6,10,17,20]
MSI status	65–73% [2,5,6]	0–16% [2,8,10–12]	9–35% [2,5,10,23,24]
Tumour cell phenotype	Intestinal [7]	Non-intestinal [7,20]	Intestinal [7]
Oncogenic viruses	Unknown	EBV latent infection [25,26]	No EBV infection [27]

CoeD-SBA, small bowel adenocarcinoma associated with coeliac disease; CrD-SBA, small bowel adenocarcinoma associated with Crohn's disease; EBV, Epstein-Barr Virus; MSI, microsatellite instability; yr, year.

Epidemiology and risk factors for small bowel adenocarcinoma

Although small bowel corresponds to the 75% of digestive tract length and the 90% of digestive absorptive surface [28], SBAs are relatively rare cancers and account for less than 5% of all gastrointestinal neoplasms [29]. Notwithstanding, they represent around 40% of all small intestine malignancies [29]. The estimated incidence of SBA ranges between 3,250 and 5,300 cases each year in the USA [30,31], whereas it is about 3,600 annual new cases in Europe [32]. The relative risk of developing SBA in CoeD and CrD raises 14 and 33 times in comparison to the general population, respectively [33,34]. Amongst all SBAs, the 13% and the 7% seem to be associated with CoeD and CrD, respectively [13,34]. The epidemiological features of SBA differ on the basis of underlying chronic immune-mediated intestinal disorder. The median age at CoeD-SBA diagnosis has been estimated from 53 to 62 years in American, British, Dutch and Italian patients [2,5,6,13,14], while that at CrD-SBA diagnosis seems to be younger varying from 42 to 53 years in most studies (Table 3) [2,8,10–12,15–21]. Conversely, in an American large-scale retrospective cohort study, CrD-SBA patients presented at a median age of 72.9 years [22]. Recently, the Small Bowel Cancer Italian Consortium also showed an older median age at CrD-SBA diagnosis, i.e. 59 years [2]. We speculate that this discrepancy might be explained by an older age at CrD diagnosis in the latter cohort (50 years) [2] and by a better clinical management of CrD over the last two decades. Sporadic SBA patients often have a higher median age at diagnosis -between 56.5 and 72.1 years- in comparison to both CoeD-SBA and CrD-SBA [2,5,6,10,17,20,22]. Risk factors for CoeD-SBA and CrD-SBA have not thoroughly assessed. According to the well-established protective effects of adherence to gluten-free diet against malignant complications in CoeD [13,35], a strict gluten-free diet also seems to reduce the risk of CoeD-SBA development. Accordingly, Elfström and colleagues [36] demonstrated that the risk of small intestine neoplasms in coeliac patients decreases, though not disappearing, after the first year of follow-up, likely by reducing intestinal inflammation and mucosal damage [37].

However, several CoeD-SBAs have been described in patients under gluten-free diet; therefore, other factors have to be involved. Interestingly, the median age at CoeD diagnosis in patients developing SBA varies from 49 to 59 years (Table 3) [2,5,6], around two-three decades higher than that of coeliac patients not evolving into neoplastic complications [38]. Therefore, the diagnostic delay, a known risk factor for refractory CoeD and, thus, for enteropathy-associated T-cell lymphoma [39], has been supposed to play a role in CoeD-SBA. Notwithstanding, only one SBA case has been hitherto reported in association with refractory CoeD [2], a finding suggesting a different pathogenesis between SBA and enteropathy-associated T-cell lymphoma in CoeD. On the other hand, there is no evidence for a role of diagnostic delay in CrD-SBA development in most studies (Table 3), although diagnostic delay is often associated with more aggressive CrD phenotypes, such as stricturing and penetrating behaviours [40]. Risk factors observed for CrD-SBA encompass a long disease duration, a small bowel involvement, a stricturing phenotype and bypassed segment(s) of small bowel [41]. As regards long disease duration, in a French study involving 1,935 patients with small bowel location at CrD diagnosis a cumulative risk of SBA has been assessed as 0.2% and 2.2% after 10 and 25 years of follow-up, respectively [17]. Although use of 6-mercaptopurine seemed to be a risk factor for CrD-SBA in an American study including seven cases [42], no medical treatment has been unquestionably found to be coupled with SBA in larger cohorts [18,43]. Conversely, small bowel resection and use of salicylates for more than two years protect against SBA in patients with CrD [18]. As regards gender, the rates of female prevalence are extremely heterogeneous in both CoeD-SBA (25–62%) [2,5,6,13,14] and CrD-SBA (29–60%) [2,8,10–12,15–22] so that it is hard to assess a gender predominance in either conditions. However, considering the strong prevalence of CoeD in women [4], these data may suggest that male gender is at higher risk to develop CoeD-SBA.

The incidence of SBA cases as a whole is doubled in African Americans (from 10.2 to 14.1 per 1,000,000) compared to Caucasians (from 4.5 to 7.2 per 1,000,000) [44,45]. On the other hand, CrD-SBA has been shown to affect more frequently Caucasians in an American large-scale retrospective study from 1992 to 2010 [22]. Similarly, CoeD-SBAs have been described exclusively in Caucasians in the only study analyzing ethnic differences in this aetiologic group [13]. It seems that more extensive investigations of epidemiology and risk factors are needed.

Table 3. Studies on small bowel carcinomas associated with coeliac disease or Crohn's disease.

Authors, Year	Pt	Age at SBC dgn (Median, Range, yrs)	Age at CoeD or CrD dgn (Median, Range, yrs)	CoeD or CrD duration at SBA dgn (Median, Range, yrs)	Stage III/IV (%)	Overall Survival (%)	Main Findings
Small bowel adenocarcinoma associated with coeliac disease (CoeD-SBA)							
Bruno JC et al., 1997 [14]	6	62, 45–75	NA	17, 0–40	NA	NA	No evidence of flat dysplasia was present
Howdle PD et al., 2003 [13]	23	62*, 47–80	NA	8.2, 0.8–36	NA	NA	CoeD-SBAs account for 13% of all SBAs
Potter DD et al., 2004 [5]	17	59.5, 42–78	53, 25–77	NA	8/17 (47)	64.2 at 5 yrs	CoeD-SBAs have a high incidence of mismatch repair deficiency
Diosdado B et al., 2010 [6]	15	61, 47–79	59, 18–79	2.5, 0–32.3	NA	NA	CoeD-SBAs have promoter hypermethylation of the *APC* gene

Vanoli A et al., 2017 [2,7]	26	53, 28–80	49, 7–79	1.4, 0–25	8/26 (31)	83 at 5 yrs	CoeD-SBAs harbour MSI, high TILs and nuclear β-catenin expression frequently and show a better outcome in comparison with CrD-SBAs
Small bowel adenocarcinoma associated with Crohn's disease (CrD-SBA)							
Michelassi F et al., 1993 [15]	7	47.7*, 33–73	24, 11–57	20, 10–30	NA	6 mos (mean)	Survival is worse in CrD-SBA than in colorectal cancer complicating CrD
Rashid A et al., 1997 [11]	8	45.5, 35–71	33.5	NA, 0–30	0/7 (0)	28.5 mos (median)	CrD-SBAs have *RAS* and *TP53* mutations
Sigel JE et al., 1999 [16]	8	42, 35–71	35, 23–52	12, 0.6–19	2/8 (25)	NA	Most CrD-SBAs have dysplasia adjacent to carcinoma
Palascak-Juif V et al., 2005 [17]	20	47, 33–72	36, 15–54	16, 0–37	11/20 (55)	35 at 5 yrs	Signet-ring cells were found in 7/20 CrD-SBAs
Piton G et al., 2008 [18]	29	45, 29–74	34, 13–63	7, 0–52	NA	NA	Small bowel resection and salicylate intake ≥2 yrs protect against CrD-SBA

Widmar M et al., 2011 [19]	29	55.4, 22–81	25, 13–63	25.2, 0.8–51.3	16/29 (55)	NA	Two clinical indicators of SBA were symptoms in longstanding quiescent CrD and obstruction refractory to medical therapy
Svrcek M et al., 2014 [8]	41	47	NA	13.5	19/41 (46)	NA	40/41 CrD-SBAs were observed in inflamed mucosal areas. Flat or raised dysplasia was found in 20/41 patients with CrD-SBA
Whitcomb E et al., 2014 [20]	11	47, 42–77	24, 6–33	25, 10–40	NA	NA	10/11 CrD-SBAs expressed at least a gastric marker and 8/11 CrD-SBAs expressed the pancreatobiliary marker CK7
Weber NK et al., 2015 [21]	34	52.9, 32–74	22.4, 13.0–69.3	22.3, 0–50.6	NA	52 at 2 yrs	Imaging features suggestive for CrD-SBA included annular mass, nodularity at the extraluminal margins of mass, and perforation
Grolleau C et al., 2017 [10]	9	46, 37–67	36, 10–67	15, 0–32	5/9 (56)	56 at 2 yrs	Adjacent dysplasia was present in 9/9 CrD-SBAs
Bojesen RD et al., 2017 [12]	23	53, 37–85	NA	NA	NA	26 at 5 yrs	79% of CrD-SBAs showed inflammation-dysplasia-carcinoma sequence

Wieghard N et al., 2017 [22]	179	72.9	NA	NA	71/179 (40)	3.9 yrs (median)	CrD-SBAs have similar overall survival compared to sporadic SBAs
Vanoli A et al., 2017 [2,7]	25	59, 33–84	50, 22–84	13, 0–41	13/25 (52)	38 at 5 yrs	CrD-SBAs exhibit a low rate of MSI and TILs CrD-SBAs are associated with dysplasia and metaplasia, both showing gastropancreatobiliary phenotype
Vanoli A et al., 2017 [26]	31	59, 33–84	NA	NA	17/31 (55)	NA	EBV^+ CrD-SBAs may occur

CoeD, coeliac disease; CK, cytokeratin; CrD, Crohn's disease; dgn: diagnosis; EBV, Epstein-Barr Virus; mo, month; MSI, microsatellite instability; NA, not available; Pt, patient; SBA, small bowel adenocarcinoma; TIL, tumour-infiltrating lymphocyte: yr, year. *, mean.

Histopathology and molecular biology of small bowel adenocarcinoma

In general, SBAs as a whole have a predominance (52–60%) of glandular histotype [7]. However, medullary-type cancers have been observed in association with CoeD-SBA [7,46], whereas poorly cohesive, diffuse-type cancers or mixed glandular/diffuse cases are more frequent in CrD-SBA in comparison to CoeD-SBA and sporadic SBA [7,17]. Most CoeD-SBAs and sporadic SBAs express intestinal phenotype markers, such as the caudal-related homeobox transcription factor (CDX)2, the goblet cell marker mucin (MUC)2, cytokeratin (CK)20 and/or the small bowel brush border marker CD10. Conversely, CrD-SBAs frequently present metaplastic gastropancreatobiliary changes, characterised by positivity for the gastric foveolar marker MUC5AC and/or the pancreatobiliary duct marker CK7 [7,20].

A high density of $CD3^+$ and $CD8^+$ TILs is typical in CoeD-SBA, while TILs are frequently low in both CrD-SBAs and sporadic SBAs [2]. This finding points to a greater host immune response against tumour in CoeD-SBA in comparison with CrD-SBA and sporadic SBA, thus leading to a better clinical outcome reported in CoeD-SBA (Table 3). Nevertheless, this does not prevent tumour growth, probably due to an increased immune tolerance. In particular, it has been hypothesized that programmed cell death ligand 1 (PD-L1) and programmed cell death protein-1 (PD-1), crucial immune checkpoints aimed at inhibiting and escaping immune surveillance, are also implicated in non-hereditary SBAs as well as in colorectal and gastric cancers with MSI and/or Epstein-Barr Virus (EBV) infection [47,48]. An American study on 42 sporadic SBAs showed PD-1 expression on intratumoural and peritumoural lymphocytes in most cases, and PD-L1 expression on neoplastic cells and immune cells, mainly histiocytes, in a minority of cases [49]. To the best of our knowledge, no study assessed clonality of TILs in CoeD-SBA.

As shown in Table 4, molecular alterations were investigated in some studies recruiting cohorts with at least 5 cases of CoeD-SBA and/or CrD-SBA. MSI, which is a consequence of defective DNA mismatch repair and is verified by mean of molecular and/or immunohistochemical analysis, is present in around one third of all non-hereditary SBAs with significant differences between CoeD-SBA (65–73% MSI) [2,5,6], CrD-SBA (0–16% MSI) [2,8,10–12], and sporadic SBA (9–35%) [2,5,10,23,24] (Tables 2 and 4). MSI causes the anti-tumour immune response supposed to play a pivotal role in inducing a more favourable outcome in these cancers. Genomic profiling of sporadic SBA showed some genetic alterations affecting most frequently *TP53* (mutated 58% of cases), *KRAS* (53.6%),

APC (26.8%), *SMAD4* (17.4%) and *PIK3CA* (16%) [50,51]. Overexpression of the *TP53* gene product has been reported in roughly half of cases in both CoeD-SBA and CrD-SBA, thus confirming the crucial role of *TP53* alterations in small bowel carcinogenesis [2,8,10], as well as in inflammatory bowel disease-associated colorectal cancers [52]. *KRAS* mutation, which is an early change in the adenoma–carcinoma sequence of colorectal cancer, has been also described in 31% of CoeD-SBA and in 12–43% of CrD-SBA [2,8,10,12,53].

Promoter hypermethylation of *APC* has been found in 73% of CoeD-SBA, whereas nonsense *APC* mutations have not been observed in CoeD-SBA [6]. Similarly, allelic loss of *APC* gene was infrequent in CrD-SBA [10]. Nevertheless, the involvement of Wnt/β-catenin pathway has been described in most CoeD-SBAs and sporadic SBAs, as suggested by aberrant nuclear β-catenin expression [7,54,55]. Conversely, nuclear translocation of β-catenin has been demonstrated only in few CrD-SBAs [7,8]. To the best of our knowledge, there are no data on *SMAD4* mutation frequency in CoeD-SBA and CrD-SBA. *BRAF* V600E mutation, which is remarkably infrequent in sporadic SBA [54], is also absent in both CoeD-SBA and CrD-SBA [2,10,11] or identified up to 7% of CrD-SBA in other studies [8,12]. Thus, unlike colorectal cancer [56,57], *BRAF* mutation does not seem to play a pivotal role in inducing *MLH1* gene methylation, a frequent finding in CoeD-SBA [2,6]. No significant difference has been observed in *PIK3CA* or *NRAS* mutation rate amongst CoeD-SBA, CrD-SBA and sporadic SBA [2,8]. Additionally, genomic profiling showed potentially targetable genetic alterations in most SBA cases (91%) [50]. Laforest and colleagues [58] described *ERBB2*/*HER2* alterations in 12% of sporadic SBAs, through mutations (7 cases) or amplifications (3 cases). *HER2* amplification was also observed in two CoeD-SBAs and in two CrD-SBAs [2].

Table 4. Molecular alterations in small bowel adenocarcinomas associated with coeliac disease or Crohn's disease.

Authors, year	Pt	MSI status N (%)	*KRAS* mutation N (%)	*NRAS* mutation N (%)	*BRAF* mutation N (%)	*PIK3CA* mutation N (%)	*HER2* amplification N (%)	p53 overexpression N (%)	Nuclear β-catenin expression N (%)
Small bowel adenocarcinoma associated with coeliac disease (CoeD-SBA)									
Potter DD et al., 2004 [5]	17	8/11 (73)	NA	NA	NA	NA	NA	NA	NA
Diosdado B et al., 2010 [6]	15	6/9 (67)	NA	NA	NA	NA	NA	NA	NA
Vanoli A et al., 2017 [2,7]	26	17/26 (65)	8/26 (31)	1/26 (4)	0/26 (0)	4/26 (15)	2/26 (8)	12/26 (46)	24/26 (92)
Small bowel adenocarcinoma associated with Crohn's disease (CrD-SBA)									
Rashid A et al., 1997 [11]	8	1/7 (14)	3/7 (43)	NA	NA	NA	NA	4/7 (57)	NA
Svrcek M et al., 2014 [8]	41	1/36 (3)	7/30 (23)	NA	1/29 (4)	0/23 (0)	NA	21/35 (60)	16/31 (52)
Grolleau C et al., 2017 [10]	9	1/9 (11)	1/8 (12.5)	NA	0/8 (0)	NA	NA	NA	NA
Bojesen RD et al., 2017 [12]	23	0/14 (0)	2/14 (14)	NA	1/14 (7)	NA	NA	NA	NA
Vanoli A et al., 2017 [2,7]	25	4/25 (16)	4/25 (12)	1/25 (4)	0/25 (0)	2/25 (8)	2/25 (8)	12/25 (48)	6/24 (25)

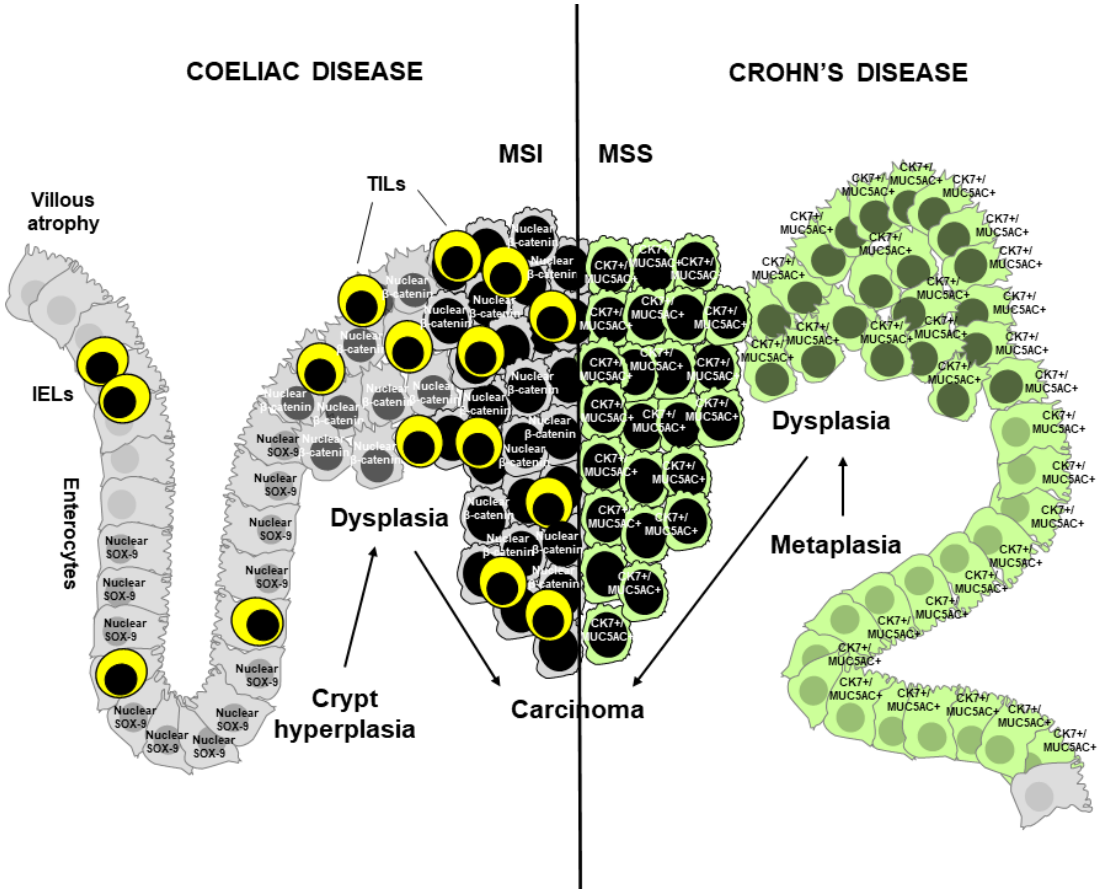
MSI, microsatellite instability; NA, not available; Pt, patient.

Pathogenesis and preneoplastic lesions of small bowel adenocarcinoma

The exact pathogenesis of non-hereditary SBA is mostly unknown due to their rarity. Dysplastic lesions close to CoeD-SBA are quite rare [7,14,59], whereas the recurrent presence of dysplasia in the superficial part of both CrD-SBA and sporadic SBA has been observed [7,8,60,61]. In addition, either in CrD-SBA or in CoeD-SBA, dysplasia has been reported as flat or raised [7,8,10-12,59]. Dysplasia is distant or adjacent to CrD-SBA [7,8,10-12], while no dysplasia has been described far from CoeD-SBA [7,59].

Dysplasia close to both CoeD-SBA and CrD-SBA is characterised by overexpression of p53 and retained reactivity for mismatch repair proteins [7]. Of note, loss of MLH1 is infrequently observed in dysplasia associated with MSI CoeD-SBA [7], thus suggesting that *MLH1*-hypermethylation-related MSI is a late event along small bowel carcinogenesis in coeliac patients. Furthermore, the rare dysplastic *foci* adjacent to the invasive CoeD-SBA have been reported to express nuclear β-catenin, whereas CrD dysplasia shows a preserved membranous expression of β-catenin [7]. Therefore, Wnt pathway activation seems to be an early process in CoeD-SBA carcinogenesis. Accordingly, overexpression of the Wnt-related transcription factor and stem cell marker Sex-determining Region Y-Box (SOX) 9 has been described in hyperplastic crypts of coeliac patients at CoeD diagnosis [62], as well as in CoeD-SBA tumour cells, in continuity with SOX-9$^+$ close dysplastic and hyperplastic crypts (Figure 1) [7]. This may suggest a histogenetic association between crypt hyperplasia and CoeD-SBA. On the other hand, a gastropancreatobiliary metaplastic phenotype has been predominantly described in dysplastic or non-dysplastic mucosa adjacent to CrD-SBA [7,20]. Although small bowel dysplasia has been found to have a low sensitivity (33%) at enteroscopy in CrD patients at high risk of SBA [63], MUC5AC-positive or CK7-positive metaplastic changes at perendoscopic biopsies should lead CrD patients to a strict endoscopic follow-up. Immature crypt hyperplasia and gastropancreatobiliary metaplasia might be reckoned as possible preneoplastic lesions, likely evolving into dysplasia and carcinoma, in CoeD and CrD, respectively (Figure 1). Thus, an inflammation-hyperplasia-dysplasia-carcinoma sequence may take place in CoeD-SBA development, whereas an inflammation-metaplasia-dysplasia-carcinoma sequence may occur in CrD-SBA pathogenesis. Further extensive and prospective studies are necessary to confirm these models of cancerogenesis in order to recognise early preneoplastic lesions, which may aid in early cancer diagnosis.

Figure 1 - Schematic representation of the pathogenic mechanisms underlying small bowel adenocarcinomas associated with chronic intestinal disorders.



Legend to Figure 1. In coeliac disease villous atrophy induces crypt hyperplasia, characterised by increased intraepithelial lymphocytes (IEL) similarly to atrophic epithelium. Nuclear Sex-determining Region Y-Box (SOX)-9-positive immature hyperplastic crypts evolve into flat nuclear β-catenin-positive dysplasia, thus leading to coeliac disease-associated adenocarcinoma (CoeD-SBA). CoeD-SBA is associated with microsatellite instability (MSI) and high number of tumour-infiltrating lymphocytes (TIL). In Crohn's disease gastric ($MUC5AC^+$)/pancreatobiliary ($CK7^+$) metaplasia evolves into dysplastic polypoid growth, which lastly becomes Crohn's disease-associated adenocarcinoma (CrD-SBA). CrD-SBA is almost always microsatellite stable (MSS).

Lytic phase of EBV infection frequently occurs in inflammatory bowel disease, particularly in patients who have overused immunomodulators, mostly corticosteroids [64]. Recently, latent phase of EBV infection, known to have a key role in gastroesophageal EBV carcinogenesis [65], has been demonstrated in two microsatellite-stable T-cell rich CrD-SBA [25,26]. In both cases EBV has been also detected in dysplastic lesions associated with CrD-SBA and in small *foci* of iuxta-tumoural epithelium apparently devoid of dysplasia [25,26]. Therefore, rarely EBV latent infection might be a very early, pivotal process along SBA pathogenesis in those patients. Up-to-now, no latent infection with EBV has been

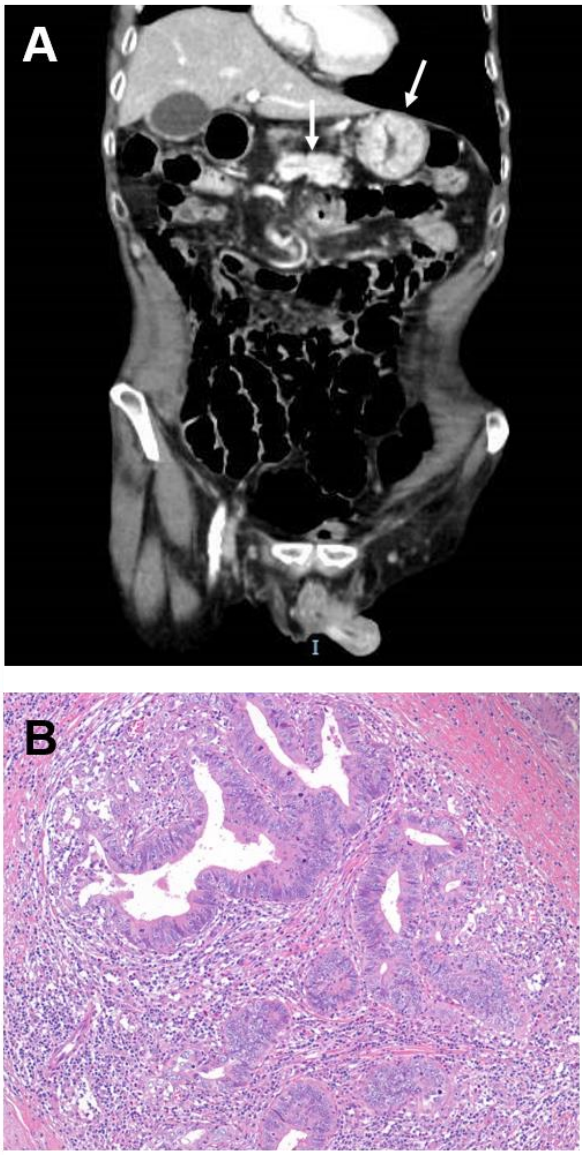
reported in CoeD-SBA, while EBV does not seem to be implicated in the carcinogenesis of sporadic SBA, as suggested by its lack in a cohort of 56 sporadic SBA [27].

Clinical presentation and diagnosis of small bowel adenocarcinoma

Duration of the underlying inflammatory intestinal disorder before SBA diagnosis differs (Table 3). CoeD-SBA presented after a median of 1.4–17 years from CoeD diagnosis in comparison to 7–25.2 years from CrD diagnosis in CrD-SBA in studies with widest cohorts of patients with CoeD-SBA and CrD-SBA, respectively [2,6,8,10,13–21]. However, SBA might be diagnosed in a few cases at the same time of underlying immune-mediated disorder for both CoeD and CrD [2,6,10,11,14,17,18,21]. The clinical spectrum of SBA at onset is wide, including bleeding with subsequent iron-deficiency anaemia, positive fecal occult blood test, maelena or coffee ground vomiting, obstruction with symptoms of nausea, vomiting, abdominal pain and unexplained weight loss, or to intussusception and perforation in the locally advanced neoplasms [1].

In coeliac patients any of the aforementioned symptoms apart from an isolated anaemia should raise the suspicion for SBA. Additionally, when diarrhoea and fever are simultaneously present, first of all enteropathy-associated T-cell lymphoma needs to be considered [39], Likewise, in case of diarrhoea and intestinal obstruction, ulcerative jejuno-ileitis has to be ruled out [4]. Once CoeD-SBA is suspected, an upper endoscopy is recommended in coeliac patients in order to identify and sample the lesion, if it is proximal to the ligament of Treitz (Figure 2). Notwithstanding, as most CoeD-SBA are jejunal, additional diagnostic tests, such as device-assisted enteroscopy, computed tomography enterography and magnetic resonance enterography, are generally needed [66]. On the contrary, capsule endoscopy should not be encouraged in symptomatic patients with SBA due to its several limitations, such as the impossibility to take biopsies for histologic diagnosis and the risk of capsule retention and of missing SBA, in particular in case of proximal site.

Figure 2 - Radiologic and histologic images of a coeliac disease-associated small bowel carcinoma.



Legend to Figure 2. (**A**) Computed tomography shows a circumferential mass with shouldered borders causing the wall thickening in the duodenum (arrows). (**B**) Haematoxylin and eosin staining shows a glandular-type carcinoma with a high tumour-infiltrating lymphocyte density. Original magnification: 100x.

In CrD patients, obstruction is more likely expected to be the manifestation of fibrostricturing phenotype [67]. Similarly, anaemia and positive fecal occult blood test are often related to active CrD [68]. Thus, apart from acute upper bleeding, all the other symptoms of SBA are hard to differentiate the neoplasm from a relapse of CrD [69]. This accounts for the fact that most CrD-SBA are diagnosed during the surgery or even post-operatively by the pathologist [70]. Failure to respond to anti-inflammatory therapies should not be considered *per se* an indicator of CrD-SBA, as it often happens in CrD patients with fibrotic strictures devoid of an inflammatory component [67]. On the

contrary, obstructive symptoms and anaemia in a patient with longstanding quiescent CrD should raise the suspicion for SBA [19]. Ileocolonoscopy is a diagnostic procedure only in CrD-SBA located in the last tract of terminal ileum. Otherwise, as nearly all CrD-SBA are more proximal, retrograde per anal device-assisted enteroscopy is the best procedure to find and sample CrD-SBA. Computed tomography enterography and magnetic resonance enterography might help in finding the correct location of SBA before enteroscopy and/or laparoscopic surgery [21]. Nevertheless, both these imaging techniques are highly indicative of CrD-SBA only in a few cases showing small bowel mass with localised lymphadenopathy and/or evidence of distant spread, such as liver metastasis or peritoneal carcinomatosis [10,21]. Notwithstanding, the review of imaging data by a gastrointestinal radiologist could improve the identification of CrD-SBA-related features, such as annular mass, nodularity at the extraluminal margins of mass and perforation [21].

In conclusion, in the absence of inherited tumour syndrome (Table 1), both CoeD and CrD should be ruled out in any patient at SBA diagnosis.

Prognosis and treatment for small bowel adenocarcinoma

SBA prognosis is frequently worse than that of large bowel cancers [71]. This seems to happen in CrD patients too, in whom SBAs have been reported to be more aggressive than colorectal carcinomas [15]. In an American retrospective study, recruiting 491 SBAs predominantly sporadic SBAs, but also CoeD-SBAs (n = 13), CrD-SBAs (n = 23) and SBAs due to familial adenomatous polyposis (n = 10), the median overall survival and the 5-year overall survival rate were 20.1 months and 26%, respectively [72]. The main primary reason for this poor outcome is that patients are generally symptom-free until late disease, when metastases are frequently already present at SBA diagnosis. Tumour stage has been reckoned the single most crucial prognostic factor in all SBAs [72]. Reduced prognosis is also related to additional features, including poor differentiation, positive margins, lymphovascular/perineural invasion, duodenal site, male gender, black ethnicity and older age at SBA diagnosis [31,73-76]. High positive lymph nodes-to-total lymph node ratio and a low number of investigated lymph nodes have been associated with a poor survival [72,76,77].

Overall survival significantly differs between patients with CoeD-SBA and those with CrD-SBA (Table 3). In particular, the predisposing immune-mediated intestinal disorder, i.e.,

CoeD or CrD, has been shown to be a stage-independent prognostic factor in patients undergoing surgery for SBA in the largest study systematically comparing CoeD-SBAs, CrD-SBAs and sporadic SBAs [2]. Five-year overall survival rate is relatively high in CoeD-SBA, that is 64.2% and 83% in an American study and in an Italian study enrolling 17 and 26 patients, respectively [2,5]. On the contrary, five-year overall survival rate seems to be poorer in CrD-SBA patients, varying from 26% to 38%, in French, Danish and Italian studies [2,12,17]. Accordingly, two-year overall survival in CrD-SBA has been observed to be 52% and 56% in an American study and in a French study, respectively [10,21], also lower than five-year overall survival in CoeD-SBA. Overall survival has been found to be more favourable in CoeD-SBC in comparison with sporadic SBA [2,5], whereas no survival difference has been shown between CrD-SBA and sporadic SBA [2,10,17,22]. Recently, Axelrad JE and colleagues [78], dealing with small bowel cancer-related mortaliy rate in patients with inflammatory bowel disease in a binational population-based cohort study from Denmark and Sweden, concluded that small bowel cancer death is higher in CrD patients than in sporadic cases. This is not in keeping with all studies published so far showing a similar death rate between patients with CrD-SBA and those with sporadic SBA (Table 5). In particular, Palascak-Juif V and colleagues [17] demonstrated a slightly, but not significantly, higher survival rate, in CrD-SBAs (54% at 2 years and 35% at 5 years) than in sporadic SBAs (37% at 2 years and 30% at 5 years) both at 2 years and 5 years. Wieghard and colleagues [22] demonstrated a better 5-year overall survival in 179 CrD-SBAs (43%) than in 1,944 sporadic SBAs (34%). In this large American study patients with CrD-SBA were diagnosed at an earlier stage (I/II) compared with sporadic SBA (55% vs. 32%, $p < 0.0001$) and were more likely to undergo surgery (81% vs. 72%, $p = 0.0016$). However, a similar cancer-specific survival was observed between the two groups, namely 65% *versus* 64%. Indeed, multivariate analysis confirmed that CrD was not significantly associated with overall survival [22]. Recently, another American investigation [79] using the National Cancer Database demonstrated a similar overall survival at 5 years between 493 CrD-SBAs (41%) and 2,175 sporadic SBAs (35%). Additionally, at multivariate analysis CrD was not a risk factor for reduced survival [79].

The disagreement between Axelrad JE and colleagues [78] and all the other studies [17,22,79] might be secondary to the fact that in the former one the death rate was calculated in the small bowel cancer cumulatively, including SBAs, neuroendocrine tumours, sarcomas and others. Mortality rate was not analysed for each small bowel cancer subtype. Furthermore, the controls recruited by Axelrad JE and colleagues was

defined as “free of IBD” [78], but this does not rule out coeliac disease or hereditary SBAs. Although Axelrad JE and colleagues [78] excluded patients with CoeD before the onset of follow-up, small bowel cancers were diagnosed after that. Moreover, CoeD diagnosis may be simultaneous to that of SBA [2], thus, if patients with CoeD were enrolled in the control group, obviously the relative mortality rate would be higher in CrD-SBA, as well as it is known in literature (Table 3). However, Axelrad JE and colleagues [80] then clarified that limiting the analysis to patients with pre-existing CrD-SBA compared to control groups the death rate was similar to that one of previous studies [17,22,79]. Although prospective studies are necessary to evaluate the impact of small bowel cancer on CrD patient survival, it is already evident that SBA is not the main cause of death in CrD patients.

Regardless of the aetiologic group, CoeD or CrD, prognostic factors for SBA include stage, tumour histotype and high TILs [2,7]. Tumour histology by itself is clinically relevant, as it has been demonstrated that diffuse-, mixed- and solid-types considered as a whole tend to have a poorer prognosis compared to glandular-type and medullary-type SBAs [7,46]. Amongst prognostic factors within the CoeD-SBAs, either MSI or high TIL density have been also found and they correlate one each other [2]. Notwithstanding, only TIL density retains a prognostic power in a multivariable model, likely due to the fact that several high-TIL SBAs showing a favourable outcome miss MSI [2]. High TIL density in SBA can be induced by further factors besides MSI status, such as oncogenic viruses. As this regards, non-MSI high-TIL SBAs with EBV latent infection reported in two CrD patients seem to be have a good prognosis [25,26], presumably due to the anti-tumour immune response triggered by abnormal peptide production from EBV. Briefly, although these findings have to be confirmed more-in-depth, EBV latent infection should be considered in CrD-SBA for a better prognostic assessment.

Currently, treatment for CoeD-SBA and CrD-SBA widely derives from recommendations for sporadic SBA [81]. Surgery is the mainstay of curative therapy for SBA without distant metastasis (M0), whose potential benefits from adjuvant chemotherapy are debated, in particular for SBA at stage II [1]. Surgical resection with appropriate lymph node sampling is mandatory for long-term survival in resectable SBA. Surgery is the unique curative treatment for SBA at stage I, whereas it should be followed by adjuvant chemotherapy, including FOLFOX4 or LV5FU2 or oral fluoropyrimidine for SBA at stage II or -to a higher extent- for SBA at stage III [81]. In particular, as the mismatch repair deficient (MMR-d), leading to MSI phenotype and high immune response in solid neoplasms, is related to a better cancer-specific survival in resected SBAs at stage II [2,51,82,83], this confirms the

National Comprehensive Cancer Network Clinical Practice guidelines, Small Bowel Adenocarcinoma, not recommending adjuvant chemotherapy for patients with resected MMR-d SBA at stage II [84]. On the contrary, within patients with mismatch repair proficient SBA at stage II, T4 neoplasms may require a more aggressive therapeutic approach [83]. Systemic chemotherapy is the therapy for non-resectable or metastatic SBC, namely those at stage IV [81]. In a meta-analysis of 14 studies, adjuvant chemotherapy provided no significant survival benefit in SBA patients [85]. Nevertheless, a recent study demonstrated that adjuvant chemotherapy was associated with a better overall survival in patients with SBA at stage II-IV in a multivariate analysis stratified by stage [86]. The international phase 3 clinical trial PRODIGE 33-BALLAD, assessing the possible benefits of adjuvant chemotherapy in patients with SBA at stage I-III, is underway [87,88].

Some molecular alterations may suggest responsiveness to novel treatments. *KRAS* wild-type mutational status has been shown to predict the response to anti-epidermal growth factor receptor monoclonal antibodies cetuximab and panitumumab alone or combined with chemotherapy in metastatic SBA in a few cases [89,90]. On the contrary, a phase 2 clinical trial demonstrated no response of panitumumab in nine patients with metastatic *KRAS* wild-type SBA, one associated to inflammatory bowel disease and two to Lynch syndrome [91]. In particular, in this study seven patients showed SBA progression, whereas the other two ones had stable SBA [91]. It has been assumed that SBA, as well as right-sided colon carcinoma, benefit less from anti-epidermal growth factor receptor agents than left-sided colon carcinomas due to their different embryologic origin, i,e, midgut for small bowel and right-sided colon and hindgut for left-sided colon [91,92]. Although *HER2* amplification is infrequent in CoeD-SBA and CrD-SBA [2], it is worth being assessed as a possible therapeutic target of anti-HER2 receptor monoclonal antibody trastuzumab [58,93]. Expression of PD-L1 on tumoural and immune cells in SBA should support clinical trials in order to investigate efficacy of anti-PD-L1 monoclonal antibodies avelumab and atezolizumab [49]. As this regards, an open-label phase 2 clinical trial of avelumab is ongoing in patients with advanced and metastatic SBA [94]. Similarly, an open-label phase 2 clinical trial has been assessing the response to atezolizumab together with the MEK inhibitor cobimetinib in advanced rare cancers, including SBAs [95]. Anti-PD-1 monoclonal antibodies pembrolizumab and nivolumab might be suitable in a subset of patients with metastatic MSI SBA [96]. An open-label phase 2 clinical trial of pembrolizumab is underway in patients with non-resectable metastatic or locally

advanced SBA [97]. Additionally, pembrolizumab has been evaluating in a large phase 1b clinical trial in combination with the Hsp90 inhibitor XL888, inhibiting Hsp90 chaperone function and promoting the proteasomal degradation of several oncogenic signaling proteins, including Her-2 and Met [98]. This study was designed for several advanced gastrointestinal cancers, including SBA, to find out the best phase 2 dose for the combination of XL888 and pembrolizumab [98]. Another clinical trial has been testing efficacy of the combination immunotherapy with nivolumab and the anti-CTLA-4 monoclonal antibody ipilimumab in advanced rare cancers, such as SBA [99]. Briefly, immunotherapy has been modifying the therapeutic approach in some solid tumours, in particular PD-1/PD-L1 pathway blockade may be considered in patients with advanced MSI SBA, as mismatch repair deficiency has been shown to predict efficacy of anti-PD-1 antibodies in eleven types of solid tumours, including SBA [100].

Table 5. Survival rate in patients with Crohn's disease-associated small bowel adenocarcinoma in comparison to that in patients with sporadic small bowel adenocarcinoma

Authors, year	**Group**	**Patients (N)**	**Overall survival at 5 years (%)**	**P-value**	**HR**	**95% CI**	**P-value**
Palascak-Juif V et al., 2005 [17]				NS			
	CrD-SBA	20	35		NA	NA	
	Sporadic SBA	40	30		NA	NA	
Wieghard N et al., 2017 [22]				0.0121			NS
	CrD-SBA	179	43		0.97	0.79-1.20	
	Sporadic SBA	1,944	34				
Fields AC et al., 2020 [79]				NS			NS
	CrD-SBA	493	41		1.01	0.99-1.02	
	Sporadic SBA	2,175	35				

CI, confidence interval; CrD-SBA, Crohn's disease-associated small bowel adenocarcinoma; HR, hazard ratio; NA, not available; NS, not significant; SBA, small bowel adenocarcinoma.

Objective of the thesis

Recent studies showed a positive correlation between PD-L1 expression and MSI-high (MSI-H) in SBAs [49,101,102]. Although MSI-H is the main determinant of tumour mutation load, causing PD-L1 expression in gastrointestinal neoplasms, other factors might be involved [103]. A recent study [104] demonstrated that PD-L1 is also expressed in several microsatellite stable endometrial carcinomas with high TILs. Furthermore, TIL density, EBV infection, and CDX2 negativity have been associated with PD-L1 positivity in gastrointestinal cancers [48,105,106]. Therefore, tumour immune microenvironment, in particular PD-L1 expression, TIL density and tumour mutation load, are under investigation, in order to identify potential markers of response to immune checkpoint blockades [107]. On the other hand, recent studies on colorectal and other gastrointestinal cancers have found that the invasive front markers tumor budding (Tb) and poorly differentiated clusters (PDCs) may significantly improve their prognostic evaluation [108-115].

On this basis, the primary objective of this thesis was to assess PD-L1 and PD-1 expression in a relatively large and well-characterised cohort of non-hereditary SBAs, associated with CoeD or CrD or sporadic, enrolled through the Small Bowel Cancer Italian Consortium. PD-L1 and PD-1 expression was then correlated with several clinical and pathological features, including the predisposing immune-mediated intestinal disorder, the MSI or EBV status, the intestinal phenotype markers CDX2 and liver fatty acid-binding protein (L-FABP), and cancer-specific survival. The secondary objective of this thesis was to investigate the invasive front markers Tb and PDCs on CrD-SBAs evaluated for the primary aim too.

Materials and Methods

Study population

This retrospective and longitudinal study involved 21 tertiary referral Italian Coeliac and/or IBD Centers taking part in the Small Bowel Cancer Italian Consortium.

CoeD diagnosis was based on positivity of serum IgA anti-endomysial and anti-tissue transglutaminase antibodies along with typical duodenal histological lesions [4]. CrD diagnosis was verified according to internationally agreed criteria [116], and the site and extent of the disease were confirmed by endoscopy, histology and imaging. A group of patients with sporadic SBA, namely without a concomitant chronic intestinal immune-mediated disorder, were recruited as controls. In patients with sporadic SBA, CoeD was ruled out (negativity of serum IgA anti-endomysial and anti-tissue transglutaminase antibodies, coupled with normal serum total IgA), while CrD was excluded by the lack of classic clinical and biochemical features. Re-assessment of the sporadic surgical samples further confirmed the absence of histologic lesions indicative of either CoeD or CrD. The main exclusion criteria for all SBA groups were Lynch syndrome, Peutz-Jeghers syndrome, familial adenomatous polyposis and juvenile polyposis. This study was approved by the Ethics Committee of the San Matteo Hospital Foundation of Pavia (protocol number 20140003980).

Histology

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 the following conventional histologic parameters: tumour histotype, World Health Organization (WHO) tumour grade (for the entire tumour), TILs and all parameters required for TNM staging [117]. Tumour histotype was classified as: a) glandular, b) diffuse, c) mixed (glandular plus diffuse), d) medullary and e) non-medullary solid types, as previously described [7,118]. WHO tumour grade was based on the proportion of gland formation and categorized as grade 1 (well differentiated, >95%), grade 2 (moderately differentiated, 50% to 95%), or grade 3 (poorly differentiated, 0% to 49%). All available H&E–stained slides from CrD-SBAs, including full-thickness sections of the tumor and encompassing the invasive front, were reviewed. In carcinomas with mucinous features, WHO grade, Tb and PDCs were assessed

in the area outside the mucinous component. An Eclipse Ci microscope (Nikon) with a standard 22-mm diameter eyepiece (specimen area of 0.950 mm^2 under an objective lens with a magnification of ×20) was used and the number of buds/PDCs was divided by 1.21 to achieve the number of buds per area of 0.785 mm^2 as recommended for colorectal cancer [111].

Definition and evaluation of Tb

A tumour bud is defined as a single tumor cell or a cell cluster of up to 4 tumour cells which develops from neoplastic glands. Tb was analyzed along the invasive parts of the tumour using the hotspot method, which is considered to be the most useful method for assessing Tb in colorectal cancer [111]. Initially, the invasive front of the tumour was screened using low magnification to find the areas with most Tb. For this purpose, cytokeratin 8-18 (monoclonal, clone EP17/EP30, Dako) immunohistochemistry was helpful in some challenging cases (ie. glandular fragmentation, strong peritumoral inflammation) to allow a better visualization of Tb-rich areas. Tb was assessed from several H&E areas and the single field with the most budding was used for quantitation. The number of buds was counted in all cancers on H&E from a single field of view using ×200 total magnification (the hotspot method). Following the International Tumor Budding Consensus Conference (ITBCC) group recommendation for colorectal cancer, we used a three-tier system: low budding (Tb1): 0-4 buds; intermediate budding (Tb2): 5-9 buds and high budding (Tb3): 10 or more buds [111].

Definition and evaluation of PDCs

PDCs were defined as clusters of ≥5 cancer cells that lacked a gland-like structure. The whole tumour was first scanned at low-power magnification to identify areas with the greatest number of PDCs at the invasive front. The number of PDCs in a single field of highest activity was then determined and graded as PDC1 (<5 PDCs), PDC2 (5 to 9 PDCs), or PDC3 (≥10 PDCs) under an objective lens with a magnification of ×20 [110,117,119].

Definition of combined invasive front (CIF)

A CIF grade was developed as high in the presence of grade 3 for either Tb or PDCs or both and as low in the remaining cases.

Immunohistochemistry

Four µm-thick sections were stained on a Dako Omnis platform with the following antibodies: CD3 (polyclonal, Dako, Carpinteria, CA), CD8 (polyclonal, Dako), MLH1 (monoclonal, clone ES05, Dako), MSH2 (monoclonal, clone FE11, Dako), MSH6 (monoclonal, clone EP49, Dako), PMS2 (monoclonal, clone EP51, Dako), PD-L1 (monoclonal, clone 22C3, Dako), PD-1 (monoclonal, clone NAT, Dako), CDX2 (monoclonal, clone DAK-CDX2, Dako) and L-FABP (monoclonal, clone EPR20464, Dako). Immunoreactions were developed using 0.03% 3,3' diaminobenzidine tetrahydrochloride and sections were then counterstained with Harris' haematoxylin. TILs were stained using CD3 and CD8 antibodies and counted in ten consecutive high-power fields (HPFs), as previously described [2]. A tumour was classified as having "high TIL density" when the mean number of TILs/HPF was greater than 15 for CD3 or greater than 9.5 for CD8 [120]. Immunostaining of DNA mismatch repair proteins MLH1, MSH2, MSH6 and PMS2 in tumour cells was evaluated as proficient (retained expression) or deficient (absent expression); only tumours showing absence of nuclear staining of all neoplastic cells in the presence of an internal positive control (intra-tumour stromal and inflammatory cells or non-tumour mucosa) were considered deficient [2]. In parallel, MSI molecular analysis was performed

PD-L1 membranous expression was evaluated using the combined positive score (CPS) measuring both tumoral cells and peritumoural/intratumoural immune cells, the mononuclear immune cell density score (MIDS) measuring peritumoural/intratumoural immune cells only and the tumour proportion score (TPS) measuring tumoral cells only, as previously described [121]. In particular, CPS was calculated as the ratio of the number of PD-L1 stained cells (tumour cells and immune cells) to the total number of viable tumour cells, multiplied by 100. Tumours were considered negative if CPS<1, positive if CPS≥1. TPS was the ratio of the number of PD-L1 stained tumour cells divided by the total number of viable tumour cells, multiplied by 100. Tumours were regarded as negative if TPS<1, positive if TPS≥1. MIDS was calculated as the ratio of the number of PD-L1 stained immune cells to the total number of viable tumour cells, multiplied by 100; the result was then scored in a scale from 0 to 4. MIDS 0 was defined as absent PD-L1 staining, while MIDS 1, MIDS 2, MIDS 3 and MIDS 4 corresponded to PD-L1^{+} immune cells per 100 viable tumour cells <1, ≥1 but <10, ≥10 but <100, ≥100, respectively. MIDS scores 2, 3 and 4 were regarded as positive, whereas scores 0 and 1 as negative. PD-1-positive immune cells were counted separately in intratumoural and peritumoural areas in ten consecutive HPFs and the mean number of PD-1-positive cells per HPF was recorded for each case.

PD-1$^+$ cells situated inside the tumour were considered as intratumoural, while PD-1$^+$ cells located in the areas adjacent to the tumour invasive front as peritumoural. In addition, the total number of PD-1-positive cells per HPF, corresponding to the sum of intratumoural and peritumoural PD-1-positive cell counts was given. L-FABP or CDX2 staining was considered positive in cases with >10% moderate-to-intense staining in tumour cells [122]. A central pathology review of each case was performed.

MSI analysis

Tumour 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, NR21, NR-22 and NR-24) by the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA), as previously reported [2].

Definition of Teng tumour microenvironment immune types

Tumour microenvironment immune types were defined based on CD3$^+$ TIL density (low versus high) and PD-L1 expression, evaluated with the CPS. Tumors were classified in four different Teng types, i.e. type I (high TIL density, CPS≥1), II (low TIL density, CPS<1), III (low TIL density, CPS≥1), IV (high TIL density, CPS<1) [123].

EBV encoded RNAs *in situ* hybridization

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), markers of latent phase EBV infection, 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 CrD-SBAs characterized in a previous investigation [26].

Gene mutation analysis

Mutation analysis of *KRAS*, *NRAS* 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 tumour 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 P319 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.

Statistical analysis

Stata 15 (StataCorp, College Station, TX, USA) was used to perform all computations. We considered a 2-sided p-value<0.05 as statistically significant. For post-hoc comparisons between etiologic groups, the significance was set at 0.017 (Bonferroni correction).

Continuous data were reported as median and 25th-75th percentiles, categorical variables are reported as counts and percent; they were compared between etiologic groups using the Kruskal Wallis test or the Fisher exact test, respectively. The Spearman R and 95% confidence intervals (CI) were computed to measure the correlation between continuous variables. For the purpose of the analysis continuous variables were dichotomized at the median value. Median follow-up (25th-75th) was computed with the reverse Kaplan Meier method. Follow-up was computed from diagnosis of cancer to death or last available follow-up for censored patients. Cumulative survival curves were plotted according to the Kaplan Meier method and compared with the logrank test. The strength of the association between series of candidate risk factors and cancer-specific mortality was assessed using Cox regression; hazard ratios (HR) and 95% CI were derived from the models. Mortality rates per 100 person year and 95% CIs were reported. We checked the proportional hazard assumption with a test based on residuals. We computed the Harrell's c statistic for discrimination (the closer to 1, the better, the closer to 0.5, the worse). Given the low number of deaths, we did not fit multivariable survival models, but only bivariable models were fitted to adjust, in turn, for aetiologic group and stage.

Results

Patient demographics and clinico-pathologic features

This retrospective study included a cohort of 121 patients with pathologically-confirmed primary non-hereditary, non-ampullary SBA, who underwent surgical resection and had complete survival data. Demographic and clinico-pathologic data of all patients investigated are reported in Table 6. We recruited 34 patients with CoeD-SBA, 49 with CrD-SBA, and 38 with sporadic SBA, a fraction of them entered previous studies from the Small Bowel Cancer Italian Consortium [2,7,26,124]. Median age at the time of SBA diagnosis among coeliac (median 53.5 years) and CrD patients (median 58 years) was significantly ($p<0.001$) lower than that of sporadic cases (median 69 years), and median duration of inflammatory disorder at cancer diagnosis was significantly ($p=0.016$) lower in CoeD-SBA (median 23.5 months) compared to CrD-SBA (median 156 months). A higher rate of male gender was found in CrD-SBA (73%) and sporadic SBA (63%) compared to the CoeD-SBA group (47%). In keeping with what is already known (Table 2), the ileum was the commonest small bowel location for CrD-SBA (94%), whereas it was the jejunum in both CoeD-SBA (70%) and sporadic SBA (63%). No significant difference was identified among the three groups regarding tumour stage at diagnosis. The majority of stage III and IV patients received systemic chemotherapy with platinum-based and 5-fluorouracil regimens after surgical intervention.

Histologically, most SBAs showed glandular differentiation in all aetiologic groups; however, medullary and diffuse/poorly cohesive cancers were more common in CoeD (17%) and CrD patients (20%), respectively. CoeD-SBAs displayed a significantly ($p<0.001$) greater number of TILs (median 25.1 TILs/HPF) compared to CrD-SBAs and sporadic SBAs (median 7.1 TILs/HPF for both). MSI-H was identified in 39 cases (32.2%), including 37 cases with loss of MLH1/PMS2 expression and two SBAs, both associated with CrD, with isolated loss of MSH6. No discordance between immunohistochemistry for mismatch repair proteins and MSI molecular analysis was observed in any case. MSI-H rate was significantly ($p<0.001$) higher in CoeD-SBAs (65%) than in both CrD-SBAs (18%) and sporadic SBAs (21%). As regards markers of intestinal differentiation, CDX2 loss was significantly ($p=0.012$) more common in CrD-SBAs (46%) compared to CoeD-SBAs (15%), while the absence of L-FABP expression was significantly ($p<0.001$) more frequent in both CoeD-SBAs (88%) and CrD-SBAs (81%) compared to sporadic SBAs (45%).

Table 6. Demographic and clinico-pathologic features of all 121 SBA patients

	CoeD-SBA	CrD-SBA	Sporadic SBA	Overall p-value	Post-hoc comparison p-value
Number	34	49	38		
Age at SBA diagnosis	53.5	58	69	<0.001	CoeD vs CrD: 0.129
Median [25th–75th IQR], yrs	[42.7-66]	[51-67.5]	[62-77]		CoeD vs Sporadic: <0.001
					CrD vs Sporadic: <0.001
Duration of inflammatory disorder at SBA diagnosis	23.5	156	NA	0.016	
Median [25th–75th IQR], mo	[12-110.25]	[6-288]			
Sex, N (%)					CoeD vs CrD: 0.014
Female	18 (53)	13 (27)	14 (37)	0.049	CoeD vs Sporadic: 0.169
Male	16 (47)	36 (73)	24 (63)		CrD vs Sporadic: 0.302
Site, N (%)*					
Duodenum	7 (21)	1 (2)	3 (8)	<0.001	CoeD vs CrD: <0.001
Jejunum	23 (70)	2 (4)	24 (63)		CoeD vs Sporadic: 0.053
Ileum	3 (9)	46 (94)	11 (29)		CrD vs Sporadic: <0.001
Stage, N (%)**					
I	3 (9)	6 (12)	2 (5)	0.550	
II	19 (60)	19 (39)	17 (46)		
III	8 (25)	18 (37)	15 (41)		
IV	2 (6)	6 (12)	3 (8)		
Histotype, N (%)					
Glandular	19 (56)	24 (50)	22 (58)	0.032	CoeD vs CrD: 0.032
Medullary	6 (17)	2 (4)	1 (3)		CoeD vs Sporadic: 0.187
Diffuse	2 (6)	10 (20)	2 (5)		CrD vs Sporadic: 0.228
Mixed	4 (12)	12 (24)	10 (26)		
Solid	3 (9)	1 (2)	3 (8)		
$CD3^+$ TILs/HPF	25.1	7.1	7.1	<0.001	CoeD vs CrD: <0.001
Median [25th–75th IQR]	[12.3-75.4]	[2-20.6]	[2.2-20.9]		CoeD vs Sporadic: <0.001
					CrD vs Sporadic: 0.962
MSI status, N (%)					
Non-MSI	12 (35)	40 (82)	30 (79)	<0.001	CoeD vs CrD: <0.001
MSI-H	22 (65)	9 (18)	8 (21)		CoeD vs Sporadic: <0.001
					CrD vs Sporadic: 0.754
CDX2 expression, N (%)***					
Negative	5 (15)	22 (46)	11 (29)	0.012	CoeD vs CrD: 0.003
Positive	28 (85)	26 (54)	27 (71)		CoeD vs Sporadic: 0.165
					CrD vs Sporadic: 0.109
L-FABP expression, N (%)****					
Negative	30 (88)	39 (81)	17 (45)	<0.001	CoeD vs CrD: 0.393
Positive	4 (12)	9 (19)	21 (55)		CoeD vs Sporadic: <0.001
					CrD vs Sporadic: <0.001

CoeD-SBA, coeliac disease-associated small bowel adenocarcinoma; CrD-SBA, Crohn's disease-associated small bowel adenocarcinoma; HPF, high-power field; IQR, interquartile range; L-FABP, liver fatty acid-binding protein; mo, month; MSI, microsatellite instability; MSI-H, microsatellite instability-high; NA, not applicable; SBA, small bowel adenocarcinoma; TIL, tumour-infiltrating lymphocyte; yr, year.

*In one CoeD-SBA the precise tumor site within small bowel was unknown.

**In two CoeD-SBAs and in one sporadic SBA the precise stage was unknown.

***In one CoeD-SBA and in one CrD-SBA, no section for CDX2 immunohistochemistry was available.

****In one CrD-SBA, no section for L-FABP immunohistochemistry was available.

Immunohistochemical expression of PD-L1 and association with clinico-pathologic features

PD-L1 staining was found in immune cells and to a variable extent in tumour cells (Figure 3). PD-L1 expression according to CPS and MIDS was positively associated with male sex, while no significant association was observed between PD-L1 expression and age at SBA diagnosis, small bowel site and tumour stage at diagnosis (Table 7, Table 8).

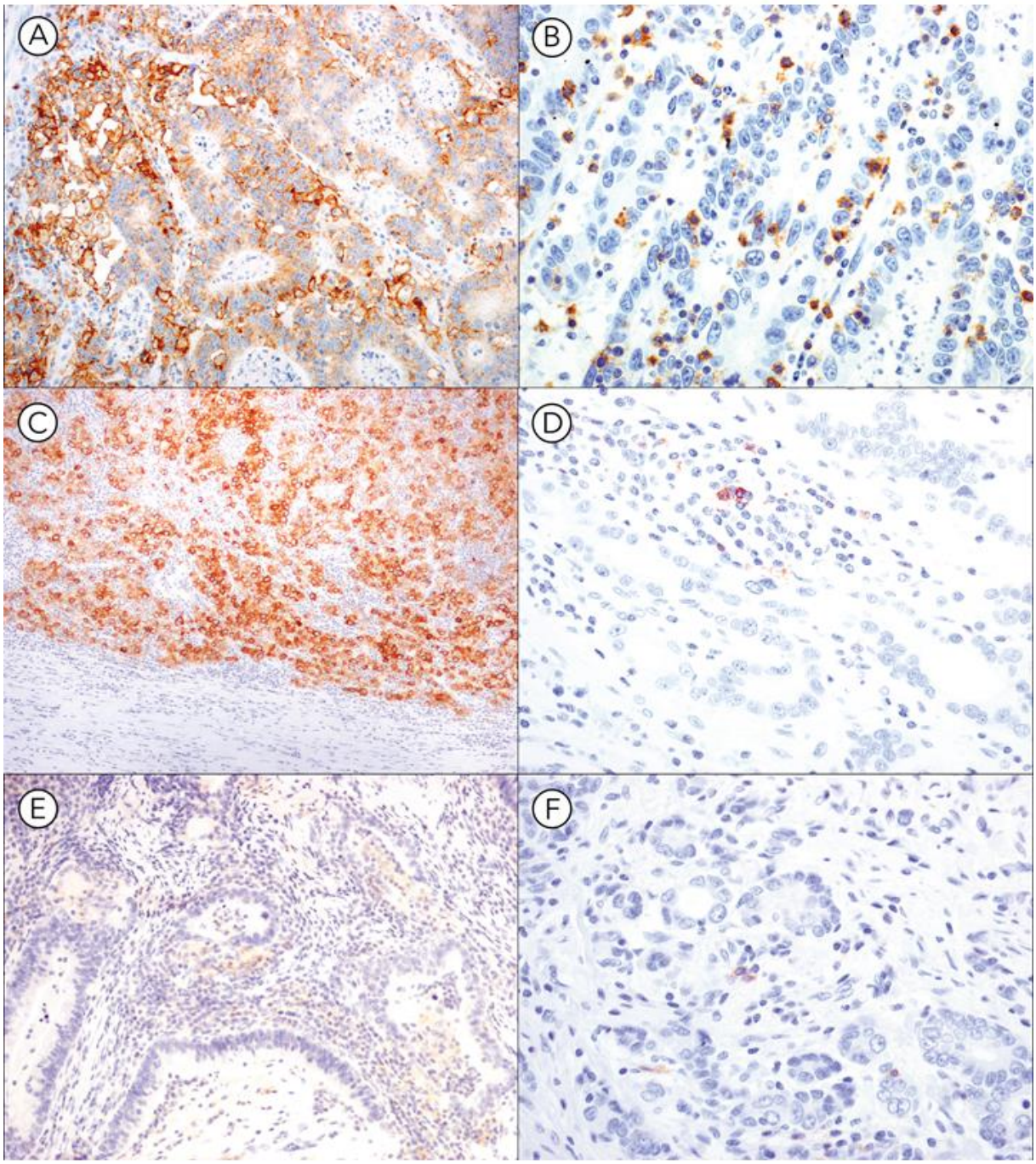
Per CPS cut-off of 1 or more (CPS≥1), the prevalence of PD-L1 expression in non-hereditary SBAs as a whole was 26%, with significantly (p=0.001) higher percentage in both CoeD-SBA (35%) and CrD-SBA (35%) than in sporadic SBAs (5%). Among CPS≥1 SBAs, 65% (20 out of 31 cases, including 8 CoeD-SBAs, 10 CrD-SBAs and 2 sporadic SBAs), 13% (four cases, including one CoeD-SBA and three CrD-SBAs) and 23% (seven cases, including three CoeD-SBAs and four CrD-SBAs) showed a 1≤CPS<10, 10≤CPS<50, and CPS≥50, respectively. Per TPS≥1, the prevalence of PD-L1 expression was 8%, with significantly (p=0.035) higher percentage in both CoeD-SBA (15%) and CrD-SBA (10%) than in sporadic SBA (0%). Per MIDS>1, the prevalence of PD-L1 expression was 22%, with significantly (p=0.005) higher percentage in both CoeD-SBAs (29%) and CrD-SBAs (31%) than in sporadic SBAs (5%). No case with MIDS 4 was identified in the whole cohort.

Patients with MSI-H SBAs had a significantly (p=0.013) higher prevalence of CPS≥1 (41%) than those with non-MSI SBAs (18%). Patients with MSI-H SBAs had a significantly (p=0.002) higher prevalence of TPS>1 (20%) than those with microsatellite stable SBAs (2%). No significant difference in terms of PD-L1-positivity according to MIDS was observed between MSI-H and microsatellite stable SBAs. In addition, PD-L1 positivity was associated with several features known to be more frequently coupled with MSI-H phenotype, including high $CD3^+$ and $CD8^+$ TIL density and medullary histotype.

SBAs with CPS≥1 exhibited L-FABP negativity significantly more frequently (p=0.036) compared to those with CPS<1. A similar trend, despite not being significant, was

observed in SBAs showing TPS≥1 or MIDS>1. L-FABP negativity was more frequent in MSI-H SBAs (82%) than in microsatellite stable cases (66%), although this did not reach statistical significance (p=0.065). No significant difference in PD-L1 expression was identified in CDX2-positive and CDX2-negative SBAs.

Figure 3 - PD-L1 and PD-1 expression in small bowel adenocarcinomas (SBAs)



Legend to Figure 3. A glandular coeliac disease-associated SBA showing a strong and diffuse PD-L1 membranous staining on both tumoural and immune cells (A) (PD-L1 staining; original magnification 200X) and a high number of PD-1^{+} intratumoural lymphocytes (B) (PD-1 staining; original magnification 400X). (C) An EBV^{+} lymphoepithelioma-like Crohn's disease-associated SBA with a diffuse PD-L1

membranous expression by tumour cells (PD-L1 staining; original magnification 200X). (D) A glandular Crohn's disease-associated SBA with a low intratumoural infiltration of PD-1$^+$ cells. (PD-1 staining; original magnification 400X). (E) A glandular sporadic SBA showing a faint membranous and cytoplasmatic PD-L1 staining on immune cells, localized either in the tumoural stroma or in the lumen of neoplastic glands. (PD-L1 staining; original magnification 200X). (F) The same sporadic case exhibiting a low number of intratumoural PD-1$^+$ lymphocytes (PD-1 staining; original magnification 400X).

Table 7. Association of PD-L1 expression according to CPS with clinico-pathologic features

	PD-L1$^-$ (CPS < 1)	**PD-L1$^+$ (CPS ≥ 1)**	**p-value**
Number, N (%)	90 (74)	31 (26)	
Age at SBA diagnosis	62 (52-71)	56 (52-67)	0.421
Median [25th–75th IQR]			
Sex, N (%)			
Female	38 (86)	6 (14)	0.029
Male	51 (67)	25 (33)	
Aetiologic group, N (%)			0.001
CoeD-SBA	22 (65)	12 (35)	
CrD-SBA	32 (65)	17 (35)	
Sporadic SBA	36 (95)	2 (5)	
Site, N (%)			0.172
Duodenum	8 (73)	3 (27)	
Jejunum	41 (84)	8 (16)	
Ileum	41 (68)	19 (32)	
Stage, N (%)			0.212
I	8 (73)	3 (27)	
II	39 (71)	16 (29)	
III	30 (73)	11 (27)	
IV	11 (100)	0 (0)	
Histotype, N (%)			0.011
Glandular	49 (75)	16 (25)	
Medullary	2 (22)	7 (78)	
Diffuse	12 (86)	2 (14)	
Mixed	21 (81)	5 (19)	
Solid	6 (86)	1 (14)	
PD-1$^+$ intratumoural cells/HPF	1.1	7.2	<0.001
Median [25th–75th IQR]	(0.1-5.5)	(2.8-28.5)	
PD-1$^+$ peritumoural cells/HPF	1.9	13.8	<0.001
Median [25th–75th IQR]	(0.4-7.8)	(5.3-29.3)	

PD-1+ total cells/HPF	2.8	23.8	<0.001
Median [25th–75th IQR]	(1.0-16.2)	(8.0-64.3)	
CD3+ TILs/HPF	7.0	21.3	<0.001
Median [25th–75th IQR]	(2.1-24.2)	(15.1-75.2)	
CD8+ TILs/HPF	5.2	52.5	0.008
Median [25th–75th IQR]	(1.0-19.3)	(10.7-93.0)	
MSI status, N (%)			
Non-MSI	67 (82)	15 (18)	0.013
MSI-H	23 (59)	16 (41)	
CDX2 expression, N (%)			
Negative	29 (76)	9 (24)	1.000
Positive	60 (74)	21 (26)	
L-FABP expression, N (%)			
Negative	59 (69)	27 (31)	0.036
Positive	30 (88)	4 (12)	

CoeD-SBA, coeliac disease-associated small bowel adenocarcinoma; CPS, combined positive score; CrD-SBA, Crohn's disease-associated small bowel adenocarcinoma; HPF, high-power field; IQR, interquartile range; L-FABP, liver fatty acid-binding protein; MSI, microsatellite instability; MSI-H, microsatellite instability-high; PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand 1; SBA, small bowel adenocarcinoma; TIL, tumour-infiltrating lymphocyte.

Table 8. Association of PD-L1 expression according to TPS and MIDS with clinico-pathologic features

	PD-L1- (TPS < 1)	**PD-L1+ (TPS ≥ 1)**	**p-value**	**PD-L1- (MIDS ≤ 1)**	**PD-L1+ (MIDS > 1)**	**p-value**
Number, N (%)	111 (92)	10 (8)		94 (78)	27 (22)	
Age at SBA diagnosis	60 (52-70)	59 (52-67)	0.732	62 (52-71)	55 (52-67)	0.209
Median [25th–75th IQR]						
Sex, N (%)						
Female	42 (95)	2 (5)	0.322	39 (89)	5 (11)	0.04
Male	68 (89)	8 (11)		54 (71)	22 (29)	
Aetiologic group, N (%)			0.035			0.005
CoeD-SBA	29 (85)	5 (15)		24 (71)	10 (29)	
CrD-SBA	44 (90)	5 (10)		34 (69)	15 (31)	
Sporadic SBA	38 (100)	0 (0)		36 (95)	2 (5)	
Site, N (%)			1.000			0.095
Duodenum	11 (100)	0 (0)		8 (73)	3 (27)	
Jejunum	45 (92)	4 (8)		43 (88)	6 (12)	
Ileum	55 (92)	5 (8)		43 (72)	17 (28)	

Stage, N (%)			0.568			0.292
I	11 (100)	0 (0)		8 (73)	3 (27)	
II	51 (93)	4 (7)		41 (75)	14 (25)	
III	36 (88)	5 (12)		32 (78)	9 (22)	
IV	11 (100)	0 (0)		11 (100)	0 (0)	
Histotype, N (%)			<0.001			0.416
Glandular	63 (97)	2 (3)		49 (75)	16 (25)	
Medullary	3 (33)	6 (67)		5 (56)	4 (44)	
Diffuse	13 (93)	1 (7)		12 (86)	2 (14)	
Mixed	25 (96)	1 (4)		22 (85)	4 (15)	
Solid	7 (100)	0 (0)		6 (86)	1 (14)	
PD-1⁺ intratumoural cells/HPF	1.3	3.3	0.061	1.2	10.9	<0.001
Median [25th–75th IQR]	(0.2-9.3)	(1.7-44.4)		(0.1-5.5)	(2.8-34)	
PD-1⁺ peritumoural cells/HPF	3.6	6	0.041	2.2	18	<0.001
Median [25th–75th IQR]	(0.6-12)	(5.2-50.1)		(0.4-7.8)	(8-33.5)	
PD-1⁺ total cells/HPF	5.5	8.9	0.055	3.4	25.6	<0.001
Median [25th–75th IQR]	(1.2-21.2)	(6.9-100.4)		(1-16.2)	(12-67.7)	
CD3⁺ TILs/HPF	10.6	77.6	0.002	7.9	20.1	0.002
Median [25th–75th IQR]	(2.8-26)	(23.2-121.4)		(2.1-26)	(15.1-47.2)	
CD8⁺ TILs/HPF	5.7	82	0.023	5.7	57.5	0.037
Median [25th–75th IQR]	(1-23.1)	(18-100)		(1-21)	(3.5-94)	
MSI status, N (%)						
Non-MSI	80 (98)	2 (2)	0.002	67 (82)	15 (18)	0.161
MSI-H	31 (80)	8 (20)		27 (69)	12 (31)	
CDX2 expression, N (%)						
Negative	35 (92)	3 (8)	1.000	32 (84)	6 (16)	0.345
Positive	75 (93)	6 (7)		61 (75)	20 (25)	
L-FABP expression, N (%)						
Negative	76 (88)	10 (12)	0.06	63 (73)	23 (27)	0.092
Positive	34 (100)	0 (0)		30 (88)	4 (12)	

CoeD-SBA, coeliac disease-associated small bowel adenocarcinoma; CrD-SBA, Crohn's disease-associated small bowel adenocarcinoma; HPF, high-power field; IQR, interquartile range; L-FABP, liver fatty acid-binding protein; MIDS, mononuclear immune cell density score; MSI, microsatellite instability; MSI-H, microsatellite instability-high; PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand 1; SBA, small bowel adenocarcinoma; TIL, tumor-infiltrating lymphocyte; TPS, tumour proportion score.

Immunohistochemical expression of PD-1

Data on PD-1 were obtained in 118 patients, 34 with CoeD-SBAs, 49 with CrD-SBAs, and 35 with sporadic SBAs. PD-1 positivity was found in both intratumoural and peritumoural immune cells. PD-1⁺ intratumoural immune cells were significantly higher in CoeD-SBAs

(median 5.4/HPF, 25th–75th IQR: 1.3-16.4) than in CrD-SBAs (1.2, 25th–75th: 0.5-3.7; p=0.002) and sporadic SBAs (0.9, 25th–75th: 0-7.7; p=0.001). Similarly, PD-1+ peritumoural immune cells were significantly higher in CoeD-SBAs (8.25, 25th–75th: 3.6-20.2) than in CrD-SBAs (3.6, 25th–75th: 1.0-9.0; p=0.007) and sporadic SBAs (1.9, 25th–75th: 0.1-9.3; p=0.001). As regards PD-1+ immune cells, no significant difference was identified between CrD-SBAs and sporadic SBAs. PD-1+ immune cells were significantly (p<0.001) higher in CPS≥1 cases in comparison with CPS<1 cases (Table 7), as well as in MIDS>1 cases compared to MIDS≤1 cases. Only PD-1+ peritumoural immune cells were significantly (p=0.041) higher in TPS≥1 cases in comparison with TPS<1 cases (Table 8). PD-1+ intratumoural, peritumoural and total cells density resulted associated with CD3+ TIL density (R=0.46, 95% CI 0.3-0.59, p<0.001; R=0.37, 95% CI 0.2-0.52, p<0.001; R=0.42, 95% CI 0.26-0.56, p<0.001, respectively).

Teng tumour microenvironment immune types

Amongst 121 non-hereditary SBAs, Teng type I, II, III and IV included 24 (20%), 62 (51%), 7 (6%), and 28 (23%) cases, respectively. Teng types according to MSI status and aetiologic group are summarised in Table 9. Distribution of Teng types was significantly (p<0.001) different amongst MSI-H and microsatellite stable SBAs. In particular, MSI-H SBAs encompassed an increased number of Teng types with high TIL density, i.e. type I (31%) and IV (46%). Most microsatellite stable SBAs (69%) had no PD-L1 expression and low TIL density, i.e. type II. However, we also found 12 microsatellite stable SBAs with high TIL density and positive PD-L1 expression (type I), including three CoeD-SBAs, eight CrD-SBAs and one sporadic SBA, as well as three type III microsatellite stable SBAs, all of them associated with CrD. Additionally, five MSI-H SBAs with low TIL density and negative PD-L1 expression (i.e. type II) were identified. Likewise, distribution of Teng types was significantly (p<0.001) different amongst CoeD-SBAs, CrD-SBAs and sporadic SBAs. In particular, an increased proportion of type I was found in patients with CoeD-SBAs (32%) and CrD-SBAs (23%) compared to sporadic SBAs (5%).

Table 9. Distribution of SBAs according to Teng type classification (in 4 tumour microenvironment immune types), MSI status and aetiologic group.

	Teng type classification				**p-value**
	Type I (PD-L1$^+$/high TIL density)	**Type II (PD-L1$^-$/low TIL density)**	**Type III (PD-L1$^+$/low TIL density)**	**Type IV (PD-L1$^-$/high TIL density)**	
Total, N (%)	24 (20)	62 (51)	7 (6)	28 (23)	
MSI status, N (%)					
Non-MSI	12 (15)	57 (69)	3 (4)	10 (12)	<0.001
MSI-H	12 (31)	5 (13)	4 (10)	18 (46)	
Aetiologic group, N (%)					<0.001
CoeD-SBA	11 (32)	9 (27)	1 (3)	13 (38)	
CrD-SBA	11 (23)	27 (55)	6 (12)	5 (10)	
Sporadic SBA	2 (5)	26 (69)	0 (0)	10 (26)	

CoeD-SBA, coeliac disease-associated small bowel adenocarcinoma; CrD-SBA, Crohn's disease-associated small bowel adenocarcinoma; MSI, microsatellite instability; MSI-H, microsatellite instability-high; PD-L1, programmed cell death ligand 1; SBA, small bowel adenocarcinoma; TIL, tumor-infiltrating lymphocyte.

For Teng type classification, PD-L1 expression was evaluated with the combined positive score.

EBV$^+$ SBAs

Amongst the 118 tumours investigated for EBER, namely 34 CoeD-SBAs, 49 CrD-SBA, and 35 sporadic SBA, only two were EBV$^+$, both CrD-SBAs, one showing a lymphoepithelioma-like and the other a glandular histology, as described in a previous study [26]. The lymphoepithelioma-like carcinoma was strongly PD-L1$^+$ with a CPS≥50, while the glandular CrD-SBA was negative for PD-L1.

Survival analysis

Three patients died peri-operatively, while the remaining 118 patients were followed-up for a median of 68 months (25th-75th: 35-117) and their cancer-specific survival data are reported in Table 10. Univariate survival analysis identified the following parameters as significant related to a better post-operative cancer-specific survival: etiologic group -in particular CoeD-SBA-, female sex, tumour site - jejunum being better-, pathological stage I and II, MSI-H, high CD3$^+$ TIL density, medullary and glandular histotype; positivity for CDX2 expression, high intratumoural PD-1$^+$ cell density, peritumoural PD-1$^+$ cell density and total PD-1$^+$ cell density (Figure 4, Figure 5). PD-L1$^+$ cases according to a CPS showed better outcome in comparison with PD-L1$^-$ cases (p=0.046, Cox analysis and Figure 6A). Teng type classification proved to be a significant prognostic parameter (p<0.001, Figure 6B); in particular, type I and IV tumours showed a significantly better cancer-specific survival in comparison to type II. However, PD-L1 positivity lost its prognostic value when combined with TIL density in Teng type classification. TPS, MIDS and L-FABP positivity were not found to be significant predictors of cancer-specific survival (Figure 7).

Bivariable survival analysis inclusive of stage confirmed the significant prognostic value of the following parameters: aetiologic group (p=0.017), sex (p=0.007), histotype (p=0.01), MSI status (p=0.002), CD3$^+$ TIL density (p=0.001), CPS (p=0.022) and Teng type classification (p=0.015). At bivariable analysis inclusive of aetiologic group, the following factors retained prognostic significance: stage (p<0.001), sex (p=0.008), histotype (p<0.001), MSI status (p=0.016), CD3$^+$ TIL density (p=0.002), and Teng type classification (p=0.018). PD1$^+$ immune cell density lost its prognostic value in a bivariable model adjusted for CD3$^+$ TIL density or MSI status.

Table 10. Cancer-specific survival of small bowel adenocarcinomas followed-up

Parameter	**N. of cases**	**N. of deaths (%)**	**Rate per 100 person-year (95% CI)**	**HR (95% CI)**	**p-value (Cox)**
Aetiologic group					p<0.001
CoeD-SBA	33	5 (15)	2.66 (1.10-6.40)	1	
CrD-SBA	47	23 (49)	13.14 (8.73-19.78)	4.37 (1.65-11.53)	
Sporadic SBA	38	23 (61)	14.49 (9.62-21,80)	4.73 (1.79-12.48)	
Sex					
Female	44	13 (29)	4.32 (2.51-7.44)	1	p<0.001
Male	74	38 (51)	17.40 (12.66-23.92)	3.00 (1.55-5.57)	
Tumor site					p=0.023
Duodenum	11	4 (36)	8.02 (3.01-21.36)	1	
Jejunum	49	17 (35)	6.25 (3.89-10.06)	0.83 (0.28-2.48)	
Ileum	58	30 (52)	15.02 (10.51-21.49)	1.86 (0.66-5.29)	
Stage					p<0.001
I	10	0 (0)	0	Not evaluable	
II	55	15 (27)	4.93 (2.97-8.18)	1	
III	40	24 (60)	23.39 (15.68-34.89)	4.66 (2.39-9.11)	
IV	11	11 (100)	76.98 (42.63-138.99)	14.61 (6.24-34.24)	
MSI status					
Non-MSI	82	44 (54)	14.55 (10.83-19.56)	1	p<0.001
MSI-H	36	7 (19)	3.19 (1.52-6.70)	0.24 (0.11-0.54)	
$CD3^+$ TIL density					
≤15/HPF	67	41 (61)	15.85 (11.67-21.52)	1	p<0.001
>15/HPF	51	10 (20)	3.81 (2.04-7.07)	0.25 (0.13-0.51)	
Histotype					p<0.001
Glandular	62	18 (29)	5.5 (3.47-8.74)	1	
Medullary	9	1 (11)	2.01 (0.28-14.28)	0.34 (0.05-2.53)	
Mixed	26	19 (73)	27.56 (17.58-43.20)	4.22 (2.23-10.34)	
Diffuse	14	10 (71)	25.14 (13.52-46.72)	3.57 (1.19-11.1)	
Solid	7	3 (43)	8.34 (2.69-25.86)	1.48 (0.27-3.63)	
CDX2 expression					
Negative	36	24 (67)	13.27 (8.89-19.81)	1	p=0.018
Positive	80	27 (34)	8.00 (5.49-11.67)	0.51 (0.29-0.89)	
L-FABP expression					

Negative	83	36 (43)	9.66 (6.97-13.39)	1	p=0.7391
Positive	34	14 (41)	9.43 (5.59-15.92)	0.90 (0.49-1.67)	
PD-L1 expression					
CPS<1	89	46 (52)	10.86 (8.13-14.49)	1	p=0.046
CPS≥1	29	5 (17)	5.11 (2.13-12.29)	0.43 (0.17-1.09)	
PD-L1 expression					
TPS<1	109	49 (44)	10.25 (7.75-13.57)	1	p=0.182
TPS≥1	9	2 (22)	4.59 (1.15-18.39)	0.43 (0.1-1.77)	
PD-L1 expression					
MIDS 0-1	93	47 (50)	10.27 (7.71-13.67)	1	p=0.16
MIDS 2-3	25	4 (16)	6.28 (2.36-16.74)	0.51 (0.18-1.43)	
PD-1$^+$ IICs*					
<1.6/HPF	59	34 (58)	13.08 (9.35-18.31)	1	p=0.004
≥1.6/HPF	56	15 (27)	6.09 (3.67-10.11)	0.43 (0.23-0.78)	
PD-1$^+$ PICs*					
<4.8/HPF	59	32 (54)	11.57 (8.18-16.37)	1	p=0.05
≥4.8/HPF	56	17 (30)	7.41 (4.60-11.91)	0.56 (0.31-1.01)	
PD-1$^+$ TICs*					
<6.8/HPF	59	32 (54)	11.94 (8.44-16.89)	1	p=0.034
≥6.8/HPF	56	17 (30)	7.14 (4.43-11.49)	0.54 (0.30-0.97)	
Teng type classification					p<0.001
Type I	23	3 (13)	3.44 (1.11-10.65)	1	
Type II	61	39 (64)	15.71 (11.48-21.5)	4.64 (1.43-15.03)	
Type III	6	2 (33)	19.08 (4.77-76.33)	4.1 (0.68-24.7)	
Type IV	28	7 (25)	3.99 (1.9-8.37)	1.27 (0.33-4.91)	

CoeD-SBA, coeliac disease-associated small bowel adenocarcinoma; CPS, combined positive score; CrD-SBA, Crohn's disease-associated small bowel adenocarcinoma; HPF, high-power field; IIC, intratumoural immune cell; L-FABP, liver fatty acid-binding protein; MIDS, mononuclear immune cell density score; MSI, microsatellite instability; MSI-H, microsatellite instability-high; PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand 1; PIC, peritumoural immune cell; TIC, total immune cell; TIL, tumour-infiltrating lymphocyte.

*The cut-off of 1.6/HPF, 4.8/HPF and 6.8/HPF were the respective medians of PD-1$^+$ IICs, PICs and TICs in the whole cohort.

Figure 4 – Kaplan-Meier cancer-specific survival estimates for small bowel adenocarcinoma (SBA) patients by aetiologic group (A), stage (B), tumour-infiltrating lymphocyte (TIL) density (C), microsatellite instability (MSI) status (D), histotype (E) and CDX2 expression (F). CoeD-SBA, coeliac disease-associated small bowel adenocarcinoma; CrD-SBA, Crohn's disease-associated small bowel adenocarcinoma.

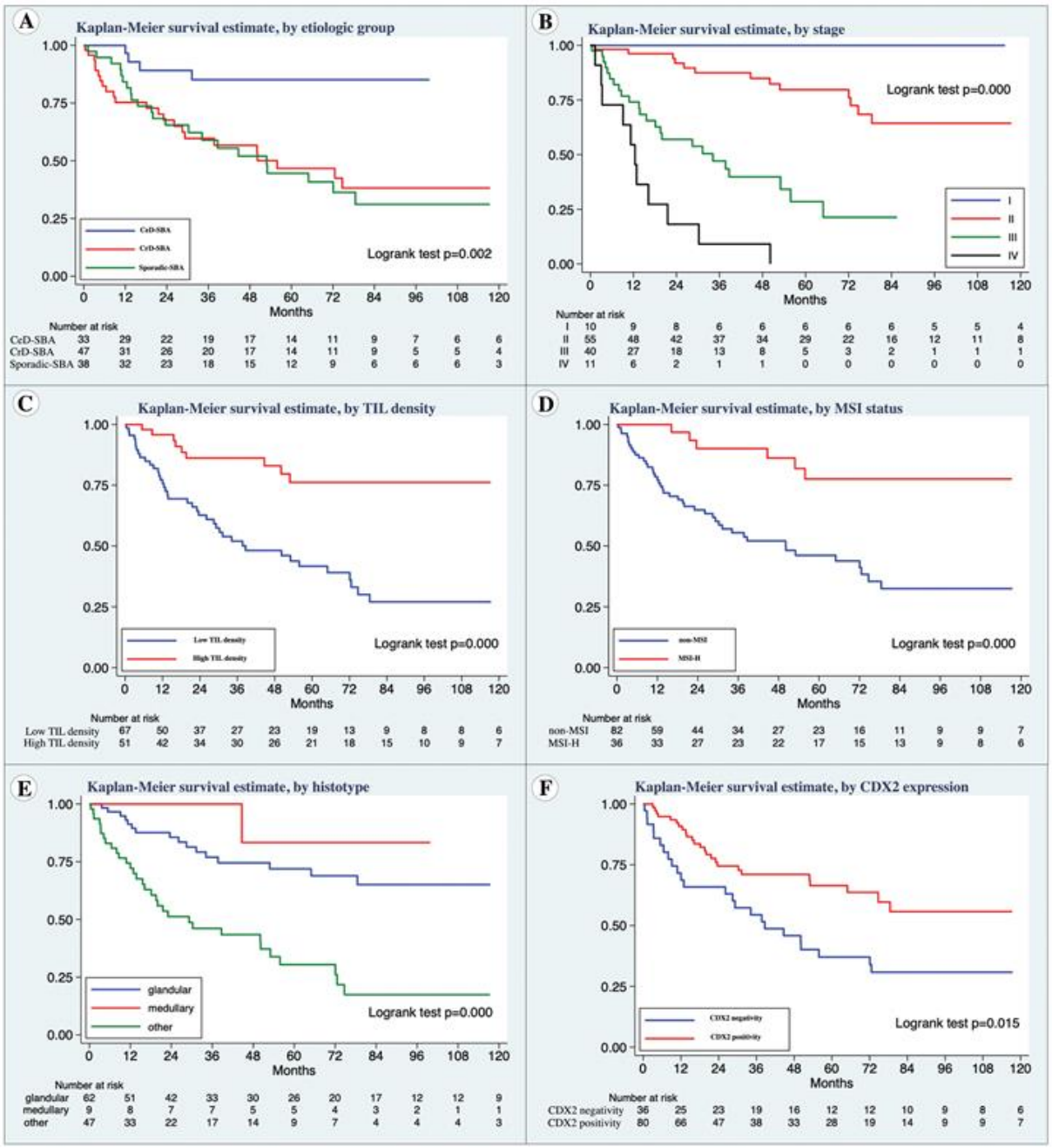


Figure 5 - Kaplan-Meier cancer-specific survival estimates for small bowel adenocarcinoma patients by intratumoural (A), peritumoural (B) and total (C) PD-1-positive immune cell counts.

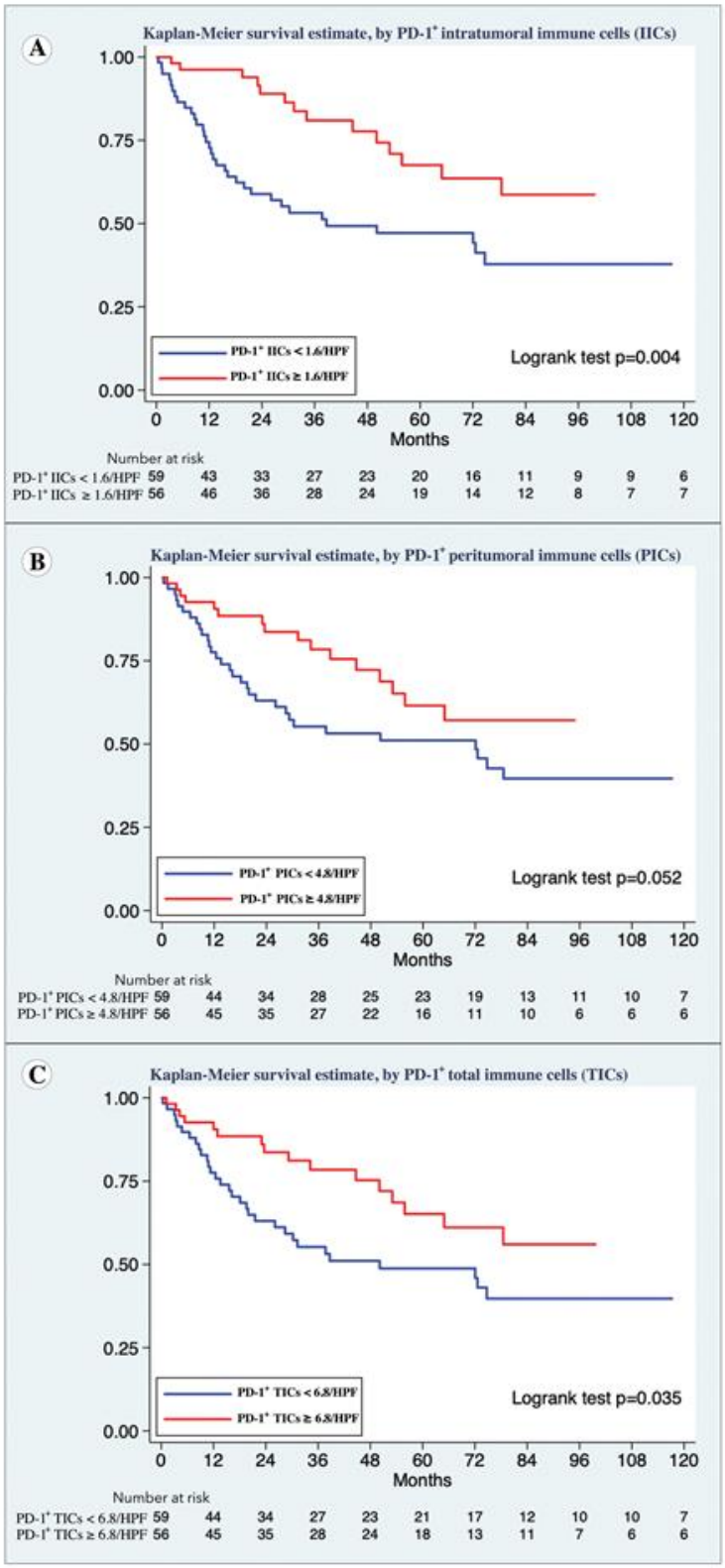


Figure 6 - Kaplan-Meier cancer-specific survival estimates for small bowel adenocarcinoma patients by PD-L1 expression according to combined positive score (CPS) (A) and Teng type classification (B).

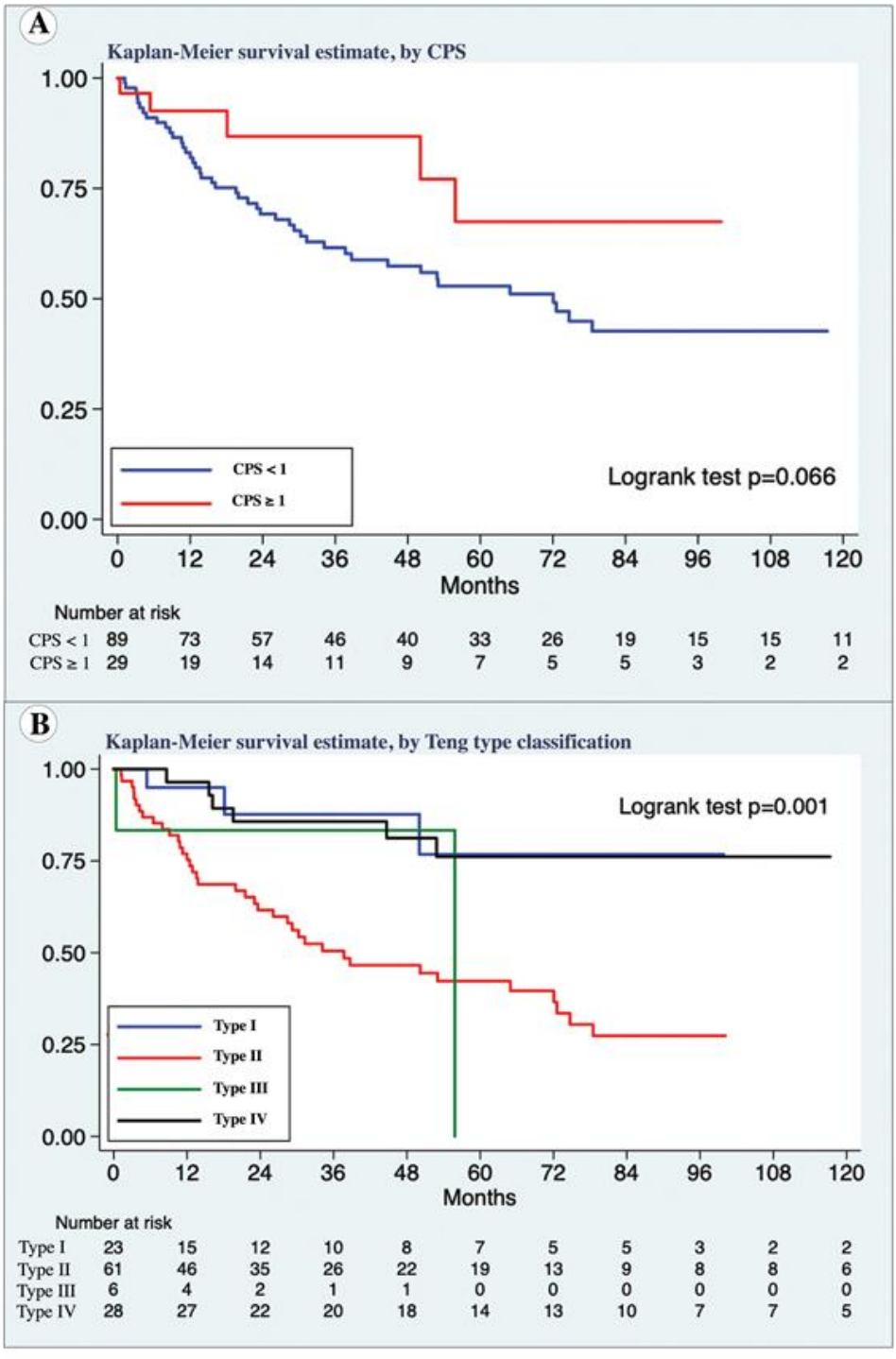
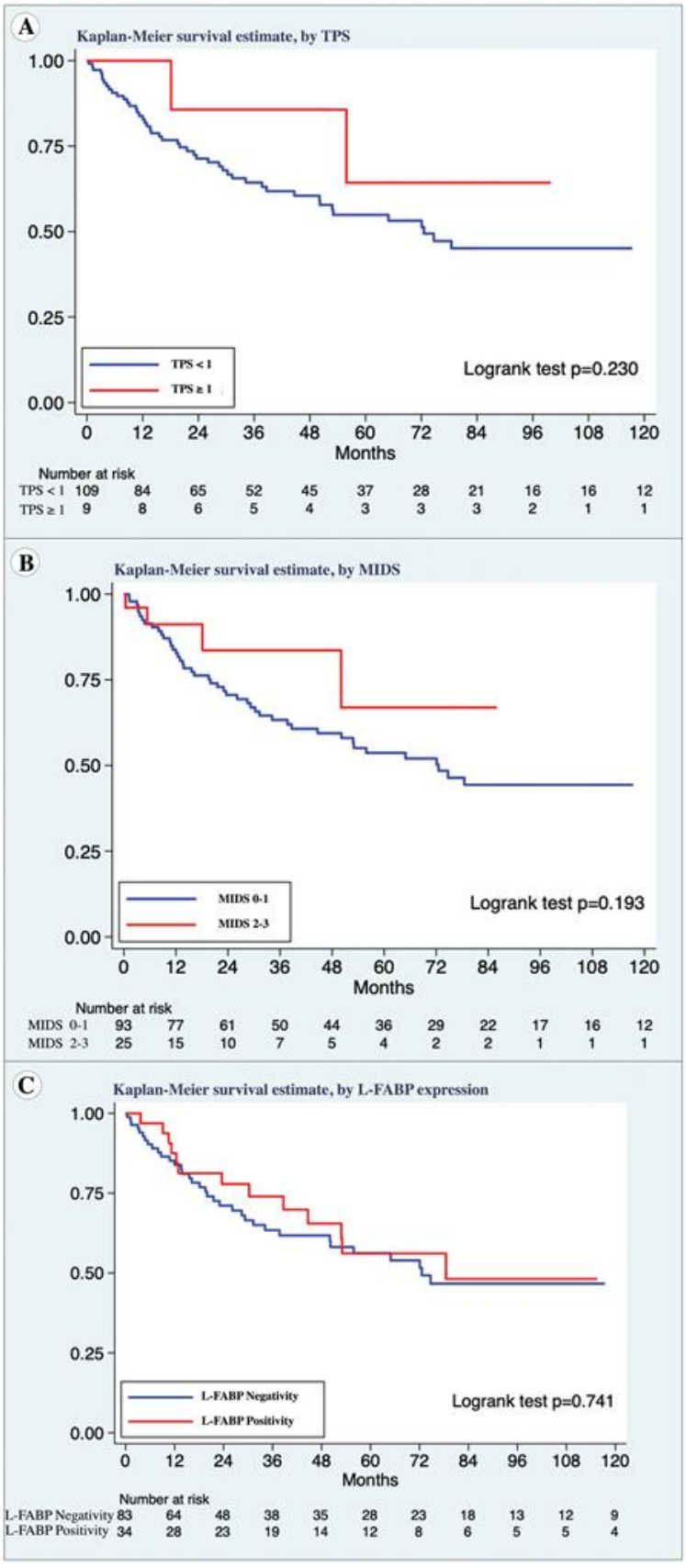


Figure 7 - Kaplan-Meier cancer-specific survival estimates for small bowel adenocarcinoma patients by PD-L1 expression according to tumor proportion score (TPS) (A) and mononuclear immune cell density score (MIDS) (B), and by liver fatty acid-binding protein (L-FABP) expression (C).



Tb and PDCs

Two CrD-SBA cases resembling in part medullary cancers, one of which EBV^+ and reinterpreted as lymphoepithelioma-like cancer [25], were omitted from this study, as recommended by Lugli and colleagues [111], owing to technical difficulties in assessing Tb and PDC status. The histologic classification of the remaining 47 CrD-SBAs and related clinico-pathologic features are reported in Table 11. A general predominance of male sex and a median age of 57 years at SBA diagnosis are worth of note. Fifteen cases showed high TIL density, while only eight were MMR-d, including seven cases with loss of MLH1/PMS2 and one case showing isolated loss of MSH6. p53 overexpression was found in 53.2% of CrD-SBCs without significant difference among histotypes. *KRAS*, *NRAS* and *PIK3CA* mutations were observed in three (12.5%), one (4%) and two (8%) out of 24 cases tested. Glandular-type cancers were predominantly well-to-moderately differentiated according to WHO grading system.

The histologic analysis of tumour invasion front parameters (Table 12 and Figure 8) showed a significant ($p<0.001$) association between histotypes and either Tb or PDC grades with a predominance of Tb1 (50%) and PDC1 (52%) among glandular-type cases, as well as of Tb3 (100%) and PDC3 (62%) among diffuse/mixed cancers. As for both Tb and PDC survival analysis (see below) gave poor separation (not significant p-values) of the relatively few grade 2 cases from the remaining grades, a two-tiered combined invasive front (CIF) grade was developed where grade 3 cases for either Tb or PDC or both defined CIF-high grade while all the remaining cases formed CIF-low grade. All diffuse and mixed tumors were placed in the CIF-high grade group, in contrast to a minority (10/24, 42%) of glandular cases. In Table 13 data on depth of tumor invasion (pT) and AJCC stage as a function of Tb, PDC and CIF grade are reported. An overall correlation was found between each of the three invasive front grading systems and invasion/stage parameters. Importantly, Tb, PDC or CIF grade were significantly ($p=0.001$, $p=0.023$ and $p<0.001$, respectively) associated with lymph node metastases, with increasing rate of lymph node metastases across grades). On the other hand, no association between invasive front markers and *KRAS*, *NRAS*, *PIK3CA* mutations, p53 overexpression or MMR-d/MSI status was observed.

Two patients died peri-operatively while the remaining 45 patients were followed for a median of 85 months (25^{th}-75^{th}: 31-121) and their cancer-specific survival data are reported in Table 14**.** As expected, stage and histotype were highly coupled with survival (Figure 9), while high TILs, found in 27% of cases, and WHO grade, with only very few

grade 1 cases, were less contributive. Both Tb and PDC invasive front analysis gave effective patient prognostication; in particular, their combination into a CIF grade separated 14 low-grade from 31 high-grade SBC patients with highly divergent outcomes (Figure 10 and Table 14). In addition, when CIF grade was applied to the 22 glandular histology cases (by themselves showing significantly better survival than 23 non-glandular cases), 13 CIF low- from 9 CIF high-grade tumors were identified, with a trend for divergent outcomes (HR=6.54, 95% CI:0.73-58.6, p=0.054), despite the limited number of available cases. CIF grade also gave significant results when applied to 24 stage I+II tumors, thus separating 13 low- (12 of which present in the CIF-low grade glandular group) from 11 high-grade cases, with significantly different outcomes (HR=7.78, 95% CI: 0.90-67.3, p=0.027). Indeed, no cancer-related death was observed, during a median follow-up of 73.5 months, among the 12 patients with CrD-SBA showing CIF-low grade, glandular structure and stage I or II. Of note, six (50%) of such tumours also showed high TILs and three were MMR-d.

Table 11. Histologic classification and clinico-pathologic features of the 47 Crohn's disease-associated SBA cases

Histotype	n (%)	Male sex, n (%)	Age at SBA diagnosis, median (25th-75th)	P53 overexpression (>50%) n (%)	MSI/MMR-d n (%)	High TILs n (%)	WHO grade, n (%)		
							1	2	3
Glandular	24 (51.1)	18 (75)	59 (54.5-69)	14 (58.3)	6 (25)	9 (37.5)	6 (25)	17 (70.8)	1 (4.2)
Mixed	11 (23.4)	9 (81.8)	56 (46-68)	6 (54.5)	2 (18.2)	2 (18.2)	0	4 (36.4)	7 (63.6)
Diffuse	10 (21.3)	7 (70)	51 (39-59)	3 (30)	0	4 (40)	0	0	10 (100)
Solid	2 (4.2)	0	53 (44-62)	2 (100)	0	0	0	0	2 (100)
Total	47 (100)	34 (72)	57 (50-68)	25 (53.2)	8 (17)	15 (31.9)	6 (12.8)	21 (44.7)	20 (42.5)

MMR-d, mismatch repair deficient; MSI, microsatellite instability; TIL, tumour-infiltrating lymphocyte.
WHO grade distribution among histotypes: p<0.001. All MMR-d SBAs also showed MSI by molecular analysis.

Table 12. Classification of 47 Crohn's disease-associated SBA cases by invasive front-based grading systems.

Histotype	Tumor budding (Tb), n (%)			Poorly differentiated clusters (PDC), n (%)			Combined invasive front grade (CIF), n (%)	
	Tb1	Tb2	Tb3	PDC1	PDC2	PDC3	Low	High
Glandular	12 (50)	4 (16.7)	8 (33.3)	13 (54.2)	6 (25)	5 (20.8)	14 (58.3)	10 (41.7)
Mixed	0	0	11 (100)	2 (18.2)	1 (9.1)	8 (72.7)	0	11 (100)
Diffuse	0	0	10 (100)	1 (10)	4 (40)	5 (50)	0	10 (100)
Solid	0	1 (50)	1 (50)	0	1 (50)	1 (50)	1 (50)	1 (50)
Total	12 (25.5)	5 (10.6)	30 (63.8)	16 (34)	12 (25.5)	19 (40.4)	15 (31.9)	32 (68.1)

Tb and CIF grade distribution among histotypes: p<0.001. PDC distribution among histotypes: p=0.016.

Figure 8 - A) A glandular Crohn's disease-associated small bowel adenocarcinoma (CrD-SBA) showing grade 1 tumor budding (Tb1) and grade 1 poorly differentiated clusters (PDC1) at the tumour invasive front (on the right) (haematoxylin and eosin; original magnification, 200x). B) A mixed-type CrD-SBC with grade 3 PDCs (arrows; haematoxylin and eosin; original magnification, 200x). C, D) A glandular CrD-SBC showing grade 3 Tb (arrows; C, haematoxylin and eosin; D, pan-cytokeratin immunostaining; original magnification, 200x).

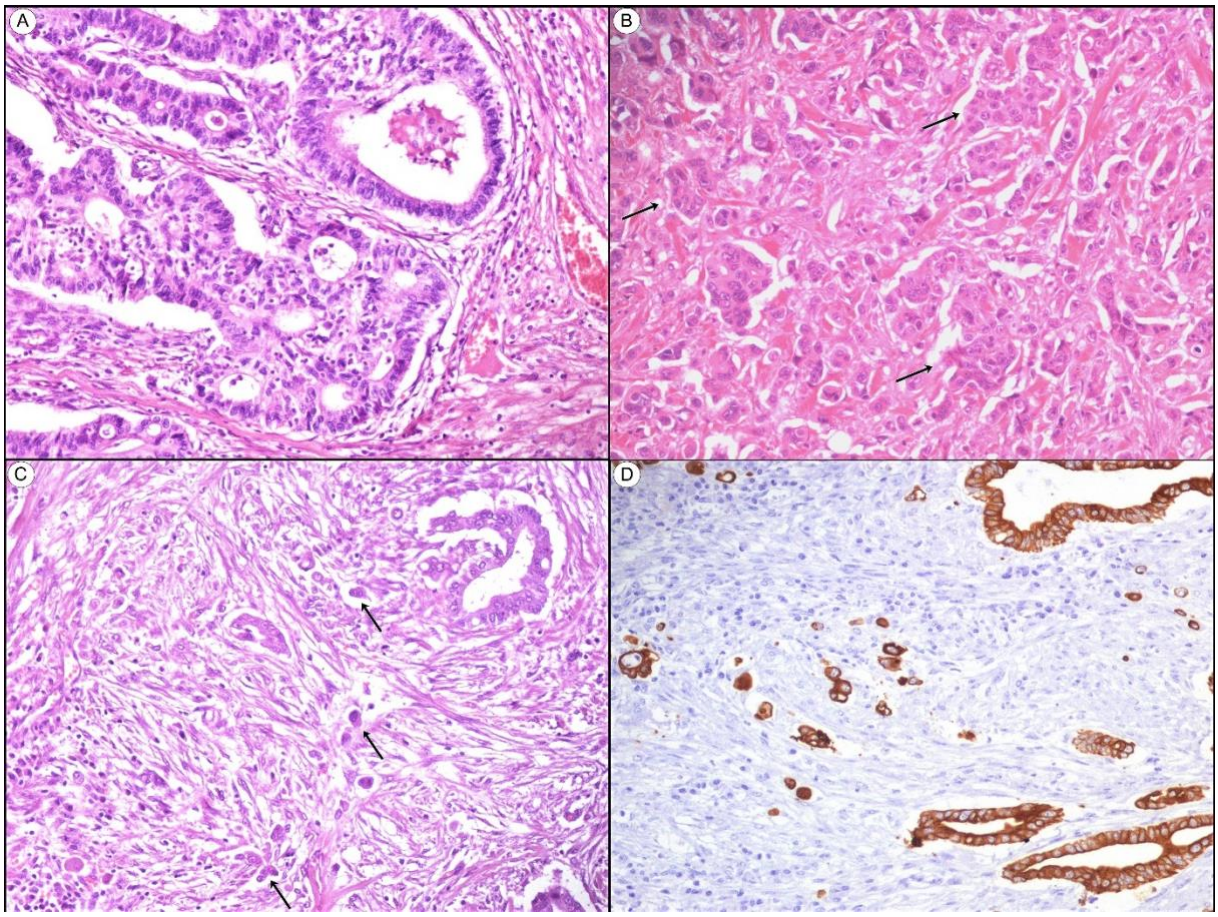


Table 13. Distribution of Tb, PDC and CIF scores among the 47 Crohn's disease-associated SBA cases classified according to pT and AJCC stage

Grading system		N. of cases, (%)	pT, n (%)				p-value	Stage, n (%)				p-value
			pT1	pT2	pT3	pT4		I	II	III	IV	
Tb	**Tb1**	12 (25.5)	2 (16.7)	4 (33)	5 (41.7)	1 (8.3)	0.001	6 (50)	5 (41.7)	1 (8.33)	0	<0.001
	Tb2	5 (10.7)	0	0	2 (40)	4 (60)		0	3 (60)	2 (40)	0	
	Tb3	30 (63.8)	0	0	16 (53.3)	14 (46.7)		0	11 (36.7)	13 (43.3)	6 (20)	
PDC	**PDC1**	16 (34.1)	2 (12.5)	4 (25)	7 (43.7)	3 (18.8)		6 (37.5)	6 (37.5)	4 (25)	0	
	PDC2	12 (25.5)	0	0	8 (66.7)	4 (33.3)	0.016	0	8 (66.7)	2 (16.7)	2 (16.7)	0.002
	PDC3	19 (40.4)	0	0	8 (42.1)	11 (57.9)		0	5 (26.3)	10 (52.6)	4 (21.1)	
CIF grade	**CIF low grade**	15 (31.9)	2 (13.3)	4 (26.7)	6 (40)	3 (20)	0.002	6 (40)	8 (53.3)	1 (6.7)	0	<0.001
	CIF high grade	32 (68.1)	0	0	17 (53.1)	15 (46.9)		0	11 (34.4)	15 (46.9)	6 (18.7)	

Tb, tumor budding; PDC, poorly differentiated clusters; CIF, combined invasive front; pT: extent of the tumor into the layers of the wall of the small intestine (according to the 8th ed. AJCC TNM staging system)

Table 14. Cancer-specific survival of 45 Crohn's disease-associated SBAs classified according to their invasion front pattern and other predictive parameters.

Parameter		N. of cases	N. of deaths (%)	Rate per 100 person-year (95% CI)	HR (95% CI)	p-value (Cox)	Harrell's c (95% CI)
Tumor budding	Tb1	11	1 (9.1)	1.33 (0.19-9.44)	1	<0.001	0.68 (0.59-0.77)
	Tb2	5	2 (40)	20.25 (5.06-80.97)	10.6 (0.95-118.7)		
	Tb3	29	20 (68.9)	25.18 (16.24-39.03)	14.72 (1.95-111.18)		
PDC	PDC1	15	3 (20)	3.69 (1.19-11.43)	1	0.004	0.69 (0.58-0.80)
	PDC2	12	8 (66.7)	23.84 (11.92-47.67)	4.88 (1.29-18.46)		
	PDC3	18	12 (66.7)	24.23 (13.76-42.67)	5.94 (1.67-21.15)		
CIF grade	low	14	2 (14.3)	2.45 (0.61-9.78)	1	<0.001	0.66 (0.57-0.75)
	high	31	21 (67.7)	25.38 (16.55-38.92)	8.27 (1.91-35.90)		
WHO	G1	5	0	0	not evaluable (-∞)	0.001	0.67 (0.56-0.78)
	G2	20	8 (40)	10.14 (5.07-20.27)	1		
	G3	20	15 (75)	29.97 (18.07-49.71)	2.08 (0.88-4.91)		
Histotype	glandular	22	5 (22.7)	4.59 (1.91-11.03)	1	<0.001	0.68 (0.58-0.78)
	non-glandular	23	18 (78.3)	32.38 (20.4-51.39)	5.02 (1.85-13.63)		
TILs	high	15	4 (26.7)	5.62 (2.11-14.98)	1	0.027	0.60 (0.50-0.70)
	low	30	19 (63.3)	20.36 (12.99-31.92)	3.01 (1.02-8.92)		
Stage	I	5	0	0	not evaluable (-∞)	<0.001	0.80 (0.72-0.87)
	II	19	6 (31.6)	5.81 (2.61-12.94)	1		
	III	15	11 (73.3)	50.24 (27.82-90.72)	8.29 (2.51-27.31)		
	IV	6	6 (100)	81.39 (36.57-181.18)	13.65 (3.67-50.83)		

Tb, tumor budding; PDC, poorly differentiated clusters; CIF, combined invasive front; TILs, tumour-infiltrating lymphocytes; HR, hazard ratio; CI: confidence interval.

Figure 9 - Kaplan-Meier survival estimates on the 45 CrD-SBCs by stage (A) and histotype (B).

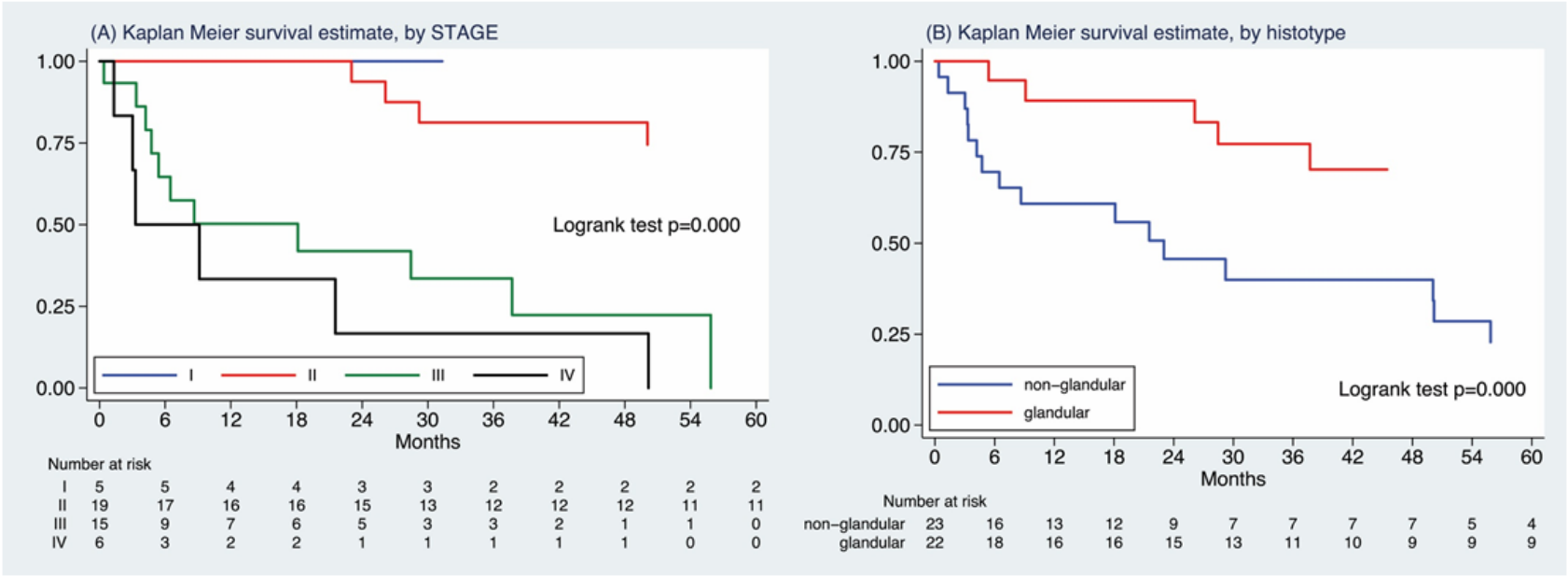
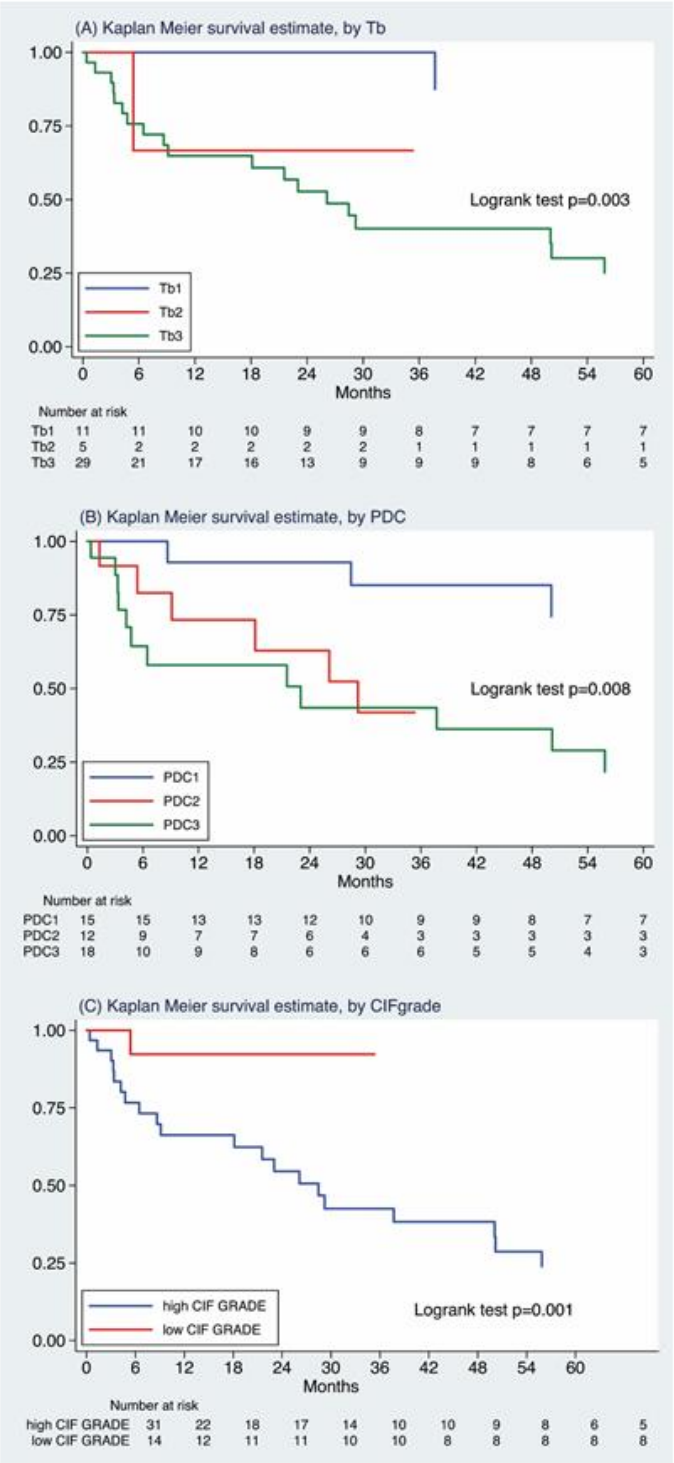


Figure 10 - Kaplan-Meier survival estimates on the 45 CrD-SBCs by tumour budding (A), poorly differentiated clusters (B) and combined invasive front grade (C).



Discussion

We herein systematically investigated the tumour immune microenvironment in a large cohort of SBAs associated with CoeD or CrD. In keeping with previous findings [49,101], we found that PD-L1 was less frequently expressed in tumour cells than in immune cells in SBAs. Moreover, in agreement with Thota and colleagues [49], we observed that PD-L1$^+$ SBAs have a higher number of intratumoural, peritumoural, and total PD-1$^+$ immune cells. We demonstrated that PD-L1 expression in non-hereditary SBAs as a whole was 25.6%, 8.3% and 22.3% according to CPS, TPS and MIDS, respectively. This is the first investigation to identify a higher proportion of PD-L1$^+$ cases in both CoeD-SBAs and CrD-SBAs in comparison with sporadic SBAs, regardless of the score used, i.e. CPS, TPS or MIDS. Additionally, we showed a significant increase in PD-1$^+$ immune cells in CoeD-SBAs in comparison with CrD-SBAs and sporadic SBAs. Indeed, a recent transcriptomic profiling study demonstrated a predominant MSI-immune subtype of CoeD-SBA characterized by high PD-1 activation [124]. Therefore, response to anti-PD-L1 or anti-PD-1 monoclonal antibodies might be more often expected in CoeD-SBAs and –likely to a lesser extent- in CrD-SBAs compared to sporadic cases. A number of clinical trials with immune checkpoint inhibitors for the treatment of SBA patients are underway. In particular, the ZEBRA trial assessed the activity of pembrolizumab in 40 patients with previously treated advanced SBA [125]. Preliminary results of the ZEBRA trial found that pembrolizumab did not significantly improve the overall response rate, although efficacy was shown in MSI-H patients and, of note, in some microsatellite stable patients too [125]. Data related to the tumour immune microenvironment are still unavailable from this study [125]. Further trials are recommended in these settings of patients, including those with CoeD-SBA and CrD-SBA.

The higher expression of PD-L1 shown in CoeD-SBAs and CrD-SBAs in comparison with sporadic SBAs and the increase in PD-1$^+$ immune cells found in CoeD-SBAs might be secondary to the underlying immune responses. In patients with untreated CoeD without SBA, PD-L1 is up-regulated in enterocytes and lamina propria mononuclear cells, whereas PD-1 is not present in the small intestine [126]. In CrD patients without SBA, PD-L1 is reduced in CD4$^+$ T cells because of high expression of the T helper 1 transcription factor T-bet *via* transfection [127], while another study [128] demonstrated overexpression of PD-L1 in macrophages and PD-1 in T cells, predominantly CD4$^+$, and B cells from the

lamina propria. It must be outlined that the expression of PD-L1 and PD-1 we scored in our cohort was strictly related topographically, within tumour tissue or immediately surrounding it, to SBA, not to the underlying inflammatory CoeD or CrD background. Whether the use of PD-L1/PD-1 pathway blockade treatments in CoeD and/or CrD patients induces changes of the underlying immune-mediated intestinal disorder, which might also affect SBA, requires more-in-depth study [129].

As the pro-inflammatory cytokine interferon-γ, known to be involved in the pathogenesis underlying both CoeD and CrD [4,9], decreased the expression *in vitro* of L-FABP from colorectal cancer cell lines [122], we investigated L-FABP in our cohort. In agreement with colorectal cancer [122], we found that L-FABP negativity is more common in MSI-H SBAs than in microsatellite stable cases. Additionally, we observed that PD-L1$^+$ SBAs correlated with L-FABP negativity. As in colorectal carcinomas the absence of expression of the intestinal marker CDX2 is coupled with PD-L1 expression [106], we also correlated PD-L1 with CDX2 expression. Nevertheless, we did not observe a significant correlation between PD-L1 expression with CDX2 negativity.

We have also confirmed the previously demonstrated association between expression of PD-L1 and MSI-H in SBAs [49,101,102], in agreement with gastric and colorectal carcinomas [47,48]. At first glance, these findings might lead to consider MSI-H as a predictor of clinical response to anti-PD-L1 or anti-PD-1 monoclonal antibodies in patients with SBA and in those with colorectal cancer [96,130] Nevertheless, in keeping with endometrial carcinoma [104], we described 15 PD-L1$^+$ microsatellite stable cases, 11 CrD-SBAs, three CoeD-SBAs, and one sporadic SBA, thus suggesting that MSI-H on its own is not enough to identify all PD-L1$^+$ SBAs. These results support the need to find additional predictors of response to anti-PD-L1 or anti-PD-1 monoclonal antibodies. TILs have been considered as a marker of host immune response against tumour [131], and their density has been described as an independent prognostic factor in non-hereditary SBAs [2,102]. High TIL density, which is typical in both Teng types I and IV, plays a role in adaptive resistance in tumours through the secretion of pro-inflammatory cytokines, which cause PD-L1 expression and ultimately induce immunosuppression in the tumour microenvironment [132]. We here showed that the majority of PD-L1$^+$ cases have a high CD3$^+$ and CD8$^+$ TIL density. However, we described seven out of 31 PD-L1$^+$ SBAs with low

TIL density, namely Teng type III. We think that tumour-specific molecular alterations might promote PD-L1 positivity in SBAs with low TIL density.

PD-L1 expression is frequently associated with EBV positivity in gastric cancer and two EBV^+ CrD-SBAs have been demonstrated [26,48]. We here evaluated latent phase EBV infection in our entire SBA cohort and confirmed EBV positivity of the two previously described SBAs, while no EBER was displayed in the other 116 cases tested. Within the two EBV^+ CrD-SBAs, only the lymphoepithelioma-like carcinoma was PD-$L1^+$ whereas the remaining case, the glandular one, was PD-L1-negative. This is the first study reporting no latent infection of EBV in CoeD-SBA. In agreement with Von Rahden and colleagues [27]. we confirmed EBV negativity in all sporadic SBAs. With regard to histotypes, PD-$L1^+$ SBAs was associated with medullary histotype, which does have a favourable outcome not only in SBAs but also in gastric and colorectal carcinomas [46,133,134].

In our entire SBA cohort, PD-L1 expression according to CPS showed a trend towards a better prognosis. This is not surprising, as PD-L1 expression was higher in SBAs with increased $CD3^+$ and $CD8^+$ TIL density, and in SBAs with medullary histotype, both features related to a more favourable prognosis [2,46]. Moreover, our results confirmed aetiologic group, histotype, TIL density, Teng tumour microenvironment immune types and stage as prognostic parameters in SBAs [2,7,101,102,135]. Although univariate analysis reported a more favourable prognosis in PD-$L1^+$ SBAs according to a CPS than that in PD-$L1^-$ cases, PD-L1 expression lost its prognostic power once adjusted for TIL density.

As regards the second objective of this thesis, we confirmed the favourable prognostic impact of glandular histotype compared to its loss to form diffuse or mixed cancerous growths in CrD-SBA, as already suggested in a previous study recruiting a smaller cohort [7]. Along with this architectural assessment on the whole cancer tissue, we demonstrated that selective evaluation of the tumour invasive front for *foci* of Tb and/or PDCs substantially improved separation of more from less aggressive CrD-SBAs. Furthermore, a significant association of Tb and PDC with lymph node metastases was observed. In particular, invasive front analysis was effective, in glandular type CrD-SBAs, in finding cases with many *foci* of cell dissociation or structural dedifferentiation -high-grade Tb or PDCs-, which were significantly associated with survival shortening

compared to those with low CIF grade, namely low-to-intermediate grade Tb and PDCs. The use of a novel CIF two tier grading system, which includes both Tb and PDC, makes this system easier to apply in comparison with the separate assessment of Tb and PDC. This is the first study assessing Tb and PDCs in non-ampullary SBAs in a cohort of 47 CrD patients. Tb was also studied in ampullary adenocarcinomas, in which high-grade Tb was found an independent predictor of overall survival [112]. Recently, Jun SY and colleagues [136] confirmed our findings, showing that high-grade Tb or PDCs are associated with an aggressive behaviour and, thus, with a worse patient outcome in SBA. Therefore, the main novelty of this Korean study was to identify the invasive front markers Tb and PDCs as prognostic indicators in sporadic SBAs [136], whereas we obtained the same results in CrD-SBAs. Finally, we do acknowledge that Jun SY and colleagues [136] are the first to analyse Tb and PDCs in the intratumoural area of SBAs.

Our findings extend to CrD-SBA the prognostic significance of Tb and PDC assessment, so far mainly described for colorectal carcinoma, mostly gland-forming, usual-type adenocarcinoma. Moreover, we demonstrated that the usefulness of CIF grade is limited to the CrD-SBA glandular histotype, which is in agreement with recent findings on gastric cancer [137]. It seems that both processes implicated in loss of structural differentiation, one occurring massively within the whole neoplasm, leading to the diffuse and mixed tumour histotypes, and the other one selectively acting at its invasive front -the high-grade Tb or PDCs-, are strongly coupled with a worse patient outcome. Briefly, persistence, even at the invasive front, of the "canonical" glandular-type structure defines a relatively less aggressive subgroup of CrD-SBAs, predominantly non-metastatic and with a restricted invasiveness.

Of note, loss of cellular/glandular differentiation to form "poorly cohesive" tumors has long been recognised within gastric cancers and suggested to worsen patient prognosis [138-140]. Molecular studies have highlighted that diffuse desmoplastic cancers of the stomach, pancreas and colon likely represent the histologic consequence of the epithelial-to-mesenchymal transition present in such neoplasms, with a severe prognostic effect [141-146]. It appears evident, particularly from recent studies on colorectal carcinoma, that cancer evaluation at its invasive front may identify an otherwise undetectable tumour proneness to cell dissociation and invasion, thus predicting a worse post-

operative behaviour [108-111,147]. From our results it seems that the same phenomenon happens in CrD-SBAs, which may add more information to stage histologic assessment of surgical samples.

High TIL density was coupled with favourable survival in CrD-SBAs too; notwithstanding, TIL density less effective in CrD-SBAs than in CoeD-SBAs [2], being present in a lower number of cases. TIL-rich medullary-type CrD-SBAs, including the EBV^+ lymphoepithelioma-like carcinoma with medullary-like histology [25], are known by themselves to generally have a better outcome [46,133,134], thus overcoming the technical difficulty for Tb and PDC evaluation reported in this rare cancer type.

Conclusions and future perspectives

As regards the primary objective of this thesis, PD-L1 expression in non-hereditary SBAs is coupled with its aetiologic group, being more frequent in CoeD-SBAs and CrD-SBAs compared to sporadic SBAs, as well as with its MSI status and TIL density. The identification of a subset of PD-L1$^+$ non-MSI cases in our cohort suggests that PD-L1 expression, together with MSI status, TIL density, and tumour mutation burden, should be recognised as potential biomarkers of response to immune checkpoint inhibitors in well-designed clinical trials focused on SBA, including CoeD-SBA and CrD-SBA.

With regard to the secondary objective of this thesis, analysis of tumour cell dissociation/de-differentiation markers at the invasive front, such as Tb and PDCs, may improve the identification of highly malignant CrD-SBAs. Indeed, as survival rate is similarly low in patients with CrD-SBA and those with sporadic SBA (Table 5), it is clinically relevant to find prognostic markers for this neoplasm, in particular in these two aetiologic groups. Briefly, these findings should encourage pathologists to describe Tb and PDCs at SBA diagnosis in both CrD patients and sporadic cases in order to separate highly malignant cancers from less aggressive SBAs. Indeed, Tb and PDCs may represent pivotal histological features together with mismatch repair status, histotype and pT [83] to identify stage II SBAs needing adjuvant chemotherapy or further surgery, as well as they have been demonstrated in colon adenocarcinoma [148].

The main limitation of this study is its retrospective nature. Notwithstanding, as SBA is a rare neoplasm, this was a necessary choice in order to recruit a relatively large cohort of SBAs. Additionally, the involvement of tertiary Italian Coeliac and/or IBD Centers with long-term referral experience in CoeD and in inflammatory bowel disease, which were following internationally agreed guidelines, was a guarantee of data quality.

Either the association of PD-L1 expression with MSI status in non-hereditary SBAs or the finding of Tb and PDCs as able to separate lower from higher CrD-SBAs confirmed some similarities between SBAs and colorectal carcinomas. Although clinico-radiologic and endoscopic distinctions between SBA and colorectal carcinoma are usually straightforward, it should be recalled that colorectal carcinoma may infiltrate or metastasise to the small bowel, mimicking a primary SBA, or be predominantly located around the ileocaecal valve, simulating a primary neoplasm of the terminal ileum, especially in CrD patients. In these challenging situations, as well as in the setting of

metastasis of unknown primary origin, immunohistochemistry may play a certain role in suggesting the site of origin. The typical immunophenotypic profile of lower gastrointestinal tract (colorectal or appendiceal) carcinomas, is $CK7^{-}/CK20^{+}/CDX2^{+}$, whereas about 60% of SBAs have been reported to co-express CK7 and CK20 and around a half of SBAs is negative for CDX2 [149-151]. Nevertheless, on one hand, CK7 is also expressed in about 10% of colorectal carcinomas [152] and CK20 and CDX2 markers can be lost in some colorectal carcinoma, especially in those harbouring MMR-d [153], while, on the other hand, a fraction of SBAs (34%) has been reported to show a colorectal cancer-like immunohistochemical profile (CK7-/CK20+/CDX2+) [154]. Thus, it is pivotal to find markers to discriminate SBAs from colorectal cancers in the aforementioned challenging contexts. The next step of this thesis is to investigate the immunohistochemical expression of special AT-rich sequence-binding protein 2 and other gastrointestinal phenotypic markers (CK7, CK20, CDX2, AMACR), in a large and etiologically well-characterized series of SBAs and to correlate the observed immunophenotypic profiles with clinico-pathologic and prognostic features, as well as with MSI status.

References

1. Raghav K, Overman MJ. Small bowel adenocarcinomas—Existing evidence and evolving paradigms. Nat Rev Clin Oncol 2013;10:534–544.
2. Vanoli A, Di Sabatino A, Furlan D, et al. Small Bowel Carcinomas in Coeliac or Crohn's Disease: Clinico-pathological, Molecular, and Prognostic Features. A Study from the Small Bowel Cancer Italian Consortium. J Crohns Colitis 2017;11:942–953.
3. Di Sabatino A, Lenti MV, Giuffrida P, et al. New insights into immune mechanisms underlying autoimmune diseases of the gastrointestinal tract. Autoimmun Rev 2015;14:1161–1169.
4. Di Sabatino A, Corazza GR. Coeliac disease. Lancet 2009;373:1480–1493.
5. Potter DD, Murray JA, Donohue JH, et al. The role of defective mismatch repair in small bowel adenocarcinoma in celiac disease. Cancer Res 2004;64:7073–7077.
6. Diosdado B, Buffart TE, Watkins R, et al. High-resolution array comparative genomic hybridization in sporadic and celiac disease-related small bowel adenocarcinomas. Clin. Cancer Res. 2010, 16, 1391–1401.
7. Vanoli A, Di Sabatino A, Martino M, et al. Small bowel carcinomas in celiac or Crohn's disease: Distinctive histophenotypic, molecular and histogenetic patterns. Mod Pathol 2017;30:1453–1466.
8. Svrcek M, Piton G, Cosnes J, et al. Small bowel adenocarcinomas complicating Crohn's disease are associated with dysplasia: A pathological and molecular study. Inflamm Bowel Dis 2014;20:1584–1592.
9. Giuffrida P, Corazza GR, Di Sabatino A. Old and New Lymphocyte Players in Inflammatory Bowel Disease. Dig Dis Sci 2018;63:277–288.
10. Grolleau C, Pote NM, Guedj NS, et al. Small bowel adenocarcinoma complicating Crohn's disease: A single-centre experience emphasizing the importance of screening for dysplasia. Virchows Arch 2017;471:611–617.
11. Rashid A, Hamilton SR. Genetic alterations in sporadic and Crohn's-associated adenocarcinomas of the small intestine. Gastroenterology 1997;113:127–135.
12. Bojesen RD, Riis LB, Høgdall E, et al. Inflammatory Bowel Disease and Small Bowel Cancer Risk, Clinical Characteristics, and Histopathology: A Population-Based Study. Clin Gastroenterol Hepatol 2017;15:1900–1907.
13. Howdle PD, Jalal PK, Holmes GK, et al. Primary small-bowel malignancy in the UK and its association with coeliac disease. QJM 2003;96:345–353.

14. Bruno CJ, Batts KP, Ahlquist DA. Evidence against flat dysplasia as a regional field defect in small bowel adenocarcinoma associated with celiac sprue. Mayo Clin Proc 1997;72:320–322.
15. Michelassi F, Testa G, Pomidor WJ, et al. Adenocarcinoma complicating Crohn's disease. Dis. Colon Rectum 1993, 36, 654–661.
16. Sigel JE, Petras RE, Lashner BA, et al. Intestinal adenocarcinoma in Crohn's disease: A report of 30 cases with a focus on coexisting dysplasia. Am J Surg Pathol. 1999;23:651–655.
17. Palascak-Juif V, Bouvier AM, Cosnes, J, et al. Small bowel adenocarcinoma in patients with Crohn's disease compared with small bowel adenocarcinoma de novo. Inflamm Bowel Dis 2005;11: 828–832.
18. Piton G, Cosnes J, Monnet E, et al. Risk factors associated with small bowel adenocarcinoma in Crohn's disease: A case-control study. Am J Gastroenterol 2008;103:1730–1736.
19. Widmar M, Greenstein AJ, Sachar DB, et al. Small bowel adenocarcinoma in Crohn's disease. J Gastrointest Surg 2011;15:797–802.
20. Whitcomb E, Liu X, Xiao SY. Crohn enteritis-associated small bowel adenocarcinomas exhibit gastric differentiation. Hum Pathol 2014;45:359–367.
21. Weber NK, Fletcher JG, Fidler JL, et al. Clinical characteristics and imaging features of small bowel adenocarcinomas in Crohn's disease. Abdom Imaging 2015;40:1060–1067.
22. Wieghard N, Mongoue-Tchokote S, Young JI, et al. Prognosis of small bowel adenocarcinoma in Crohn's disease compares favourably with de novo small bowel adenocarcinoma. Colorectal Dis 2017;19:446–455.
23. Warth A, Kloor M, Schirmacher P, et al. Genetics and epigenetics of small bowel adenocarcinoma: The interactions of CIN, MSI, and CIMP. Mod Pathol 2011;24:564–570.
24. Overman MJ, Pozadzides J, Kopetz S, et al. Immunophenotype and molecular characterisation of adenocarcinoma of the small intestine. Br J Cancer 2010;102:144–150.
25. Vanoli A, Di Sabatino A, Biancone L, et al. Small bowel Epstein-Barr virus-positive lympho-epithelioma-like carcinoma in Crohn's disease. Histopathology 2017;70:837–839.

26. Vanoli A, Di Sabatino A, Martino M, et al. Epstein-Barr virus-positive ileal carcinomas associated with Crohn's disease. Virchows Arch 2017;471:549–552.
27. Von Rahden BH, Langner C, Brücher BL, et al. No association of primary adenocarcinomas of the small bowel with Epstein-Barr virus infection. Mol Carcinog 2006;45:349–352.
28. DeSesso JM, Jacobson CF. Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. Food Chem Toxicol 2001;39:209–228.
29. Aparicio T, Zaanan A, Svrcek M, et al. Small bowel adenocarcinoma: Epidemiology, risk factors, diagnosis and treatment. Dig Liver Dis 2014;46:97–104.
30. Kummar S, Ciesielski TE, Fogarasi MC. Management of small bowel adenocarcinoma. Oncology 2002;16:1364–1369.
31. Bilimoria KY, Bentrem DJ, Wayne JD, et al. Small bowel cancer in the United States: Changes in epidemiology, treatment, and survival over the last 20 years. Ann Surg 2009;249:63–71.
32. Faivre J, Trama A, De Angelis R, et al. Incidence, prevalence and survival of patients with rare epithelial digestive cancers diagnosed in Europe in 1995–2002. Eur J Cancer 2012;48:1417–1424.
33. Han Y, Chen W, Li P, et al. Association Between Coeliac Disease and Risk of Any Malignancy and Gastrointestinal Malignancy: A Meta-Analysis. Medicine 2015;94:e1612.
34. Canavan C, Abrams KR, Mayberry J. Meta-analysis: Colorectal and small bowel cancer risk in patients with Crohn's disease. Aliment Pharmacol Ther 2006;23:1097–1104.
35. Silano M, Volta U, Vincenzi AD, et al. Effect of a gluten-free diet on the risk of enteropathy-associated T-cell lymphoma in celiac disease. Dig Dis Sci 2008;53:972–976.
36. Elfström P, Granath F, Ye W, et al. Low risk of gastrointestinal cancer among patients with celiac disease, inflammation, or latent celiac disease. Clin Gastroenterol Hepatol 2012;10:30–36.
37. Mukherjee R, Kelly CP, Leffler DA. Gastrointestinal cancer in celiac disease: "The first days are the hardest days, don't you worry anymore?". Clin Gastroenterol Hepatol 2012;10:4–6.
38. Silano M, Volta U, Mecchia AM, et al. Delayed diagnosis of coeliac disease increases cancer risk. BMC Gastroenterol 2007;7:8.

39. Di Sabatino A, Biagi F, Gobbi PG, et al. How I treat enteropathy-associated T-cell lymphoma. Blood 2012;119:2458–2468.
40. Cantoro L, Di Sabatino A, Papi C, et al. The Time Course of Diagnostic Delay in Inflammatory Bowel Disease Over the Last Sixty Years: An Italian Multicentre Study. J Crohns Colitis 2017;11:975–980.
41. Cahill C, Gordon PH, Petrucci A, et al. Small bowel adenocarcinoma and Crohn's disease: Any further ahead than 50 years ago? World J Gastroenterol 2014;20:11486–11495.
42. Lashner BA. Risk factors for small bowel cancer in Crohn's disease. Dig Dis Sci 1992;37:1179–1184.
43. Solem CA, Harmsen WS, Zinsmeister AR, et al. Small intestinal adenocarcinoma in Crohn's disease: A case-control study. Inflamm Bowel Dis 2004;10:32–35.
44. Schottenfeld D, Beebe-Dimmer JL, Vigneau, FD. The epidemiology and pathogenesis of neoplasia in the small intestine. Ann Epidemiol 2009;19:58–69.
45. Goodman MT, Matsuno RK, Shvetsov YB. Racial and ethnic variation in the incidence of small-bowel cancer subtypes in the United States, 1995–2008. Dis Colon Rectum 2013;56:441–448.
46. Brcic I, Cathomas G, Vanoli A, et al. Medullary carcinoma of the small bowel. Histopathology 2016;69:136–140.
47. Rosenbaum MW, Bledsoe JR, Morales-Oyarvide V, et al. PD-L1 expression in colorectal cancer is associated with microsatellite instability, BRAF mutation, medullary morphology and cytotoxic tumor-infiltrating lymphocytes. Mod Pathol 2016;29:1104–1112.
48. De Rosa S, Sahnane N, Tibiletti MG, et al. EBV^{+} and MSI Gastric Cancers Harbor High PD-L1/PD-1 Expression and High $CD8^{+}$ Intratumoral Lymphocytes. Cancers 2018;10:102.
49. Thota R, Gonzalez RS, Berlin J, et al. Could the PD-1 Pathway Be a Potential Target for Treating Small Intestinal Adenocarcinoma? Am J Clin Pathol 2017;148:208–214.
50. Schrock AB, Devoe CE, McWilliams R, et al. Genomic Profiling of Small-Bowel Adenocarcinoma. JAMA Oncol 2017;3:1546–1553.
51. Aparicio T, Svrcek M, Zaanan A, et al. Small bowel adenocarcinoma phenotyping, a clinicobiological prognostic study. Br J Cancer 2013;109:3057–3066.

52. Yaeger R, Shah MA, Miller VA, et al. Genomic Alterations Observed in Colitis-Associated Cancers Are Distinct from Those Found in Sporadic Colorectal Cancers and Vary by Type of Inflammatory Bowel Disease. Gastroenterology 2016;151:278–287.
53. Younes N, Fulton N, Tanaka R, et al. The presence of K-12 ras mutations in duodenal adenocarcinomas and the absence of ras mutations in other small bowel adenocarcinomas and carcinoid tumors. Cancer 1997;79:1804–1808.
54. Bläker H, Helmchen B, Bönisch A, et al. Mutational activation of the RAS-RAF-MAPK and the Wnt pathway in small intestinal adenocarcinomas. Scand J Gastroenterol 2004;39:748–753.
55. Lee HJ, Lee OJ, Jang KT, et al. Combined loss of E-cadherin and aberrant β-catenin protein expression correlates with a poor prognosis for small intestinal adenocarcinomas. Am J Clin Pathol 2013;139:167–176.
56. Fang M, Ou J, Hutchinson L, et al. The BRAF oncoprotein functions through the transcriptional repressor MAFG to mediate the CpG Island Methylator phenotype. Mol Cell 2014;55:904–915.
57. Serra RW, Fang M, Park SM, et al. A KRAS-directed transcriptional silencing pathway that mediates the CpG island methylator phenotype. Elife 2014;3:e02313.
58. Laforest A, Aparicio T, Zaanan A, et al. ERBB2 gene as a potential therapeutic target in small bowel adenocarcinoma. Eur J Cancer 2014;50:1740–1746.
59. Rampertab SD, Forde KA, Green PH. Small bowel neoplasia in coeliac disease. Gut 2003;52:1211–1214.
60. Perzin KH, Bridge MF. Adenomas of the small intestine: A clinicopathologic review of 51 cases and a study of their relationship to carcinoma. Cancer 1981;48:799–819.
61. Genta RM, Feagins LA. Advanced precancerous lesions in the small bowel mucosa. Best Pract Res Clin Gastroenterol 2013;27:225–233.
62. Senger S, Sapone A, Fiorentino MR, et al. Celiac Disease Histopathology Recapitulates Hedgehog Downregulation, Consistent with wound Healing Processes Activation. PLoS ONE 2015;10:e0144634.
63. Simon M, Cosnes J, Gornet JM, et al. Endoscopic Detection of Small Bowel Dysplasia and Adenocarcinoma in Crohn's Disease: A Prospective Cohort-Study in High-Risk Patients. J Crohns Colitis 2017;11:47–52.
64. Ciccocioppo R, Racca F, Scudeller L, et al. Differential cellular localization of Epstein-Barr virus and human cytomegalovirus in the colonic mucosa of patients with active or quiescent inflammatory bowel disease. Immunol Res 2016;64:191–203.

65. Genitsch V, Novotny A, Seiler CA, et al. Epstein-barr virus in gastroesophageal adenocarcinomas—single center experiences in the context of current literature. Front Oncol 2015;5:73.
66. Tomba C, Sidhu R, Sanders DS, et al. Celiac Disease and Double-Balloon Enteroscopy: What Can we Achieve? The Experience of 2 European Tertiary Referral Centers. J Clin Gastroenterol 2016;50:313–317.
67. Rieder F, Latella G, Magro F, et al. European Crohn's and Colitis Organisation Topical Review on Prediction, Diagnosis and Management of Fibrostenosing Crohn's Disease. J Crohns Colitis 2016;10:873–885.
68. Bergamaschi G, Di Sabatino A, Corazza GR. Pathogenesis, diagnosis and treatment of anaemia in immune-mediated gastrointestinal disorders. Br J Haematol 2018;182:319-329.
69. Baars JE, Thijs JC, Bac DJ, et al. Small bowel carcinoma mimicking a relapse of Crohn's disease: A case series. J Crohns Colitis 2011;5:152–156.
70. Collier PE, Turowski P, Diamond DL. Small intestinal adenocarcinoma complicating regional enteritis. Cancer 1985;55:516–521.
71. Overman MJ, Hu CY, Kopetz S, et al. A population-based comparison of adenocarcinoma of the large and small intestine: Insights into a rare disease. Ann Surg Oncol 2012;19:1439–1445.
72. Halfdanarson TR, McWilliams RR, Donohue JH, et al. A single-institution experience with 491 cases of small bowel adenocarcinoma. Am J Surg 2010;199:797–803.
73. Mahul B. Amin, Donna M. Gress. AJCC Cancer Staging Manual, Eight Edition. New York: Springer, 2017.
74. Aydin D, Sendur MA, Kefeli U, et al. Evaluation of prognostic factors and adjuvant chemotherapy in patients with small bowel adenocarcinoma who underwent curative resection. Clin Colorectal Cancer 2017;16:220-227.
75. Huffman BM, Jin Z, Yadav S, et al. Novel Prognostic Factors in Resected Small Bowel Adenocarcinoma. Clin Colorectal Cancer. 2019;18:218-225.
76. Overman MJ, Hu CY, Wolff RA, et al. Prognostic value of lymph node evaluation in small bowel adenocarcinoma: Analysis of the surveillance, epidemiology, and end results database. Cancer 2010;116:5374–5382.
77. Dabaja BS, Suki D, Pro B, et al. Adenocarcinoma of the small bowel: Presentation, prognostic factors, and outcome of 217 patients. Cancer 2004;101:518–526.

78. Axelrad JE, Olén O, Sachs MC, et al. Inflammatory Bowel Disease and Risk of Small Bowel Cancer: A Binational Population-Based Cohort Study From Denmark and Sweden. Gut 2020 May 30. [Epub ahead of print]
79. Fields AC, Hu FY, Lu P, et al. Small Bowel Adenocarcinoma: Is There a Difference in Survival for Crohn's Versus Sporadic Cases? J Crohns Colitis 2020;14:303-8.
80. Axelrad JE, Olén O, Sachs MC, et al. Reply: Survival in Crohn's disease-associated small bowel adenocarcinoma. Gut 2020 Sep 1 [Epub ahead of print]
81. Locher C, Batumona B, Afchain P, et al. Small bowel adenocarcinoma: French intergroup clinical practice guidelines for diagnosis, treatments and follow-up (SNFGE, FFCD, GERCOR, UNICANCER, SFCD, SFED, SFRO). Dig Liver Dis 2018;50:15–19.
82. González I, Goyal B, Xia MD, et al. DNA mismatch repair deficiency but not ARID1A loss is associated with prognosis in small intestinal adenocarcinoma. Hum Pathol 2019;85:18-26.
83. Vanoli A, Grillo F, Guerini C, et al. Prognostic Role of Mismatch Repair Status, Histotype and High-Risk Pathologic Features in Stage II Small Bowel Adenocarcinomas. Ann Surg Oncol 2020 Aug 5 [Epub ahead of print]
84. Benson AB, Venook AP, Al-Hawary MM, et al. Small Bowel Adenocarcinoma, Version 1.2020, NCCN Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw 2019;17:1109–1133.
85. Ye X, Zhang G, Chen H, et al. Meta-analysis of postoperative adjuvant therapy for small bowel adenocarcinoma. PloS One 2018;13:e0200204.
86. Akce M, Jiang R, Zakka K, et al. Clinical Outcomes of Small Bowel Adenocarcinoma. Clin Colorectal Cancer 2019;18:257–268.
87. Evans J, Aparicio T, Le Malicot K, et al. GLOBAL BALLAD: an International Rare Cancers Initiative trial to evaluate the potential benefit of adjuvant chemotherapy for small bowel adenocarcinoma (IRCI 002). J Clin Oncol 2016;34:15_suppl. TPS4154.
88. U.S. National Library of Medicine. Phase III Trial Investigating the Potential Benefit of Adjvant Chemotherapy for Small Bowel Adenocarcinoma (BALLAD). ClinicalTrials.gov: 2015. https:// clinicaltrials.gov/ct2/show/NCT02502370. Accessed 07 Sep 2020.
89. Santini D, Fratto ME, Spoto C, et al. Cetuximab in small bowel adenocarcinoma: A new friend? Br J Cancer 2010;103:1305.

90. Falcone R, Roberto M, Filetti M, et al. Anti epidermal growth factor receptor therapy in small bowel adenocarcinoma: Case report and literature review. Medicine 2018;97:e9672.
91. Gulhati P, Raghav K, Shroff R, et al. Phase II Study of Panitumumab in RAS Wild-Type Metastatic Adenocarcinoma of Small Bowel or Ampulla of Vater. Oncologist 2018;23:277-e26.
92. Venook AP. Right-sided vs left-sided colorectal cancer. Clin Adv Hematol Oncol 2017;15:22–24.
93. Hamad A, Singhi AD, Bahary N, et al. Neoadjuvant Treatment with Trastuzumab and FOLFOX Induces a Complete Pathologic Response in a Metastatic ERBB2 (HER2)-Amplified Duodenal Cancer. J Natl Compr Canc Netw 2017;15:983–988.
94. U.S. National Library of Medicine. ClinicalTrials.gov. 2018. Available online: https://clinicaltrials.gov/ct2/show/NCT03000179. Accessed 07 Sep 2020.
95. U.S. National Library of Medicine. ClinicalTrials.gov. 2018. Available online: https://clinicaltrials.gov/ct2/show/NCT03108131. Accessed 07 Sep 2020.
96. Le DT, Uram JN, Wang H, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med 2015;372:2509–2520.
97. U.S. National Library of Medicine. ClinicalTrials.gov. 2018. Available online: https://clinicaltrials.gov/ct2/show/NCT02949219. Accessed 07 Sep 2020.
98. U.S. National Library of Medicine. ClinicalTrials.gov. 2018. Available online: https://clinicaltrials.gov/ct2/show/NCT03095781. Accessed 07 Sep 2020.
99. U.S. National Library of Medicine. ClinicalTrials.gov. 2018. Available online: https://clinicaltrials.gov/ct2/show/NCT02834013. Accessed 07 Sep 2020.
100. Le DT, Durham JN, Smith KN, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science 2017;357:409–413.
101. Watari J, Mitani S, Ito C, et al. Molecular alterations and PD-L1 expression in non-ampullary duodenal adenocarcinoma: Associations among clinicopathological, immunophenotypic and molecular features. Sci Rep 2019;9:10526.
102. Noh BJ, Hong SM, Jun SY, et al. Prognostic implications of immune classification in a multicentre cohort of patients with small intestinal adenocarcinoma. Pathology 2020;52:228-235.
103. Salem ME, Puccini A, Grothey A, et al. Landscape of Tumor Mutation Load, Mismatch Repair Deficiency, and PD-L1 Expression in a Large Patient Cohort of Gastrointestinal Cancers. Mol Cancer Res 2018;16:805-812.

104. Crumley S, Kurnit K, Hudgens C, et al. Identification of a subset of microsatellite-stable endometrial carcinoma with high PD-L1 and CD8+ lymphocytes. Mod Pathol 2019;32:396-404.

105. Lee LH, Cavalcanti MS, Segal NH, et al. Patterns and prognostic relevance of PD-1 and PD-L1 expression in colorectal carcinoma. Mod Pathol 2016;29:1433-1442.

106. Inaguma S, Lasota J, Wang Z, et al. Clinicopathologic profile, immunophenotype, and genotype of CD274 (PD-L1)-positive colorectal carcinomas. Mod Pathol 2017;30:278-285.

107. Taube JM, Galon J, Sholl LM, Rodig SJ, Cottrell TR, Giraldo NA, et al. Implications of the tumor immune microenvironment for staging and therapeutics. Mod Pathol 2018;31:214-234.

108. Puppa G, Senore C, Sheahan K, et al Diagnostic reproducibility of tumour budding in colorectal cancer: a multicentre, multinational study using virtual microscopy. Histopathology 2012;61:562-575.

109. Ueno H, Hase K, Hashiguchi Y, et al. Site-specific tumor grading system in colorectal cancer: multicenter pathologic review of the value of quantifying poorly differentiated clusters. Am J Surg Pathol 2014;38:197-204.

110. Barresi V, Reggiani Bonetti L, Branca G, et al. Colorectal carcinoma grading by quantifying poorly differentiated cell clusters is more reproducible and provides more robust prognostic information than conventional grading. Virchows Arch 2012;461:621-628.

111. Lugli A, Kirsch R, Ajioka Y, et al. Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. Mod Pathol 2017;30:1299–1311.

112. Ohike N, Coban I, Kim G E, et al. Tumor budding as a strong prognostic indicator in invasive ampullary adenocarcinomas. Am J Surg Pathol 2010;34:1417-1424.

113. Cappellesso R, Luchini C, Veronese N, et al. Tumor budding as a risk factor for nodal metastasis in pT1 colorectal cancers: a meta-analysis. Hum Pathol 2017;65:62-70.

114. Karamitopoulou E, Wartenberg M, Zlobec I, et al. Tumour budding in pancreatic cancer revisited: validation of the ITBCC scoring system. Histopathology 2018;73:137-146.

115. Dawson H, Galuppini F, Träger P, et al. Validation of the International Tumor Budding Consensus Conference 2016 recommendations on tumor budding in stage I-IV colorectal cancer. Hum Pathol 2019;85:145-151.

116. Gomollón F, Dignass A, Annese V, et al. 3rd European Evidence-based consensus on the diagnosis and management of Crohn's disease 2016: Part 1: Diagnosis and medical management. J Crohns Colitis 2017;11:3-25.
117. Ueno H, Mochizuki H, Hashiguchi Y, et al. Risk factors for an adverse outcome in early invasive colorectal carcinoma. Gastroenterology 2004;127:385-394.
118. Chiaravalli A M, Klersy C, Vanoli A, et al. Histotype-based prognostic classification of gastric cancer. World J Gastroenterol 2012;18: 896-904.
119. Konishi T, Shimada Y, Lee L H, et al. Poorly Differentiated Clusters Predict Colon Cancer Recurrence: An In-Depth Comparative Analysis of Invasive-Front Prognostic Markers. Am J Surg Pathol 2018;42:705-714.
120. Chiaravalli AM, Feltri M, Bertolini V, et al. Intratumor T cells, their activation status and survival in gastric carcinomas characterized for microsatellite instability and Epstein-Barr virus infection. Virchows Arch 2006;448:344-353.
121. Kulangara K, Zhang N, Corigliano E, et al. Clinical Utility of the Combined Positive Score for Programmed Death Ligand-1 Expression and the Approval of Pembrolizumab for Treatment of Gastric Cancer. Arch Pathol Lab Med 2019;143:330-337.
122. Wood SM, Gill AJ, Brodsky AS, et al. Fatty acid-binding protein 1 is preferentially lost in microsatellite instable colorectal carcinomas and is immune modulated via the interferon γ pathway. Mod Pathol 2017;30:123-133.
123. Teng MW, Ngiow SF, Ribas A, et al. Classifying cancers based on T-cell infiltration and PD-L1. Cancer Res 2015;75:2139-2145.
124. Rizzo F, Vanoli A, Sahnane N, et al. Small-bowel carcinomas associated with celiac disease: transcriptomic profiling shows predominance of microsatellite instability-immune and mesenchymal subtypes. Virchows Arch 2020;476:711-723.
125. Pedersen K, Foster N, Overman M, et al. ZEBRA: an ACCRU/IRCI multicenter phase 2 study of pembrolizumab in patients with advanced small bowel adenocarcinoma (SBA). Ann Oncol 2019;30(Suppl 4).
126. Ponce de León C, Angel López-Casado M, Lorite P, et al. Dysregulation of the PD-1/PD-L1 pathway contributes to the pathogenesis of celiac disease. Cell Mol Immunol 2019;16:777-779.
127. Ma F, Zhao M, Song Z, et al. T-bet interferes with PD-1/PD-L1-mediated suppression of CD4(+) T cell inflammation and survival in Crohn's disease. Clin Exp Pharmacol Physiol 2019;46:798-805.

128. Kanai T, Totsuka T, Uraushihara K, et al. Blockade of B7-H1 suppresses the development of chronic intestinal inflammation. J Immunol 2003;171:4156-4163.
129. Cortellini A, Buti S, Santini D, et al. Clinical Outcomes of Patients with Advanced Cancer and Pre-Existing Autoimmune Diseases Treated with Anti-Programmed Death-1 Immunotherapy: A Real-World Transverse Study. Oncologist 2019;24:327-337.
130. Chen C, Zhang F, Zhou N, et al. Efficacy and safety of immune checkpoint inhibitors in advanced gastric or gastroesophageal junction cancer: a systematic review and meta-analysis. Oncoimmunology 2019;8:e1581547.
131. Mantovani A, Romero P, Palucka AK, et al. Tumor immunity: effector response to tumor and role of the microenvironment. Lancet 2008;371:771-83.
132. Sharpe AH, Pauken KE. The diverse functions of the PD1 inhibitory pathway. Nat Rev Immunol 2018;18:153-167.
133. Minamoto T, Mai M, Watanabe K, et al. Medullary carcinoma with lymphocytic infiltration of the stomach. Clinicopathologic study of 27 cases and immunohistochemical analysis of the subpopulations of infiltrating lymphocytes in the tumor. Cancer 1990;66:945-952.
134. Friedman K, Brodsky AS, Lu S, et al. Medullary carcinoma of the colon: a distinct morphology reveals a distinctive immunoregulatory microenvironment. Mod Pathol 2016;29:528-541.
135. Jun SY, Park ES, Lee JJ, et al. Prognostic Significance of Stromal and Intraepithelial Tumor-Infiltrating Lymphocytes in Small Intestinal Adenocarcinoma. Am J Clin Pathol 2020;153:105-118.
136. Jun SY, Chung JY, Yoon N, et al. Tumor Budding and Poorly Differentiated Clusters in Small Intestinal Adenocarcinoma. Cancers (Basel) 2020;12:E2199
137. Kemi N, Eskuri M, Ikäläinen J, et al. Tumor Budding and Prognosis in Gastric Adenocarcinoma. Am J Surg Pathol 2019;43:229-234.
138. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt to a histo-clinical classification. Acta Pathol Microbiol Scand 1965;64:31-49.
139. Carneiro F, Seixas M, Sobrinho-Simões M. New elements for an updated classification of the carcinomas of the stomach. Pathol Res Pract 1995;191:571-584.

140. Solcia E, Klersy C, Mastracci L, et al. A combined histologic and molecular approach identifies three groups of gastric cancer with different prognosis. Virchows Arch 2009;455:197-211.
141. Kim M A, Lee H S, Lee H E, et al. Prognostic importance of epithelial–mesenchymal transition-related protein expression in gastric carcinoma. Histopathology 2009;54:442-451.
142. Collisson EA, Sadanandam A, Olson P, et al. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. Nat Med 2011;17:500-503.
143. Wu Y, Grabsch H, Ivanova T, et al. Comprehensive genomic meta-analysis identifies intra-tumoural stroma as a predictor of survival in patients with gastric cancer. Gut 2013;62:1100-1111.
144. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature 2014;513:202-209.
145. Cristescu R, Lee J, Nebozhyn M, et al. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med 2015;21:449-456.
146. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. Nat Med 2015;21:1350-1356.
147. Reggiani Bonetti L, Barresi V, Bettelli S, et al. Poorly differentiated clusters (PDC) in colorectal cancer: what is and ought to be known. Diagn Pathol 2016;11:31.
148. Lee VWK, Chan KF. Tumor budding and poorly-differentiated cluster in prognostication in Stage II colon cancer. Pathol Res Pract 2018;214:402-407.
149. Chen ZM, Wang HL. Alteration of cytokeratin 7 and cytokeratin 20 expression profile is uniquely associated with tumorigenesis of primary adenocarcinoma of the small intestine. Am J Surg Pathol 2004;28:1352–1359.
150. Xue Y, Vanoli A, Balci S, et al. Non-ampullary-duodenal carcinomas: clinicopathologic analysis of 47 cases and comparison with ampullary and pancreatic adenocarcinomas. Mod Pathol 2017;30:255–266.
151. Zhang MQ, Lin F, Hui P, et al. Expression of mucins, SIMA, villin, and CDX2 in small-intestinal adenocarcinoma. Am J Clin Pathol 2007;128:808–816.
152. Lee MJ, Lee HS, Kim WH, et al. Expression of mucins and cytokeratins in primary carcinomas of the digestive system. Mod Pathol 2003;16:403–410.
153. Lugli A, Tzankov A, Zlobec I, et al. Differential diagnostic and functional role of the multi-marker phenotype CDX2/CK20/CK7 in colorectal cancer stratified by mismatch repair status. Mod Pathol 2008;21:1403–1412.

154. Kim CJ, Baruch-Oren T, Lin F, et al. Value of SATB2 immunostaining in the distinction between small intestinal and colorectal adenocarcinomas. J Clin Pathol 2016;69:1046–1050.

Scientific production arisen from this thesis

Peer-reviewed publications

1. **Giuffrida P**, Vanoli A, Arpa G, Bonometti A, Luinetti O, Solcia E, Corazza GR, Paulli M, Di Sabatino A. Small bowel carcinomas associated with immune-mediated intestinal disorders: the current knowledge. Cancers (Basel) 2018;11:31.
2. **Giuffrida P**, Arpa G, Grillo F, Klersy C, Sampietro G, Ardizzone S, Fociani P, Fiocca R, Latella G, Sessa F, D'Errico A, Malvi D, Mescoli C, Rugge M, Nesi G, Ferrero S, Furlan D, Poggioli G, Rizzello F, Macciomei MC, Santini D, Volta U, De Giorgio R, Caio G, Calabrò A, Ciacci C, D'Armiento M, Rizzo A, Solina G, Martino M, Tonelli F, Villanacci V, Cannizzaro R, Canzonieri V, Florena AM, Biancone L, Monteleone G, Caronna R, Ciardi A, Elli L, Caprioli F, Vecchi M, D'Incà R, Zingone F, D'Odorico A, Lenti MV, Oreggia B, Reggiani Bonetti L, Astegiano M, Biletta E, Cantoro L, Giannone AG, Orlandi A, Papi C, Perfetti V, Quaquarini E, Sandri G, Silano M, Usai P, Barresi V, Ciccocioppo R, Luinetti O, Pedrazzoli P, Pietrabissa A, Viglio A, Paulli M, Corazza GR, Solcia E, Vanoli A, Di Sabatino A. PD-L1 in small bowel adenocarcinoma is associated with etiology and tumor-infiltrating lymphocytes, in addition to microsatellite instability. Mod Pathol 2020;33:1398-1409.
3. Arpa G, Grillo F, **Giuffrida P**, Nesi G, Klersy C, Mescoli C, Lenti MV, Lobascio G, Martino M, Latella G, Malvi D, Macciomei MC, Fociani P, Villanacci V, Rizzo A, Ferrero S, Sessa F, Orlandi A, Monteleone G, Biancone L, Cantoro L, Tonelli F, Ciardi A, Poggioli G, Rizzello F, Ardizzone S, Sampietro G, Solina G, Oreggia B, Papi C, D'Incà R, Vecchi M, Caprioli F, Caronna R, D'Errico A, Fiocca R, Rugge M, Corazza GR, Luinetti O, Paulli M, Solcia E, Di Sabatino A, Vanoli A. Separation of low versus high grade Crohn's disease-associated small bowel carcinomas is improved by invasive front prognostic marker analysis. J Crohns Colitis 2020;14:295-302.
4. **Giuffrida P**, Vanoli A, Di Sabatino A. Survival in Crohn's disease-associated small bowel adenocarcinoma. Gut 2020 Jul 24. [Epub ahead of print]
5. **Giuffrida P**, Arpa G, Vanoli A, Di Sabatino A. Comment on Jun, S.Y. et al., "Tumor Budding and Poorly Differentiated Clusters in Small Intestinal Adenocarcinoma" Cancers 2020, 12, E2199. Submitted for publication.

Additionally, during my PhD I also published the following peer-reviewed papers:

1. Di Sabatino A, Aronico N, **Giuffrida P**, Cococcia S, Lenti MV, Vanoli A, Guerci M, Di Stefano M, Corazza GR. Association between defective spleen function and primary eosinophilic gastrointestinal disorders. J Allergy Clin Immunol Pract 2018;6:1056-8.
2. **Giuffrida P**, Corazza GR, Di Sabatino A. Old and new lymphocyte players in inflammatory bowel disease. Dig Dis Sci 2018;63:277-88.
3. **Giuffrida P**, Vanoli A, Biletta E, Klersy C, Bergamaschi G, Sampietro GM, Ardizzone S, Luinetti O, Martino M, Lenti MV, Aronico A, Curti M, Manca R, Fociani P, Truffi M, Corsi F, Corazza GR, Di Sabatino A. Increase in chromogranin A- and serotonin-positive cells in pouch mucosa of patients with ulcerative colitis undergoing proctocolectomy. Dig Liver Dis 2018;50:1205-13.
4. Naviglio S, **Giuffrida P**, Stocco G, Lenti MV, Ventura A, Corazza GR, Di Sabatino A. How to predict response to anti-tumour necrosis factor agents in inflammatory bowel disease. Expert Rev Gastroenterol Hepatol 2018;12(8):797-810.
5. **Giuffrida P**, Caprioli F, Facciotti F, Di Sabatino A. The role of interleukin-13 in chronic inflammatory intestinal disorders. Autoimmun Rev 2019;18:549-555.
6. **Giuffrida P**, Cococcia S, Delliponti M, Lenti MV, Di Sabatino A. Controlling gut inflammation by restoring anti-inflammatory pathways in inflammatory bowel disease. Cells 2019 30 Apr;8(5).
7. Lenti MV, Miceli E, Cococcia S, Klersy C, Staiani M, Guglielmi F, **Giuffrida P**, Vanoli A, Luinetti O, De Grazia F, Di Stefano M, Corazza GR, Di Sabatino A. Determinants of diagnostic delay in autoimmune atrophic gastritis. Aliment Pharmacol Ther 2019;50:167-175.
8. **Giuffrida P**, Curti M, Al-Akkad W, Biel C, Crowley C, Frenguelli L, Telese A, Hall A, Tamburrino D, Spoletini G, Fusai G, Tinozzi FP, Pietrabissa A, Corazza GR, De Coppi P, Pinzani M, Di Sabatino A, Rombouts K, Mazza G. Decellularized human gut as a natural 3D platform for research in intestinal fibrosis. Inflamm Bowel Dis 2019;25:1740-1750.
9. De Re V, Repetto O, De Zorzi M, Casarotto M, Tedeschi M, **Giuffrida P**, Lenti MV, Magris R, Miolo G, Mazzon C, Zanette G, Alessandrini L, Canzonieri V, Caggiari L, Zanussi S, Steffan A, Di Sabatino A, Cannizzaro R. Polymorphism in Toll-like receptors and

Helicobacter pylori motility in autoimmune atrophic gastritis and gastric cancer. Cancers (Basel) 2019 May 10;11(5).

10. Fiorino G, Sturniolo GC, Bossa F, Cassinotti A, Di Sabatino A, **Giuffrida P**, Danese S. A Phase 2a, Multicenter, Randomized, Double-Blind, Parallel-Group, Placebo-Controlled Trial of IBD98-M Delayed-Release Capsules to Induce Remission in Patients with Active and Mild to Moderate Ulcerative Colitis. Cells 2019 May 30;8(6).

11. **Giuffrida P**, Aronico N, Rosselli M, Lenti MV, Cococcia S, Roccarina D, Saffioti F, Delliponti M, Thorburn D, Miceli E, Corazza GR, Pinzani M, Di Sabatino A. Defective spleen function in autoimmune gastrointestinal disorders. Intern Emerg Med 2020;15:225-229.

12. Franzè E, Monteleone I, Laudisi F, Rizzo A, Dinallo V, Di Fusco D, Colantoni A, Ortenzi A, **Giuffrida P**, Di Carlo S, Sica GS, Di Sabatino A, Monteleone G. Cadherin-11 is a regulator of intestinal fibrosis. J Crohns Colitis 2020;14:406-417.

13. Barbaro MR, Cremon C, Morselli-Labate AM, Di Sabatino A, **Giuffrida P**, Corazza GR, Di Stefano M, Caio G, Latella G, Ciacci C, Fuschi D, Mastroroberto M, Bellacosa L, Stanghellini V, Volta U, Barbara G. Serum zonulin and its diagnostic performance in non-coeliac gluten sensitivity. Gut 2020 Feb 14. [Epub ahead of print]

14. **Giuffrida P**, Di Sabatino A. MicroRNAs in celiac disease diagnosis: a miR curiosity or game-changer? Dig Dis Sci 2020;65:1877-1879.

15. Curciarello R, Sobande T, Jones S, **Giuffrida P**, Di Sabatino A, Docena GH, MacDonald TT, Kok K. Human neutrophil elastase proteolytic activity in ulcerative colitis favours loss of function of anti-TNF-α agents. J Inflamm Res 2020;13:233-243.

16. Schiepatti A, Sanders DS, Aziz I, De Silvestri A, Goodwin J, Key T, Quaye L, **Giuffrida P**, Vanoli A, Paulli M, Cross SS, Vergani P, Betti E, Maiorano G, Ellis R, Snowden JA, Di Sabatino A, Corazza GR, Biagi F. Clinical phenotype and mortality in patients with idiopathic small bowel villous atrophy: a dual-centre international study. Eur J Gastroenterol Hepatol 2020 Apr 10. [Epub ahead of print]

17. Holm Nielsen S, Mortensen JH, Willumsen N, Rasmussen DGK, Mogensen DJ, Di Sabatino A, Mazza G, Jørgensen LN, **Giuffrida P**, Pinzani M, Klinge L, Kjeldsen J, Leeming DJ, Karsdal MA, Genovese F. A Fragment of Collagen Type VI alpha-3 chain is Elevated in Serum from Patients with Gastrointestinal Disorders. Sci Rep 2020;10(1):5910.

18. Franzè E, Dinallo V, Laudisi F, Di Grazia A, Di Fusco D, Colantoni A, Ortenzi A, **Giuffrida P**, Di Carlo S, Sica GS, Di Sabatino A, Monteleone G. Interleukin-34 Stimulates Gut Fibroblasts to Produce Collagen Synthesis. J Crohns Colitis 2020 Apr 9. [Epub ahead of print]

19. Sedda S, Dinallo V, Marafini I, Paoluzi OA, Izzo R, **Giuffrida P**, Di Sabatino A, Corazza GR, Monteleone G. mTOR sustains inflammatory response in celiac disease. Sci Rep 2020;10(1):10798.

20. **Giuffrida P**, Di Sabatino A. Targeting T cells in inflammatory bowel disease. Pharmacol Res 2020;159:105040.

21. Vanoli A, Grillo F, Guerini C, Neri G, Arpa G, Klersy C, Nesi G, **Giuffrida P**, Sampietro G, Ardizzone S, Fociani P, Fiocca R, Latella G, Sessa F, D'Errico A, Malvi D, Mescoli C, Rugge M, Ferrero S, Poggioli G, Rizzello F, Macciomei MC, Santini D, Volta U, De Giorgio R, Caio G, Calabrò A, Ciacci C, D'Armiento M, Rizzo A, Solina G, Martino M, Tonelli F, Villanacci V, Cannizzaro R, Canzonieri V, Florena AM, Biancone L, Monteleone G, Caronna R, Ciardi A, Elli L, Caprioli F, Vecchi M, D'Incà R, Zingone F, D'Odorico A, Oreggia B, Reggiani Bonetti L, Giannone AG, Orlandi A, Barresi V, Ciccocioppo R, Amodeo G, Biletta E, Luinetti O, Pedrazzoli P, Pietrabissa A, Corazza GR, Paulli M, Solcia E, Di Sabatino A. Prognostic role of mismatch repair status, histotype and high-risk pathologic features in stage II small bowel adenocarcinomas. Ann Surg Oncol 2020 Aug 5. [Epub ahead of print]

22. Di Fusco D, Marafini I, Stolfi C, Troncone E, Onali S, Lolli E, Caprioli F, Mazza S, Cascella R, Manzo L, Borgiani P, **Giuffrida P**, Di Sabatino A, Monteleone I, Monteleone G. A Novel Smad7 Genetic Variant Mapping on the Genomic Region Targeted by Mongersen Is Associated with Crohn's Disease. Biomedicines 2020;8:E234.

Abstracts at international meetings

Giuffrida P, Vanoli A, Martino M, Persichella A, Viglio A, Furlan D, Klersy C, Grillo F, Fiocca R, Mescoli C, Rugge M, Nesi G, Fociani P, Sampietro GM, Ardizzone S, Luinetti O, Calabrò A, Tonelli F, Volta U, Santini D, Caio G, Elli L, Ferrero S, Latella G, Ciardi A, Caronna R, Solina G, Rizzo A, Ciacci C, D'Armiento FP, Salemme M, Villanacci V, Cannizzaro R, Canzonieri V, Reggiani Bonetti L, Biancone L, Monteleone G, Orlandi A, Santeusanio G, Macciomei MC, D'Incà R, Perfetti V, Sandri G, Silano M, Florena AM, Giannone AG, Papi C, Coppola L, Usai P, Maccioni A, Astegiano M, Migliora P, Lenti MV, Rovedatti L, Sessa F, Solcia E, Corazza GR, Paulli M, Di Sabatino A. Programmed cell death ligand-1 expression is associated with microsatellite instability in non-hereditary small bowel carcinomas: results from the Small Bowel Cancer Italian Consortium. 26th United European Gastroenterology Week, October 20th-24th 2018 in Vienna, Austria.

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